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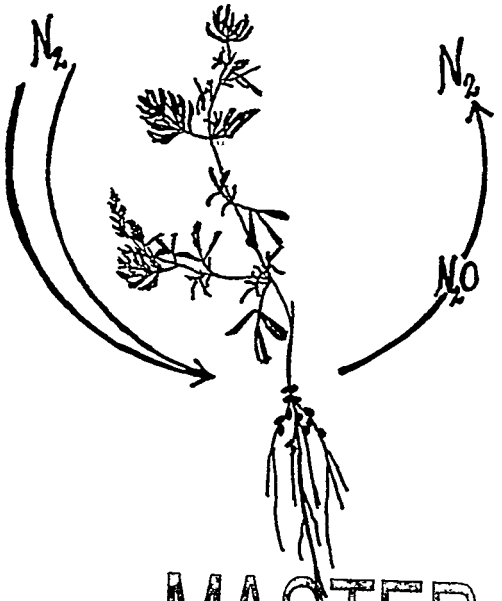
Denitrification by *Rhizobium meliloti*

Agneta Rosén



Licentiatavhandling

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Institutionen för mikrobiologi
Uppsala genetikcentrum

Rapport 65
Report
Uppsala 1996

Swedish University of Agricultural Sciences
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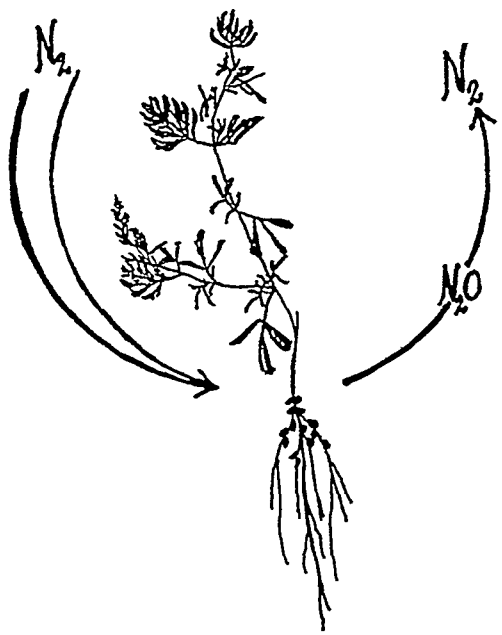

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Det finns en förunderlig måne
som manar till ebb och till flod
skulle jag tänkas gå vilse,
är den förunderligt god.

Det finns en förunderlig aning
vart vägar och stigar gå,
skulle jag tänkas gå vilse,
lyser nog månen ändå.

Malte Rosén

ABSTRACT

Rhizobium meliloti strains were investigated for their denitrification activity as free-living cells and in nodules on lucerne (*Medicago sativa*) roots. They were also investigated for presence of nitrous oxide reductase (Nos) activity and for genes using a *nos*Z probe derived from the *Pseudomonas stutzeri*. To decide whether *R. meliloti* strains used as inoculants contribute to the total denitrification activity in a lucerne ley, strains with different denitrifying capacities were used in field and laboratory experiments. The nitrate reduction activity of *R. meliloti* during anaerobic respiration was compared with that of a strain of *Pseudomonas aeruginosa*.

A great diversity in the denitrification activity was found within strains of *R. meliloti*, and four of thirteen investigated strains showed an obvious denitrification activity. Two denitrifying bacteria were used as references, one strain each of *Bradyrhizobium japonicum* and *P. aeruginosa*. The highest denitrification rate in investigated *R. meliloti* strains was half that of the activity in *B. japonicum* and a quarter compared to the *P. aeruginosa* strain. Most investigated strains were much lower.

All but one of the *R. meliloti* strains hybridised to the *Pst*I-fragment of the *nos*Z-gene from *P. stutzeri*. Two sizes of the hybridising fragment, 5 and 7 kb, were noticed. Nos activity was only shown in three *R. meliloti* strains, and these were all characterised by a high denitrification activity.

No contribution to the total denitrification activity in the lucerne ley was found by inoculum strains. The actual denitrification activity in the lucerne ley was about 4 times higher than in the grass ley or fallow, independent of inoculation. The potential denitrification activity was about 20, 40 and 80 times higher than the actual denitrification activity for lucerne, fallow, and grass, respectively. The potential denitrification activity was almost the same in lucerne and grass planted soils. Compared with the unplanted soil, the presence of lucerne roots in the soil increased the actual denitrification activity, while roots of both plant species, grass and lucerne, increased the potential denitrification activity in the soil.

PREFACE

This thesis consists of a summary and the following two papers:

I Rosén, Agneta; Lindgren, Per-Eric & Ljunggren, Hans: Denitrification by *Rhizobium meliloti*. 1. Studies of free-living cells and nodulated plants. Submitted.

II Rosén, Agneta & Ljunggren, Hans: Denitrification by *Rhizobium meliloti*. 2. Field and laboratory studies with soil. Submitted.

The papers are referred to in the text by their respective roman numerals.

CONTENTS

INTRODUCTION

Nitrogen

Nitrogen (N) is one of the major elements needed for biosynthesis of cell components in all living organisms. The oxidation state of N may vary from -3 to +5. Air consists of about 78% molecular N, N_2. Other common forms of N in the environment are inorganic N as ammonia (NH_3), and nitrate (NO_3^-) or organic compounds, e.g. amino acids (Fig. 1).

N_2-fixation, nitrification and denitrification are three major microbiological processes in the N cycle. N_2-fixation is a process found only within prokaryotes, either as free-living organisms or in symbiosis with an eukaryotic organism. An example of symbiotic N_2-fixation is the *Rhizobium*-leguminous symbiosis. *Rhizobium meliloti* bacteria for example form an endosymbiosis with lucerne plants where bacteroids in the nodules fix atmospheric N. The N_2-fixation process contributes to an input of NH_3-N into the soil (Sprent & Sprent 1990).

