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Root exudate effects on the bacterial communities, CO_2 evolution, nitrogen transformations and ATP content of rhizosphere and bulk soils

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Abstract

The ATP content, soil respiration, bacterial community composition, and gross N mineralization and immobilization rates were monitored under laboratory condition at 25 °C for 28 d in a model system where low molecular weight root exudates (glucose and oxalic acid) were released by a filter placed on the surface of a forest soil also treated with ^{15}N, so as to simulate rhizosphere conditions. Periodically, the soil was sampled from two layers, 0–2 and 6–14 mm below the filter's surface, which were indicated as rhizosphere and bulk soils, respectively. The isotope dilution technique was used to determine the effect of these low molecular weight organic compounds (LMWOCs) on gross N mineralization and immobilization rates. From 0 to 3 d both glucose and oxalic acid amended soils showed a rapid evolution of CO_2, more pronunced in the latter treatment together with a decrease in the amount of mineral N of the rhizosphere soil, probably due to N immobilization. Nevertheless, these changes were accompanied by a very small increase in the net ATP content probably because the low C application rate stimulated microbial activity but microbial growth only slightly. A positive 'priming effect' probably developed in the oxalic acid amended soil but not in the glucose amended soil. Gross N mineralization and immobilization rates were only observed in the rhizosphere soil, probably due to the greater C and N concentrations and microbial activity, and were a little higher in both amended soils than in the control soil, only between 1 and 7 d. Both glucose and oxalic acid influenced the bacterial communities of the rhizosphere soil, as new bands in the DGGE profiles appeared at 3 and 7 d. Glucose induced lower changes in the bacterial community than oxalic acid, presumably because the former stimulated a larger proportion of soil microorganisms whereas the latter was decomposed by specialized microorganisms. Peaks of net daily soil respiration and net ATP content and the appearence of new dominant bacterial populations were shifted in time, probably because there was less ATP synthesis and DGGE patterns changed after complete substrate mineralization.

Keywords: CO_2 evolution; ATP content; Root exudates; Gross N transformation rates; Soil bacterial community

1. Introduction

The transformation of organic N to NH_4^+ by heterotrophic microorganisms is the main process by which soil N is made available to plants. Both N mineralization and immobilization occur simultaneously and it is only possible to measure the net rates of these processes (Jansson and Persson, 1982; Landi et al., 1995), unless the ^{15}N dilution technique is used (Kirkham and Bartholomew, 1954; Barraclough et al., 1985; Nishio et al., 1985).

It is well established that N transformations in the rhizosphere soil are related to C dynamics and release of available C from roots. For example, in the presence of maize roots, 67% more soil mineral N was immobilized into organic N than without plants, despite an higher competition by plants for mineral N (Qian et al., 1997). However, it is not clear how root-derived C affects microbial N transformations in soil (Badalucco and Kuikman, 2001). Studies on the effect of root exudates on the microbial processes and the composition of the soil microflora are complicated by the fact that the exudates 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 problem can be overcame by monitoring microbial activity or microbial composition in a system where the solution of a single compound (present in root exudates), added on a cellulose filter located on

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the surface of the soil, diffuses from this model root surface into soil, placed inside a rigid PVC cylinder, simulating a concentration gradient and permitting sampling of soil at distint and know distance from the root surface (Badalucco and Kuikman, 2001; Falchini et al., 2003). Using this simple microcosm Falchini et al. (2003) observed a gradient of diffusion into soil of glucose, oxalic acid and glutamic acid because the concentration of these root exudates decreased by increasing the distance from the cellulose filter. However, the mineralization of oxalic acid showed a lag phase, probably due to the presence, in the early stages of exposure, of few microorganisms able to mineralize this substrates. The release of oxalic acid and glutamic acid, but not that of glucose, induced changes in the denaturing gradient gel electrophoresis profiles (DGGE) of bacterial communities, with the appearance of a few extra-bands in the rhizosphere soil layer. On the other hand, Duineveld et al. (1998) observed that the effect of root exudates on the bacterial community was small or undetectable when PCR-DGGE approach was used. However, it could be important to examine the effect of low molecular weight root exudates on microbial N transformation rates using the same model system used to simulate the diffusion of root exudation in the rhizosphere soil (Falchini et al., 2003), so that our results could be used to develop a conceptual model of rhizosphere C and N interactions.

