CHANGES IN INORGANIC N AND CO2 EVOLUTION IN SOIL INDUCED BY L-METHIONINE-SULPHOXIMINE

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Summary—Short-term (up to 48 h) incubation assays were conducted in the presence of L-methionine-DL-sulphoximine (MSX), an inhibitor of glutamine synthetase, to assess its effect on exchangeable NH4+-N, NO3-N production, and CO2 evolution. A sandy-clay-loam (Pistoia) and a sandy (Romola) soil were moistened and either amended with glucose (200 pmol glucose·g-1 soil), glucose + (NH4)2SO4 [50 pmol N·g-1 soil as (NH4)2SO4] or left unamended. In the two unamended soils, NH4+-N concentration was increased by the highest MSX level (1 pmol g-1 soil), while the lowest inhibitor concentration (0.5 pmol g-1 soil) had less influence. NH4+-N concentrations were higher with 1 pmol MSX than without the inhibitor in the glucose-only-treated Pistoia soil in the 0–12 h period; thereafter the opposite situation was observed. Probably the CO2 evolution increased as a result of inhibitor mineralization after 12 h. In the glucose-treated Romola soil both MSX concentrations were generally effective in increasing NH4+-N concentrations with respect to the same amendment without the inhibitor. These increases were probably due to glutamine synthetase inhibition by MSX and not to the presence of mineralization of the inhibitor because CO2 evolution was only slightly increased at 48 h by MSX. Probably, as a result of this inhibition, NO3-N was used as an alternative N source in the glucose-amended Romola soil. The inhibitor had no significant effect on NH4+-N concentrations when both soils were amended with glucose + (NH4)2SO4 probably because, in the presence of high NH4+-N concentrations, NH3 assimilation occurred more through glutamate dehydrogenase than through glutamine synthetase–glutamate synthase enzymes. NO3-N concentrations were decreased by MSX in the glucose-amended Romola soil but not in the glucose-amended Pistoia soil.

INTRODUCTION

Ammonium (NH4+), the end product of N mineralization process, is often re-used and incorporated into organic molecules by soil microorganisms. Thus, N mineralization and immobilization occur simultaneously and for this reason it is very difficult to determine the gross rates of the two processes (Jansson, 1958; Nannipieri et al., 1983; Stevenson, 1986). NH4+ reacts with glutamate to give glutamine through the reaction catalysed by the glutamine synthetase (GS); then glutamine reacts with α-ketoglutarate producing glutamate through glutamate synthase, known as glutamine oxoglutarate amino transferase (GOGAT). However, when the NH4+ concentration is high (>1 mM), the NH4+ is incorporated as glutamate via glutamate dehydrogenase (GDH). In some microorganisms, NH4+ is also incorporated by the reaction producing asparaginase from aspartate and catalysed by asparagine synthetase (Reitzer and Magasanik, 1987). Moreno-Vivian et al. (1983) showed that when GS is inactive, reductive amination of pyruvate is the obligatory way for NH4+ incorporation in phototrophic bacteria. The Km for GS is less than those of both GDH and asparagine synthetase, indicating a relatively higher affinity of GS for NH4+. In fact, when NH4+-N concentrations are <0.1 mM, NH4+-N is only incorporated through the reaction catalysed by GS (Magasanik and Neidhardt, 1987). Since NH4+ concentrations in the soil solution are usually very low, microbial N immobilization in soil probably occurs through the GS/GOGAT pathway.

L-Methionine-DL-sulphoximine (MSX) is an effective GS inhibitor and is commonly used in pure culture experiments to study the pathways of NH4+-N assimilation and to measure nitrate reductase activity (Rowe and Meister, 1970; Rigano et al., 1982; Arp and Zumft, 1983). It is more efficient than the corresponding D-isomer and other putative GS inhibitors (Brenchley, 1973). However, MSX could also be utilized as a N and C source by some microorganisms, as found for some antibiotics (e.g. streptomycin and cycloheximide) applied to soil (Badalucco et al., 1994). The effect of MSX on N metabolism can therefore be rather complex: for example, the addition of MSX to a NO3--assimilating phototrophic bacteria prevented both growth and NO3 uptake with simultaneous excretion of NH3 to the medium (Moreno-Vivian et al., 1983). The GS activity was inactivated by MSX while NO3 reductase was unaffected.
In soil this inhibitor has been used to study the effect of various N compounds on the microbial synthesis of urease (McCarty et al., 1992), the regulation of assimilatory NO$_3^-$ reductase activity (McCarty and Bremner, 1992a,b) and the microbial uptake of NH$_4^+$ (Schimel and Firestone, 1989a). MSX inhibited NH$_4^+$ incorporation by 24 and 13% in submerged O$_2$ and O$_2$ subhorizons of a forest soil, respectively (Schimel and Firestone, 1989a). Soil slurries permit less variability than aerated soils, but waterlogged conditions are markedly different with respect to those occurring in situ. Particularly, the submergence of soil in water severely limits O$_2$ diffusion to the microflora with the consequent shift from aerobic to anaerobic metabolism.