The oxidation of NH_3 to NO_2^-, and NO_2^- to NO_3^- is called nitrification. It is performed by lithotrohpic nitrifying bacteria for which the oxidation process yields energy, but the process is also found in some heterotrophic bacteria (Schmidt & Belser 1982).

The nitrification products, NO_3^- and NO_2^-, are both substrates for the denitrification process. Denitrification is a dissimilatory reduction of oxidised N compounds NO_3^-, and NO_2^-, to the gaseous compounds of N, nitric oxide (NO), nitrous oxide (N_2O), and N_2. This process occurs generally in micro-aerophilic or anaerobic conditions (Tiedje 1988).

The *Rhizobium*-leguminous symbiosis

The genera *Rhizobium, Bradyrhizobium* and *Azorhizobium* belong to the family Rhizobiaceae (Table 1). Bacteria of this family are all Gram-

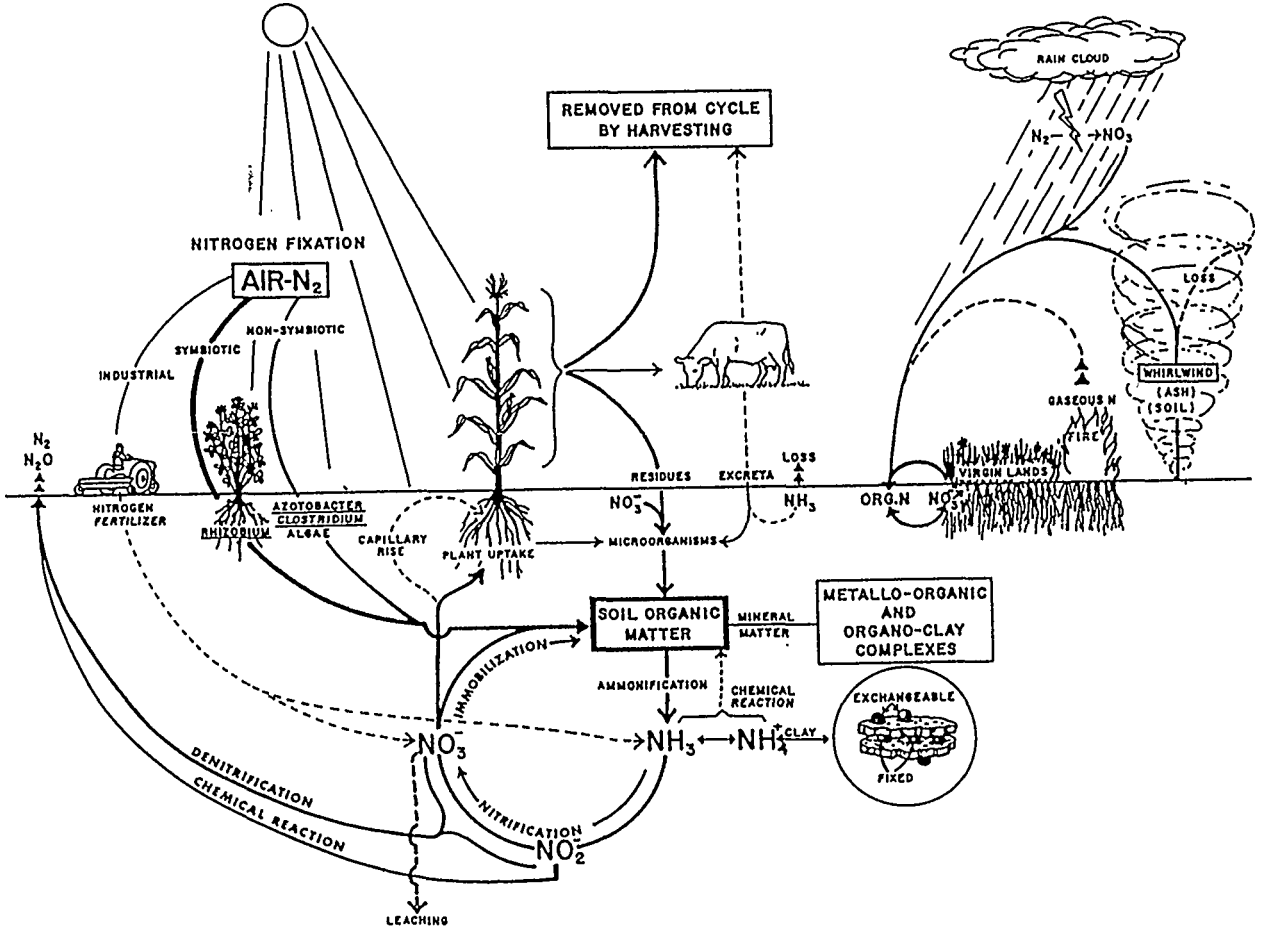


Figure 1. The N cycle in soil (Stevenson 1986).

negative, aerobic, and motile rods. The leguminous plants belong to the family Fabaceae. It is a large family defined as plants which bear seeds in pods. N_2-fixation by rhizobium occur when the bacteria live symbiotically in nodules on roots of the leguminous host plant.

Taxonomic classification of rhizobia is based on the symbiont host and the names of the bacterial species are in most cases based on the corresponding host plant. This classification indicates that the symbiosis is species-specific, but the situation is much more complex and the degree of host specificity varies tremendously (Sprent & Sprent 1990, van Rhijn & Vanderleyden 1995).

Table 1. *Rhizobium*-plant associations (van Rhijn & Vanderleyden 1995).

