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Effects of fractions of coal-derived humic substances on seed germination and growth of seedlings (*Lactuga sativa* and *Lycopersicum esculentum*)

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Summary. Lettuce and tomato seeds were treated in Petri dishes with a humic acid derived from an oxidized coal and with fractions of the same humic acid obtained by ultrafiltration through membranes of known molecular cutoff and by extracting with buffers set at pH 4 and pH 5. The unfractionated humic acid was applied at 40, 100, 1000, and 5000 mg l^{-1} whereas the humic fractions were applied at 40, 100, and 200 mg l^{-1}. Germination parameters such as the number of total germinated seeds, the velocity of seed germination, the fresh weight and dry weight of total seedlings were measured and related to the chemical and physicochemical properties of the humic material. No increase in the germination percentage or the germination rate was observed for either lettuce or tomato seeds. The fresh weight of total seedlings and per seedling increased in treatments with unfractionated humic acid with increasing concentrations for both lettuce and tomato plants without showing signs of growth inhibition up to 5000 mg l^{-1}. This was attributed to cell elongation and more efficient water uptake. For the lettuce, the fresh weight both of total seedlings and per seedling was enhanced by treating the seeds with fractions of low molecular weight and high content of acidic functions, whereas the dry weight both of total seedlings and per seedling did not change with the humic fraction used. For the tomato seeds in contrast, the dry weight both of total seedlings and per seedling was increased by the use of unfractionated humic acid and by some of the humic fractions. An uptake of humic material by growing tomato seedlings was inferred.

Key words: Humic substances – Molecular sizes – Fractionation – Seed germination – Seedling growth – Lettuce – Tomato

Humic substances are known to stimulate the germination of several varieties of agricultural seeds (Dixit and Kishore 1967; Azam and Malik 1982). The immersion of seeds in a sodium humate solution was reported to increase germination, water absorption, and respiration (Smidova 1962), the length of roots and shoots (Rauthan and Schnitzer 1981; Malik and Azam 1985), and the fresh and dry mass of roots and shoots (Vaughan and Linehan 1976). Crop yields and nutrient uptake (Pagel 1960; Varshney and Gaur 1974) may also improve as a result of direct or indirect effects of humic substances. Despite the recognition of these effects, relationships with the structural nature of the humic material used remained neglected. Recent work on humic substances derived from a coal oxidation process (Rausa et al. 1989) led van de Venter et al. (1991) to examine the effect of coal-derived sodium humate on the stimulation of seedling growth. The results showed that this compound stimulated the primary root growth of cantaloupe, lettuce, and onion seedlings at different rates of application. In agreement with Vaughan and Linehan (1976), the stimulatory effect was attributed to the humate itself rather than to microbial breakdown products, but the results were not related to any molecular property of the humic substances.

The aim of the present study was to evaluate the effects on seed germination and seedling growth of treating lettuce and tomato seeds with fractions of coal-derived humic substances varying in the physicochemical property of molecular size.

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Materials and methods

Humic substances were obtained by dry-phase oxidation of a sample of Sulcis sub-bituminous coal (Calemma and Rausa 1987) in a fluidized bed at 230 °C for 9 h. The raw oxidized coal was extracted, under N_2, with a 1 mol l^{-1} NaOH solution at 100 °C for 7 h. The pH of the solution was adjusted to 1 with 6 mol l^{-1} HCl and the precipitated humic acid was dialyzed until chloride-free, and air-dried under a vacuum at 70 °C. Total acidity (900 cmol kg^{-1}) was measured by the $Ba(OH)_2$ method and the content of COOH groups (340 cmol kg^{-1}) was determined by the $Ca(CH_3COO)_2$ method (Piccolo 1984).

A sample of humic acid (4 g) was then completely dissolved in 0.5 mol l^{-1} KOH and the pH adjusted to 7. The solution was placed in a 400-ml Amicon ultrafiltration cell under N_2 pressure and desalted through a 500-molecular weight cutoff membrane. Ultrafiltration membranes with progressively higher molecular weight cutoffs (1000, 5000, 10000, 20000, 100000, 500000) were then used to obtain humic fractions of a known range at a nominal molecular size.

