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Chemical changes in humic substances from compost due to incubation with ligno-cellulolytic microorganisms and effects on lettuce growth

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Abstract Differences in the chemical properties of the organic matter from a highly lignocellulosic compost after incubation with two ligno- and cellulolytic microorganisms were studied in this work. Inoculation with either of the two microorganisms assayed, *Trichoderma viride* or *Bacillus* sp., of soil-compost mixtures enhanced degradation processes and the degree of organic matter humification. According to the humification index, inoculation with *T. viride* produced the highest humification rate in all the compost-soil proportions studied (10, 20 and 30%). To evaluate the quality of the extracted humic substances according to their electrofocusing behaviours a new index was established. This index showed an increased yield of humic substances of the lowest electrophoretic mobility (highest molecular weight) in treatments inoculated with *Bacillus* sp., whereas inoculation with *T. viride* enhanced the formation of molecules of the fastest electrophoretic mobility. These results, together with the fibre analysis performed, showed that the nature of the humic substances produced after incubation appeared to depend greatly on the degradation pathway carried out by the inoculated microorganism, *T. viride* or *Bacillus* sp.. Both degradation-humification pathways beneficially affected lettuce growth, demonstrating that inoculation with any of these two microorganisms may be a useful tool to modify agronomic properties of unripe composts.

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Introduction

Reincorporation of organic matter into the soil improves soil fertility, enhances microbial growth, and buffers the soil environment from sudden changes. Fresh organic matter should not be added directly, however, because the initial degradation processes produce considerable microbial growth, the possible formation of allelopathic products and exothermal reactions which can damage plant roots (Zucconi and De Bertoldi 1986). Microorganisms may compete with plants for soil nutrients under such conditions, thereby inducing nutrient starvation effects on plants.

There are many types of agroindustrial organic refuse which can be transformed and applied to soil as crop amendments, such as compost, thus reducing the need for chemical fertilizer inputs. During the composting process, the organic substrate present in the refuse is mainly transformed oxidatively and converted into a stabilized organic matter (Chen et al. 1994). Humification is the transformation process of the organic matter, which becomes progressively enriched in phenolic groups, or complexed into a phenolic matrix (De Nobili et al. 1989). Refuse with a high lignocellulosic content, however, does not decompose easily, and contributes mainly to the formation of more stable soil organic matter fractions, different from those formed from more available compounds such as polysaccharides and simple nitrogen-containing molecules (Haider 1994). Thus, Almendros et al. (1983) described how structures mainly derived from lignins and celluloses existing in composts have a high molecular weight and an average size greater than those existing in soil. The slow transformation of the lignocellulosic compounds results in products which, in many cases, can be

phytotoxic even after composting (Gallardo-Lara and Nogales 1987). According to Lynch (1986), inoculation of a compost with lignocellulolytic microorganisms is potentially a strategy for improving the product for agronomic purposes. Thus, microbial inoculation of a semi-stabilized compost could allow the inoculated microorganisms to dominate the endogenous microbiota and successfully develop an appropriate degradation.

Our aim was to improve the agronomic effectiveness of an unripe compost with two microorganisms of known ligno- or cellulolytic activity: *Trichoderma viride* and *Bacillus* sp.. We designed, for that purpose, an incubation process to be carried out after inoculation of three soil-compost proportions with any of these two microorganisms. At the end of the incubation process, several chemical parameters were analysed in all the treatment groups, i.e. control (with no extra-microbiota added) and inoculated ones. Effects on plant growth of *Lactuca sativa* were determined as a definitive assessment of the agronomic effectiveness of the inoculation-incubation process.