Rhizobium	Host plant(s)
Rhizobium meliloti	*Medicago, Melilotus* and *Trigonella* spp.
Rhizobium leguminosarum	
bv. viciae	*Pisum, Vicia, Lathyrus* and *Lens* spp.
bv. trifolii	*Trifolium* spp.
bv. phaseoli	*Phaseolus vulgaris*
Rhizobium loti	*Lotus* spp.
Rhizobium haukuii	*Astragalus sinicus*
Rhizobium ciceri	*Cicer arietinum*
Rhizobium tropici	*Phaseolus vulgaris, Leucaena* spp., *Macroptilium* spp.
Rhizobium etli	*Phaseolus vulgaris*
Rhizobium galegae	*Galega officinalis, G. orientalis*
Rhizobium fredii	*Glycine max, G. soja* and other legumes
Bradyrhizobium japonicum	*Glycine max, G. soja* and other legumes
Bradyrhizobium elkanii	*Glycine max, G. soja* and other legumes
Bradyrhizobium sp. strain *Parasponia*	*Parasponia.* spp.
Azorhizobium caulinodans	*Sesbania* spp. (stem nodulating)

Bacterial infection and nodule development

The nodulation process is controlled by genetic information from both symbionts. The infection and development of root nodules can be divided into several steps (Fig. 2). First there is a **recognition** of both symbionts, then the bacterium **attaches** to the root hair. The root hair is curled to facilitate the bacterium's entry into the root hair. There is a formation of an **infection tread** in which the bacterium moves towards the main root. The nodule is initiated and vegetative bacterial cells are transformed to enlarged pleomorphic forms called **bacteroids** which fix N. The plant produces **leghaemoglobin** to protect the N_2-fixing enzyme, nitrogenase, which is sensitive to oxygen. The presence of leghaemoglobin gives the nodules a pink colour (Sprent & Sprent 1990, van Rhijn & Vanderleyden 1995).

N_2-fixation and nitrogenase activity

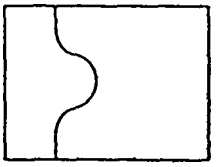
The N_2-fixing bacteria reduce N_2 to ammonia NH_3 (Fig. 3). As the triple bond of the N_2 molecule is very stable, N_2-fixation is an energy demanding process. For industrial production of N fertiliser the Haber-Bosch process is most frequently used, and it operates at 300-400°C at a pressure of 35 to 100 MPa.

The nitrogenase enzyme complex in rhizobia and in most N_2-fixing organisms is large, slow acting, and expensive in terms of ATP. It consists of two distinct proteins, the Mo-Fe protein and the Fe protein, and is inactivated by oxygen. This inactivation may be reversible or irreversible (Sprent & Sprent 1990). The summary of the N_2-reduction to NH_3 is:

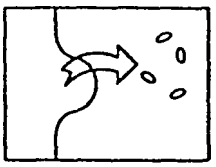
$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

The denitrification process

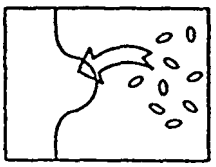
Denitrification is the major biological process through which fixed N in the



nitrogen limitation

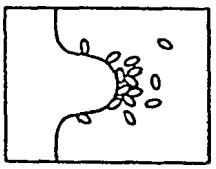


release of plant signals
(flavonoids)

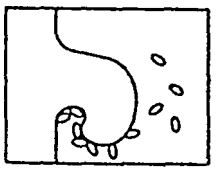


release of rhizobial host-plant-specific signals (oligosaccharides)

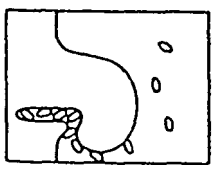
synthesis of (specific) extracellular polysaccharides (EPS, CPS, LPS, β-1,2-glucan)



rhizobial attachment
(rhicadhesin, plant lectin, cellulose microfibrils)



root hair curling
(oligosaccharides, extracellular polysaccharides, plant lectin)



infection thread formation
(oligosaccharides, extracellular polysaccharides, plant lectin)

Figure 2. Steps in the infection process of *Rhizobium* bacteria. Molecules possibly involved in the various steps are indicated. The order in which the steps are drawn is not necessarily the order in the nodulation process. (Smit et al. 1992.)