The aims of this work were to study:

(1) the effect of C input with either glucose or oxalic acid, two compounds present in root exudates, on the immobilization of available NH_4^+ and mineralization of organic N;
(2) the response of microbial biomass and microbial activity as determined by the ATP content and CO_2 evolution;
(3) the changes in the eubacterial community in the rhizosphere soil, as determined by DGGE profiles.

The experiment was carried out under laboratory conditions by using the system described above (Badalucco and Kuikman, 2001; Falchini et al., 2003), with application of low C inputs to mimic the release of root exudates in situ (Falchini et al., 2003); ^{15}N enriched NH_4^+ was used to discriminate N immobilization from mineralization of organic N so as to calculate the gross rates of these processes.

2. Materials and methods

2.1. Soil and experimental design

A forest soil (Fragic Distrudepts) was sampled from the Ao horizon at Vallombrosa, Tuscany (Central Italy); the soil contained 3.7% organic C, 0.21% total N, 78% sand, 20% silt and 2% clay, and had a $pH_{(H2O)}$ of five. It was sieved moist (2 mm), after removing visible coarse plant material, and stored at 4 °C. Prior to use, soil was moistened to 40% of the water holding capacity (WHC) and conditioned at 25 °C for 7 d to allow microbial activity to stabilize before the addition of substrates. Soil samples were incubated in units simulating the rhizosphere as described by Falchini et al. (2003). Briefly, 60 g of moist soil were placed in plastic cylinders (68 mm dia, 20 mm high), at a bulk density of 1.2 Mg m^{-3}, and treated with 1 ml of $^{15}NH_4NO_3$ solution (30 μg N g^{-1}dry soil, as $^{15}NH_4NO_3$ at 10 atom% excess ^{15}N). The ^{15}N solution was applied uniformly over the soil surface using a fine tipped pipette. Then a cellulose filter (Whatman 41, ∅ 68 mm) was placed on the top of soil and treated immediately with 1 ml of glucose solution (glucose amended soil) or oxalic acid solution (oxalic acid amended soil), drop by drop, at rate of 300 μg C cm^{-2}. The controls were treated in the same way using deionized water. Soil moisture after treatments was approximately 50% of the WHC. Each cylinder was placed in a 1l airtight jar, with 3 ml of water at the bottom to avoid soil dessication and a vial with 4 ml of 1 M NaOH to trap the evolved CO_2; cylinders were incubated for 0, 1, 3, 7, 14 and 28 d at 25 °C. Flasks containing only the same amount of water and NaOH were used as blanks for correction of background CO_2 concentration. Soil was sampled from two different layers (0–2 and 6–14 mm below the filter's surface), which were designated as rhizosphere (0–2 mm) and bulk (6–14 mm) soils. Each fraction (rhizosphere and bulk soil) was immediately assayed for inorganic N concentration and isotopic ratio, and then stored at −15 °C before being analysed for the ATP content and bacterial community structure.

All treatments and measurements were replicated three times.

2.2. Analyses

2.2.1. Microbial respiration and ATP content

The CO_2-C trapped in 1 M NaOH was measured by back-titration, after precipitating the carbonate with 0.75 N $BaCl_2$. The excess of NAOH was titrated with 0.1 M HCl by an automatic titrator.

The ATP content was measured according to Ciardi and Nannipieri (1990).

The daily CO_2-C evolution and ATP content were showed as net values, calculated by subtracting the values of the control soils (treated with $^{15}NH_4NO_3$ only) from those of soils treated with LMWOCs.

2.2.2. Inorganic N content

Inorganic N (NH_4^+-N and NO_3^--N) was extracted by shaking 5 g fresh soil for 1 h with 1 M KCl (ratio of 1:5 soil-solution) (Keeney and Nelson, 1982). Soil suspensions were filtered through glass fibre filter (Whatman GF/A) and analysed for NH_4^+-N and NO_3^--N by a Flow Injection

Analyser System (FIAS 300 Perlin-Elmer) coupled to a spectrophotometer (Lambda 2 Perkin-Elmer).