Materials and methods

Experimental design and general procedure

A loamy soil, from the province of Granada, Spain, was sieved (2 mm), mixed with medium coarse quartz sand (1:1, v:v), and steam-sterilized in 5-kg cloth bags (100°C for 1 h on 3 consecutive days). Soil characteristics were: pH 7.8 (in water extract); 2.07% organic matter; 1 mg N g^{-1}; 1.8 μg NH_4^+ g^{-1}; 4.6 μg NO_3^- g^{-1}; 0.03 mg P g^{-1} (Olsen and Dean 1965); 0.3 mg K g^{-1}. The compost used was a commercially available compost, named HUMUS FFONG produced in Nigüelas, province of Granada. This compost, with a high lignocellulosic content, was produced from undergrowth material mainly formed by shrubs which are removed to avoid forest fires. The composting process is described by the factory as an extremely short process (4 months) using aerobic conditions initially and anaerobic ones at the end of the process. Temperature conditions during the composting process were unknown, but presumably high enough during the anaerobic stage to kill most of the microbial components in the mixture. The degree of ripeness was assessed according to the cress seed germination test (Baca et al. 1990) and the compost was found to have a low maturity level (data not shown). Compost chemical characteristics were: pH 6.87 (in water extract); electric conductivity: 1.04 μS/cm; initial moisture: 66%; lignin: 43.36%; cellulose 24.11% hemicellulose 9.65% (lignocellulosic analyses according to Goering and Van Soest 1970); 11.5 mg N g^{-1}; 1 mg P g^{-1} (Olsen and Dean 1965); 1.9 mg K g^{-1}; 23.2 mg Ca g^{-1}; 3 mg Mg g^{-1}. Compost was sieved (6 mm) and homogenized in an automatic blender before being mixed with the sterile soil in three different ratios: 10%, 20% and 30% (v:v) of compost. The different soil-compost mixtures were kept (≈ 1.5 kg of each) in propylene plastic bags.

Microbial cultures

T. viride strain 2423 was purchased from the Colección Española de Cultivos Tipo, University of Valencia, Spain. *Bacillus* sp. was isolated from soil and screened for lignolytic activity according to Rajan and Srinivasan (1992).

Cultures of *T. viride* or *Bacillus* sp., were grown on a rotary shaker at 28°C for 1 week and 2 days respectively in 250-ml flasks containing 75 ml of Czapek Dox Broth modified medium (DIFCO). These were then centrifuged (3000 *g*) for 15 min, and the cultures resuspended in sterile tapwater (1:1). This suspension (10 ml) was used as inoculum for each bag of soil-compost. The control treatment comprised 10 ml of sterile tap water. Final moisture content was adjusted to 75% of the water-holding capacity of each soil-compost mixture. The inoculated soil-compost mixtures and the controls were kept for 45 days in the dark at 28°C.

Identification of microorganisms present in the compost was carried out before compost inoculation and in the different treatment groups at the end of the incubation process. Aliquots (5 g) were suspended in 100 ml of saline solution (NaCl, 0.85%) and shaken overnight at 28°C. Serial dilutions were sown in Malt Extract agar or King's B medium to assess fungal or bacterial populations in the compost respectively. Malt extract agar was prepared with 20 $g l^{-1}$ of malt extract; 20 $g l^{-1}$ of D-glucose; 1 $g l^{-1}$ of peptone and 2% agar, supplied with 0.5 $g l^{-1}$ of streptomycin sulphate and 0.25 $g l^{-1}$ of chloramphenicol. King's B medium was prepared with 20 $g l^{-1}$ of proteose peptone (DIFCO); 1.5 $g l^{-1}$ of K_2HPO_4; 1.5 $g l^{-1}$ of $MgSO_4$; 10 $ml l^{-1}$ of glycerol; 1.5% agar, to which 100 $\mu g \cdot ml^{-1}$ of cycloheximide was added to avoid fungal growth.