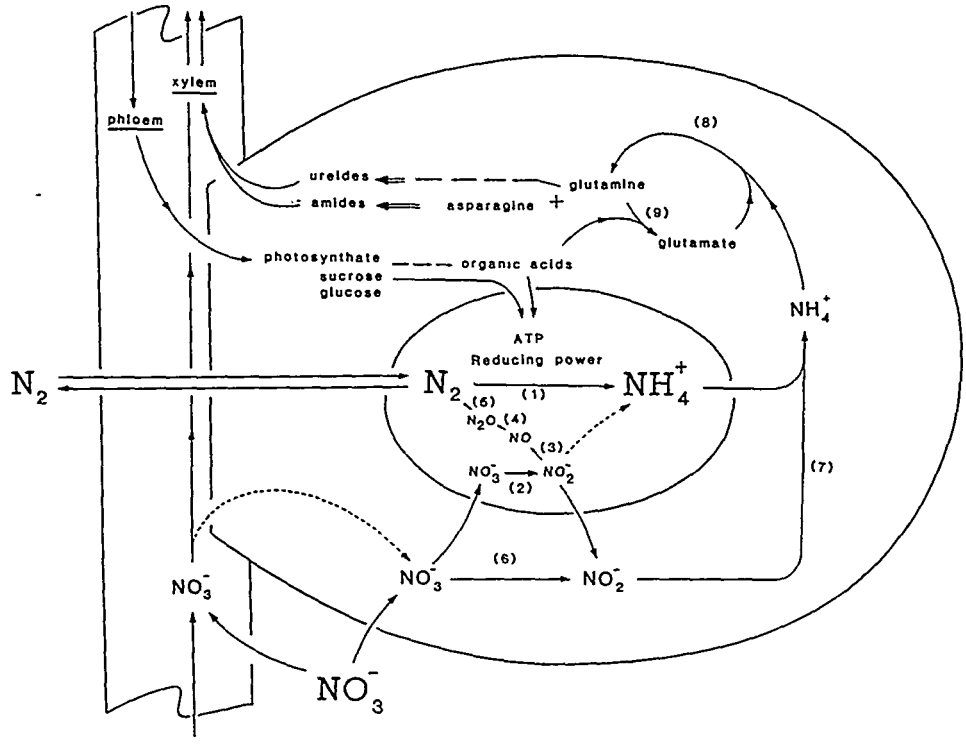


Figure 3. Diagram representing the interactions among N_2-fixation, NO_3^-- and NO_2^--reduction, and NH_4^+-assimilation in legume root nodules. Dashed lines indicate steps of minor importance. Enzymes in bacteroids: (1) N_2-ase, (2) NR, (3) NiR, (4) NO-reductase and (5) N_2O-reductase. Enzymes in the nodule plant fraction: (6) NR, (7) NiR, (8) glutamine synthetase (GS), and (9) glutamate synthase (GOGAT). (Becana & Sprent 1987.)

soil returns to the atmosphere. The general requirements for denitrification are: (1) the presence of bacteria possessing the relevant metabolic capacity; (2) suitable electron donors such as organic compounds or inorganic compounds such as reduced sulphur or molecular hydrogen, H_2; (3) anaerobic conditions or restricted O_2 availability and (4) available N-oxides, NO_3^- or NO_2^- as terminal electron acceptors (Firestone 1982).

The rate of denitrification is influenced by the **temperature**, and the denitrification activity increases with increasing temperature within the

range of enzyme activities. Within the range of neutral **pH** (pH 6-8) there is little effect on the denitrification rate (Firestone 1982), but an increase of the pH from 3.5 to 6.5 strongly stimulates the denitrification activity (Klemedtsson et al. 1987). Similarly, increased concentrations of both **oxidised N** and **reductant C** (organic substrate) accelerate the denitrification rate. Denitrification is a bacterial respiratory process that couples electron transport phosphorylation to the stepwise, sequential reduction of nitrogenous oxides (Zumft 1991).

Consequences of denitrification

The great interest in denitrification in soil is due to (1) the loss of fertiliser-N, (2) the contribution that atmospheric N-oxides, especially N_2O, are thought to have in actively depleting the stratospheric ozone layer, of acting as green house gases, and (3) the process being one of the major steps in the global cycling of N (Firestone 1982, Knowles 1982, Klemedtsson 1986).

Nitrous oxide

Nitrification, denitrification and nitrate ammonification are regarded as sources of the N_2O evolved from soils. Agriculture is one of the main producers of anthropogenic N_2O-emissions. The emission of N_2O derived from biologically fixed N is thought to be of the same magnitude or larger than that derived from fertiliser-N (Isermann 1994). In clay soils 65% (R^2) of the variation in N_2O-emission rates in Swedish soils could be explained by the soil NH_4^+-content and soil moisture (Robertson 1994). High N_2O-fluxes have been reported in tropical forests and savannas and rhizobium may contribute to these both directly, through denitrification by rhizobia, and indirectly through N-fixation (Anonymous 1990).

Air pollution by N_2O is not desirable. In the troposphere N_2O acts as a green house gas with a high global warming potential. The main sink for

N_2O is in the stratosphere through photochemical reactions with ozone. The residence time for N_2O in the atmosphere is long, 100-200 years. The concentration in air is 300 ppb and the annual rise about 0.2-0.3% (Anonymous 1990, Insermann 1994). Nitrification is an oxidising process and denitrification a reduction process and the emission of N_2O related to each of these processes depends on the level of water in the soil pore-space (Fig. 4).

Denitrification in soil

Denitrifying bacteria have a high energy requirement. The effects of plant roots on soil denitrification are two-fold: they take up oxygen for their own use and excrete easily decomposible organic matter (Stefanson 1972, Woldendorp 1962). The denitrification activity is greater in rhizosphere soils than in non-rhizosphere soils, but the total amount of denitrification is lower in those rhizosphere soils competing with plants for nitrogen (Smith & Tiedje 1979).

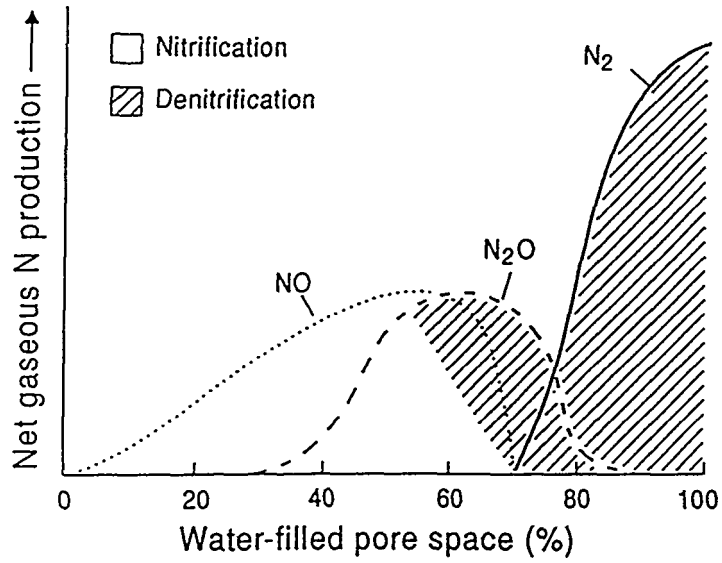


Figure 4. Model of the relationship between water-filled pore space of soil and relative fluxes of N-gases (Yoshinari 1993).

