Aquatic Humic Substances Inhibit Clastogenic Events in Germinating Seeds of Herbaceous Plants

Giuseppe Ferrara, Elisabetta Loffredo,* and Nicola Senesi

Dipartimento di Biologia e Chimica Agroforestale ed Ambientale, University of Bari, Via Amendola 165/A, 70126 Bari, Italy

One humic acid (HA) and two fulvic acids (FAs) of aquatic origin have been tested for their capacity to inhibit clastogenic events caused by maleic hydrazide (MH) in germinating seeds of the herbaceous plant species *Allium cepa* and *Vicia faba*. Either HA or FA at concentrations of 50 and 500 mg L^{-1} was interacted with 10 mg L^{-1} MH for 24 h before addition to the seeds. The evaluation of genotoxic activity was made by counting micronuclei (MN) and aberrant anatelophases (AT) in root tip cells after treatment with HA or FA alone, MH alone, and interacted HA + MH and FA + MH. Regular AT were also counted as an index of mitotic activity. In all cases HA and FA interacted with MH showed an evident anticlastogenic action indicated by the marked reduction of genetic anomalies. In *A. cepa*, the anticlastogenic effect of HA and FA was more significant for aberrant AT than for MN, whereas the opposite was true in the case of *V. faba*. The protective effect exhibited for both anomalies by HA was slightly higher than that of the corresponding FA in *A. cepa*, whereas no significant differences between these HA and FA treatments were observed in the case of *V. faba*. The two FAs generally showed similar anticlastogenic behaviors with slight quantitative differences observed as a function of the type of anomaly and the plant species. The effects of HA and FA concentration differed depending on the type of anomaly observed, the plant species, and FA origin. In *V. faba*, cell division, that is, the number of regular AT, was generally depressed by HA and FA at either concentration with respect to the control. In *A. cepa*, HA and FA produced either stimulating or inhibiting effects on regular AT depending on their nature, origin, and concentration.

Keywords: *Anticlastogenic activity; micronuclei; aberrant anatelophases; maleic hydrazide; humic acid; fulvic acids; Allium cepa; Vicia faba*

INTRODUCTION

Humic substances (HS) are heterogeneous natural compounds ubiquitous in all terrestrial and aquatic environments. Aquatic HS, including fulvic acids (FAs) and humic acids (HAs), represent 40−60% of dissolved organic carbon and are the largest fraction of natural organic matter in water (*1*). Aquatic HAs and FAs are colloidal, polydispersed, polyelectrolyte organic compounds of mixed aliphatic and aromatic nature originated from soil humus and terrestrial and aquatic plants (*2*, *3*). They have variable composition, structure, and properties depending on the soil types from which waters run off and drain, aquatic biota, local climatic conditions, and anthropogenic inputs (*4*). In general, HAs and FAs of various origins and natures are known to influence markedly several biochemical, physiological, and genetic processes in plants (*5*, *6*).

Several natural and xenobiotic organic compounds, generally defined as environmental mutagens, possess the capacity of altering some genetic processes, such as mitotic division, which occur in meristematic plant cells (*7*). In particular, some mutagens can behave as clastogens for their ability to produce breakage of chromosomes. Clastogenic agents can act in two different ways: as DNA synthesis (S)-independent agents, such as X-rays, which are able to produce aberrations independently from DNA replication; and as S-dependent agents, such as many chemical agents, which can produce aberrations during DNA replication (*8*).

Anticlastogenic activity consists of the suppression of clastogenesis processes and can occur by different mechanisms either inside or outside the cell. A variety of genetic assays have been used to evaluate qualitatively and quantitatively the clastogenic activity of a compound. These include the micronucleus test and the aberrant anatelophase assay in various living organisms (*9*, *10*) and in particular in plants (*11*−*13*).

Although a mutagenic behavior has been found for aquatic HAs and FAs in the chlorination and ozonation processes of drinking waters (*14*−*16*), very few data are available on the antimutagenic action of HS and other natural organic compounds in microorganisms and animal and plant cells. An HA isolated from wood leaf mold was shown to be able to inhibit genetic aberrations in *Salmonella typhimurium* cultured in the presence of benzo[*a*]pyrene, 2-aminoanthracene, 2-nitrofluorene, and 1-nitropyrene (*17*, *18*). In experiments with *Arabidopsis thaliana* potassium humate was able to inhibit the formation of the mutagens *N*-nitrosopropoxur and *N*-methyl-*N*-nitrosourea produced as metabolites of some compounds used in agriculture (*6*, *19*). A reduced number of genetic anomalies were shown by seedlings of *Vicia faba* treated with various herbicides when grown in an organic soil with respect to a sandy soil (*13*, *20*, *21*). Cozzi et al. (*22*) reported that natural HAs can inhibit the mutagenicity action of maleic hydrazide

* Author to whom correspondence should be addressed (telephone +39/080/5442282; fax +39/080/5442813; e-mail loffredo@agr.uniba.it).