2.2.3. ^{15}N analyses and gross N transformation rates

The NH_4^+-N of the soil extracts were deprotonated to NH_3 by alkaline reaction with MgO, and NH_3 was trapped on 5 mm glass fibre discs (Whatman GF/D) acidified with 10 µl of 2.5 M $KHSO_4$ placed in the lid of the Erlenmeyer flasks (Brooks et al., 1989). After 5 d the discs were removed, dried over anhydrous $CaSO_4$ and the ^{15}N enrichment of the NH_4^+-N was measured by mass spectrometry. The solution remaining in the flask was not stoppered overnight to remove any traces of NH_4^+-N and then Devarda's alloy was added to reduce NO_3^--N to NH_4^+-N, and the flask immediately capped with a new acidified glass fibre disc. The ^{15}N enrichment of the NO_3^--N was determined as described above.

The gross rate of N mineralization and NH_4^+-N immobilization were calculated by using ^{15}N isotope dilution techniques as described by Powlson and Barraclough (1993); Nishio et al. (1985).

2.2.4. Extraction of soil total DNA and PCR-DGGE analysis of bacterial community structure

The eubacterial community structure was monitored by the 16S rDNA PCR-DGGE approach, after amplification of total soil DNA (extracted by FastDNA SPIN Kit for soil, Bio 101 Inc., USA), with the primer set GC-968f and 1401r, as described by Felske et al. (1997). The denaturing gradient gel electrophoresis (DGGE) was performed in duplicate using the DCode System (Biorad), on a 6% polyacrylamide gel containing a denaturant gradient of 46-56% made of urea and formamide (100% denaturant contains 7 M urea and 40% formamide). The DGGE gels were stained with SybrGreen I (FMC Bio Products, Rockland, ME USA), and the bands were detected by UV illumination and described from digital images (Polaroid Gel Cam, Elect; Polaroid Type 667 Film ISO 3000) of the gels.

2.2.5. Statistical analysis

Analysis of variance (ANOVA) and Tukey honest significant differences (HSD) were used to assess the significance of the means ($n=3$). Differences obtained a levels of $P<0.05$ were considered significant.

3. Results

3.1. Microbial respiration and ATP content

The addition of organic compounds immediately increased soil respiration after 1 d when no differences were observed between the glucose and oxalic acid amended soils (Fig. 1). However, after prolonged incubation the cumulative respiration was higher in the oxalic acid than in the glucose amended soil.

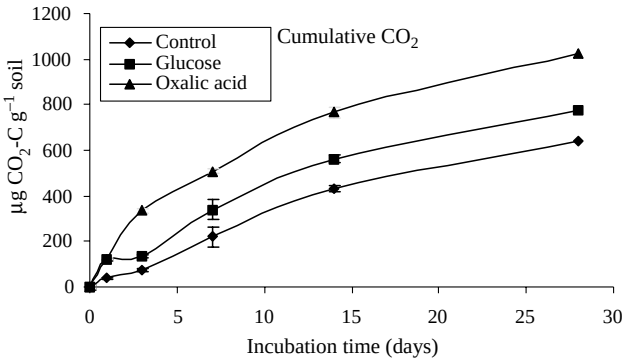

Fig. 1. Cumulative CO_2-C evolution in control, glucose and oxalic acid treated soils during incubation. The error bars are the standard deviation of the means ($n=3$).

The ATP content did not show significant ($P>0.05$) differences between all treatments at each time of incubation in both rhizosphere and bulk soil (data not shown).

In the glucose amended soil net daily respiration was maximum after 1–d (Fig. 2) whereas the net ATP content in the rhizosphere and bulk soil peaked after 1 and 3 d, respectively (Fig. 2). The oxalic acid stimulated the soil respiration up to 3 d and the net ATP content of both the rhizosphere and bulk soil peaked after 3 d (Fig. 2). However, both of the increases were negligible if compared with the relative ATP contents (data not shown).

