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CO_2 evolution and denaturing gradient gel electrophoresis profiles of bacterial communities in soil following addition of low molecular weight substrates to simulate root exudation

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Abstract

Simulating the evolution of both ^{14}C and ^{12}C-CO_2 in the rhizoplane was monitored during the diffusion of ^{14}C-labelled glucose, oxalic acid, or glutamic acid into soil from a filter placed on the surface of a sandy loam. After 3 and 7 d, soil was sampled from four layers (0–2, 2–4, 4–6, and 6–14 mm) to determine residual ^{14}C in each layer. The mineralisation pattern of oxalic acid was characterised by a lag phase probably due to the presence, in the early stages of exposure, of a few microorganisms able to mineralise this substrate. Glucose and glutamic acid showed a positive priming effect with a CO_2 flush from native organic matter. Oxalic and glutamic acids changed the denaturing gradient gel electrophoresis profiles of soil bacterial communities with the appearance of a few extra-bands in the 0–2 mm soil layer. The addition of the substrates onto the soil surface formed a gradient due to their diffusion in soil. That of oxalic acid was specific probably because almost all of this compound reacted with $CaCO_3$ and was localised in the 0–2 mm soil layer.
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1. Introduction

It is well known that the rhizosphere is chemically, physically, and biologically complex (Bowen and Rovira, 1987; Bazin et al., 1990; Campbell and Greaves, 1990) and that plant roots are able, at least partially, to induce a selection of a specific microbial community by their exudates (Swinnen, 1994; Lemanceau et al., 1995; Latour et al., 1996). Root exudates are low molecular weight (LMW) and generally water soluble organic compounds (sugars, organic acids, and amino acids), that leak passively from the root cells giving a concentration gradient in the rhizosphere soil (Bowen and Rovira, 1987). These compounds are readily available C sources for the soil heterotrophs (Whipps, 1990) and their effects depend on the distance that they can diffuse away from the rhizoplane (Bazin et al., 1990). This diffusion rate into soil is a function of the size and charge of the compound and soil properties such as soil texture, organic matter content, pH, soil moisture and temperature (Nye and Tinker, 1977; Darrah, 1991a,b).

Carbohydrates, carboxylic acids and amino acids are among those root exudates mainly responsible for discrimination between C sources utilised by microbial communities of different plant species (Grayston et al., 1998). However, studies on the effects of root exudates on the composition and activity of soil microflora are complicated by the fact that they include a complex mixture of organic compounds and it is difficult to sample soil at distinct and known distances from the root surface (Sørensen, 1997). The former problem can be overcome by comparing the effects of the main LMW compounds added to soil. The second problem can be overcome by setting up a suitable system which allows the formation of a concentration gradient to permit soil sampling at various distances from the rhizoplane. It was shown that a concentration gradient was formed in soil inside a rigid PVC cylinder when a cellulose

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paper with a solution of glucose was located on the top of the soil; the concentration glucose decreased by increasing the distance from the cellulose paper (Badalucco and Kuikman, 2001). A similar system was also used by Darrah (1991a,b).

Our lack of knowledge of microbial diversity in soil has been partly overcome by using molecular techniques to detect and identify microorganisms by molecular markers, such as 16s RNA or its corresponding gene (16s rDNA) (Liesack et al., 1997; Van Elsas et al., 1998). After DNA extraction and purification, PCR followed by denaturing gradient gel electrophoresis (DGGE) is an approach to study complex microbial populations in soil (Heuer and Smalla, 1997).

Our aims were to study:

(1) the diffusion gradient into soil of glucose, oxalic acid and glutamic acid. These model substances are representative of carbohydrates, organic acids and amino acids of root exudates, respectively (Curl and Truelove, 1986). We have used a simple microcosm (Badalucco and Kuikman, 2001) to simulate the release of root exudates by an hypothetical 'root surface';
(2) the oxidation of these compounds to CO_2 by soil microorganisms during and following their diffusion into soil;
(3) the effects of these compounds on the composition of the rhizosphere microflora as determined by DGGE.