10.1021/jf0011438 CCC: $20.00
Published on Web 02/10/2001

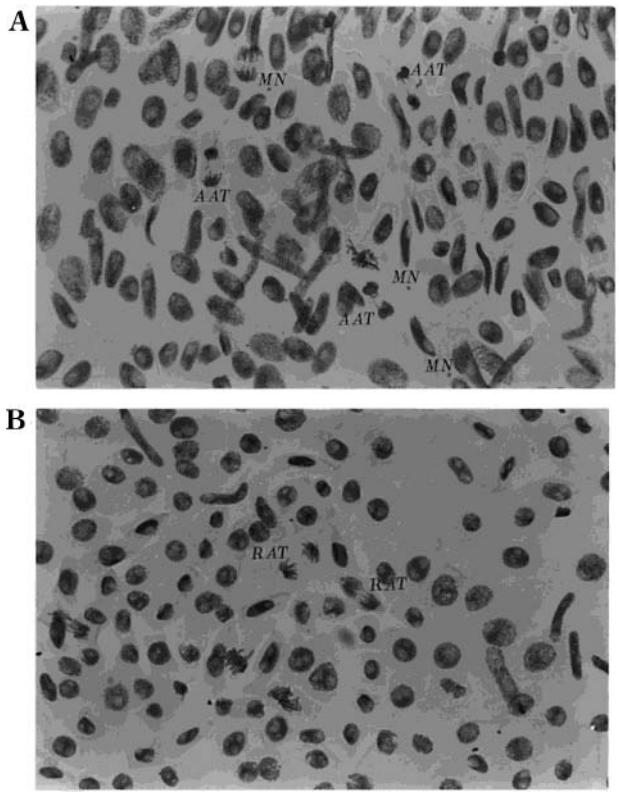

Figure 1. Presence of micronuclei (MN) and aberrant anatelophases (AAT) (A) and regular anatelophases (RAT) (B) in root tip cells of *A. cepa* treated with 10 mg L^{-1} maleic hydrazide. Magnification 400×. (Figure is reproduced here at 50% of its original size.)

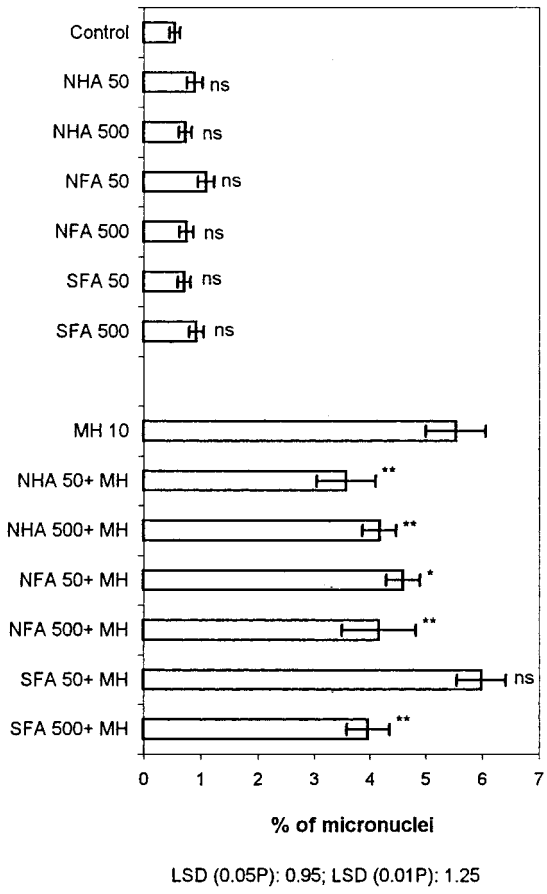

Figure 2. Frequency (percent) of micronuclei in *A. cepa* root tip cells. The frequency is calculated on 30000 cells for each treatment. The symbols **, *, and ns refer, respectively, to a difference significant at 0.01*P*, a difference significant at 0.05*P*, and nonsignificant difference, according to LSD test. The horizontal line on each bar indicates the standard error for three replicates.