Organic matter transformation: evaluation techniques

Humification index

The humification index (HI), calculated as the ratio of non-humified (non-phenolic) to humified (phenolic) organic carbon after extraction with alkaline sodium pyrophosphate, provides an estimate of the sample humification after treatment with the lignocellulolytic microorganisms. Organic matter extraction was carried out with 0.1 M $Na_4P_2O_7$ and 0.1 M NaOH solution under N_2 flux for 1 h with a sample:extractant ratio of 1:10 (Sequi et al. 1986; De Nobili and Petrussi 1988). After centrifugation at 25 000 *g* for 25 min, the supernatant was filtered through a 0.2-μm Millipore membrane filter. Humic acids were precipitated by acidifying the extract with 8N H_2SO_4, separated by centrifugation at 5000 *g* for 20 min and stored after freeze-drying. HI was calculated after separating the phenolic and non-phenolic fractions of the supernatant by using small polyvinylpyrrolidone columns containing about 8 cm^3. The resin was washed with 0.5 N NaOH and equilibrated before use with 0.01 N H_2SO_4. The non-retained (non-phenolic) fractions were eluted from the columns with 0.01 N H_2SO_4. The adsorbed fractions (phenolic) were eluted with 0.5 N NaOH and joined to the humic acid precipitates (humified fraction). HI is calculated as the ratio of the total organic carbon in the non-phenolic fraction to the total organic carbon in the humified fraction (De Nobili and Petrussi 1988; De Nobili et al. 1989; Ciavatta et al. 1990). The organic carbon concentration was measured by a wet oxidation method using a Mettler Memo Titrator DL 40 RC (De Nobili and Petrussi 1988).

Electrofocusing

Analytical electrofocusing (EF) was performed on humic acid extracts (shaken for 24 h in 0.5N NaOH under the same conditions as for the organic matter), which had been previously purified by passing them through 0.2-μm filter membranes and concentrated by precipitation-centrifugation of humic acids. Extracts were then desalted with Amberlite IR 120 H^+ (Serva) and neutralized to pH 7 with 0.1N NaOH. Electrophoretic carrier ampholytes (pH range 2.5–7) were purchased from Pharmacia Biotech (Sweden). EF analyses were carried out using a water-cooled 4°C Pharmacia Biotech Ultrophor electrophoresis cell (De Nobili et al. 1986). The intensity of bands on the gel slabs were read with a Pharmacia Biotech Ultroscan laser densitometer at 460 nm.