Other humic acid samples (10 g) were treated with solutions buffered at pH 4 (998 ml of potassium hydrogen phthalate 0.1 mol l^{-1} and 2 ml of HCl 0.1 mol l^{-1}) and at pH 5 (689 ml of potassium hydrogen phthalate 0.1 mol l^{-1} and 311 ml of HCl 0.1 mol l^{-1}) in order to extract two more humic fractions, named p4 and p5, respectively. The extracts were desalted on a 500-molecular weight ultrafiltration membrane. All fractions were then freeze-dried.

Seeds of lettuce (*Lactuga sativa,* S. Anna local variety) and tomato (*Lycopersicum esculentum,* S. Marzano local variety) were placed, equally spaced, on a filter paper adjusted on a 9-cm Petri dish (32 seeds per dish). One volume (10 ml) of each humic fraction at concentrations of 0, 40, 100, and 200 mg l^{-1} (pH 7) was added to the Petri dishes. Similarly, 10 ml of the original humic acid solution (pH 7) at concentrations of 0, 40, 100, 1000, and 5000 mg l^{-1} was added to another set of Petri dishes. After the additions the Petri dishes were sealed with a glass dish and incubated at room temperature. From the 3rd day onward the number of seeds in each dish showing radicle emergence were counted daily, up to the 6th day. The daily count was used to calculated the mean germination rate as N_i/T_i where N_i is the number of germinated seeds on the ith day and T_i is the ith counting day. After the 6th day the seedlings were washed with water and the total fresh weight of all seedlings was obtained. The dry weight of all seedlings was recorded after 48 h of drying in an oven at 65 °c. The fresh weight and the dry weight per seedling were calculated by dividing the total fresh and dry weights by the number of germinated seeds on the 6th day.

The treatments were replicated five times in each experiment, using a completely random design. In order to compare the different treatment means, the data were subjected to analysis of variance and to Duncan's test.

Results and discussion

The coal-derived humic acid was fractionated by ultrafiltration through eight nominal molecular weight ranges of 500–1000, 1000–5000, 5000–10000, 10000–20000, 20000–50000, 50000–100000, 100000–500000, and >500000. Two more fractions were obtained by extracting the humic acid with buffer solutions set at either pH 4 or pH 5. Data on the elemental composition of the unfractionated humic acid and of the ultrafiltered and buffer fractions are reported in Table 1.

The original humic acid was particularly high in C and low in H compared to a "model" soil humic acid (Schnitzer 1978), suggesting a large degree of aromaticity.

Table 1. Composition of humic acid (HA) fractions

Fraction	MW (×1000)	C (%)	N (%)	H (%)	S (%)	C:H
HA	–	62.9	3.0	1.9	5.6	20.9
p4	–	50.0	1.1	2.8	2.6	17.9
p5	–	49.6	1.1	2.8	3.0	17.5
1	0.5–1	29.4	1.9	1.5	2.5	15.3
2	1–5	30.4	2.0	1.3	2.3	15.5
3	5–10	29.1	1.9	0.8	1.1	15.6
4	10–20	21.8	2.0	0.9	<0.1	15.9
5	20–50	32.2	1.9	0.8	<0.1	17.1
6	50–100	34.1	2.1	0.6	<0.1	16.2
7	100–500	36.3	2.2	1.0	<0.1	16.3
8	>500	37.9	2.1	0.9	<0.1	17.6

MW, molecular weight

This characteristic was confirmed by the ^{13}C-nuclear magnetic resonance spectrum (Piccolo et al. 1992a), which had the following C distribution: 18% aliphatic C (0–110 ppm); 72% aromatic C (110–160 ppm); and 10% COOH groups (160–190 ppm). The relatively low content of C and N in the ultrafiltered fractions can be explained by carbonatation of the humic acid solution during the course of a lengthy ultrafiltration.

The general progressive increase in the C:H ratio in fractions of increasing molecular dimensions may suggest that the aromatic character is less pronounced in the low molecular-size fractions. An evalutation of the apparent molecular weight and infrared spectroscopy features of the different fractions was conducted in a previous study (Piccolo et al. 1992b). The apparent molecular weight, determined by high performance size exclusion chromatography technique ranged from 10000 to 240000 for the ultrafiltered fractions and the infrared spectra revealed that the lower molecular weight fractions were chemically more homogeneous than the higher molecular weight fractions, with a number of bands being suggestive of a highly hydroxylated aliphatic and aromatic material.