(MH) and mitomycin C in Chinese hamster ovary cells. In a recent work (*23*), an apparent desmutagenic behavior was exhibited by HAs and FAs originated from soil and leonardite on germinating seeds of *V. faba* treated with MH.

The objective of this work was to evaluate the anticlastogenic activity of aquatic HAs and FAs in germinating seeds of two herbaceous plant species, *Allium cepa* and *Vicia faba*, which were treated with the ascertained clastogen MH. This compound behaves as a chromosome-breaking agent able to induce chromosomal aberrations preferentially in heterochromatic segments and as an inhibitor of mitotic activity in plant cells (*24*). In this work, genetic aberrations were estimated using the root micronucleus test and the aberrant anatelophase assay.

MATERIALS AND METHODS

Three aquatic HS samples were used in this work, Nordic HA (NHA), Nordic FA (NFA), and Suwannee River FA (SFA), which belong to the Standard and Reference Collection of HAs and FAs of the International Humic Substances Society (IHSS). The samples NHA and NFA originated from a freshwater lake in Norway, and the sample SFA was isolated from Suwannee River waters collected near Fargo, GA. All samples were extracted, fractionated, and purified according to the official methods proposed by Thurman and Malcolm (*2*) and adopted by IHSS. The compound MH was obtained from Sigma-Aldrich S.r.l., Milano, Italy. Seeds of *Allium cepa* L. cv. Southport white globe and *Vicia faba* L. var. *maior* cv. Aguadulce were germinated in Petri dishes in a Phytotron growth chamber at 21 ± 1 °C in the dark.

Twenty seeds of *A. cepa* and seven seeds of *V. faba* were allowed to germinate in Petri dishes in the presence of 8 and 12 mL, respectively, of the following test solutions: (a) distilled H_2O, used as positive control; (b) MH at a concentration of 10 mg L^{-1}, used as negative control; (c) NHA, NFA, or SFA at concentrations of 50 and 500 mg L^{-1}; and (d) mixtures of NHA, NFA, or SFA at 50 and 500 mg L^{-1} and MH at 10 mg L^{-1}, which were interacted by mechanical shaking for 24 h at room temperature (20 ± 2 °C) before addition to the seeds. The pH value of all solutions used ranged from 6 to 7. Each solution was applied to the seeds in two successive aliquots to avoid seed flooding and consequent ipooxygenated conditions. All experiments were triplicated.

Root tips were collected after 5 days of germination and prepared adequately for the observation at the microscope according to the following procedure: (a) fixing in Carnoy's solution I (ethyl alcohol and acetic acid, 3:1, v/v); (b) hydrolyzation with 1 M HCl at 60 °C for 10−12 min; (c) rinsing with distilled water; (d) staining overnight with Schiff reagent; (e) rinsing with distilled water; (f) placing 2-mm cut root tips on a slide, addition of a drop of 45% acetic acid, and squashing of root tips between the cover glass and the slide; (g) removing the cover glass after freezing in dry ice; (h) two successive immersions in two baths of 95% ethanol and then in two histolemon Erba baths; and (i) air-drying of slides and mounting in Canada balsam (Eukitt). Fifteen root tips (5 × 3 replicates) and 30000 cells (2000 cells per root tip) were prepared as described above for each treatment and observed at an Olympus CX40 microscope using a magnification of 400×.

The genotoxic effect was estimated by counting the micronuclei (MN) and the aberrant anatelophases (AT). The MN are portions of extranuclear DNA and consist of chromosome