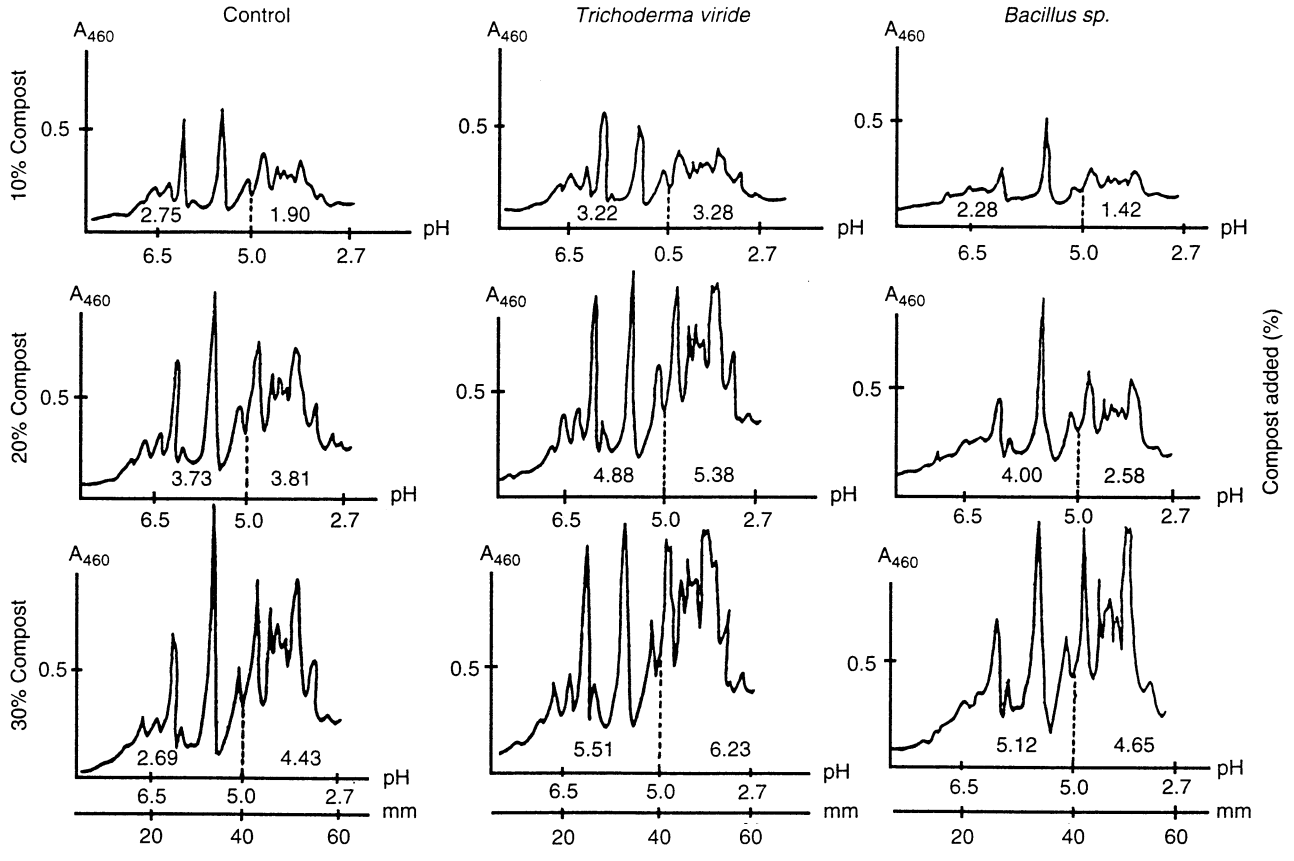

Fig. 1 Electrofocusing profiles of humic substances extracted with 0.5 N NaOH, in the different compost-soil mixtures after treatment with the ligno-cellulolytic microorganisms (A_{460} is absorbance at 460 nm). *Figures* under the *curve* indicate areas above and below pH 5.0 of the profile

Lignocellulosic content

The lignocellulosic content in the different treatments after the incubation process was calculated as described by Goering and Van Soest (1970).

Plant growth test

The effects of the different microbial treatments on growth of lettuce plants were determined at the end of the incubation process. Single plants (from seed) were grown in 100-ml pots, filled with the different soil-compost mixtures, for 60 days in a green-house under controlled conditions of light, humidity and temperature. The relative humidity was 70% to 90%, the temperature ranged from 10°C to 25°C and the photoperiod was 16:8 h light:dark. Pots were irrigated with tap water at 75% of the water-holding capacity of each soil-compost proportion. Shoots and roots were harvested and dried at 60°C for 24 h and the dry weight determined.

Statistical analysis

All chemical determinations were made in triplicate and the relative standard deviations of the data were less than 5%. Data relating to the lignocellulosic content were analysed using one way-analysis of variance (ANOVA) for a completely randomized design. Significant differences between treatments (means for three replicates) were differentiated using Duncan's multiple range test. Results from the plant growth test were expressed with the standard error of the treatment means (five replicates) for 95% confidence limits.

Results

Among the bacterial isolates found, most of the species belonged to the genera *Bacillus*, *Enterobacter*, *Pseudomonas* or to the actinomyces group. All the species were also found at the end of the incubation process. Fungal population of the compost was mainly formed by *Aspergillus niger*, *Cunninghamella* sp., *Mucor* sp., *Rhizopus nigricans* and *T. viride*. Treatments inoculated with *T. viride* had a larger proportion of this fungus at the end of the incubation process.

The EF analyses of the extracted humic acids of the compost mixtures after incubation showed a general trend in which the area under the curve of the EF profiles increased with an increase in the percentage of compost. It seems to indicate that the total amount of humic substances extracted after treatment with ligno- or cellulolytic microorganisms depends on the initial organic matter content (Fig. 1). Treatment with *T. viride* produced more humic substances in all the compost proportions studied, as compared to control treatment