2. Material and methods

2.1. Experimental design

An Italian grassland sandy loam soil from Tuscany (sand 66%, silt 20%, clay 14%, organic C 1.66%, total N 0.17%, pH (H_2O) 7.9, $CaCO_3$ 3.5%) was sampled (0–15 cm) sieved (2 mm) and stored at 4 °C until use. The soil was conditioned for 7 d at 25 °C under aerobic conditions to allow microbial activity to stabilise before the addition of the substrates. Then 60 g of soil was placed in a plastic cylinder (20 mm height; ∅ 68 mm) and carefully pressed to a bulk density of 1.2 g dm^3. Cellulose paper (Whatman 41, ∅ 68 mm) moistened with a suitable amount of deionized water was placed on the top of the soil. Then, 1 ml of deionized water (control soil), ^{14}C[U]-glucose (0.57 kBq g^{-1} soil), ^{14}C[U]-oxalic acid (0.75 kBq g^{-1} soil) or ^{14}C[U]-glutamic acid (0.54 kBq g^{-1} soil) solution was added, drop by drop, to the filter to give a rate of 300 μg C cm^{-2} of the filter surface. After these additions soil moisture was approximately 50% of the water holding capacity. The amended samples were incubated at 25 °C in 1.5 l airtight jars containing 10 ml of 0.5 M NaOH to trap the evolved CO_2 and 3 ml of water to avoid soil desiccation. Each treatment was replicated three times.

2.2. Measurement of CO_2 and ^{14}C evolution

Total and ^{14}C-labelled CO_2 evolution were determined after 1, 2, 3, and 7 d by trapping the evolved CO_2 in 2 ml of 0.5 M NaOH and titrating the residual NaOH solution with 0.1 M HCl, in the presence of excess 1.5 M $BaCl_2$, with an automatic titrator. The ^{14}C-content of the NaOH solutions was determined by mixing 0.5 ml aliquots with 3 ml liquid scintillation cocktail (Ultima Gold, Packard). Counting was carried out on a liquid scintillation counter (Tri-carb 2100 TR, Packard) and counts were corrected for background and counting efficiency.

2.3. Measurement of ^{14}C in soil and filter papers

After 3 and 7 d of incubation, soil was sampled from different layers (0–2, 2–4, 4–6, and 6–14 mm below the filter's surface). A sub-sample (about 1 g) of the 0–2 and 6–14 mm layers was used for the DGGE-analysis to determine the composition of the soil bacterial community. The remaining soil was oven dried at 70 °C and analysed for total ^{14}C content by mixing 0.3 g of soil with 6 ml of digestion mixture, obtained by mixing concentrated H_2SO_4 and H_3PO_4 (3:2 by volume) and $K_2Cr_2O_7$ (100 g l^{-1}), in sealed digestion tubes (Amato, 1983; Bremer and Kuikman, 1994). The residual ^{14}C present at 3 and 7 d on filter papers was determined by a TRI-CARB Oxidizer (Packard Instruments; L'Annunziata, 1979).

2.4. DNA analyses

Soil DNA was extracted and purified by CsCl precipitation (van Elsas and Smalla, 1995). Then, soil DNA was amplified by PCR using a DNA Thermal Cycler 480 (Perkin Elmer) with universal bacterial primers of 16S rRNA (341F with 40 bp GC at the 5′ end and 534R of *Escherichia coli*) as reported by Muyzer et al. (1993) by using 40 instead of 30 cycles; PCR products were analysed by DGGE on a polyacrilamide gel (8%, w/v) containing a range of denaturants (100% denaturants is urea 7 M and 40% deionized formamide) from 45 to 75%. Electrophoresis was performed at 60 °C and with a voltage of 75 V for 16 h. After electrophoresis, the gels were reacted for 30 min in a TAE buffer (20 mM Tris acetate at pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2-EDTA), containing ethidium bromide (5 mg l^{-1}), rinsed for 5 min with demineralised water and photographed with an UV-transilluminator at 302 nm and a video image processor P67E (Mitsubishi Co., Japan). The images were analysed by gel analysis software (ONE-Dscan™, version 1.2, Scanalytics, MA, USA).

2.5. Statistics

Standard error values of means were calculated to assess significant differences during the incubation time.