3.2. N dinamics

Changes in the amount of inorganic N and in the percentage of N in the soil derived from the $^{15}NH_4NO_3$

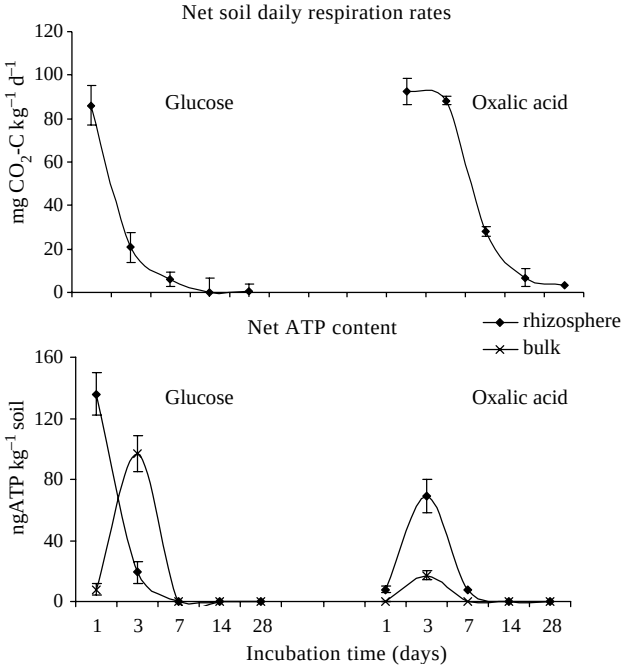

Fig. 2. Net daily respiration rates and net ATP content of the soil treated with LMWOCs during incubation. The error bars are the standard deviation of the means ($n=3$).

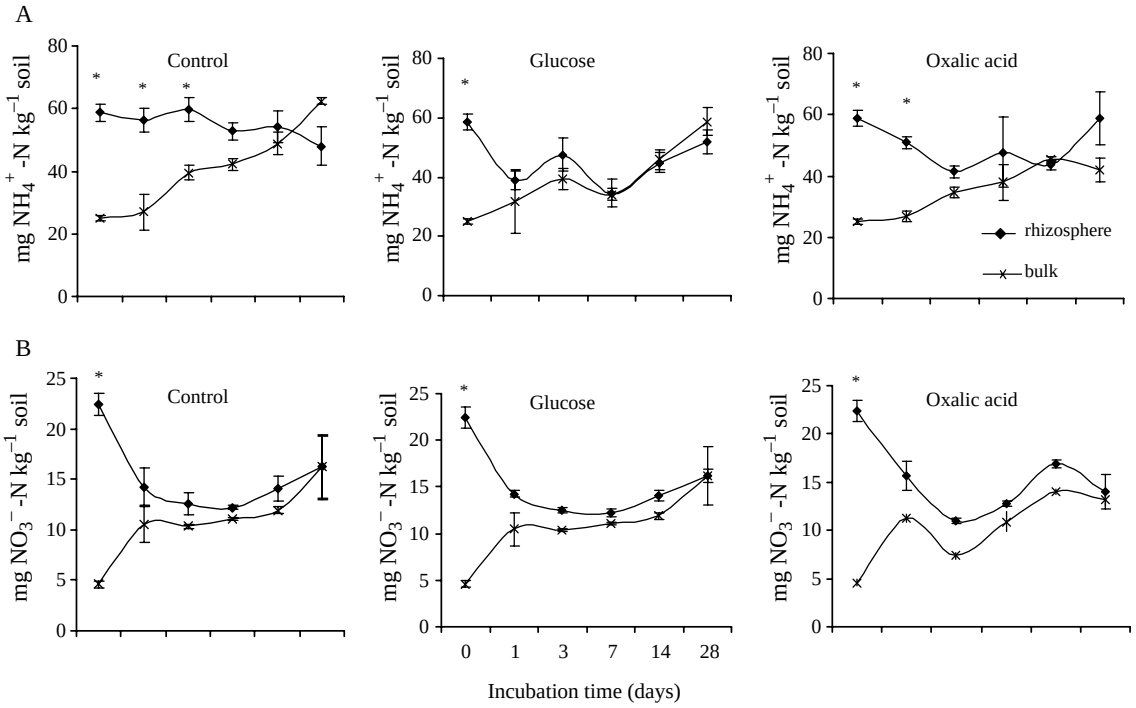

Fig. 3. Changes in the NH_4^+-N (A) and NO_3^--N (B) concentrations during the incubation in rhizosphere and bulk soils. The error bars are the standard deviation of the means ($n=3$).