Our work was to follow changes in exchangeable NH$_4^-$, NO$_3^-$ production and CO$_2$ evolution in soils treated with MSX to test the possibility of developing a method for determining gross rates of N mineralization by blocking the N immobilization process. Soils were also treated with glucose so as to favour N immobilization.

**MATERIALS AND METHODS**

A sandy-clay-loam (Pistoia) and a sandy (Romola) textured soil were sampled (0–20 cm), air-dried, sieved (2 mm) and stored (Table 1). Particle size was determined by using the pipette method (Day, 1965); pH by a glass electrode with a 1:2 soil-to-water ratio; organic C by the Walkley-Black procedure (Nelson and Sommers, 1982) and total N by Kjeldahl digestion (Bremner and Mulvaney, 1982).

In a short-term incubation experiment, each soil was treated with distilled water (unamended), dissolved glucose (glucose-amended) (200 pmol glucose-C g$^{-1}$ d.w. soil) or glucose + (NH$_4$)$_2$SO$_4$ [50 pmol N g$^{-1}$ d.w. soil as (NH$_4$)$_2$SO$_4$] in the presence of 0, 0.5 or 1.0 pmol MSX g$^{-1}$ d.w. soil. In each treatment after all the additions and prior to incubation, the soil moisture content corresponded to 50% of the water holding capacity (WHC). Each treatment was replicated 3 times. MSX was purchased from Sigma Chemical Co. Glass beakers with soil samples (20 g oven d.w.), were placed in airtight jars (1) containing a vial with 4 ml 1 N NaOH to adsorb CO$_2$, and a vial with 5 ml distilled water to maintain a moist atmosphere. Soils were kept at 25°C for 48 h. After 6, 12, 24, 36 and 48 h of incubation, the evolved CO$_2$ was determined and 10 g of soil were transferred to 100 ml centrifuge tubes and extracted with 40 ml 2 M KCl solution. Tubes were shaken for 1 h, centrifugated for 10 min at 4000g, filtered through Whatman No. 40 paper and the extracts stored at 4°C. The NH$_4^+$ and NO$_3^-$ contents were determined by steam distillation of the soil extracts (30 ml) with MgO and Devarda’s alloy (Keeney and Nelson, 1982).

CO$_2$ evolved during the incubation was determined using a Radiometer autotitrator after precipitating the carbonate with 8 ml of 0.75 N BaCl$_2$; the excess of base was titrated with 0.1 M HCl; the end-titration point was pH 8.8. A control without soil was used for each incubation time to correct for atmospheric CO$_2$ and to standardize the NaOH (Tinsley et al., 1951).

**RESULTS**

**NH$_4^+$-N concentration**

Without glucose, the NH$_4^+$-N concentration was immediately greater in the presence of 1 pmol MSX in Pistoia soil and after 12 h in Romola soil (Figure 1). The effect of the lowest MSX concentration in increasing NH$_4^+$-N concentration was not always significant.

The addition of glucose only caused a significant decrease in NH$_4^+$-N concentration in both soils (Figure 1); this decrease was more marked in Romola than in Pistoia soil. In the glucose-treated Pistoia soil the addition of the inhibitor caused a greater decrease further with the exception of the 0–12 h incubation period at the highest MSX concentration (Figure 1). However, the 0.5 pmol treatment only slightly affected the NH$_4^+$-N concentration with respect to control values. By contrast, in the glucose-treated Romola soil NH$_4^+$-N concentration was generally higher with than without MSX. The greatest difference in NH$_4^+$-N concentration was observed at 12 h, then the difference decreased and at the end of the incubation all the three treatments were the same (Figure 1).

Higher concentrations of NH$_4^+$-N were generally observed with than without the inhibitor in the glucose + (NH$_4$)$_2$SO$_4$-treated Pistoia soil (Figure 1). The differences in NH$_4^+$-N concentration between the two MSX concentrations were only significant at 48 h. The addition of both inhibitor concentrations to the glucose + (NH$_4$)$_2$SO$_4$-treated Romola soil significantly increased the NH$_4^+$-N concentration in comparison to the respective control values only after 24 h.