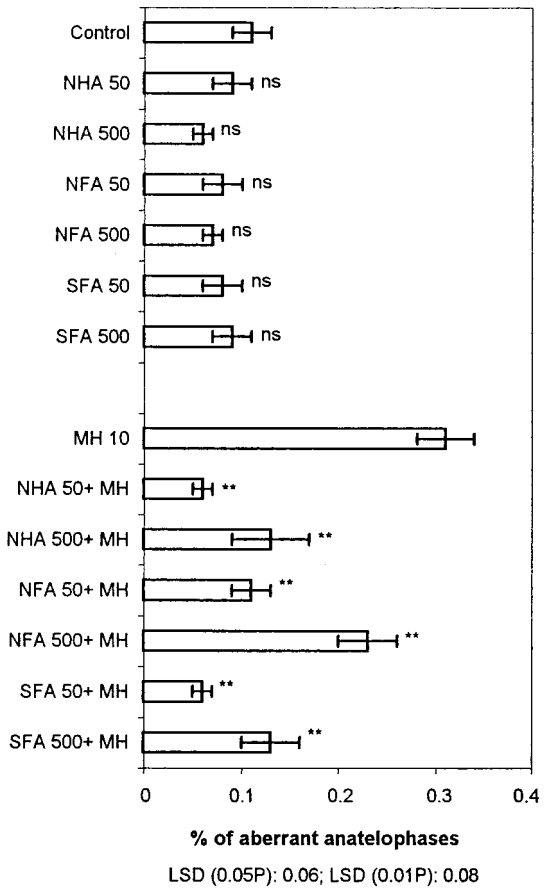

Figure 3. Frequency (percent) of aberrant anatelophases in *A. cepa* root tip cells. The frequency is calculated on 30000 cells for each treatment. The symbols ** and ns refer, respectively, to a difference significant at 0.01*P* and nonsignificant difference, according to LSD test. The horizontal line on each bar indicates the standard error for three replicates.

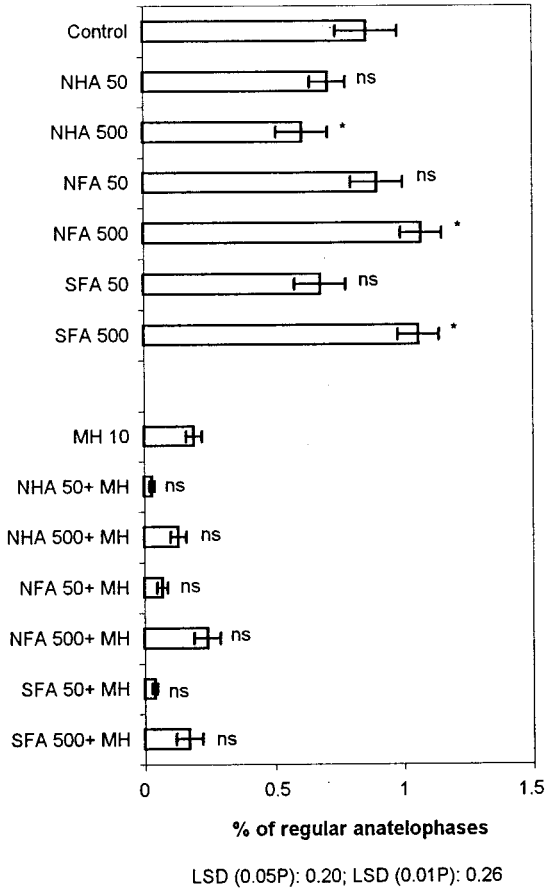

Figure 4. Frequency (percent) of regular anatelophases in *A. cepa* root tip cells. The frequency is calculated on 30000 cells for each treatment. The symbols * and ns refer, respectively, to a difference significant at 0.05*P* and nonsignificant difference, according to LSD test. The horizontal line on each bar indicates the standard error for three replicates.

fragments or complete chromosomes that did not segregate correctly. According to Countryman and Heddle (*25*), the following criteria were used to identify MN: (a) diameter no larger than one-third of the main nucleus; (b) nonrefractility; (c) same color of the nucleus or lighter; (d) location inside the cell cytoplasm but separated from the nucleus; and (e) no consideration of multinucleated cells. The aberrant AT are abnormal cell divisions showing chromosomal bridges and/or isolated DNA fragments and/or lagging and sticking chromosomes. To evaluate the rate of mitotic activity of root tip cells in each treatment, regular AT were also counted.

The frequencies of MN, aberrant AT, and regular AT, expressed as percentages, have been statistically analyzed by one-way analysis of variance (ANOVA) at both 95 and 99% confidence levels. The mean values were separated by using the least significant difference (LSD) test. Data obtained for NHA, NFA, and SFA treatments were statistically compared to the positive control (H_2O) values, whereas data of HS and MH combinations were compared to the negative control (MH).

RESULTS AND DISCUSSION

Two representative microphotographs are presented in panels A and B of Figure 1, respectively showing MN and aberrant AT and regular AT occurring in root tip cells of *A. cepa* treated with MH. Both anomalies are observed also in MH-treated *V. faba* cells, but with frequencies generally higher than in *A. cepa*. In agreement with previous results (*20*, *23*, *26*), cytogenetic anomalies are detected also in control (H_2O) experiments. The frequencies measured for MN and aberrant AT in controls are, respectively, 0.54 and 0.11% for germinating seeds of *A. cepa* and 1.84 and 0.12% for *V. faba*.