(Ndff) are shown in Figs. 3 and 4. The NH_4^+-N concentration of the rhizosphere layer of the glucose or oxalic acid amended soils decreased from 0 to 3 d incubation period whereas that of the control soil remained almost constant (Fig. 3A). Up to 3 d, the NH_4^+-N concentration was significantly higher in the rhizosphere soil than in the bulk soil for all treatments, except for the glucose treated soil at 1 and 3 d. Later no significant differences were observed between both rhizosphere and bulk soils (Fig. 3A).

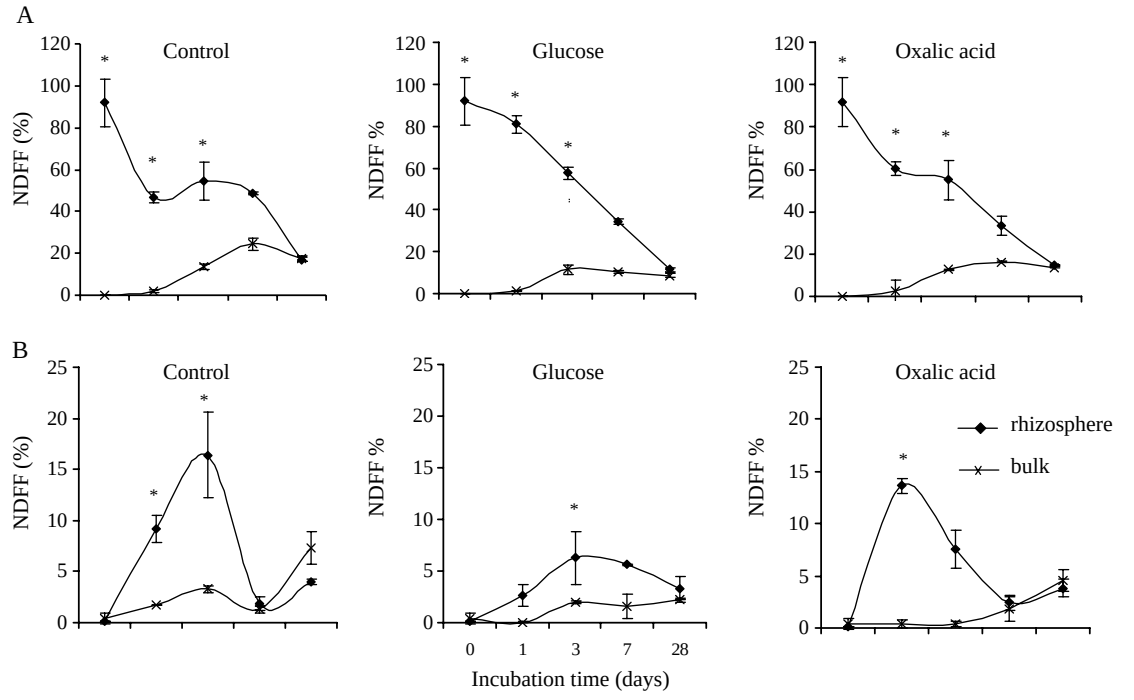

Fig. 4. Changes in Ndff values in the NH_4^+-N (A) and NO_3^--N (B) pools during the incubation in rhizosphere and bulk soils. The error bars are the standard deviation of the means ($n=3$).

Table 1
Effect of glucose and oxalic acid on gross N mineralization and immobilization rates (ng N g^{-1} soil h^{-1}) in rhizosphere and bulk soils during incubation period

Interval time (days)	Gross N mineralization rate				Gross N immobilization rate			
	0–1	1–3	3–7	7–28	0–1	1–3	3–7	7–28
Control								
Rhizosphere	540 (81)	259 (91)	118 (73)	199 (17)	705 (77)	190 (55)	112 (32)	85 (9)
Bulk	9	−430	−66	17	−90	−349	21	5
Glucose								
Rhizosphere	426 (96)	303 (68)	219 (11)	176 (20)	750 (56)	323 (9)	203 (21)	70 (6)
Bulk	9	−1615	35	31	−946	−560	7	-89
Oxalic acid								
Rhizosphere	430 (67)	330 (96)	232 (44)	164 (13)	248 (39)	287 (65)	289 (23)	71 (8)
Bulk	8	−545	−95	26	−342	−313	43	23

The NO_3^--N concentrations of the rhizosphere soil were significantly higher than those of the bulk soil at 0 d for all treatments (Fig. 3B). Then NO_3^--N concentrations decreased up to 1 d, but they remained higher than those of the bulk soil up to 14 d, but no significant differences were observed.

