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Evolution of humic substances from unripe compost during incubation with lignolytic or cellulolytic microorganisms and effects on the lettuce growth promotion mediated by *Azotobacter chroococcum*

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Abstract Some recalcitrant organic wastes, which contain a large proportion of lignin or cellulose, are not changed much by composting, and thus the effectiveness of the compost as fertilizer is usually low. In this study, incubation of unripe compost with ligno-cellulolytic microorganisms – *Trichoderma viride* or *Bacillus* spp. – was investigated to increase the degree of humification of the organic matter present, and improve its quality as a soil amendment. High-performance liquid chromatography (HPLC) analyses together with humification indices and electrofocusing patterns were used to monitor the evolution of the humic substances during the incubation process. Plant growth effects exerted by *Azotobacter chroococcum* on lettuce plants growing on the previously incubated compost were affected by the length of incubation and by changes in the composition of humic substances. Higher organic matter content and better humification seem to be important factors for predicting *A. chroococcum* hebaviour in the rhizosphere.

Key words Ligno-cellulolytic microorganisms · Humic substances · *Azotobacter chroococcum* · Compost · *Lactuca sativa* · *Trichoderma viride* · *Bacillus* spp. · Ligno-cellulolysis

Introduction

Management of soil-plant microbial interactions has been suggested as an appropriate strategy to improve the capture and cycling of nutrients, thus leading to a more rational use of land resources by reducing the use-abuse of chemical fertilizers (Barea and Jeffries 1995). Nitrogen-fixing free-living microorganisms have frequently been reported as plant growth promoters (Barea and Brown 1974; Döbereiner and Pedrosa 1987; Bashan and Levanony 1990; Abbass and Okon 1993). However, N_2 fixation rates by diazotrophic bacteria are known to be dependent on the specific plant-bacteria interaction. Thus, in tropical legumes, associative N_2-fixing bacteria contribute significant amounts of N to the plant while in temperate climates these contributions appear to be insignificant (Michiels et al. 1989). Plant growth promoting effects observed in the latter case should be attributed to the production of hormone-like substances by the bacteria rather than to N_2 fixation (see Martinez-Toledo et al. 1988; Nieto and Frankenberger Jr 1991). Whatever the mechanisms involved in plant promotion by diazotrophic bacteria, it is known that these bacteria require large amounts of available carbon for their survival in soil (Azcón 1993). In this respect, the addition of compost and other organic amendments to agricultural soils may be of special importance in restoring optimal levels or organic matter for plant growth and for microbial activity.

Composting can be defined as the process by which fresh organic matter is partially oxidized and the labile organic compounds transformed into a biologically stable material enriched in humic substances (Baca et al. 1992). Compost quality is sometimes diminished when the nature of the waste to be composed limits its transformation, for instance, waste with a high lignocellulosic content. Organic matter not completely stabilized, that is, that may undergo further important chemical changes, can result in phytotoxicity if it is added to soil. The degree of stabilization by the organic matter after waste composting is thus an important parameter for assessing the efficacy of compost as an organic amendment to the soil (Zucconi and De Bertoldi 1986). However, it is difficult to define the exact degree of stabilization that a compost should have at the end of the composting process (De Nobili et al. 1986). Under such circumstances, it is difficult to predict how diazotrophic bacteria might behave in competition with other

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microorganisms in the compost. In addition, organic matter breakdown may produce N immobilization by the microbiota involved and consequent starvation of plant growing on such substrates.

Lynch (1986) suggested that incubation with ligno-cellulolytic microorganisms of non-mature compost containing lignocellulosic waste may improve compost quality, making it more appropriate as a fertilizer. Kostov et al. (1991) have described an increase in the N_2-fixing activity of free-living bacteria from wood wastes inoculated with cellulolytic microorganisms, and a parallel increased development of tomato plants grown on those substrates. Requena et al. (1996) have found that such an incubation procedure increases organic matter humification from the compost and has a positive effect on plant growth. The aim of this work was to intensify the transformation process of non-mature compost organic matter by incubation with two lignolytic or cellulolytic microorganisms throughout the incubation period. We also looked at the possible modification of the promotion of lettuce growth by *Azotobacter chroococcum* in relation to changes in the humic properties of the compost.