The percentages of MN, aberrant AT, and regular AT measured in root tip cells subjected to various treatments, together with results of statistical analysis of data, are presented in Figures 2−4 for *A. cepa* and in Figures 5−7 for *V. faba*. In agreement with results obtained using various humic compounds at concentrations ranging from 20 to 9000 mg L^{-1} (*23*, *26*, *27*), treatments with NHA, NFA, and SFA result in MN and aberrant AT frequencies not significantly different from the corresponding values of the control. These results indicate no clastogenic behavior of HS in both species tested. The values of both anomalies are, however, generally higher for *V. faba* than for *A. cepa*. These results agree well with previous findings showing that the genotoxic responses to MH and to aqueous extracts from various contaminated soils are generally more intense for *V. faba* than for *A. cepa* (*11*).

The presence of MH causes an evident clastogenic effect in both species, as expected and shown by the marked increase of both MN and aberrant AT frequencies. However, a marked reduction ($P \leq 0.01$) of both MN and aberrant AT frequencies, and thus an apparent anticlastogenic activity, is generally shown for both species in experiments with interacted MH and HS. In *A. cepa* the effect is more apparent on aberrant AT than on MN. In particular, aberrant AT frequency measured

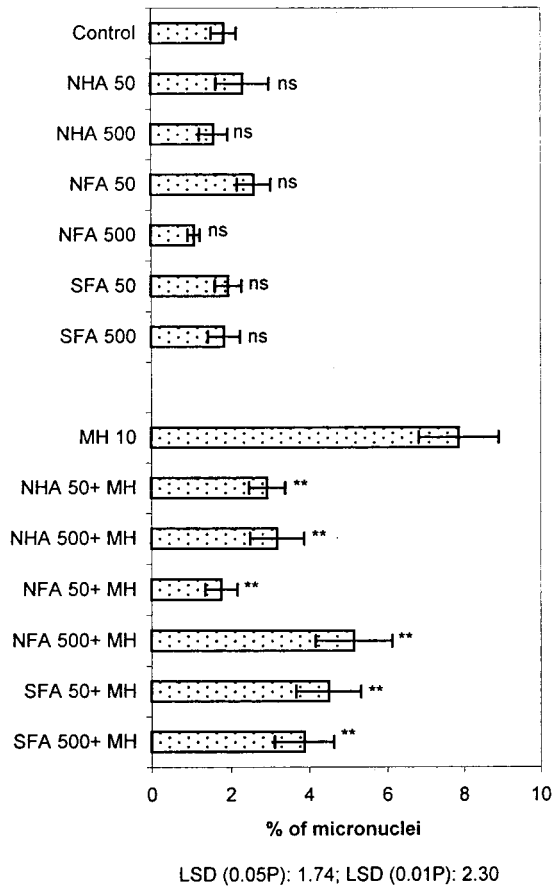

Figure 5. Frequency (percent) of micronuclei in *V. faba* root tip cells. The frequency is calculated on 30000 cells for each treatment. The symbols ** and ns refer, respectively, to a difference significant at 0.01*P* and nonsignificant difference, according to LSD test. The horizontal line on each bar indicates the standard error for three replicates.