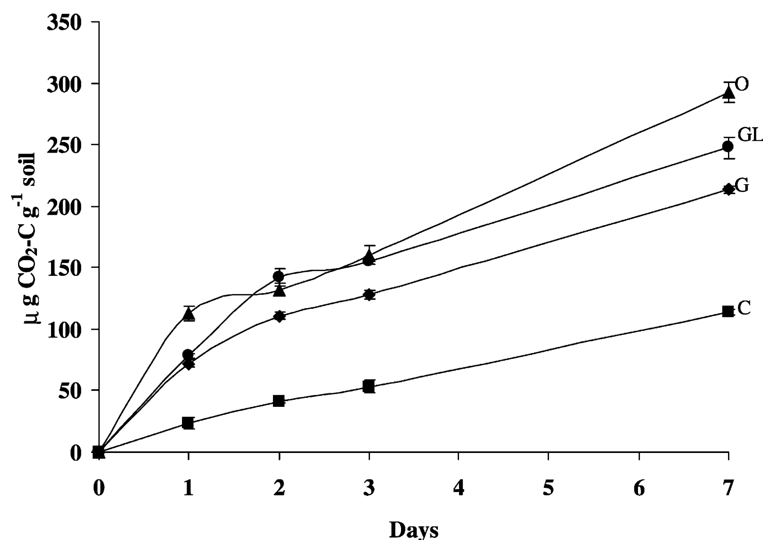

Fig. 1. Total CO_2-C evolution in control soil (C) and in glucose (G), oxalic acid (O) and glutamic acid (GL) treated soils. The error bar is the standard error of the means ($n = 3$).

3. Results

3.1. Soil respiration

Soil amendments increased the amount of the total CO_2-C evolved, but mineralisation patterns differed depending on the added C source (Fig. 1). In the oxalic acid amended soil the total CO_2 evolution after 7 d was higher than in the two other treatments and the control soil. However, oxalic acid oxidation showed a lower mineralisation rate (10% of the added ^{14}C evolved as CO_2-C) than the added glucose-C and glutamic acid-C, about 30 and 43% of the added ^{14}C was oxidised to CO_2-C, respectively (Fig. 2). Most of the evolved CO_2-C from the oxalic acid treatment was probably due to the dissolution of carbonates due to the addition of the acid solution. At the end of the incubation the amount of oxalic acid evolved as ^{14}C-CO_2 (38.4%) was equivalent to that evolved from ^{14}C-glucose treated soil. After 7 d, the percentage respired from the glutamic acid was 59.8%.

The amounts of CO_2-C derived from native soil organic C (^{12}C) was determined by difference between total CO_2-C evolved minus ^{14}C-CO_2. In the glucose amended soil, during the first 3 d of incubation, glucose and soil native C were mineralised at the same rate (Fig. 3). However, towards the end of the incubation, when the added C source was depleted, the ^{12}C-CO_2 respiration rate increased. On the contrary, in the glutamic acid amended soil, during the first 3 d, the amount of ^{14}C-CO_2 evolved was higher than that of the ^{12}C-CO_2 but at the end of the incubation the amount of the evolved ^{12}C was the same as that derived from the substrate (Fig. 3). The ^{12}C-CO_2 evolved was much higher than the ^{14}C-CO_2 in the oxalic acid amended soil during the

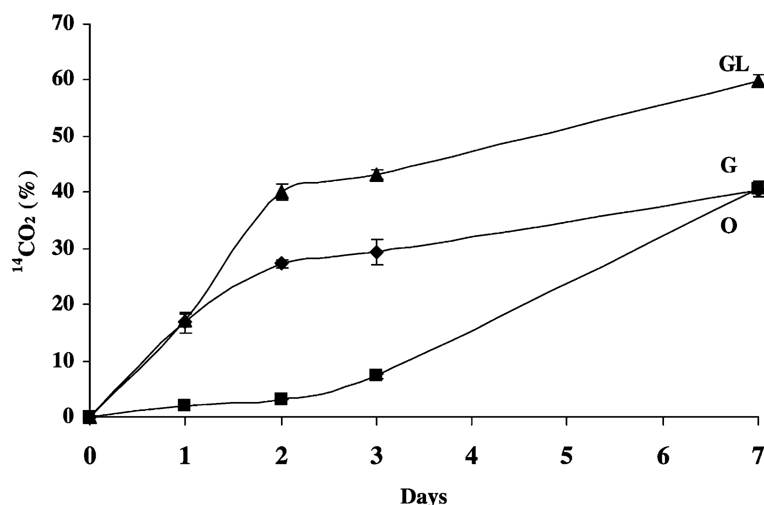

Fig. 2. Total ^{14}C-CO_2 evolution from the control soil (C) and the glucose (G), oxalic acid (O) and glutamic acid (GL) treated soils. The error bar is the standard error of the means ($n = 3$).