Materials and methods

Preparation of the sand-compost mixtures

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, mainly 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 conditions at the end of the process. Temperatures during the composting process were unknown, but were presumably high enough during the anaerobic stage to kill most of the microbial components in the mixture. The degree of ripeness was assessed by 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); electrical conductivity 1.04 μS cm^{-1}; 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 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 sterile quartz sand (1–2 mm particle size) in three different proportions: 10%, 20% and 30% (v:v) of compost. The sand was washed in with tap water for 20 min and then sterilized by autoclaving at 121 °C for 30 min.

Microbial incubation

From each sand-compost mixture, 1.5 kg was taken and placed in 5-l propylene plastic bags (three bags for each compost proportion). The top of each bag was folded, and held together with a rubber band. Two microorganisms, *Bacillus* sp. and *Trichoderma viride*, with lignolytric and cellulolytic activity, respectively, on lignocellulosic wastes (Requena et al. 1996) were used in this experiment. *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 (Rajan and Srinivasan 1992). *T. viride* and *Bacillus* sp. were grown on a rotary shaker at 28 °C for 1 week or 2 days, respectively, in 250-ml flasks containing 75 ml Czapek Dox Broth Modified medium (DIFCO). The microbial culture was then centrifuged (3000 *g*) for 15 min, and the pellet resuspended in sterile tap water (1:1; v:v). Ten millilites of the suspension was used as inoculum for each bag of sand-compost. The control treatment comprised 10 ml sterile tap water. Final moisture content was ajusted to 75% of the water-holding capacity calculated for each sand-compost mixture. Incubation was carried out for up to 95 days in the dark at 28 °C. Aliquots of 100 g each mixture were periodically sampled.

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 a measure of the humification of the sample after treatment with ligno-cellulolytic 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 24 h with a sample:extractant ratio of 1:10 (Sequi et al. 1986; De Nobili and Petrussi 1988). After centrifugation at 25000 *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 8 *N* H_2SO_4, separated by centrifugation at 5000 *g* for 20 min and stored at 4 °C. The humification index 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 before use 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). The humification index 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 Nobi-

Table 1 Shoot dry weight (mg) of *Lactuca sativa* L. plants grown in the different compost mixtures for 60 days, and inoculated or not with *Azotobacter chroococcum*

		Length of compost incubation					
		35 days			95 days		
Amount of compost (%)	Bacterial treatment	Control	*Trichoderma viride*	*Bacillus* sp.	Control	*Trichoderma viride*	*Bacillus* sp.
10	None	28	56	68	106	118	74
	A. chroococcum	32	62	70	87	150	102
20	None	42	46	92	142	140	175
	A. chroococcum	48	92	102	147	177	107
30	None	50	68	85	76	66	67
	A. chroococcum	84	98	115	114	104	102

LSD 30 ($P<0.05$)

li and Petrussi 1988; De Nobili et al. 1989; Ciavatta et al. 1990a). The organic carbon concentration was measured by a wet oxidation method using a Mettler Memo Titrator DL 40 RC. This index was calculated during incubation at 0, 15, 35, 47 and 95 days.

HPLC of humic substances

Molecular weight distribution was calculated from size exclusion chromatography data. Analyses were performed on humic substances extracted with 0.5 *N* NaOH under N_2 flux for 1 h with a sample:extractant ratio of 1:10. The extract was centrifuged at 25000 *g* for 25 min and the supernatant Millipore-filtered (0.2 µm) and desalted with Amberlite IR 120 H^+ (Serva) to pH 7.5, and filtered again (0.2 µm). It was then analysed by high-performance liquid chromatography (HPLC) with a Waters 160 pump and a Waters UV-Vis detector set at 400 nm, on a Biosec-250 column (Biorad) using 0.025 *M* TRIS-phosphate, pH 7.5, as eluent (Tsutsuki and Kuwatsuka 1984). The column was calibrated using as standards a series of humic acid fractions in the range 100000–50000, 50000–30000, 30000–10000 and 10000–5000 Da, obtained by ultrafiltration on Amicon membranes (M. De Nobili, M.T. Baca, and N. Milani, unpublished data).