The effects of different concentrations of the unfractionated humic acid on seed germination and seedling growth of lettuce and tomato are reported in Table 2. The lettuce results showed no increase in the number of germinated seeds (per cent germination) with any of the humic acid treatments whereas the mean germination rate was significantly different form the control for all concentrations except 5000 ppm. Apart from this concentration, the humic acid treatments appeared to slow down the rate of germination. Both the fresh weight of all seedlings and the fresh weight per seedling increased progressively and significantly with the humic acid concentration. In fact, seedlings treated with humic acid were significantly longer than for the control and the length increased with the humic acid concentration. Conversely, the total dry weight and the dry weight per seedling did

Table 2. The effect of different concentrations of unfractionated humic acid (HA) at pH 7 on germination parameters

HA (ppm)	Germination (%)	MGR	Total seedling weight (mg)		Weigth per seedling (mg)	
			Fresh	Dry	Fresh	Dry
Lettuce plants						
0	83.13a	3.14c	316.38c	26.10a	10.28c	0.85a
40	76.88a	3.25ab	327.84bc	26.16a	10.64c	0.85a
100	76.25a	3.25a	363.22b	25.88a	11.89b	0.85a
1000	79.38a	3.24ab	412.42a	27.50a	13.22a	0.88a
5000	83.13a	3.15bc	424.96a	26.32a	14.14a	0.88a
Tomato plants						
0	96.88a	3.19c	607.30c	56.56b	19.61c	1.73c
40	80.00b	4.85b	425.18d	45.08c	16.60d	1.76c
100	75.63bc	5.24a	552.06c	49.88bc	22.76b	2.06b
1000	70.63c	5.22a	707.14b	49.66bc	31.25a	2.20a
5000	83.75b	5.20a	875.24a	60.54a	32.81a	2.26a

Means in the same column followed by the same letter are not significantly different ($P = 0.05$). MGR, Mean germination rate not differ from the control at any humic acid concentration.

For tomato seeds both the germination percentage and the mean germination rate were significantly different from control at all humic acid concentrations (Table 2). The humic acid generally decreased the percentage of germination and slowed down the germination rate. Contrary to the results for lettuce, not only the total fresh weight and fresh weight per seedling were significantly different from control but also the total dry weight and dry weight per seedling showed significant increase with increasing humic acid concentrations. This discrepancy suggests that while the only humic acid effects on the lettuce were to promote cell elongation and a more efficient water uptake, in the tomato seedlings, which were also longer than the control, some humic acid uptake may have also occurred.

The effect of the single humic acid fractions (all concentrations were pooled in the statistical analysis) on the different germination parameters is shown in Table 3. Lettuce germination percentages determined for the various fractions and for the control did not differ significantly. The rate of germination was similar to that of the control for the lower molecular weight fractions (1, 2 and p4 in Table 3) whereas it appeared slower for fractions of increasing molecular sizes. Piccolo et al. (1992b), using infrared spectroscopy, have shown that fractions 1 and 2 had a high content of acidic functions. Also, p4 is likely to have contained a large number of acidic functional groups since it was extracted with a buffer solution at pH 4. In fact, only the most acidic humic molecules would have been dissolved by a buffer set at pH 4. Similarly, the total fresh weight and fresh weight per seedling were significantly higher for fraction 1, 2, and p4 than for the others. Both total fresh weight and fresh weight per seedling for fraction p4 were similar to those of fraction 7. As previously observed by Piccolo et al. (1992b), this fraction, though its molecular size should have been set high by the ultrafiltration membranes (100000–500000), it showed, oddly, a longer retention time on high performance size exclusion chromatography than that of fraction 4 and a nominal molecular weight of 42000 which was lower than the value (49000) for fraction 4. This molecular discrepancy in fraction 7 was reflected in the fresh weight of the lettuce seedlings. These fresh weight results indicate that fractions with a low molecular weight and a high content of acidic functional groups generally promoted cell elongation compared to the control whereas fractions with a high molecular weight did not behave differently from the control. The lettuce dry weight data, although showing less variation than the fresh weights, also produced values significantly different from control for fractions 1, 2, and p4 only. The tomato seed results were rather different from those described for the lettuce (Table 3). The germination percentages and the velocity of germination were both significantly lower than those of the control and the lettuce, for all humic fractions applied. The slowest tomato-seed germination rate occurred for seeds treated with fractions 7 and p5. Total and seedling fresh weights were generally higher than for the lettuce and did not indicate any particular fractional trend except for the larger values shown by fractions 7 and p5. Similar results were obtained for the dry weights, which were clearly greater than for lettuce, the largest effects being seen with fractions 7 and p5. Thus the effects of different molecular weight fractions on tomato and lettuce seeds were to enhance cell elongation and water uptake in both lettuce and tomato seeds and, in the latter only, a probable uptake of humic molecules.