The percentage of NH_4^+-N derived from $^{15}NH_4NO_3$ (Ndff) markedly decreased with incubation time in the rhizosphere soil whereas increased in the bulk soil up to 3–7 d in all treatments (Fig. 4A). Up to 7 d, Ndff value was significantly higher in the rhizosphere soil than in the bulk soil for all treatments. However, Ndff value was higher in the rhizosphere of the glucose soil at 0–1 d; while not significant differences ($P > 0.05$) were observed between treatments at other time of incubation. By contrast, the percentage of NO_3^--N derived from $^{15}NH_4NO_3$ (Ndff) of the rhizosphere layer of the glucose treated soil was lower than that of the other treatments in the 0–3 d incubation time (Fig. 4B). At 3 d, Ndff values were lower in the glucose and oxalic acid treatded soils than in the control soil, in both layers. Generally, in all treatments, the percentage of NO_3^--N derived from $^{15}NH_4NO_3$ (Ndff) was significantly higher in rhizosphere soil at 1–3 d; thereafter, not significant differences were observed between the rhizosphere and the bulk soils (Fig. 4B).

Gross N mineralization and immobilization rates calculated as reported by Powlson and Barraclough (1993); Nishio et al. (1985) are shown in Table 1. Generally, gross N mineralization rates decreased during the incubation and they were higher in the rhizosphere than in the bulk soil. Usually the presence of available C, either as glucose or as oxalic acid, increased the rates of the rhizosphere soil respect to the rates of the control soil in the 3–7 d periods (Table 1).

The gross N immobilization rates, based on changes in the NH_4^+-N pool (Nishio et al., 1985), of the rhizosphere soil were higher in the glucose and oxalic acid treated soils than in the respective layer of the control soil in the 1–3 and 3–7 d incubation period (Table 1). The highest rates were observed in the control and glucose amended soil in the 0–1 d incubation period.

The gross N mineralization and immobilization rates of the bulk soils were generally negative or come near zero (Table 1).

3.3. Bacterial community structure

The DGGE fingerprinting patterns of the eubacterial community structure of the rhizosphere layer of both glucose and oxalic acid treated soils showed the disappearance of two bands after 3 d incubation period (Fig. 5). The same two bands also disappeared in the rhizosphere layer of the control soil after 7 d. In the layer of the oxalic acid treated soil four new bands appeared after 7 d and only one was in common with the DGGE profile of the glucose amended soil layer. Nevertheless, these characteristic bands did not persist and at 28 d no differences were observed in the eubacterial community structure of all treatments.

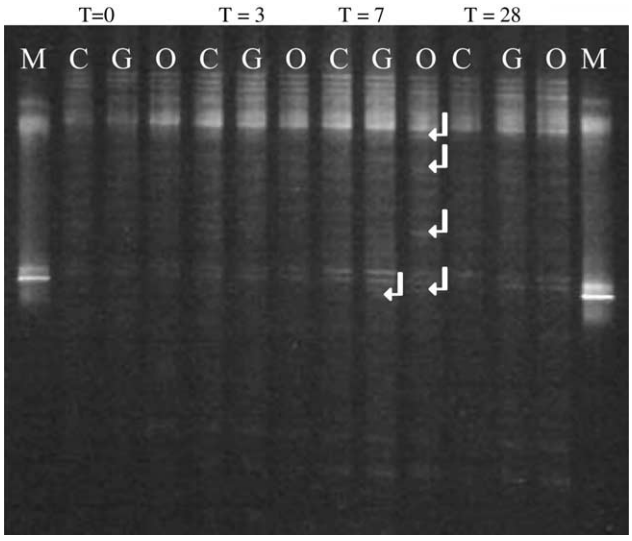

Fig. 5. DGGE profiles of the rhizosphere soil. T indicate the incubation time (days) and C, G and O indicate the control, glucose and oxalic acid treated soils.The additional bands described in the text are indicated by arrows.