Electrofocusing of humic substances

Analytical electrofocusing was performed on the humic acid extracts of samples containing 20% of compost extracted with 0.5 *N* NaOH, as above, previously purified by passing through 0.2-µm Millipore membrane filters and concentrated by precipitation-centrifugation of humic acids. Extracts were then desalted with Amberlite IR 120 H^+ (Serva) and neutralized to pH 7 with 0.1 *N* NaOH. Electrophoretic carrier ampholites (pH range 2.5–7) were purchased from LKB (Sweden). Electrofocusing analyses were carried out using a water-cooled 4 °C LKB Ultrophor electrophoresis cell (De Nobili et al. 1986; De Nobili et al. 1990). The intensity of bands on the gel slabs was read with an LKB Ultroscan laser densitometer at 460 nm.

Plant growth and *Azotobacter* inoculation

The effects of the different compost incubation treatments on growth of lettuce plants were determined after 35 and 95 days of incubation with the ligno-cellulolytic microorganisms (Table 1). Single plants (from seed) were grown in 100-ml pots filled with the different sand-compost mixtures after the two incubation periods. Half of the pots were inoculated with 5 ml of a suspension of *A. chroococcum*, ca. 5×10^6 cfu g^{-1} sand-compost mixture. The bacteria were grown in Burk's medium (Wilson and Knight 1952), and then centrifuged (10000 *g* for 10 min) and resuspended in sterile tap water. The other half of the pots (control) received 5 ml sterile tap water. Plants were grown for 60 days in the greenhouse under controlled conditions of light, humidity and temperature. The relative humidity was 70–90%, 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 calculated for each sand-compost proportion. Leaves and shoots of lettuce plants were harvested and dried at 60 °C for 24 h and the dry weight was determined.

Statistical analysis

All chemical determinations were made in triplicate and the relative standard deviations of the data were less than 5%. Data of the plant growth test (five replicates) were differentiated using the least significant difference ($P<0.05$).