The increase in cell elongation in the lettuce seedlings appeared to be promoted especially by fractions with a low molecular size and high content of acidic groups. In the tomato seeds the uptake of humic material was suggested by the increase in dry weight obtained by treating the seeds with fractions 7 and p5. The effect of these two fractions must be attributed to a combination of higher molecular dimension and lower acidic functionality in respect to the fractions active on lettuce seeds.

Table 4 reports the cumulative effects of all humic fractions (ultrafiltered plus buffer-extracted fractions) at different rates of addition. These data show that the germination percentage and the germination rate for tomato seeds decreased with increasing concentrations of humic fractions. The lettuce seeds behaved similarly, except for the germination percentage, which did not change significantly with the fraction used. Fresh weight values generally increased with the fraction concentration in both lettuce and tomato seedlings. The lettuce dry weight did not differ with any of the humic fractions used but the tomato dry weight generally increased with increasing humic

Table 3. The effect of humic acid fractions (HA fr.) on germination parameters

HA fr.	Germination (%)	MGR	Total seedling weight (mg)		Weight per seedling (mg)	
			Fresh	Dry	Fresh	Dry
Lettuce plants						
0	96.06 a	3.09 c	370.76 c	26.37 ab	12.04 d	0.86 a
1	97.19 a	3.07 c	462.24 a	25.71 bc	14.87 a	0.83 b
2	96.56 a	3.06 c	461.28 a	25.63 c	14.92 a	0.83 b
3	96.72 a	3.15 b	336.01 d	26.96 a	10.84 e	0.87 a
4	97.34 a	3.15 b	349.46 d	27.26 a	11.21 de	0.88 a
5	96.09 a	3.16 ab	357.17 d	27.00 a	11.59 d	0.88 a
6	95.47 a	3.21 a	343.30 d	26.55 ab	11.24 de	0.87 a
7	95.31 a	3.17 ab	414.69 bc	27.02 a	13.61 b	0.89 a
8	95.16 a	3.18 ab	394.10 c	26.80 a	12.93 c	0.88 a
p5	96.88 a	3.16 ab	399.48 c	27.43 a	12.88 c	0.88 a
p4	96.09 a	3.09 c	428.59 b	24.97 c	13.94 b	0.81 b
Tomato plants						
0	83.13 a	3.86 c	467.36 b	44.12 a	17.34 cd	1.65 cd
1	73.75 c	4.01 bc	458.94 bc	41.59 cb	19.59 b	1.77 b
2	80.31 ab	3.87 c	484.97 b	42.57 b	18.88 b	1.66 cd
4	73.59 c	4.16 b	376.98 d	36.36 e	16.00 d	1.54 e
5	72.81 c	4.08 bc	424.83 c	39.14 cde	18.28 bc	1.68 bc
6	73.44 c	4.00 bc	434.18 c	40.12 bcd	18.61 b	1.71 bc
7	79.38 ab	4.62 a	573.88 a	48.60 a	22.60 a	1.92 a
8	75.78 bc	4.17 b	463.93 bc	38.22 de	19.14 b	1.58 de
p5	82.19 a	4.62 a	569.45 a	48.95 a	21.84 a	1.87 a
p4	76.41 bc	4.15 b	420.84 cd	37.74 de	17.10 cd	1.54 e