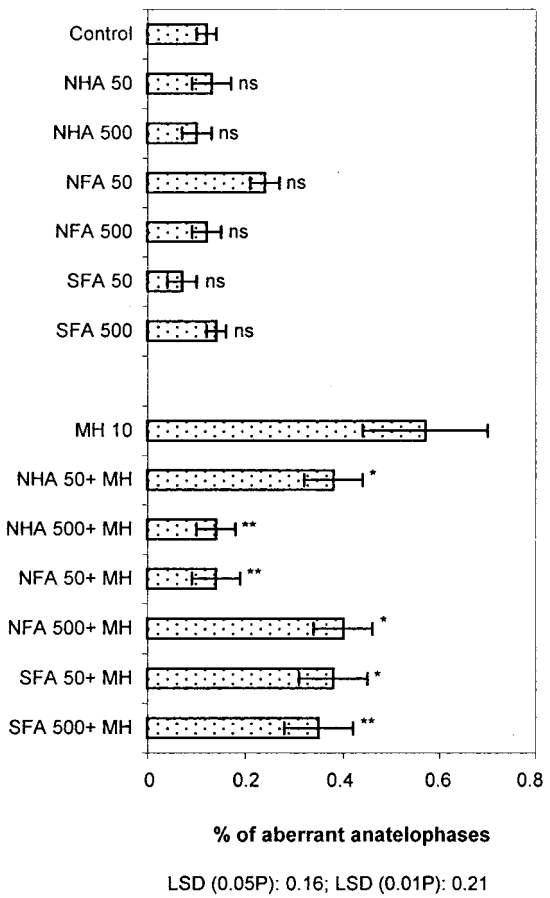

Figure 6. Frequency (percent) of aberrant anatelophases in *V. faba* root tip cells. The frequency is calculated on 30000 cells for each treatment. The symbols **, *, and ns refer, respectively, to a difference significant at 0.01*P*, a difference significant at 0.05*P*, and nonsignificant difference, according to LSD test. The horizontal line on each bar indicates the standard error for three replicates.

for MH interacted with NHA and SFA at 50 mg L^{-1} is 5 times lower than that for the MH treatment, whereas the reduction of MN frequency is significant ($P \leq 0.05$) only for combinations of MH and NFA at 50 mg L^{-1} and is not observed for MH + SFA at the same concentration. Differently, in germinating seeds of *V. faba* combinations of MH and HS show an anticlastogenic behavior that is more evident for MN than for aberrant AT. In this species, the reduction of MN frequencies is highly significant ($P \leq 0.01$) for all combinations, and the maximum effect is exhibited by the combination MH + NFA at 50 mg L^{-1}, for which the MN frequency decreases to 1.76% from the value of 7.86% observed for the MH treatment. However, the reduction of frequencies of aberrant AT in *V. faba* is significant at the 95% confidence level only for combinations of MH with NHA at 50 mg L^{-1}, with NFA at 500 mg L^{-1}, and with SFA at 50 mg L^{-1}.

Among the various combinations of MH with HS, the anticlastogenic action of NHA is slightly more intense than that of the corresponding NFA for both anomalies in the case of *A. cepa*, whereas in *V. faba* no relevant difference is observed for both anomalies. Although NFA and SFA in combination with MH show a generally similar anticlastogenic effect, NFA at low concentration appears to be more efficient in *V. faba*, whereas SFA is more efficient at both concentrations in *A. cepa* for aberrant AT.

In general, HS combined with MH at different concentrations affects differently the two plant species and the two anomalies. In *A. cepa*, the higher concentration of both FAs is more efficient for MN than for aberrant AT, whereas in *V. faba* SFA and NFA exhibit higher anticlastogenic actions at 500 and 50 mg L^{-1}, respectively.

As expected, a lower number of regular AT, that is, a mitodepressive effect, are observed in both species treated with MH. In *V. faba* cells, regular AT are generally reduced by any HS, although the effect appears to be significant ($P \leq 0.01$) when HS are used alone and not significant when HS are used in combination with MH. In the case of *A. cepa*, the presence of HS, either alone or in combination with MH, yields contrasting results for regular AT. In particular, the higher concentration produces a significant depression of mitotic activity in the case of NHA and a significant enhancement of cell divisions in the cases of NFA and SFA.

Results of this study confirm that aquatic HAs and FAs are capable of inhibiting clastogenic events induced by MH in plants; thus, these substances can be used in agriculture as protecting agents of plants by genotoxic environmental pollutants. Furthermore, the plants used in this work, *V. faba* and *A. cepa*, have been shown to be appropriate, simple, and efficient test species for the evaluation of anticlastogenic effects of aquatic HS.

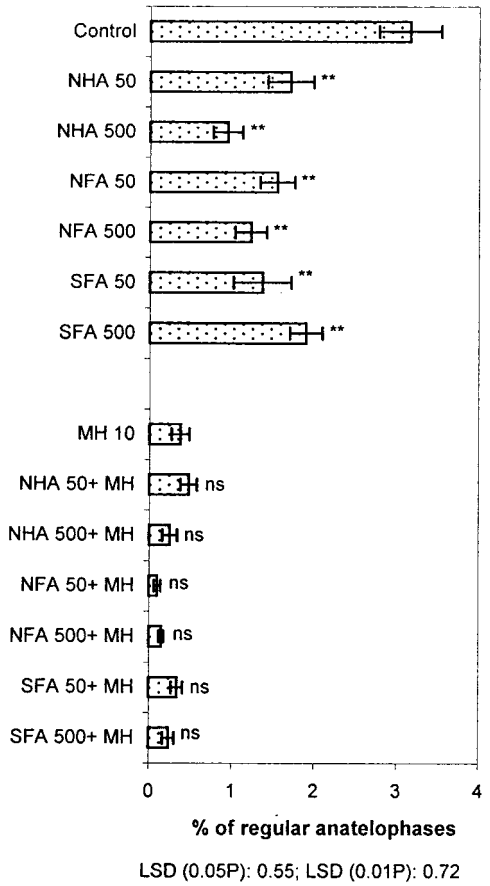

Figure 7. Frequency (percent) of regular anatelophases in *V. faba* root tip cells. The frequency is calculated on 30000 cells for each treatment. The symbols ** and ns refer, respectively, to a difference significant at 0.01*P* and nonsignificant difference, according to LSD test. The horizontal line on each bar indicates the standard error for three replicates.