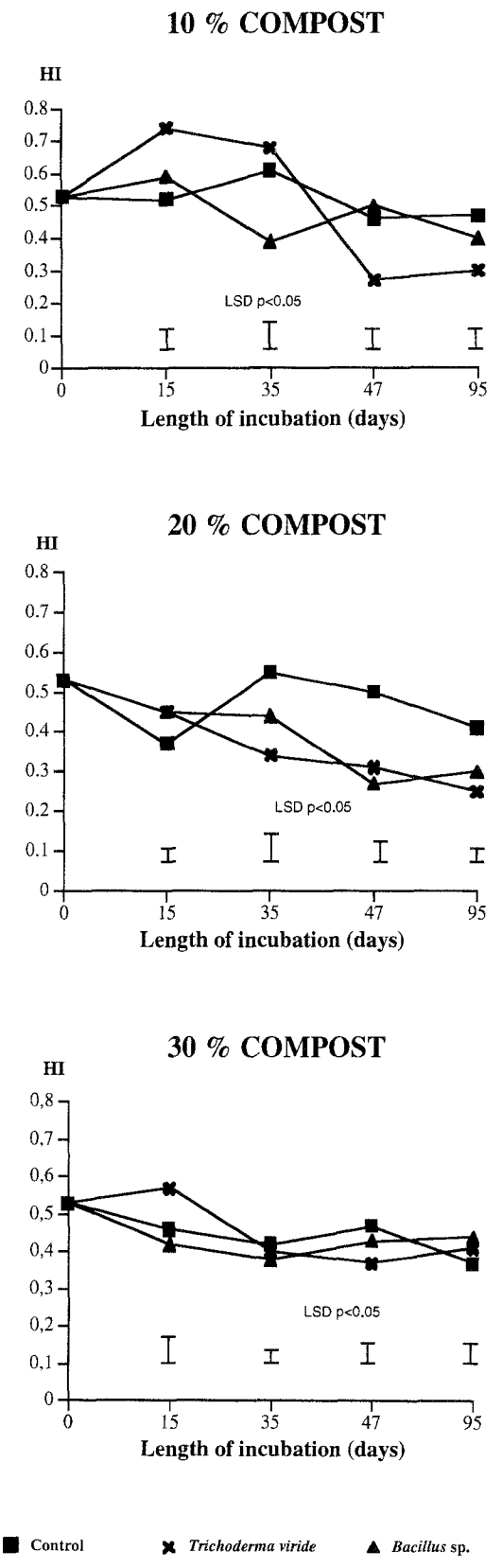

Fig. 1 Evolution of humification indices during the period of incubation, in the different compost proportions, according to the lignolytic or cellulolytic microorganism inoculated