Parsons et al. (1991) found that measurements of neither the denitrifying enzyme activity nor of the denitrifying population with MPN were good predictors for the denitrification rate in the soil. Soil respiration and soil moisture were the most important influences on the denitrification rate in some soils, but not all. None of the parameters measured accounted for the variation in N gas losses from the soil.

Svensson et al. (1991) pointed to differences in the denitrification activity in soil sampled in different cropping systems in Sweden. Denitrification losses were greater in a lucerne ley (1.7 g N m^{-2} yr^{-1}) compared to a grass ley (0.4-1.4 g N m^{-2} yr^{-1}) and a barley crop (0.3-0.6 g N m^{-2} yr^{-1}). Related to fixed N in the lucerne ley the measured N loss was about 4%.

Erich et al. (1984) measured the N_2O-reduction under anaerobic conditions in fresh soil samples. They found no reduction activity in the lucerne planted soil, but in three other soils cropped with corn, grass and a forest soil, the N_2O was reduced.

Rhizobial denitrification

In 1888 when Beijerinck isolated the legume bacteria *Rhizobium leguminosarum* from a nodule of a garden pea, one of the reported characteristics of this bacterium was the ability to reduce NO_3^- to NO_2^- (Wilson 1947).

Zablatowics et al. (1978) were the first to demonstrate that species of *Rhizobium* are able to denitrify and Daniel et al. (1982) extended the testing and found the ability in some additional *Rhizobium* species.

NO_3^- and NO_2^- have a negative effect on root infection by rhizobia. The capacity to denitrify could be an advantage for the rhizobium symbiont, since denitrification decreases the concentration of NO_3^- and NO_2^- in the root zone and thus promotes the rhizobial infection. An ability to denitrify also increases the ability to survive and perhaps grow in the soil under periods of low oxygen pressure.

A survey of rhizobial denitrification showed considerable variation between species and between strains. Slow-growing rhizobia seem to be more common denitrifiers than fast-growing species, and generally, they also denitrify at a higher rate (O'Hara & Daniel 1985).

O'Hara et al. (1984) suspected N-losses by rhizobial denitrification in New Zealand soils of a similar magnitude to the N gained by symbiotic rhizobial N_2-fixation even when the numbers of bacteria were quite moderate.

Bertelsen (1990) was looking for a correlation between the denitrification potential and the N_2-fixing efficiency in *R. leguminosarum* without success. The denitrification potential of these strains was of the magnitude of 0.01 to 0.08 mg N g^{-1} bacteria h^{-1}, which was about a quarter of that of a denitrifying *Pseudomonas clororaphis* strain under identical conditions.

Garcia-Plazaola et al. (1993a) found that most of the thirteen *R. meliloti* strains investigated were able to reduce NO_3^- to N_2O or N_2. However, the bacteria were not able to use NO_3^- as an electron acceptor for ATP generation and growth at low oxygen tensions.

The denitrification activity in some fast growing rhizobium species that do not grow anaerobically, is considered to be a detoxifying process that reduces the concentration of the toxic compound NO_2^- (Casella et al. 1988, Garcia-Plazaola et al. 1993a).

The contribution of *R. meliloti* to the soil denitrification activity was investigated by Garcia-Plazaola et al. (1993b). They inoculated the rhizobium into unplanted soil and no contribution by inoculant strains was found in the non-sterilised soil. In the sterilised (autoclaved) soil they found an increase in the denitrification activity caused by increased soil moisture, temperature, and number of inoculant bacterial cells.

AIMS OF THE STUDY

The high denitrification activity in lucerne planted soil, reported by Svensson et al. (1991), and other reports regarding rhizobial denitrification (O'Hara & Daniel 1985) were the basis for the present investigation of denitrification by *R. meliloti*. The aim of my study was to investigate whether some strains of *R. meliloti*, used as inoculants, were responsible for the considerable denitrification activity in lucerne cultivated soils in Sweden.

EXPERIMENTS

Thirteen *R. meliloti* strains, called 23, 27, 29, 36, 50, 52, 53, 56, 57, 58, 59, 1021, 6963, and two reference strains *B. japonicum* 526 and *P. aeruginosa* 10261, were used. They were all obtained from the bacterial culture collection at the Department of Microbiology, Swedish University of Agricultural Sciences (SLU). In experiments with a host plant we used seeds of lucerne *Medicago sativa* L. (cv. SW Vertus). (**I.**)

The denitrification activity of *R. meliloti* was examined in free-living cells and in nodules on roots of intact plants (**I**).

The nitrogenase activity in *R. meliloti* strains was evaluated with the acetylene reduction assay (**I**).

In a closed bioreactor one strain each of *R. meliloti* and *P. aeruginosa* were investigated separately for oxygen consumption, NO_3^--respiration, and production and consumption of intermediates in the denitrification process (**I**).

The presence of the nitrous oxide reductase (Nos) activity was studied in most of the strains (**I**). Bacterial DNA from these strains was examined for the presence of hybridisation signals to the *nosZ* probe from *Pseudomonas stutzeri* Zobell (ATCC14405; **I**).

In a field plot experiment four inoculant strains with different denitrifying capacities were used, two strains each with a "low" and with a "high" capacity to denitrify. The treatments were randomly distributed in 3 blocks. Rye grass was used as a reference crop and soil cores were sampled after harvest the second year. Actual denitrification activity was investigated in soil cores, while the potential denitrification activity was measured in a soil slurry, as was the potential nitrification activity (**II**). Before sowing and bacterial inoculation of the field plot area, the numbers of indigenous *R. meliloti* bacteria were studied in the soil using the MPN method (**II**).