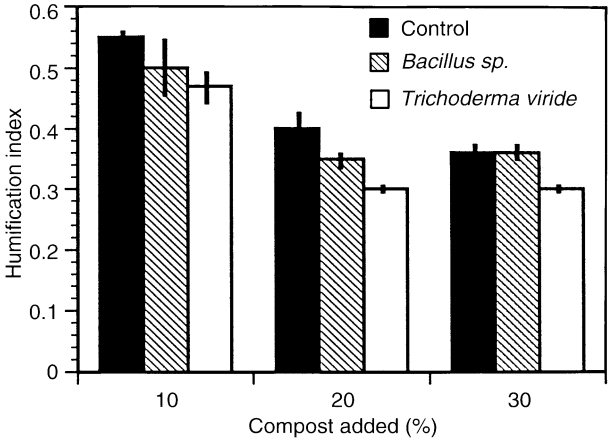

Fig. 2 Humification indices of the different soil-compost mixtures at the end of incubation with the ligno-cellulolytic microorganisms

or the treatment inoculated with *Bacillus* sp., which appeared to bring about a decrease in the amount of humic substances except for mixtures containing 30% of compost.

The HI values obtained in our experiment (Fig. 2) decreased with the amount of organic matter present as a result of a higher phenolic content, and showed that inoculation with *T. viride* produced the highest humification rate, correlating with the EF analyses. Inoculation with *Bacillus* sp. lowered the HI value at compost contents of 10% or 20%, but had no effect on the 30% compost. The increases in the humic substance content for substrates inoculated with *Bacillus* sp. were less than those produced by inoculation with *T. viride*.

EF patterns reflect qualitative changes during the stabilization of organic matter (De Nobili et al. 1986). A humic substance distribution on the pH gradient of the EF profiles in the range between pH 5.0 and 6.5 would be similar to that of humic substances present in soils. In order to compare the different patterns of EF obtained, we established a ratio *A*-to-*B*, since *A* and *B* were the areas under the curves of the different profiles corresponding to the zone above and below

Table 1 *A*-to-*B* ratios of electrofocusing profiles, calculated as the proportion of humic substances of highest molecular weight (placed in the zone above pH 5.0 of the profile) to those of lowest molecular weight (below pH 5.0)

	Compost (%)		
	10	20	30
Control	1.46	0.98	0.61
Trichoderma viride	0.98	0.91	0.88
Bacillus sp.	1.60	1.55	1.10

pH 5.0 respectively. This ratio would express the relative quantity of humic substances similar to those existing in soil with respect to other less humified substances. This would provide an index of the quality of the humic substances extracted. Results in Table 1 show that when the incubation of the soil-compost mixture was performed in the presence of inoculum of *Bacillus* sp., the *A*-to-*B* ratio was highest, whereas mixtures inoculated with *T. viride*, except for the 30% compost-soil mixture, had lower values than control treatments. The *A*-to-*B* ratio was inversely proportional to the amount of compost added. These data suggest that more compounds of high molecular weight, i.e., the lowest electrophoretic mobility fraction in the EF profiles as demonstrated by Maggioni and Cacco (1977) and by Ceccanti et al. (1986), were produced after inoculation with *Bacillus* sp. than those produced by the *T. viride*-treated and control groups. In contrast, in the latter treatment groups the proportion of molecules of lower molecular weight was comparatively larger than in the group treated with *Bacillus* sp..

Analysis of the lignocellulosic content of the samples after the incubation process showed that the hemicellulose had been completely degraded in all cases, including the control incubation group. Also observed were considerable reductions in the cellulose content, mainly in the samples inoculated with *T. viride*. The lignin content was significantly modified by inoculation with *Bacillus* sp.. Smaller amounts of compost showed higher lignin degradation rates (Fig. 3).