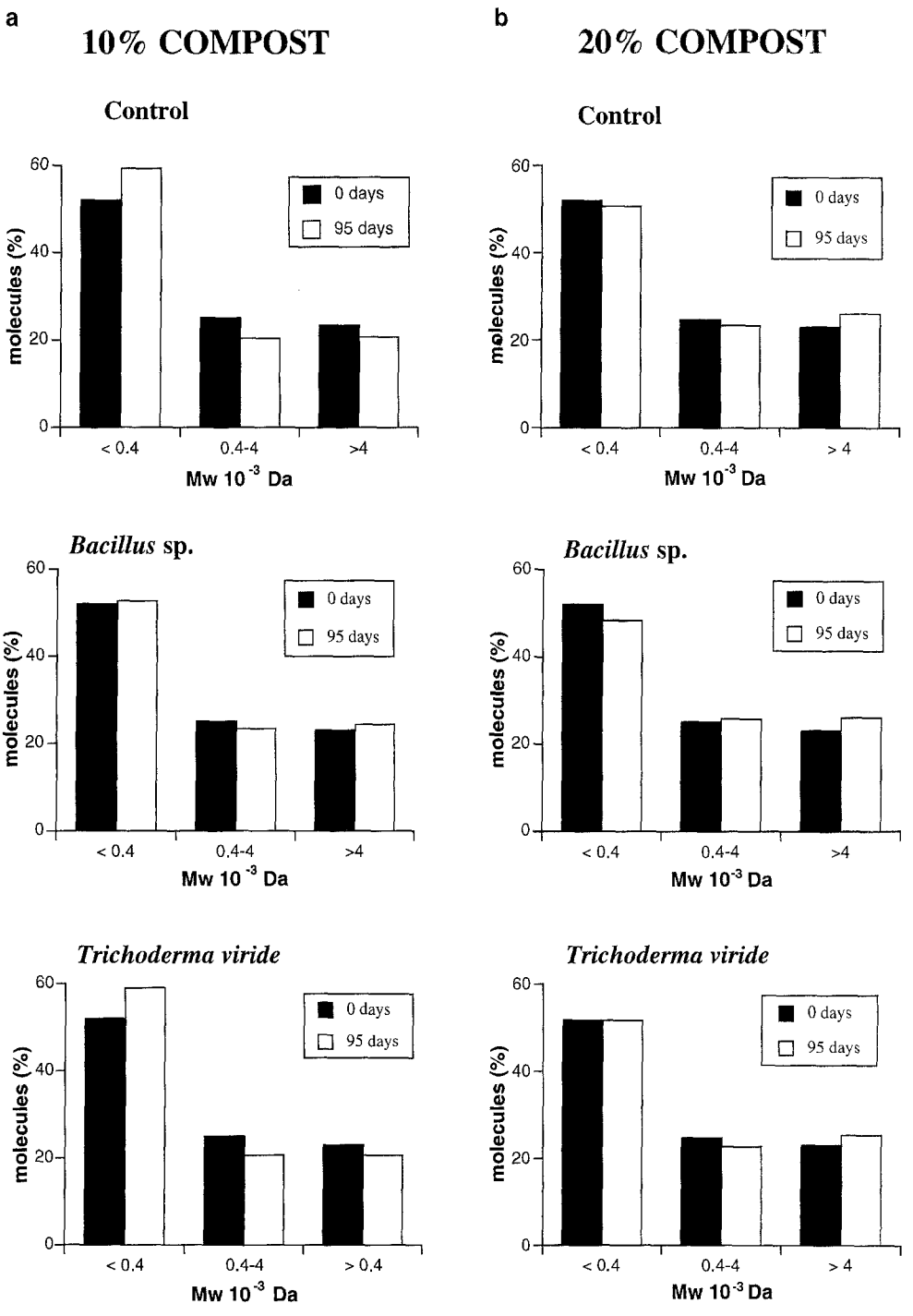

Fig. 2a–c Molecular weight distribution of humic acids extracted with 0.5 *N* NaOH and passed through an HPLC column, in the different compost mixtures before and after incubation (for 95 days) with lignolytic or cellulolytic microorganisms

Results and discussion

The humification index (Sequi et al. 1986; De Nobili and Petrussi 1988) has been described in other studies as a useful parameter for characterizing organic matter of different origins (Petrussi et al. 1988; Saviozzi et al. 1988; Ciavatta et al. 1990a; Baca et al. 1992). The progressive decrease observed for the HI of the different sand-compost mixtures during incubation (Fig. 1) implies an increase in the humification level, and therefore a higher stabilization degree of the organic matter. Once the compost is stabilized, further decomposition is delayed and its nutrient availability for the plant increases because of the reduction of competition between plants and compost microbiota (Poincelot 1975). This may result in better plant growth due to the cessation of fast metabolic processes that can damage plant roots, and to the decrease of phytotoxic substances characteristic of the fresh organic matter (Zucconi and De Bertoldi 1986). The results obtained using the different sand-compost mixtures (Table 1) showed that plant growth was better in sand-compost mixtures incubated for 95 days than in those from mixtures incubated for 35