Although the mode of action of HS as anticlastogenes is not yet clearly understood, it can be hypothesized that the MH mutagen molecules can be adsorbed and/or inactivated by interaction with some reactive groups of HAs and FAs, thus resulting in a decreased availability for root absorption.

ABBREVIATIONS USED

HS, humic substances; HA, humic acid; FA, fulvic acid; MH, maleic hydrazide; MN, micronuclei; AT, anatelophases; LSD, least significant difference; NHA, Nordic humic acid; NFA, Nordic fulvic acid; SFA, Suwannee River fulvic acid.

ACKNOWLEDGMENT

We thank Dr. Antonio De Marco and Dr. Claudio De Simone for useful suggestions in the evaluation of genetic aberrations.

LITERATURE CITED

(1) Thurman, E. M. Aquatic Humic Substances. In *Organic Geochemistry of Natural Waters*; Nihoff/Junk: Dordrecht, The Netherlands, 1986; pp 273−361.

(2) Thurman, E. M.; Malcolm, R. L. Preparative isolation of aquatic humic substances. *Environ. Sci. Technol.* **1981**, *15*, 463−466.

(3) Senesi, N.; Loffredo, E. The Chemistry of Soil Organic Matter. In *Soil Physical Chemistry*; Sparks, D. L., Ed.; CRC Press: Boca Raton, FL, 1999; pp 239−370.

(4) Watt, B. E.; Clark, N. W. E.; Hayes, M. H. B.; Chipman, J. K.; Skjemstad, J. O.; Swift, R. S. Aquatic humic substances from Pristine watersheds: chemistry and post-chlorination mutagenicity. In *Humic Substances and Organic Matter in Soil and Water Environments: Characterization, Transformations and Interactions*; Clapp, C. E., Hayes, M. H. B., Senesi, N., Griffith, S. M., Eds.; Proceedings of the 7th International Conference IHSS, St. Augustine, Trinidad and Tobago; International Humic Substances Society: St. Paul, MN, 1996; pp 389−390.

(5) Chen, Y.; Aviad, T. Effects of Humic Substances on Plant Growth. In *Humic Substances in Soil and Crop Sciences: Selected Readings*; MacCarthy, P., Clapp, C. E., Malcolm, R. L., Bloom, P. R., Eds.; American Society of Agronomy: Madison, WI, 1990; pp 161−186.

(6) Gichner, T.; Badaev, F.; Pospisil, F.; Veleminsky, J. Effects of humic acids, para-aminobenzoic acid and ascorbic acid on the N-nitrosation of the carbamate insecticide propoxur and on the mutagenicity of nitrosopropoxur. *Mutat. Res.* **1990**, *229*, 37−41.

(7) Grant, W. F. The present status of higher plant bioassays for the detection of environmental mutagens. *Mutat. Res.* **1994**, *310*, 175−185.

(8) Palitti, F. Mechanisms of the origin of chromosomal aberrations. *Mutat. Res.* **1998**, *404*, 133−137.

(9) Békaert, C.; Rast, C.; Ferrier, V.; Bispo, A.; Jourdain, M. J.; Vasseur, P. Use of in vitro (Ames and Mutatox tests) and in vivo (Amphibian Micronucleus test) assays to assess the genotoxicity of leachates from a contaminated soil. *Org. Geochem.* **1999**, *30*, 953−962.

(10) Vijayalaxmi, K. K.; Venu, R. In vivo anticlastogenic effects of L-ascorbic acid in mice. *Mutat. Res.* **1999**, *438*, 47−51.

(11) Cotelle, S.; Masfaraud, J. F.; Férard, J. F. Assessment of the genotoxicity of contaminated soil with the *Allium*/*Vicia*-micronucleus and the *Tradescantia*-micronucleus assays. *Mutat. Res.* **1999**, *426*, 167−171.