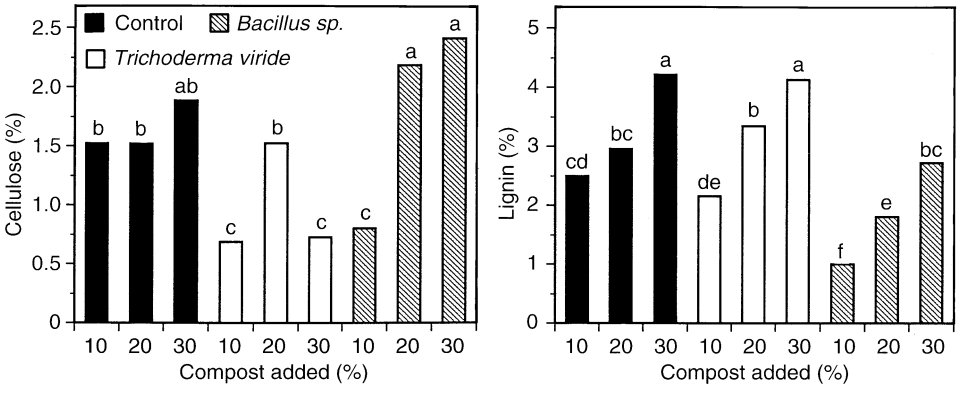

Fig. 3 Differences in the lignocellulosic content of the soil-compost mixtures at the end of incubation according to the microbial treatment inoculated. Cellulose content before incubation was ≈ 2.5, 5 and 7% for the 10, 20 and 30% soil-compost mixtures respectively. Lignin content before incubation was ≈ 4, 8.5 and 12% for the 10, 20 and 30% soil-compost mixtures respectively. *Columns* sharing any *letter* are not significantly different for $P < 0.05$ according to Duncan's test

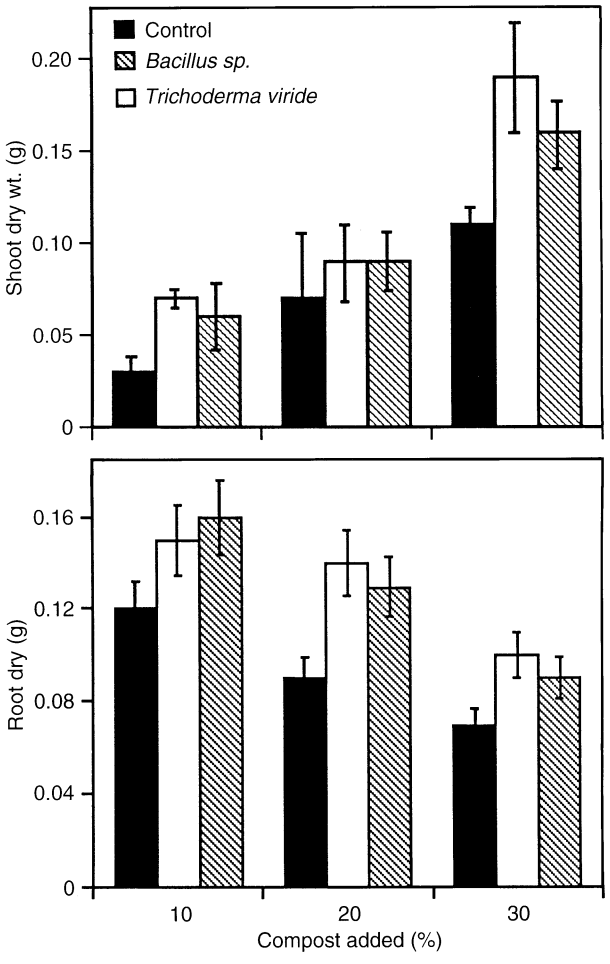

Fig. 4 Shoot and root dry weight of lettuce plants grown on the different soil-compost mixtures after incubation with the lignocellulolytic microorganisms. *Vertical bars* represent the standard error

With respect to the biological effects of the different incubation treatments, there was a consistent lettuce shoot development of plants grown on compost mixtures incubated with any of the microbial treatments as compared to the control incubation except for 20% compost mixtures (Fig. 4). Plants grown on soil-compost incubated with *T. viride* showed, however, no different shoot promotion than those grown on mixtures inoculated with *Bacillus* sp.. According to the root data observed, the percentage of compost correlated negatively the root growth (Fig. 4). Incubation of the compost mixtures with any of the two microorganisms assayed consistently enhanced root promotion of lettuce plants grown on such mixtures.