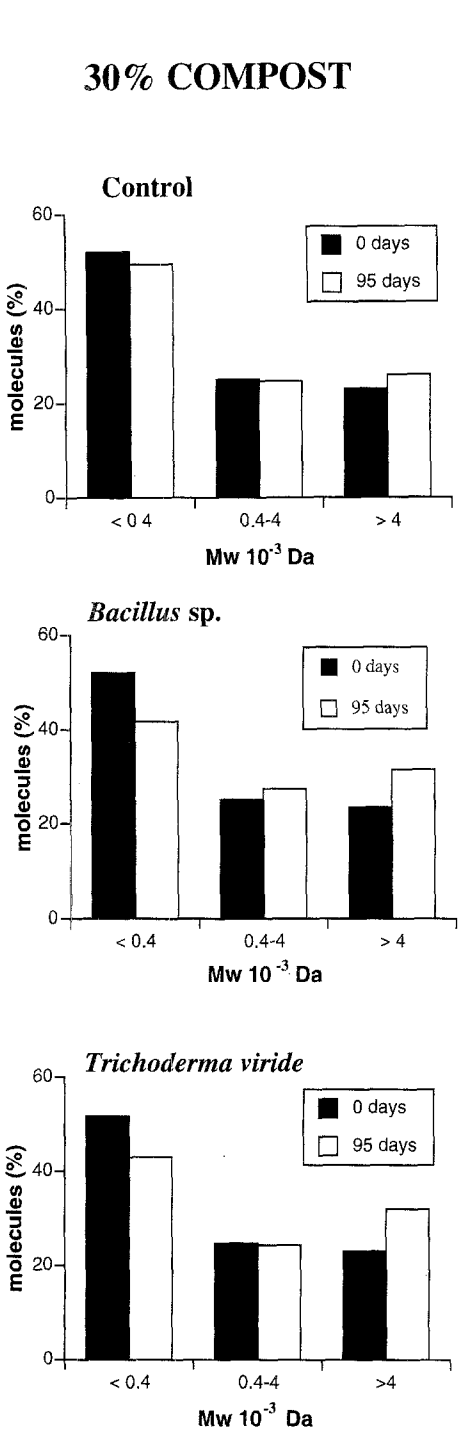

Fig. 2c

days, meaning that at least 95 days of compost incubation are needed to achieve an optimal transformation level for plant growth using this source of compost. In mixtures containing 20% compost, greater differences were recorded between 35 and 95 days of incubation for all the treatments. Electrofocusing of humic substances contained in 20% compost mixtures was therefore carried out to study possible changes in their structure after 95 days of incubation.

The amount of compost present in the incubated mixtures also determined the degree of stabilization of the organic matter at the end of the process. With 30% compost, transformation of the organic matter is apparently delayed, and there are no great differences between plants grown in compost incubated for 35 or 95 days. One possible explanation could be that mixtures containing larger quantities of organic matter also have a larger microbiota that could compete for nutrients with the inoculated ligno-cellulolytic microorganisms, thus reducing their stabilizing activity on organic matter (Lynch 1986). The determination of the most probable number of microorganisms present in the different compost mixtures, carried out on potato dextrose agar, supported this idea (data not shown). This hypothesis could also explain why only the control incubation showed a slight decrease in the humification index at 95 days (30% compost), possibly due to the lack of competition between the endogenous microbiota and other microorganisms.

After 95 days of incubation, an increase in the amount of compost produced a rise in number of the largest humic molecules (>4 kDa) and a decrease in the smallest ones (<0.4 kDa) (Fig. 2a–c). The molecular weight distribution after 95 days was almost the same for both the control and *T. viride* inoculation, except for 30% compost mixtures, in which incubation performed with *T. viride* increased the proportion of molecules of highest molecular weight. In mixtures incubated with *Bacillus* sp., the molecular weight distribution after 95 days incubation is similar for 10% and 20% compost proportions: 50% of the molecules have sizes less than 0.4 kDa; 24% are between 0.4 and 4 kDa; and 26% have sizes greater than 4 kDa. In mixtures with 30% compost, there was a remarkable increase in the proportion of molecules of highest molecular weight compared with the control incubation due to a decrease in the proportion of those of the lowest molecular size. These determinations showed that in samples with 10% compost some depolymerization occurred during the incubation process. Depolymerization is carried out by the endogenous microbiota in the compost, and is apparently not modified by the presence of *T. viride*. The inoculation of *Bacillus* sp., however, seems to compensate for such depolymerization as new humic substances are formed of greater molecular size. In contrast, when mixtures with higer amounts of compost are incubated, some changes in humic molecules occur and there is formation of humic substances of greater molecular size instead of depolymerization processes, especially in the case of mixtures inoculated with *T. viride*.

The effect of *T. viride* can also be seen in the electrofocusing profiles. In control and *Bacillus* inoculated sand-compost mixtures incubated for 95 days, there was no apparent change in the distribution of molecules across the pH gradient. This was probably due to the type of molecules extracted. Some pseudohumic molecules may interfere with organic matter extraction (Ciavatta et al. 1990b). In mixtures inoculated with *T. viride*, however, the size of some peaks (a, b and c; Fig. 3) from the basic zone increased with incubation time. In contrast, the height of peak d, from the acid zone, had decreased by the end of the incubation period. These results indicate that inocula-