4. Discussion

4.1. Microbial respiration and ATP content

Both organic substrates increased CO_2 evolution. The addition of oxalic acid, but not glucose, produced a positive 'priming effect' (Dalenberg and Jager, 1989). Falchini et al. (2003), simulating the conditions of soil rhizosphere with the same system, found that in a calcareous soil the cumulative evolution of total ^{12}C-CO_2 showed positive priming effects in the glucose and glutamic acid amended soil, while in the oxalic acid amended soil the effect was masked by CO_2 evolved from the acidification of soil carbonates. Our observation that small additions of glucose did not cause a 'priming effect' confirms the report of Wu et al. (1993), who showed that large additions of glucose (5000 μg C g^{-1} soil), but not small additions (500 μg C g^{-1} soil), caused a priming effect. The 'priming effect' can be caused by an enhanced turnover of microbial C and/or by enhanced decomposition of organic matter (Jenkinson et al., 1985; Kuzyakov et al., 2000; Bell et al., 2003). With these data it is difficult to explain why oxalic acid but not glucose caused the priming effect. By considering that glucose can be used by a large proportion of soil microorganisms (Anderson and Domsch, 1978), whereas oxalic acid decomposition is carried out by specialized microorganisms (Messini and Favilli, 1990), it is reasonable to hypothesize that the priming effect is caused by the former addition. Indeed there are more chances to have a priming effect when a large rather than a small proportion of soil microflora is stimulated by C addition. A possible explanation might be that the microbial species degrading the oxalic acid are capable of decomposing some of the native soil organic matter; whereas the application of glucose reduced the decomposition of more complex soil organic material because the soil microflora preferentially used the easily decomposable C substrate. Further research based on the use of C labelled substrate is required to confirm these data.

The fact that net ATP increases were negligible as compared to the ATP content of soil may suggest that increases in microbial biomass were not important. Indeed soil microflora can metabolize glucose without any proliferation (Blagodatsky et al., 2000). Peaks of net daily soil respiration and net ATP content were shifted in time leading us to hypothesize that small amounts of ATP are synthesized only after complete substrate mineralization and that the low C application rate can stimulate microbial activity but microbial growth only slightly, which may be N limited.

4.2. N dynamics

The decrease in the NH_4^+-N concentration observed in the rhizosphere soil of glucose and oxalic acid amended soils in the 0–3 d period may be the result of diffusion of added NH_4^+-N into bulk soil, nitrification and N immobilization. Nitrification is stimulated by the NH_4^+-N addition (Recous et al., 1999; Willison et al., 1998), but in this acid forest soil, nitrification was probably not important because NO_3^--N concentration also decreased during the first 3 d of incubation. The decrease of NH_4^+-N concentrations and Ndff values of the rhizosphere soil and their increase in the bulk soil suggest the formation of a NH_4^+-N diffusion gradient Even if we did not measure the glucose and oxalic acid concentration in soil it is reasonable to suggest a diffusion gradient for both organic compounds. Falchini et al. (2003) showed a diffusion gradient for both glucose and oxalic acid by using ^{14}C-labelled compounds, the presence of the highest amount of the added C in the rhizosphere soil, probably stimulated microbial N immobilization (Table 1).