Strains with different denitrifying capacities were used in a complementary soil experiment using soil from the experimental field. The soil was γ-sterilised or non-sterililised and planted with lucerne or unplanted and supplied with rhizodeposition from lucerne roots (**II**).

RESULTS AND DISCUSSION

Pure culture studies with *Rhizobium meliloti*

A number of inoculant strains of *R. meliloti* were tested, with aim of investigating whether this species was responsible for the high denitrification activity in soil cropped with lucerne, as reported by Svensson et al. (1991).

The studies of potential denitrification activity in pure cultures with free-living cells and cells in nodules on lucerne plants, showed a great variety among the thirteen *R. meliloti* strains investigated. Four of the strains were more active than the others, and this difference was shown in the two experiments with free-living cells and nodulated plants. Compared with the two reference strains, *Bradyrhizobium japonicum* and *Pseudomonas aeruginosa*, the highest activity in *R. meliloti* was one-half and one-fourth, respectively. However, most *R. meliloti* strains were very much lower or even without detectable activity (Table 1 in **I**).

All strains except one showed nitrogenase activity. The Fix$^-$ strain 50 induced many white nodules containing bacterial cells but no bacteroids, and no acetylene reduction activity was detected. This strain is one of the four with high denitrification capacity (Table 1 in **I**).

The *nos*Z-probe derived from *P. stutzeri* hybridised to *Pst*I fragments of DNA from 8 of the 9 investigated *R. meliloti* strains. The size of the fragments was 5 or 7 kb. Nos activity was only found in three strains and these all showed a high denitrification activity. The Fix$^-$ strain 50, which was "high" in denitrification activity, was Nos$^-$ and did not hybridise to the *nos*Z-derived probe (Table 2 in **I**).

In the bioreactor experiment we found similarities and differences between the two strains belonging to *R. meliloti* and *P. aeruginosa*, respectively (Fig. 1 in **I**). They both consumed almost all dissolved oxygen before the reduction of NO_3^- started. There were differences regarding the accumulation of NO_2^-. *P. aeruginosa* accumulated high concentrations of NO_2^-, while *R. meliloti* only accumulated a very small amount. They both accumulated N_2O, but the pseudomonad did not reduce the N_2O, not even when all NO_3^- was consumed. However, the rhizobium strain reduced N_2O even when there was NO_3^- left. The pseudomonad is phenotypically Nos$^+$, but the reduction of N_2O was not as fast as for the Nos$^+$ rhizobium strains (Table 2 in **I**).

Denitrification activity in soil inoculated with *Rhizobium meliloti*

No contribution from inoculant strains to the total denitrification activity in the field plot experiment was found. The denitrification activity was high in all lucerne planted soils independent of inoculation. The actual denitrification activity was high in lucerne planted soil and the difference between actual and potential denitrification was low. In grass planted soil on the other hand, actual denitrification activity was lower than in unplanted soil, but potential denitrification was equal to the activity in lucerne. Both crops increased potential denitrification activity in the soil, while the

presence of lucerne also increased actual denitrification activity (Fig. 1 in **II**).

Direct emission of N_2O in the field plot experiment was measured with closed chambers (Fig. 5). The frame was fixed in the soil and covered for 1 h for sampling of gas to investigate the N_2O-emission. The emission was measured on five occasions during 1993 (Fig. 6). No differences were found within inoculated lucerne treatments, but compared with grass and uninoculated lucerne, we found small differences (Fig. 6b). The emission rate in grass and uninoculated lucerne were highest 27 August, when the emission rate in inoculated lucerne was relatively low. The highest emission rates in inoculated lucerne were found at the latest sampling occasion, 7 October. Grass and uninoculated lucerne was fertilised with more N than inoculated lucerne, to compensate for N, fixed by the inoculants. There might be a N-limitation in these systems in October. The N_2O-emission was low in all treatments in June and July when the precipitation was small. Too few investigations were made to evaluate the N_2O-emission in the field but the results we got was not alarmingly high in any treatment.

In the experiment with γ-sterilised and non-sterilised soil from the experimental field, we found a great influence on the denitrification rate, which was doubled, when rhizodeposition from lucerne roots was added to soil inoculated with *R. meliloti* (Table 4 in **II**). The rhizodeposition was also utilised for denitrification by the pseudomonad and the indigenous soil population (Tables 4 and 5 in **II**).

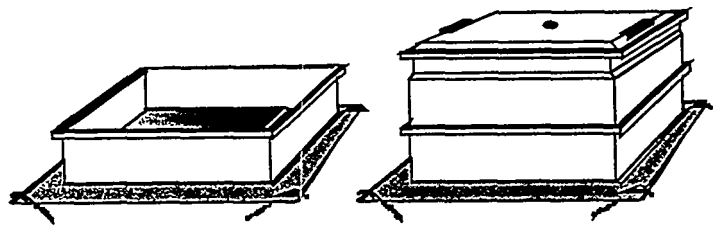


Figure 5. A frame (0.45 x 0.45 m) enclosed each subplot that were covered for 1 h during sampling of gas to measure the direct emission of N_2O in field. Frame and cover were both made in stainless steel.