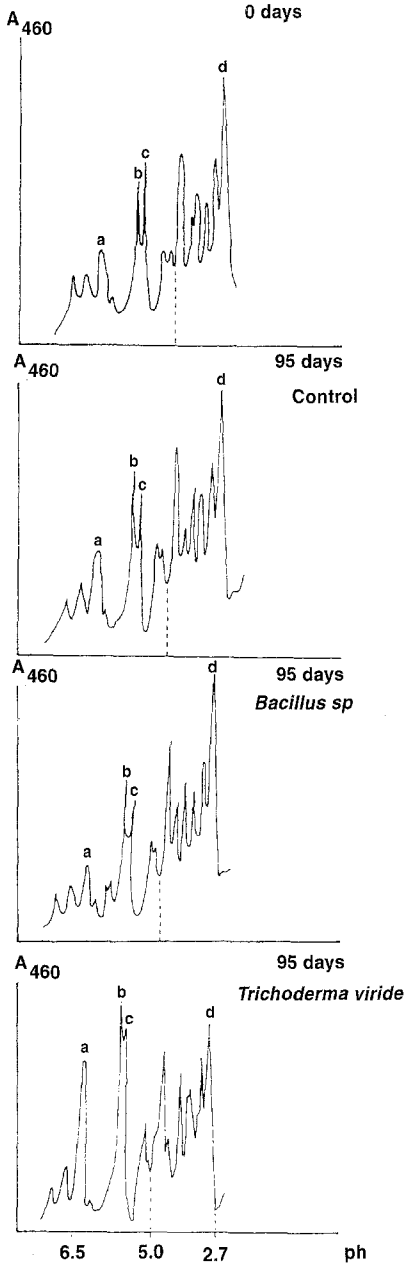

Fig. 3 Electrofocusing profiles of humic substances extracted with 0.5 *N* NaOH, in 20% compost-sand mixtures after 0 and 95 days of incubation with lignolytic or cellulolytic microorganisms (*A* absorbance)

tion with *T. viride* promotes the humification of the organic matter, increasing the amount of humic substances of higher molecular weight. These results are not consistent with the HIs, showing that humification degree does not correlate with the quality of humic molecules. This can be explained according to the results of Requena et al. (1996), which showed that the nature of the humic substances produced after incubation appeared to depend greatly on the degradation pathway characteristic of the inoculated microorganism, *T. viride* or *Bacillus* spp.

The duration of compost incubation with the ligno-cellulolytic microorganisms exerts a great influence on the size of the lettuce plants grown on the different mixtures. When the length of incubation was long (95 days), the dry weight of the lettuce plant was higher in most cases. Differences greatly depended on the amount of compost used. The largest differences between the plants grown on mixtures that had been incubated for 35 days and those grown on 95-day incubation mixtures were recorded when the amount of compost used was 10% or 20% (Table 1). The effect of *A. chroococcum* on plant growth was also influenced by the percentage of compost in the mixtures. Asymbiotic bacteria have considerable requirements for available carbon for their survival and activity in the soil (Azcón 1993), which is why these bacteria grow more extensively in the rhizosphere or in the rhizoplane of several plants. Bacteria use the root exudates (organic acids and other substances), and provide the plant with hormone-like substances that enhance plant growth. It has also been reported (Jodice and Nappi 1987; Halsall 1993) that the addition of compost or other organic materials to culture substrates increases asymbiotic N_2 fixation. Independently of the mechanism of plant promotion exerted by *A. chroococcum* in this experiment, it seems that control incubation mixtures of 30% compost provide *Azotobacter* with enough energy to enhance plant growth. Previous incubation of such mixtures with *Trichoderma* or *Bacillus* may even provide sufficient energy with an incubation of only 35 days.

With smaller percentages of organic matter, an increase in the length of incubation is necessary to obtain an effect of *Azotobacter* on plant growth, or in some cases (20% compost) 35 days of incubation with *T. viride* may have the same effect. Some hydrocarbon substrates could be released to the medium through the cellulolytic activity of *Trichoderma*, and could be used by *Azotobacter*. This "combined" treatment could act synergistically (Lynch 1983) and thus optimize plant growth. These results correlate with the idea proposed by some authors that a community-level control underlies all microbial activities in the population (Janzen et al. 1995a, b). In conclusion, we can say that organic matter content and its degree of humification are important factors for the understanding of *Azotobacter* behaviour in the rhizosphere.

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