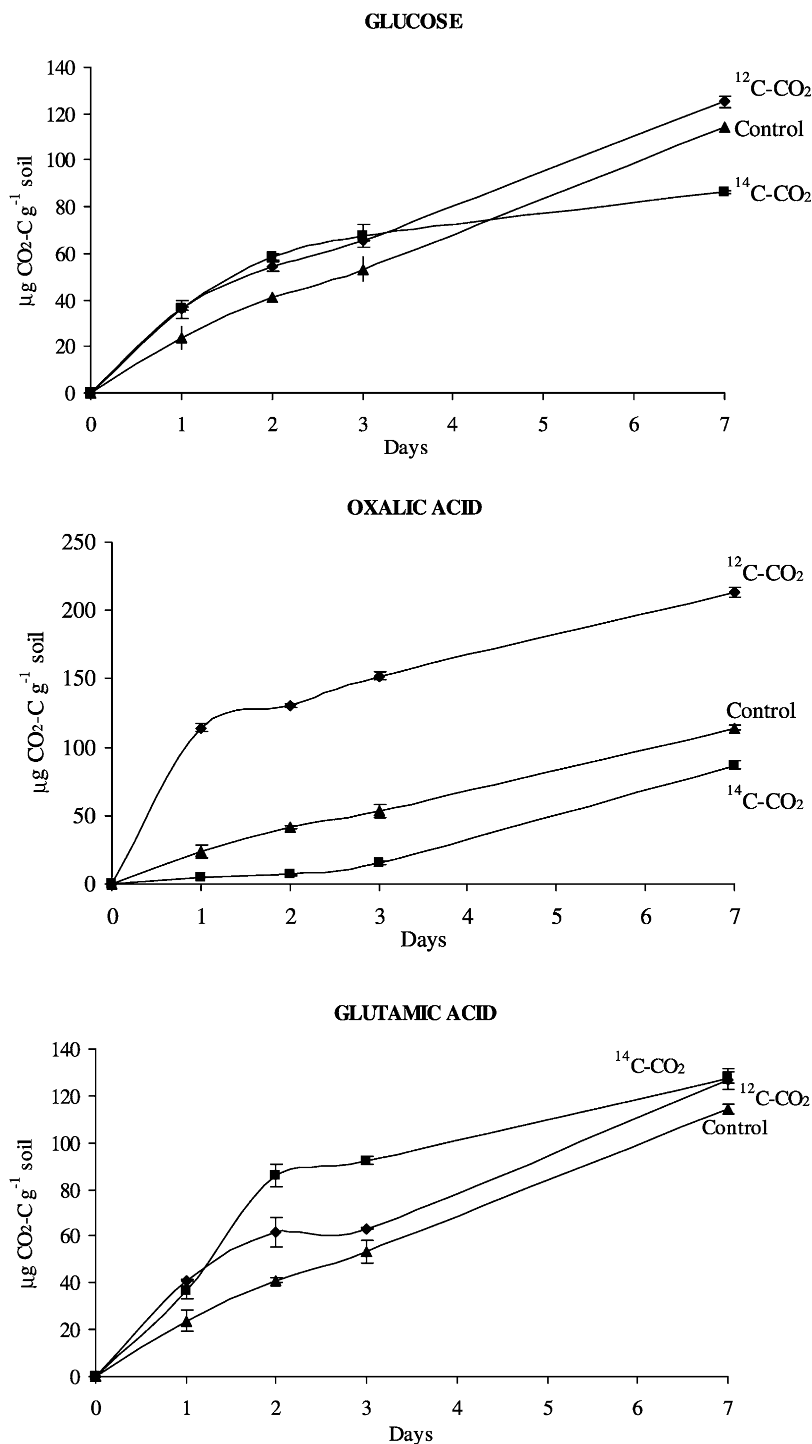

Fig. 3. Cumulative ^{12}C- and ^{14}C-CO_2 respiration in the glucose, oxalic acid and glutamic acid treated soils. The error bar is the standard error of the means ($n = 3$).

first day of incubation, most likely as the result of the reaction of the organic acid with the soil carbonates. Thereafter both CO_2 evolution rates were similar as it is shown by the two parallel plots.