**NO$_3^-$-N concentration**

The content of NO$_3^-$-N in the unamended Pistoia soil without the inhibitor decreased rapidly during the first 12 h, increased until 24 h and then remained almost constant (Figure 2). Greater NO$_3^-$-N concentrations were observed in the Pistoia soil amended with

| Soil    | Sand (%) | Silt (%) | Clay (%) | Organic-C (%) | Total-N (%) | pH | CEC (mequiv 100 g$^{-1}$) |
|---------|----------|----------|----------|---------------|-------------|----|--------------------------|
| Pistoia | 63.2     | 17.6     | 19.2     | 3.64          | 0.26        | 6.7| 6.1                      |
| Romola  | 90.05    | 3.5      | 5.6      | 0.86          | 0.08        | 7.2| 5.9                      |

*Table 1. Properties of soils*
Effect of MSX on N mineralization-immobilization turnover

both MSX concentrations with respect to the control from 6 to 12 h, no significant differences were observed between the two MSX treatments. After 24 h the two inhibitor treatments presented different patterns; the greatest MSX concentration showed greater NO₃⁻-N values with respect to the 0.5 μmol MSX treatment and to the respective control. By contrast, in the unamended Romola soil the inhibitor decreased the NO₃⁻-N content with respect to the control and the major decline was observed with the highest MSX concentration after 24 h (Figure 2). However, due to the initial lower NO₃⁻-N concentration, NO₃⁻-N fluctuations were much smaller in the unamended and glucose-amended Romola soil than in the respective Pistoia treatments.

The NO₃⁻-N content of the glucose-treated Pistoia soil markedly decreased during the incubation. By 24 h it had fallen to about 7% of its initial value, then it remained constant until the end of experiment (Figure 2). The only significant inhibitor effect on the NO₃⁻-N concentration was observed at 6 h with the highest MSX concentration (Figure 2). In Romola soil, the glucose addition also decreased NO₃⁻-N concentration; the presence of both MSX concentrations lowered NO₃⁻-N concentrations with respect to control values after 12 h.

NO₃⁻-N concentrations also decreased in the glucose + (NH₄)₂SO₄-treated Pistoia soil during the incubation (Figure 2). The addition of 1 μmol MSX gave the highest NO₃⁻-N concentrations during the 0–12 h. Then a marked decrease was observed and values were lower than those without the inhibitor. The 0.5 μmol MSX in glucose + (NH₄)₂SO₄-treated Pistoia soil showed lower NO₃⁻-N contents than the respective control but significant differences were only observed after 6 h. In the glucose + (NH₄)₂SO₄-treated Romola soil, the NO₃⁻-N concentration decreased at 6 h and then increased up to 12 h (Figure 2). After 12 h, the NO₃⁻-N concentration fluctuated but almost reached the initial value at the end of the incubation. The addition of the inhibitor decreased the NO₃⁻-N concentration with respect to control values and the most marked effect was generally observed with the highest MSX concentration.

CO₂ evolution

The CO₂ evolution plots of both unamended soils are shown together with those of glucose-amended soils so as to maintain the same presentation of NH₄⁺-N and NO₃⁻-N concentration patterns. Due to the scale used, differences in CO₂ evolution among unamended soils are often not clear (Figure 3). The
CO₂ evolution of the unamended Romola soil was not affected by both of MSX concentrations, with the exception of respiration values at 12 h (both an 18% increase) and 24 h (22 and 37% increase for the lowest and the highest MSX concentrations, respectively). On the contrary, the inhibitor affected respiration immediately in the unamended Pistoia soil. However, the highest MSX concentration showed the most marked effect throughout (41, 100, 77, 74 and 53% increases over the control at 6, 12, 24, 36 and 48 h, respectively).

The addition of 0.5 μmol MSX to the glucose or glucose + (NH₄)₂SO₄-treated Pistoia soil did not alter CO₂ evolution in comparison with the respective control up to 24 h (Figure 3). Thereafter this MSX concentration inhibited CO₂ evolution with the exception of the glucose + (NH₄)₂SO₄ treatment at 48 h. The presence of 1 μmol MSX rapidly and markedly stimulated CO₂ evolution after 6 h in both glucose and glucose + (NH₄)₂SO₄-amended Pistoia soil (about 34 and 45% increase over the control, respectively). By contrast, in the glucose-treated Romola soil, the inhibitor did not influence the CO₂ evolution rate with the exception of 1 μmol MSX at 12 and 48 h and of 0.5 μmol MSX at 12 h (Figure 3). Both concentrations of MSX depressed the respiration in the glucose + (NH₄)₂SO₄-amended Romola soil after 12 h (Figure 3). No significant differences were observed between the two MSX treatments except at the end of incubation.