For explanations, see Table 2

Table 4. The effect of concentrations of humic acid (HA) fractions on germination parameters

HA (ppm)	Germination (%)	MGR	Total seedling weight (mg)		Weight per seedling (mg)	
			Fresh	Dry	Fresh	Dry
Lettuce plants						
0	96.06a	3.09b	370.76c	26.37a	12.04c	0.86a
40	96.56a	3.17a	367.82c	26.46a	11.90c	0.86a
100	96.19a	3.16a	403.28b	26.56a	13.11b	0.86a
200	96.31a	3.14a	436.66a	26.73a	14.17a	0.87a
Tomato plants						
0	83.13a	3.86b	467.36b	44.12a	17.34c	1.65c
40	72.85b	4.32a	400.38c	38.81c	17.22c	1.66bc
100	74.44b	4.23a	471.55b	40.94bc	19.74b	1.72ab
200	75.21b	4.34a	530.92a	42.03ab	22.16a	1.75a

For explanations, see Table 2

fraction concentrations. This latter result further confirmed the different response by the two seeds to the addition of humic fractions. In contrast to the lettuce, the tomato seeds apparently took up humic material and the rate of uptake grew with increasing humic fraction concentrations.

In order to classify the behavior of the different humic fractions into functional classes, all data related to seed germination and seedling growth were pooled to construct separate dendrograms (cluster analysis as single-linkage grouping; Sneath 1957) for the lettuce and the tomato plants. The dendrogram for lettuce (Fig. 1) shows that the different humic fractions can be grouped into three classes which are directly related to molecular size, the C percentage and the C:H ratio. Fractions 1, 2 and p4, which increased the fresh weight values, were grouped in one dendrogram class. Fraction 7, which also increased

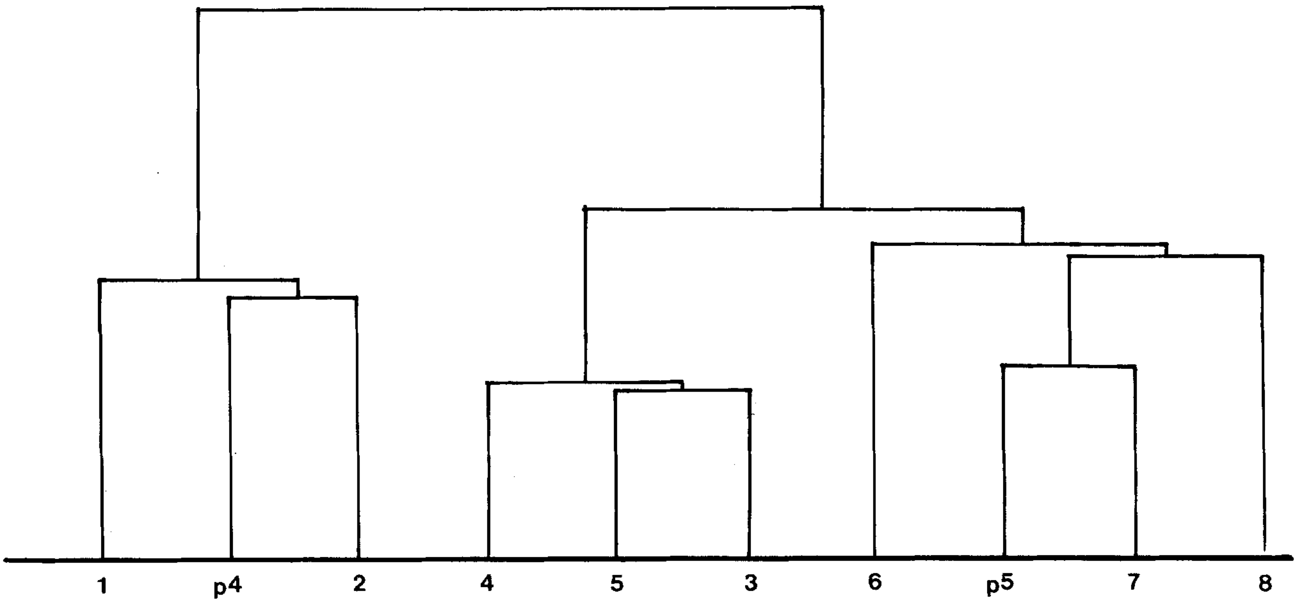

Fig. 1. Dendrogram for the cumulative effects of different humic fractions at 0, 40, 100, and 200 ppm on lettuce seeds