Cumulative evolution of total ^{12}C-CO_2 evolution showed positive priming effects in all treatments (Fig. 3). As already mentioned CO_2 evolution in the oxalic acid treatment was increased due to the CO_2 evolved from soil carbonates.

3.2. Concentration gradients of the added LMW carbon substrates in soil

The C sources showed distinct patterns with respect to the concentration gradient in soil. The highest proportion of the added C was found in all treatments in the 0–2 mm layer (Fig. 4). However, in the case of the oxalic acid treatment the residual ^{14}C was almost completely present in the first

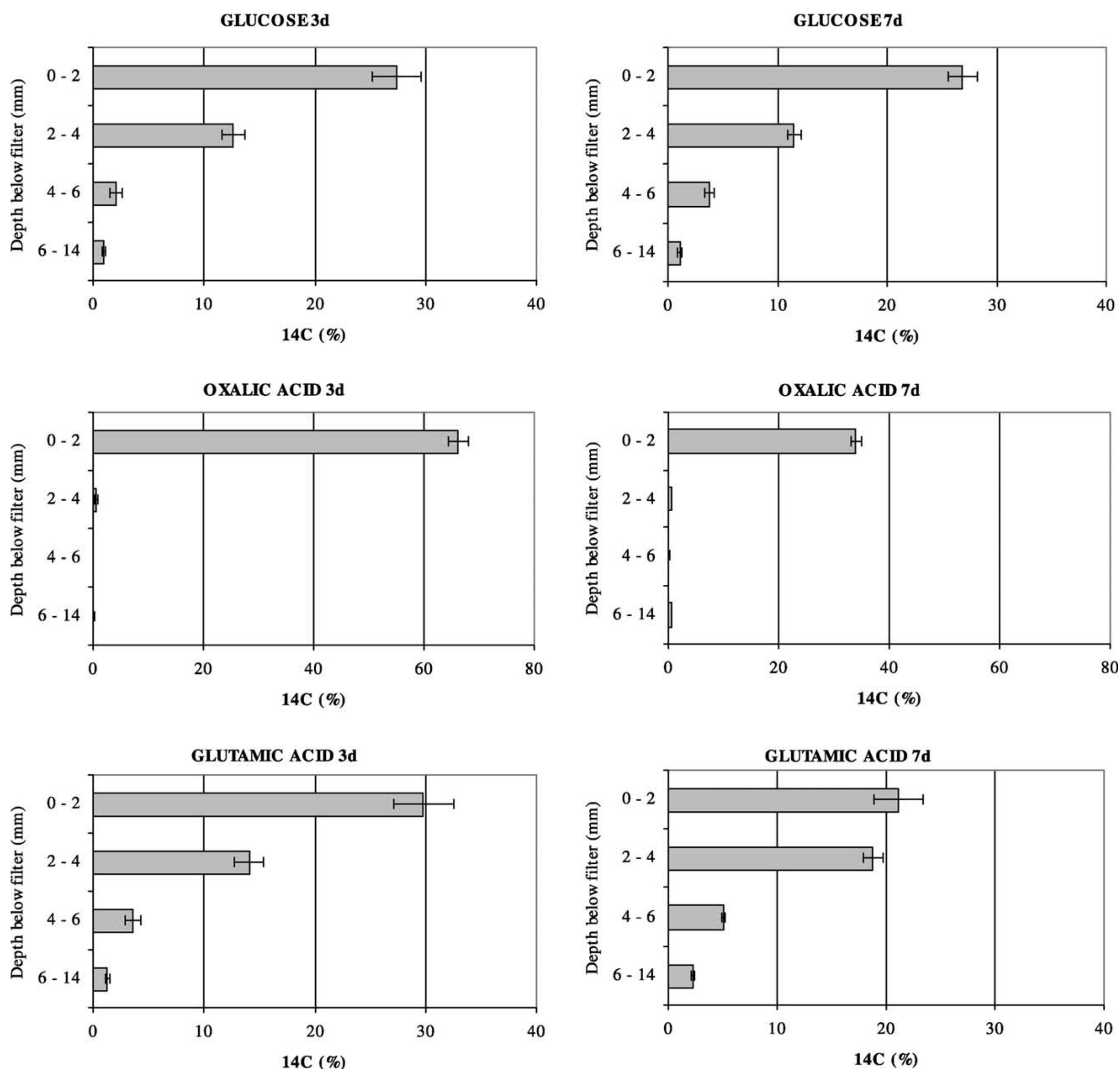

Fig. 4. Concentration gradients of glucose, oxalic acid, and glutamic acid, at days 3 and 7, in the different soil layers. The error bar is the standard error of the means ($n = 3$).

layer being the percentage of the applied compound lower than 1% in the 2–4, 4–6 and 6–14 mm layers both at 3 and 7 d. Glucose and glutamic acid also formed a steep gradient but both compounds were present in higher concentration than oxalic acid in the deeper soil layers. These data seem to confirm our hypothesis that the oxalic acid reacted rapidly with carbonates to produce insoluble Ca-oxalates. At the