Discussion

Despite of the large volume of research done to ascertain the origin, structure and formation of the humic substances, this topic still remains a controversial aspect of the study of organic matter. Several theories have been proposed to explain the main pathways of formation of humic substances (Flaig 1988; Hatcher and Spiker 1988; Hedges 1988). But nevertheless, as Schnitzer (1978) stated, probably all of them are possible depending on the origin of the organic matter and the conditions under which the humification process takes place. Evolution of humic substances during the humification process can be shown by observing the changes in their focusing behaviours within a pre-established pH gradient (De Nobili 1988; De Nobili et al. 1990). Through the process, substances of low molecular weight with a high ratio of carboxylic groups are formed first and focus at lower pH values, while more complex molecules of a less-negative charge density are formed later and focus at higher pH values.

According to Haider (1994), lignocellulosic material in soil is mainly transformed into a stabilized humus pool which is barely removable. Other soil organic components, such as polysaccharides or proteins, however, are easily mineralized or transformed into a microbial biomass, becoming a more available carbon pool. This latter source of carbon may be used in addition by other soil microorganisms as a precursor for melanic, humic-like materials.

Lignin and cellulose contents of the different mixtures after incubation showed that, as expected, *T. viride* exhibited cellulolytic activity, whereas *Bacillus* sp. showed some lignolytic activity on the compost organic material. If, as stated by Haider (1994), lignin degradative microbes cannot use lignin as a source of carbon and energy (due to the complex nature of the biopolymer), *Bacillus* sp. activity during the incubation process was probably limited to small modifications of the lignin molecule. Demethoxylation processes as described for other microorganisms (Bowen 1990) and/or irregular splittings of bonds in the side chains or in the rings probably occurred (Haider 1994; Ramunni et al. 1994). The resulting products would have, then, similar characteristics to lignin (Stevenson 1982) and would, consequently, focus at a higher pH in the EF analysis due to their lower negative charge density. This should explain the *A*-to-*B* ratio results observed for mixtures inoculated with *Bacillus* sp.. As the modification and transformation pathways of lignin are very slow (Zucconi and De Bertoldi 1986), the total amount of humic-like substances produced by *Bacillus* sp. would be rather small, as shown by the HI.

A different humification pathway may have occurred in the compost mixtures inoculated with *T. viride*. It is known that polymerization and condensation of quinones and sugars with nitrogen compounds produce dark-coloured molecules described as humic substance precursors (Giovanozzi-Sermani 1986; Baca et al. 1992). The cellulose degradation performed by *T. viride*, resulting in small molecules such as dextrins and glucans, may have contributed to the formation of

humic-like substances by undergoing further polymerization and condensation reactions. In addition, *T. viride* would play an important role in the initial steps of microbial humification by producing an available carbon source for the rest of microorganisms involved in the lignocellulosic material degradation. The nature of this latter humification pathway also determines the quality of the humic substances formed, the mass-to-superficial charge ratio of which is always lower than those found in the compost-mixtures inoculated with *Bacillus* sp..

Concerning the effects on plant growth, different studies have shown a positive correlation between the amount of humic substances and promotion of plant growth. Effects are more noticeable on root development, but shoot promotion follows similar trends (Chen et al. 1994). That was not the case with our results. A higher amount of compost in the incubation mixture correlated with greater shoot growth promotion, probably through the provision of the plant with more nutrients. However, a very large amount of compost reduced root development, in spite of the degree of humification, which, as determined by HI, increased proportionately with the amount of compost. Because information about the ways in which humic materials affect plant growth is very scarce (Piccolo et al. 1992), it is difficult to interpret these results and give a detailed explanation about the mechanisms involved. We believe that the nutritive effect of compost is widely determined by its humification degree. However, the lack of significant differences in shoot or root growth promotion between the two microbial treatments assayed did not allow us to correlate qualitative differences of the humic substances with specific effects on plant growth as described by some other authors (Piccolo et al. 1992; Dell'Amico et al. 1994).

The results described here suggest that inoculation with selected microorganisms and further incubation of lignocellulosic wastes can be a useful tool to modify the chemical structure and the properties of their organic matter by changes in the humification and mineralization pathways. That may improve the agricultural value of the resulting product probably by making nutrients more available to plants. More studies are needed, however, to understand this complex process in detail.

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