**DISCUSSION**

Although organic C and total N contents were greater in the sandy-clay-loam Pistoia than in the sandy Romola soil (Table 1), higher mineralization rates of soil organic matter, as determined by CO₂ evolution, were generally found in the sandy than in the sandy-clay-loam soil when both soils were incubated without glucose from 0 to 48 h. The lower mineralization rate in sandy-clay-loam soil may be due to the higher physical protection (e.g. by clays) of organic material or the lower protozoan predator activity in Pistoia than in Romola soil (Heynen et al., 1988). In fact, the degradation rate of organic matter in soil is strongly influenced by the physico-chemical properties of soil (Catroux et al., 1987; Hassink, 1992; Kretzschmar and Ladd, 1993). With glucose the...
cumulative CO₂ evolved at 48 h was greater from the Pistoia than from the Romola soil. This behaviour probably did not depend on the presence of more available N in the Pistoia than in the Romola soil. Despite the fact that the addition of a N source as (NH₄)₂SO₄ with glucose increased the cumulative CO₂ evolved from Romola at 48 h with respect to the glucose-amended soil, the total CO₂ evolved was still lower than that of the glucose + (NH₄)₂SO₄-amended Pistoia soil. The difference in CO₂ evolution between the two glucose-treated soils may depend on the different size of the microbial biomass or the different composition of soil microflora rather than on the amount of available N. Opportunistic microorganisms (zymogenous according to Winogradsky) exhibit high rates of activity and rapid growth on easily utilizable substrates (Atlas and Bartha, 1981). The number of these microorganisms might be greater in the Pistoia than in the Romola soil.

Fig. 3. Changes in cumulative CO₂ evolved during the incubation: □, no MSX; ◊, +0.5 µmol MSX; △, +1 µmol MSX. Bars represent SDs; SDs not reported are smaller than symbol size.

Incorporation in amino acids was found to increase the N mineralization of cytoplasmic protein-N (Reitzer and Magasanik, 1987). Both net N mineralization and CO₂ evolution were increased from an organic sandy soil amended with MSX, or L-methionine or methionine sulfoxide another methionine analogue (Hopkins et al., 1995). These increases were attributed to the mineralization of the added compounds by soil microflora. Thus, the increases in NH₄⁺-N concentration and CO₂ evolution may both be indicative of inhibitor mineralization and not GS inhibition in soil. This behaviour was evident in the unamended Pistoia soil with the highest MSX concentration but not in the unamended Romola soil, where both MSX concentrations positively affected the NH₄⁺-N concentration after 12 h (Figure 1) and CO₂ evolution only at 12 and 24 h (Figure 3). The increase in the NH₄⁺-N concentration in the unamended Romola soil might be due to the release of only NH₄⁺-N by the deamination of the inhibitor. When glucose was applied to the Romola soil, NH₄⁺-N concentrations were higher with both MSX concentrations than without the inhibitor in the 0–24 h period (Figure 1). No differences in CO₂ evolution were observed in the glucose treatment except a decrease at 12 h for both MSX concentrations.
and an increase at 48 h for the highest MSX concentration. The observed patterns in NH$_4^+$-N concentrations and CO$_2$ evolution seem to indicate that microbial NH$_4^+$ incorporation had been inhibited by MSX in the glucose-treated Romola soil before 24 h. The deamination of MSX was probably not responsible for the differences in NH$_4^+$-N concentrations observed in the glucose-amended Romola soil between the MSX treatments and the respective control because any available inorganic N source would have been immediately immobilized by microorganisms during the rapid oxidation of glucose. The addition of 1 μmol MSX to the glucose-treated Pistoia soil gave slightly higher NH$_4^+$-N concentrations in comparison to the respective control in the 0–12 h period. Probably, the highest MSX concentration slightly inhibited GS enzyme activity during this period (Figure 1); then, the inhibitor was used as an N and C source by soil microorganisms as it seems to be proved by the higher CO$_2$ evolution over the control after 12 h. The mineralization of the inhibitor was not reflected in higher NH$_4^+$-N concentrations probably because NH$_4^+$ was incorporated by microorganisms in the presence of glucose, that is under conditions favouring N immobilization. Probably, microbial NH$_4^+$ was incorporated in the glucose-amended Pistoia soil with 0.5 μmol MSX; however, it is difficult to explain the inhibition of CO$_2$ evolution observed after 24 h in this treatment.