end of the incubation the percentage of residual oxalic acid-C found in the 0–2 mm layer was 20% lower than that observed at day 3. This amount was equivalent to that respired as CO_2 indicating that the organic acid-C was rapidly mineralised from day 3 to 7. After 7 d the concentration gradient in the glucose treated soil was similar to that observed at day 3. However, in the glutamic acid amended soil the gradient at 7 d was different as residual ^{14}C content decreased in the top layer and increased in the underlying 2–4 mm layer. This is most likely due to a slower diffusion in soil of the amino acid because of its lower water solubility.

3.3. Bacterial community structure

The DGGE separation patterns of PCR amplified 16S rDNA fragments after 3 d of incubation showed marked differences in soil bacterial community structure. Glutamic and oxalic acids favoured the development of a few specific bacterial species (Fig. 5). These effects were only observed in the 0–2 mm layer with the highest concentration of the

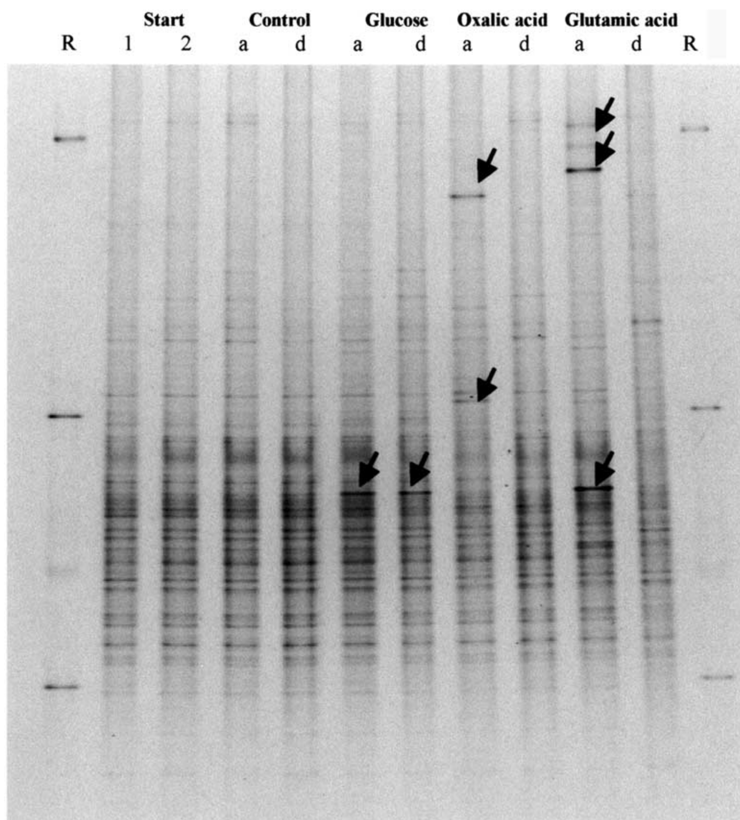

Fig. 5. The DGGE separation pattern of PCR amplified 16S rDNA fragments (position 341–534) before (start) and after 3 d of incubation (layers 0–2 mm (a) and 6–14 mm (d)).