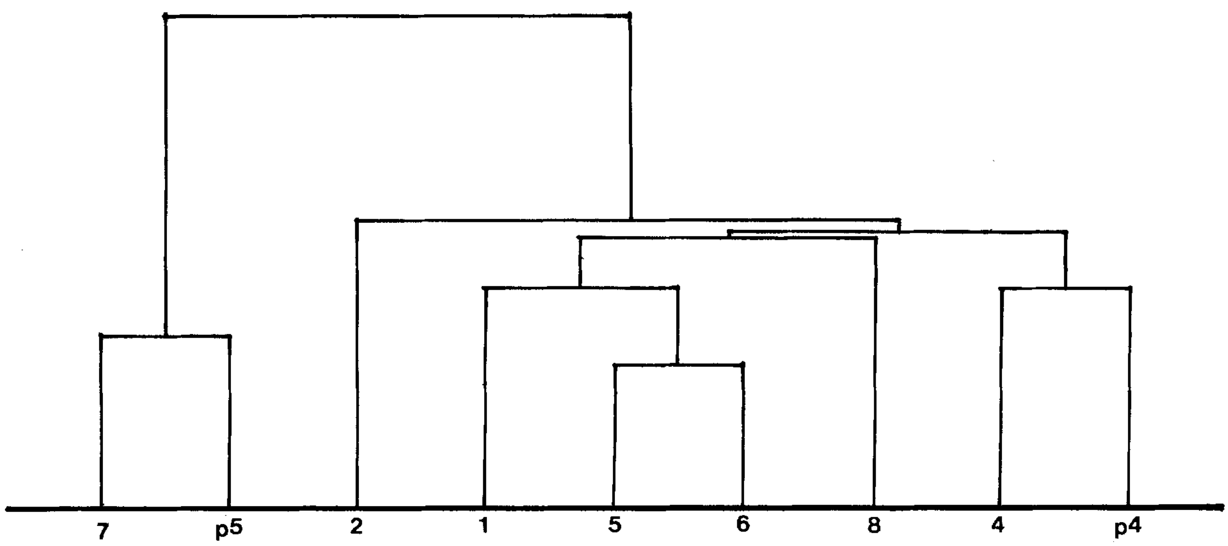

Fig. 2. Dendrogram for the cumulative effects of different humic fractions at 0, 40, 100, and 200 ppm on tomato seeds

the fresh weight values, fell instead into class three together with other high molecular-size fractions. The dendrogram for the action of humic fractions on tomato seeds did not show the same clear relationship between molecular size and effects (Fig. 2). However, fractions 7 and p 5, which gave higher fresh and dry weights of tomato seedlings, fitted into one class that was distinct from the rest of the fractions.

Conclusions

This study on the effect of humic substances from oxidized coal on seed germination indicated that neither unfractionated humic acid nor humic fractions applied at different rates improved the germination percentage and the mean germination rate of lettuce and tomato seeds compared with control values. However, these coal-derived humic substances had a positive effect on the fresh weight of total seedlings and the fresh weight per seedling. This effect was evident for unfractionated humic acid at increasing rates of addition up to 5000 ppm. Thus, the growth inhibition reported by Vaughan and Malcolm (1985) with high humate concentrations was not observed here, in agreement with recent work by Krogmeier and Bremner (1989).

The increase in total fresh weight and in fresh weight per seedling (Table 4) at increasing rates of humic fraction concentrations was particularly evident for lettuce (Table 3) treated with fractions of low molecular weight and a high content of acidic groups. Vaughan (1974) attributed the cell elongation promoted by humic substances to the formation of strong Fe complexes with humic acids, resulting in a reduction in wall-bound hydroxyproline. The findings of the present study may support this explanation since fractions of low molecular weight and high in acidic groups are strong complexing agents (Stevenson and Ardakani 1972). Although the results for tomato seedlings were less straightforward, the largest increase in total fresh weight and fresh weight per seedling was obtained with fractions of relatively low molecular weight in which a sufficiently high content of acidic groups had been preserved.