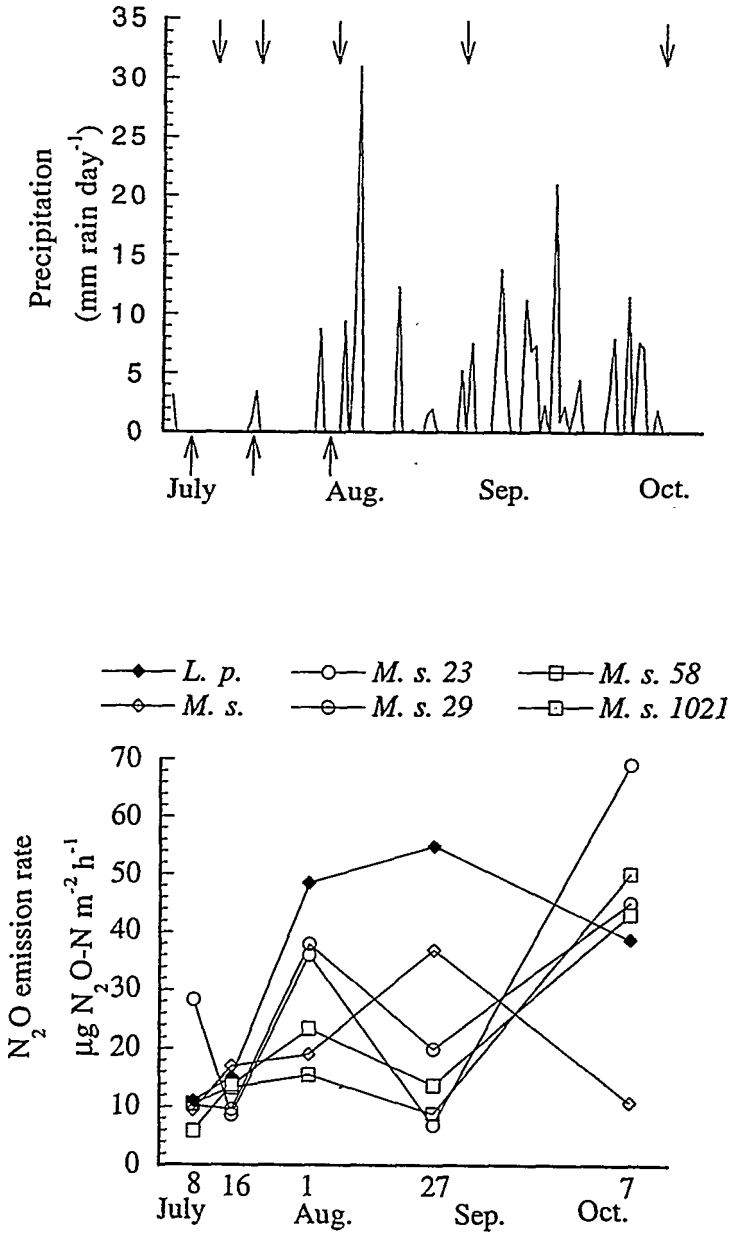


Figure 6. The field plot experiment was divided into three blocks and subplots were planted with lucerne *Medicago sativa* (*M. s.*) or ryegrass *Lolium perenne* (*L. p.*). Lucerne was uninoculated or inoculated with one of four different *R. meliloti* strain: 23, 29, 58, and 1021, of which the two former are "low" denitrifying strains and the two latter are "high" denitrifying strains. The figure (A) shows precipitation, sampling of gas (↓), and N-fertilisation (↑) during the establishing year 1993. The N_2O emission rates (B) are mean values of the three blocks. The subplots were fertilised 2, 15 and 30 July, and gas was sampled 8, 16 July; 1, 27 August and 7 October. The crops were harvested twice; 30 July and 30 August.

The acetylene inhibition method (Yoshinari & Knowles 1976) was used to investigate the denitrification activity. Acetylene is used to inhibit the reduction of N_2O to N_2 and accumulated N_2O is analysed on a gas chromatograph (**I**). We have used this method for all denitrification studies since it is a common method, and cheap and easy to use.

When acetylene was added in the middle of the incubation period, we found that the denitrification activity in different strains was influenced in different ways. Two of four *R. meliloti* strains were negatively influenced (Fig. 7). When acetylene was added the denitrification rate decreased by ca. 60% for the two *R. meliloti* strains 50 and 1021. On the other hand the rate increased by ca. 50% in the *B. japonicum* strain, which is Nos^- (Table 2 in **I**). The *R. meliloti* strain 50, is a Fix^- and Nos^- strain, that did not hybridise to the *nosZ*-derived probe from *P. stutzeri* (Table 2 in **I**), while strain 1021 is Fix^+, Nos^+ and $nosZ^+$ like the other two *R. meliloti* strains 58 and 6963 in the test. Further investigations are needed to understand the effect of acetylene on the denitrification process in *R. meliloti* strains 50 and 1021 and *B. japonicum* 526.

CONCLUDING REMARKS

The results obtained in this investigation showed a great difference regarding the denitrifying capacity in strains of *R. meliloti.* Most investigated strains denitrified with a very low rate. The N_2-fixing *R. meliloti* strains with a high denitrification activity reduced effectively N_2O to N_2. In soil experiments the addition of rhizodeposition from lucerne roots markedly increased the denitrification rate by *R. meliloti* strain 1021. However, when this strain was used as inoculant in the field plot experiment, we found no contribution to the total denitrification activity in the soil. The carbon source is of a great importance for the denitrification rate. Rhizodeposition from lucerne roots seems to be a preferable carbon source for *R. meliloti*. For some denitrifying strains a disadvantage by using the acetylene inhibition method was found. For reliable estimates of the potential denitrification capacity, attention should be paid to these observations in future studies.