added C sources, thus following the pattern of residual ^{14}C-distribution with depth. The effect persisted until 7 d (Fig. 6). Further research is needed to characterise the new DNA bands of the oxalic and glutamic acid fingerprints. Fingerprints of DNA extracted from the glucose-treated soil were similar to those of the control soil even in the deepest layer; however, one band was more intense in the fingerprints of the glucose-treated soil than that of the control. Both glutamic acid- and glucose-responsive bacteria were involved in the degradation of native organic matter or microbial cells, with the acceleration of the relative turnover, the cause of the observed priming.

Fig. 3 shows only one profile per sample without replications. The fact that profiles of soil layers sampled at 3 and 7 d were similar (Fig. 6) are indicative of a good reproducibility of the used DGGE technique. Then, it can be concluded that the extra bands of glutamic and oxalic acid treated samples are real and not artifacts. In addition, profiles of glucose treated soil are similar to those of control soil (Figs. 5 and 6).

4. Discussion

The mineralisation and the diffusion into soil of oxalic acid, glucose and glutamic acid was studied in this soil because LMW organic acids, carbohydrates and amino acids are mainly responsible for discrimination between C sources utilised by microbial communities of different plant species (Grayston et al., 1998). The LMW compounds released by a cellulose paper, simulating the root surface, formed a concentration gradient into soil.

Oxalic acid is a poorer C source for soil microorganisms than the other two substrates because it has only two atoms of C in its molecule. Moreover few microorganisms are able to utilise oxalic acid (Messini and Favilli, 1990; Morris and Allen, 1994). The observed 3 d lag phase probably indicates that the original soil microbial community had only few microorganisms capable of mineralising oxalic acid. This hypothesis is supported by the fact that after 3 and 7 d the relative DGGE profiles showed a few bands in the 0–2 mm soil layer that were not present in the control soil. After 7 d the amount of oxalic acid-C mineralised to CO_2-C was not significantly different in respect to that evolved from the mineralisation of glucose-C.

Glucose and glutamic acid showed different mineralisation patterns. The glutamic acid was the most mineralised substrate at the end of the incubation. However, after 1 d the amount of $^{14}CO_2$-C evolved from the amino acid amended soil was the same of that evolved from the glucose amended soil. Marstorp (1996), observed that the lag phases of glucose and glutamic acid are similar but the amino acid gave a slight higher specific growth rate than the carbohydrate. The amount of ^{14}C-glucose mineralised at