The dry weight of total lettuce seedlings and the dry weight per seedling showed no significant differences from control whether the seeds were treated with unfractionated humic acid or with the various humic fractions at different rates. Conversely, the treatment of tomato seeds generally increased the dry weights. These results suggest that cell elongation was the only effect on lettuce seeds whereas an uptake of humic material must have also occurred in the case of tomato seeds.

References

Azam F, Malik KA (1982) Effect of humic acid on seedling growth of wheat (*Triticum aestivum L.*) under different conditions. Pak J Bot 14:47–48

Calemma V, Rausa R (1987) EP Patent no. 0272737 A2

Dixit VK, Kishore N (1967) Effect of humic acid and fulvic acid fraction of soil organic matter on seed germination. Indian J Sci 1:202–206

Krogmeier MJ, Bremner JM (1989) Effects of phenolic acids on seed germination and seedling growth in soil. Biol Fertil Soils 8:116–122

Malik KA, Azam F (1985) Effect of humic acid on wheat (*Triticum aestivum L.*) seedling growth. Environ Exp Bot 25:245–252

Pagel M (1960) Über den Einfluss von Humusstoffen auf das Pflanzenwachsen. I. Einfluss von Humusstoffen auf Keimung und Wurzelwachstum. Albrecht Thaer Arch 4:450–468

Piccolo A (1984) Caratterizzazione chimica e chimico-fisica di un acido umico da torba. Uno studio di purificazione. Ann Ist Stud Dif Suolo 25:121–131

Piccolo A, Nardi S, Concheri G (1992a) Structural characteristics of humic substances as related to nitrate uptake and growth regulation in plant systems. Soil Biol Biochem 24:373–380

Piccolo A, Rausa R, Celano G (1992b) Characteristics of molecular size fractions of humic substances derived from oxidized coal. Chemosphere 24:1381–1387

Rausa R, Calemma V, Girardi E (1989) Humic acids by dry oxidation of coal with air under pressure. Analytical and spectroscopical characteristics. In: Proc Int Conf on coal science, vol 1 Tokyo, pp 237–240

Rauthan BS, Schnitzer M (1981) Effects of a soil fulvic acid on the growth and nutrient content of cucumber (*Cucumis sativus*) plants. Plant and Soil 63:491–495

Schnitzer M (1978) Humic substances: Chemistry and reactions. In: Khan SU, Schnitzer M (eds) Soil organic matter. Elsevier, Amsterdam, pp 1–57

Sneath PHA (1957) The application of computers to taxonomy. J Gen Microbiol 17:201–226

Smidova M (1962) Über den Einfluss von Na-Humat auf die Oxydations-Reduktionsprozesse in den Wurzeln von Winterweizenpflanzen. In: Studies about humus (Symp). Czechoslovak Akademy of Sciences, Prague, pp 291–304

Stevenson FJ, Ardakani MS (1972) Organic matter reactions involving micronutrients in soils. In: Mortvedt JJ, Giordano PM, Lindsay WL (eds) Micronutrients in agriculture. Am Soc Agron, Madison, Wisconsin, pp 79–114

van de Venter HA, Mari Furter, Dekker J, Cronje IJ (1991) Stimulation of seedling root growth by coal-derived sodium humate. Plant and Soil 138:17–21

Varshney TN, Gaur AC (1974) Effect of spraying sodium humate and hydroquinone on *Glycine max L.* var Bragg and *Solanum Lycopersicum* var Heiz 1370. Curr Sci 43:95–96

Vaughan D (1974) A possible mechanism for humic acid action on cell elongation in root segments of *Pisum sativum* under aseptic conditions. Soil Biol Biochem 6:241–247

Vaughan D, Linehan DJ (1976) The growth of wheat plants in humic acid solutions under axenic conditions. Plant and Soil 44:445–449

Vaughan D, Malcom RE (1985) Influence of humic substances on growth and physiological processes. In: Vaughan D, Malcom RE (eds) Soil organic matter and biological activity. Kluwer Academic Publisher, Amsterdam, The Netherlands, pp 37–46