The addition of MSX to both soils treated with glucose + (NH$_4$)$_2$SO$_4$ did not influence significantly NH$_4^+$-N concentrations. Under non-limiting NH$_4^+$-N concentrations, NH$_4^+$ is probably incorporated through reactions catalysed not only by both GS/GOGAT enzymes but also by the GDH enzyme, which is unaffected by the MSX (Magasanik and Neidhardt, 1987).

The concentration of NO$_3^-$ in soil depends on various processes: NO$_3^-$ is formed by nitrification while it is consumed by denitrification and microbial and plant uptake (Stevenson, 1986). Higher NO$_3^-$-N concentrations with respect to the control were observed with the highest MSX concentration in unamended Pistoia soil (Figure 2). The addition of 1 μmol MSX to glucose + (NH$_4$)$_2$SO$_4$-amended Pistoia soil increased the NO$_3^-$-N concentration only in the 0–12 h period (Figure 2). This increase might be due to the inhibition of NO$_3^-$ uptake by microorganisms as it has been observed for NO$_3^-$-assimilating phototrophic bacteria treated with MSX (Moreno-Vivian et al., 1983) and not as the result of higher nitrification rates due to the slightly higher NH$_4^+$-N concentrations with respect to control values (Figure 1). If the increased nitrification rate had been caused by the slight increase in NH$_4^+$-N concentration, higher NO$_3^-$-N contents would have been also observed in the glucose + (NH$_4$)$_2$SO$_4$-amended Pistoia soil with 0.5 μmol MSX. In fact, this treatment also showed slightly higher NH$_4^+$-N concentrations than the respective control (Figure 1). Probably, MSX at the lowest concentration was ineffective in inhibiting NO$_3^-$-N uptake by microorganisms because at this level most of the inhibitor was adsorbed by soil particles. Both MSX applications decreased NO$_3^-$-N concentrations in the unamended and glucose-amended Romola soil in comparison with the respective control, while this did not occur in the respective Pistoia treatments. NO$_3^-$ might have been used as an alternative N source in the unamended and glucose-amended Romola soil because GS was inhibited by the MSX, as has been hypothesized by Magasanik and Neidhardt (1987). However, as discussed before, GS was probably inhibited in the glucose-amended but not in the unamended Romola soil. In addition, with the present data we cannot exclude that differences in the composition as well in the physiological state of the nitrifiers may be the cause of the different NO$_3^-$ patterns between the two soils. No published data are available on the effect of MSX on the nitrifying activity of soil.

The addition of glucose in Pistoia soil caused a marked decrease of NO$_3^-$-N as a result of N immobilization (Nannipieri et al., 1983) or denitrification (Lalisser-Grundman et al., 1988). If NO$_3^-$ immobilization is the process responsible for the decrease of NO$_3^-$-N it remains to provide an explanation of why NH$_4^+$-N was not completely utilized by microorganisms. It has been demonstrated that NO$_3^-$ is utilized by microorganisms when the NH$_4^+$ concentration is lower than 0.1 μg N g$^{-1}$ soil (Rice and Tiedje, 1989). Probably, in the glucose-amended Pistoia soil anaerobic conditions prevailed and NO$_3^-$ was used as an electron acceptor, while in the glucose-amended Romola soil NO$_3^-$ was used partly as an alternative N source in the presence of MSX. O$_2$ shortage due to intense microbial activity promoted by glucose may be more severe in fine than in coarse textured soil (Atlas and Bartha, 1981).

In conclusion, MSX was probably effective in inhibiting GS enzymes in glucose-amended Romola soil up to 24 h. It might have been also effective in the glucose-amended Pistoia soil at the highest concentration only in the 0–12 h period. Probably, in the unamended and glucose-amended Pistoia soil (after 12 h for the highest MSX concentration) MSX was mineralized to CO$_2$ and NH$_4^+$-N, while in the unamended Romola soil it was deaminated to NH$_4^+$-N. However, more studies are needed to ascertain the complex effect of this inhibitor on N metabolism and to monitor the fate of MSX in soil. By using the $^{15}$N-dilution technique (Nishio et al., 1989; Barraclough, 1991; Geens et al., 1991) it may be possible to determine the gross rates of the N mineralization and immobilization processes and how these processes are affected by MSX. In addition, more direct evidence of the MSX effect on the GS activity of soil is required through an enzyme assay to determine the GS activity in soil.
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