(12) Miao, M.; Fu, R.; Yang, D.; Zheng, L. *Vicia* root micronucleus assay on the clastogenicity of water samples from the Xiaoqing River in Shandong province of the People's Republic of China. *Mutat. Res.* **1999**, *143*, 143−145.

(13) De Marco, A.; De Simone, C.; Raglione, M.; Lorenzoni, P. Influence of soil characteristics on the clastogenic activity of maleic hydrazide in root tips of *Vicia faba*. *Mutat. Res.* **1995**, *344*, 5−12.

(14) Meier, J. R.; Lingg, R. D.; Bull, R. J. Formation of mutagens following chlorination of humic acids: a model for mutagen formation during drinking water treatment. *Mutat. Res.* **1983**, *118*, 25−41.

(15) Watt, B. E.; Malcolm, R. L.; Hayes, M. H. B.; Clark, N. W. E.; Chipman, J. K. Chemistry and potential mutagenicity of humic substances in waters from different watersheds in Britain and Ireland. *Water Res.* **1996**, *30* (6), 1502−1516.

(16) Matsuda, H.; Ose, Y.; Nagase, H.; Sato, T.; Kito, H.; Sumida, K. Mutagenicity of the components of ozonated humic substance. *Sci. Total Environ.* **1991**, *103*, 129−140.

(17) Sato, T.; Ose, Y.; Nagase, H. Desmutagenic effect of humic acid. *Mutat. Res.* **1986**, *162*, 173−178.

(18) Sato, T.; Ose, Y.; Nagase, H.; Hayase, K. Mechanism of the desmutagenic effect of humic acid. *Mutat. Res.* **1987**, *176*, 199−204.

(19) Gichner, T.; Badaev, F.; Pospisil, F.; Veleminsky, J. The effect of humic acids, fractionated according to molecular weight, on the formation and mutagenicity of *N*-methyl-*N*-nitrosourea. *Biol. Plant.* **1989**, *31*, 392−399.

(20) De Marco, A.; De Simone, C.; Raglione, M.; Testa, A.; Trinca, S. Importance of the type of soil for the induction of micronuclei and the growth of primary roots of *Vicia*

faba treated with herbicides atrazine, glyphosate and maleic hydrazide. *Mutat. Res.* **1992**, *279*, 9−13.
(21) De Simone, C.; Piccolo, A.; De Marco, A. Genotoxic effect induced by herbicides atrazine and glyphosate in plants of *Vicia faba* grown in different soils. *Sci. Total Environ.* **1992**, *123/124*, 233−240.
(22) Cozzi, R.; Nicolai, M.; Perticone, P.; De Salvia, R.; Spuntarelli, F. Desmutagenic activity of natural humic acids: inhibition of mitomycin C and maleic hydrazide mutagenicity. *Mutat. Res.* **1993**, *299*, 37−44.
(23) Ferrara, G.; Loffredo, E.; Simeone, R.; Senesi, N. Evaluation of antimutagenic and desmutagenic effects of humic and fulvic acids on root tips of *Vicia faba*. *Environ. Toxicol.* **2000**, *15*, 513−517.
(24) Osiecka, R.; Janas, K. M. Mitodepressive and clastogenic effects of some aminophosphonates, inhibitors of phenylalanine ammonia-lyase. I. 2-Aminoindan-2-phosphonic acid. *Plant Physiol. Biochem.* **1998**, *36* (11), 805−808.
(25) Countryman, P. I.; Heddle, J. A. The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutat. Res.* **1976**, *41*, 321−332.
(26) De Marco, A.; De Simone, C.; D'Ambrosio, C.; Owczarek, M. Buthionine sulfoximine prevents the reduction of the genotoxic activity of maleic hydrazide by soil humic substances in *Vicia faba* seedlings. *Mutat. Res.* **1999**, *438*, 89−95.
(27) De Simone, C.; Piccolo, A.; De Marco, A.; D'Ambrosio, C. Antimutagenic activity of humic acids of different origin. In *The Role of Humic Substances in the Ecosystems and in Environmental Protection*; Drozd, J., Gonet, S. S., Senesi, N., Weber, J., Eds.; Proceedings of the 8th International Conference IHSS; Polish Society of Humic Substances: Wroclaw, Poland, 1996; p 276.

Received for review September 15, 2000. Revised manuscript received December 29, 2000. Accepted December 29, 2000. Research supported by the National Research Council (CNR) of Italy, Bilateral Project Grant 97.193.06. G.F. expresses gratitude to the European Commission for partial financial support of his Ph.D. work, partly described in this paper.

JF0011438