Total NO_3^--N concentration of the rhizosphere soil decreased in all treatments from 0 to 3 d, and it can be the result of NO_3^--N immobilization and/or denitrification. At 3 d the percentage of NO_3^--N derived from $^{15}NH_4^+$-N (Ndff) was lower in the glucose and oxalic acid amended soils than in the control soil probably due to NO_3^--N immobilization in presence of the low molecular weight substrates. However, soil conditions are not favourable to NO_3^- immobilization. Indeed NO_3^--N is immobilized when NH_4^+-N concentrations are lower than 0.1 μg g^{-1} (Rice and Tiedje, 1989) and NH_4^+-N concentrations in all treatments were always higher than this value. In addition, Recous and Mary (1990) observed that NO_3^--N added as NH_4NO_3 was poorly immobilized in the presence of low amount of glucose, because these amounts did not allow complete NH_4^+-N immobilization, while NO_3^--N was intensively immobilized when applied alone. On the other hand, if NO_3^--N was immobilized it remains to be explained why NH_4^+-N was not completely utilized by microorganisms. The preferential immobilization of NH_4^+-N over that of NO_3^--N by the soil microflora biomass has been demonstrated in several studies (Recous and Mary, 1990; Recous et al., 1999; Puri and Ashman, 1999). Probably soil conditions were not anaerobic as organic substrates were readily mineralized to CO_2 and the amount of the applied C source was not enough to stimulate high rates of microbial activity unaccompanied by O_2 shortage in soil microhabitats. Thus, the lower Ndff values of both amended soils, with respect to the control soil, can be due to a lower rate of nitrification.

Our gross N mineralization and immobilization rates were comparable to those measured in a forest soil by Davidson et al. (1991); Myrold and Tiedie (1986). Gross N mineralization and immobilization rates of the rhizosphere soil were higher than those of the bulk soil in all treatments. This is due to higher microbial activity in the rhizosphere than in the bulk soil. In the case of the control soil the higher inorganic N concentrations of the rhizosphere than bulk soil probably stimulated the decomposition of native soil organic matter.

In the rhizosphere soil, the higher gross N immobilization rates in the glucose and oxalic acid amended soils than

in the control soil incubated for longer than 1 d may be due to the stimulation of the process by organic C. However the fact that the N immobilization rate of the control rhizosphere soil was high and similar to that of glucose amended rhizosphere soil at 0–1 d may be indicative that the system is not C-limited. The available C for NH_4^+-N immobilization in our soil may derive from natural sources such as the easily degradable soil organic C, microbial metabolites or microbial debris. However, it has been reported that microbial N can be immobilized without the microbial use of a C organic source (Woods et al., 1987; Recous and Mary, 1990).

4.3. Bacterial community structure

The molecular approach showed that both glucose and oxalic acid influenced the bacterial communities of the rhizosphere soil. Glucose induced lower changes in the bacterial community than oxalic acid, presumably because the first stimulates a larger proportion of soil microorganisms (Anderson and Domsch, 1978), whereas the latter is decomposed by specialized microorganisms (Messini and Favilli, 1990). This confirms the finding by Falchini et al. (2003), who simulating the diffusion gradient within calcareous soil observed that oxalic and glutamic acids but not glucose changed the DGGE profiles of soil bacterial communities in the 0–2 mm soil layer. On the other hand, Jjemba and Alexander (1999) reported that periodic additions to a readily-available C source to soil did not greatly affect microbial colonization of the rhizosphere.

5. Conclusions

Data obtained with this model system seem to indicate that soil microbes were N limited. In fact a soil that is immobilizing N suggests microbial N limitation; however, adding LMWOCs enhances respiration suggesting C limitation (Vance and Chapin, 2001). Thus, microorganisms can appear to be both C and N limited simultaneously. The normal argument to explain C limitation in organic-rich forest soil is that the low quality of soil substrates limits their availability to soil microorganisms, and thus, even if the microorganisms are not truly C limited, they can be energy limited.

Higher gross N mineralization and N immobilization rates were observed in rhizosphere soil than in bulk soil, probably due to the greater C and N concentrations and microbial activity. Even if microbial activity was stimulated by both C and N additions, microbial growth was negligible.

However, oxalic acid and in minor part glucose changed the DGGE profiles indicating that also small amounts of root exudates can also affect different bacterial communities and confirming that glucose but not oxalic acid is used by the majority of the soil bacterial community. These data suggest that microbial activity and community composition and N turnover in forest soils may depend on the pool of LMWOCs, particularly in the rhizosphere, and this may be relevant for forest ecosystem management. Experiments now in progress are intended to provide more realistic information on ecosystem function by using a continuous flux of low molecular weight organic compounds to simulate the quantity of exudates that might flow into an intact rhizosphere.

Acknowledgements

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