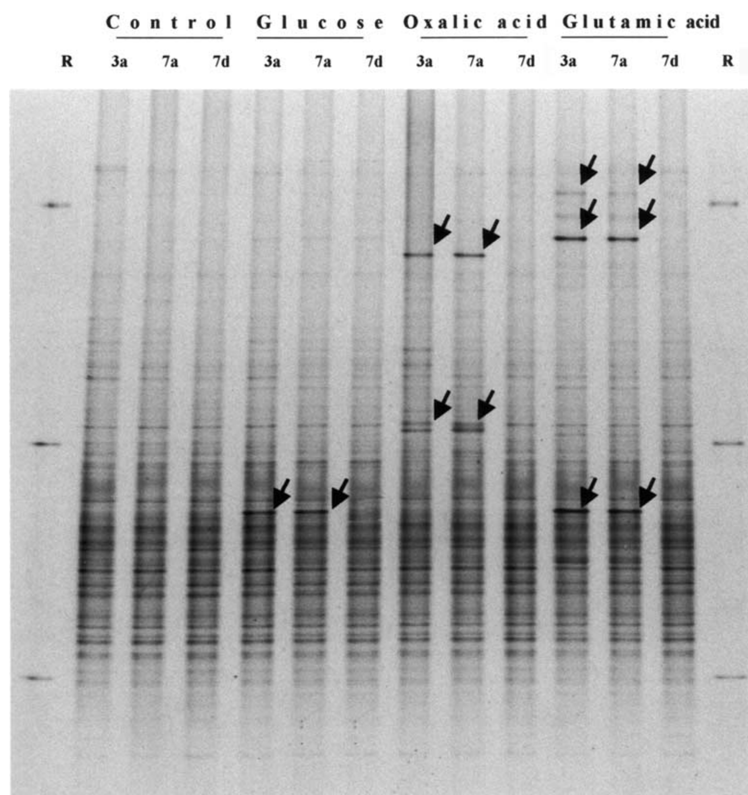

Fig. 6. The DGGE separation pattern of PCR amplified 16S rDNA fragments (position 341–534) after 3 (layer 0–2 mm (a)) and 7 d (layers 0–2 mm (a) and 6–14 mm (d)) of incubation with different C substrates.

the end of incubation (38.4%) was consistent with that found by Bremer and Kuikman (1994) in a similar textured soil. Saggar et al. (1999), using soils with different texture, found that from 38 to 51% of glucose-C was mineralised to CO_2-C after 7 d at 25 °C and a gravimetric moisture content adjusted to 10 kPa. The higher mineralisation of glutamate during the first 3 d, may indicate a preference for a source containing both C and N by the soil microflora. A complex relationship links C and N mineralisation in the rhizosphere soil; growth of bacteria in response to release of exudates, promotes firstly N and C immobilisation from native organic matter followed by protozoan or other predatory activities. Digestion of bacteria by protozoa is followed by release of inorganic N which can be taken up by plant roots (Clarholm 1983; Kuikman and Van Veen, 1989).

Priming effects probably occurred when glucose or glutamic acid were oxidised in soil and these results agree with those in the literature. For example, Dalenberg and Jager (1989) observed that amino acids like glutamate and aspartate leaded to a positive priming effect. Kuzyakov et al. (2000) proposed several mechanisms to interpret the mechanism of the priming effect; in the case of a flush of CO_2-C it was hypothesised the acceleration of organic matter turnover in response to the addition of an easily decomposable organic compound. Bremer and Kuikman (1994) suggested that the mineralised soil C may result from higher turnover of microbes including reserve materials stored in microbial cells.

The concentration gradient of the added oxalic acid, glucose and glutamic acid in soil depended on both water solubility of the LMW compound and its reactivity with soil constituents. Indeed oxalic acid probably reacted rapidly with Ca^{2+} ions to produce insoluble Ca-oxalates. According to Škrtić et al. (1984) and Bramley et al. (1997) this relatively fast reaction is influenced by the oxalate ion concentration. Glutamate diffused at a lower rate than glucose, probably because it interacted with soil matrix being a charged molecule. Darrah (1991a,b) has also shown a slighty slower diffusion of glutamic acid than glucose in soil.

Our findings seem to contradict the hypothesis that the effect of exudates can be small or undetectable when the whole bacterial community is analysed at molecular level. This hypothesis was proposed by Duineveld et al. (1998) because similar bacterial communities were observed between chrysanthemum rhizosphere and bulk soil using the PCR-DGGE approach. They concluded that the effect of root exudates on the dominant bacterial species can be marginal as most organisms are oligotrophic and thus responding slowly to root exudation. Our results show that some bacteria, possibly copiotrophs, are clearly favoured by the addition of LMW substrates. However, real roots exude a spectra of substrates following a certain dynamic pattern (Kuzyakov and Cheng, 2001), thus masking the effect of the individual compound.

In conclusion, ^{14}C-labelled glucose, oxalic acid and glutamic acid added to soil in a model system simulating the

conditions of soil rhizosphere showed distinct mineralisation patterns with a lag phase, which in the case of oxalic acid probably due to its reaction with $CaCO_3$. Glucose and glutamic acid both caused a positive priming effect with a CO_2 flush from native C sources. Glutamic acid and oxalic acid but not glucose changed the DGGE profile indicating that probably different communities responded to their addition. This may be explained by the hypothesis that glucose and glutamic acid were utilised by different microbial communities yet both lead to the respiration of possibly different soil C sources. The addition of the substrates on the soil surface formed a gradient due to their diffusion in soil. That of oxalic acid was specific probably because almost all compound reacted with $CaCO_3$ before it was mineralised and remained localised in the top soil layer.

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