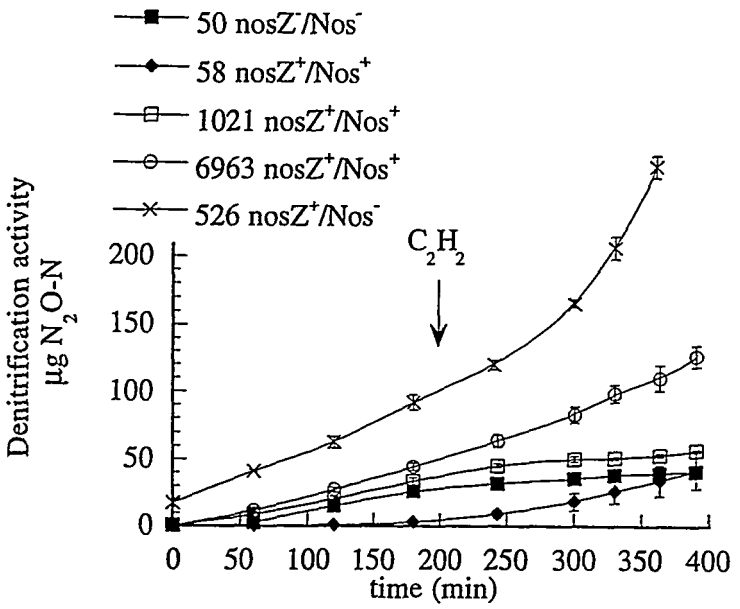


Figure 7. The effect of acetylene (10%) on the denitrification activity by rhizobium strains. The accumulated denitrification product is expressed in µg N_2O-N. The bacteria used were *R. meliloti* strains 50, 58, 1021, and 6963, and *B. japonicum* 526. Mean ± SE (n = 3) of the total amount of N_2O-N in the experimental vessel.

From an environmental point of view the *Rhizobium*-leguminous symbiosis is preferable compared with use of industrial N-fertilisers, that require much energy to produce, transport and spread. Our results showed a higher actual denitrification capacity in lucerne planted soil than in grass subplots fertilised with N. No high emissions of N_2O were found in any crop or inoculant treatments. The total denitrification activity was not influenced by using high denitrifying *R. meliloti* strains as inoculants. Preferably legumes would be cultivated mixed with a non-fixing crop, followed by a catch crop to effectively utilise the N fixed by rhizobium to avoid accumulation of high quantities of N in the soil. However, more investigations are desirable regarding the nitrogen transformation, NO_3^--leakage, N_2O-emission, and denitrification by rhizobia in leguminous planted soils.

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I

Denitrification by *Rhizobium meliloti*

1. Studies of free-living cells and nodulated plants

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ABSTRACT

The denitrification activity was investigated in thirteen *Rhizobium meliloti* strains, of which most are proposed inoculant strains and one is deficient in nitrogen fixation (Fix$^-$). One strain each of the species *Pseudomonas aeruginosa* and *Bradyrhizobium japonicum* were used as references. We found a great variety regarding the denitrification capacity within *R. meliloti* strains, most of them with low or no detectable activity. Four strains showed a denitrification rate 200 times higher or more than the strains with low activity, and the highest rate was found in *R. meliloti* 6963. The average denitrification rate (μg N_2O-N mg^{-1} protein h^{-1}) by free-living bacteria was 100, 50, and 26 for *P. aeruginosa*, *B. japonicum*, and *R. meliloti* 6963, respectively. Of nine *R. meliloti* strains tested, three expressed nitrous oxide reductase (Nos) activity, while eight strains gave positive hybridisation signals to a gene probe derived from *nosZ* of *Pseudomonas stutzeri*. The Fix$^-$ strain, with a great denitrification capacity, was phenotypically Nos$^-$ and did not hybridise to the *nosZ* gene. Bacterial growths during nitrate respiration as well as consumption and production of nitrogenous oxides were investigated in a closed bioreactor with a limited access for oxygen. The growth rate of *R. meliloti* was more influenced by decreasing oxygen pressure than *P. aeruginosa*. Both bacteria accumulated detectable levels of nitrite and nitrous oxide during the nitrate respiration, but *R. meliloti* seems to be much more sensitive for accumulation of nitrite than the pseudomonad.

Key words: *Rhizobium meliloti*, denitrification, acetylene inhibition, N_2O, *Medicago sativa*, lucerne, nitrogen fixation, Nos, *nosZ*, hybridising restriction fragment, bioreactor, anaerobic growth, *Pseudomonas aeruginosa*, *Bradyrhizobium japonicum*, *Pseudomonas stutzeri*.

INTRODUCTION

Rhizobium and *Bradyrhizobium* are genera that consist of diazotrophic bacterial species living in symbiosis with leguminous plants. Symbiotic nitrogen fixation is a process of ecological importance for input of nitrogen into agriculture and natural ecosystems. In Swedish agriculture *Rhizobium meliloti* is the most important rhizobium inoculant species, since the number of indigenous bacteria in the soil is low or even non-existent, and lucerne is a common leguminous crop.

Denitrification is a dissimilatory reduction of ionic nitrogen oxides as nitrate (NO_3^-) and nitrite (NO_2^-) via gaseous oxides of nitrogen, nitric oxide (NO) and nitrous oxide (N_2O), to dinitrogen (N_2). Many soil bacteria are able to use nitrogen oxides as terminal electron acceptors for the generation of energy in the absence of oxygen (Tiedje, 1982). There are many reports dealing with denitrification by rhizobia (O'Hara & Daniel, 1985), particularly concerning the slow-growing species *Bradyrhizobium japonicum*. Reports on denitrification in *R. meliloti* by Chan *et al.* (1989) and Garcia-Plazaola *et al.* (1993) show that the denitrification rate among strains differed widely. In half the number of strains investigated by Garcia-Plazaola *et al.* (1993) denitrification stopped with N_2O as the final product, while dinitrogen earlier was considered to be the predominant final product in *R. meliloti* denitrification (Daniel *et al.*, 1982).

Denitrification activities in cropping systems in Sweden, were investigated by Svensson *et al.* (1991), who found higher denitrification activity in soil sampled in the lucerne ley. They proposed, among other things, that the inoculated rhizobia could be responsible for the increased activity. To verify this a study of denitrification in *R. meliloti* was initiated.

The objectives of the present study were: (a) to investigate the denitrification capacity in a number of proposed inoculant strains of

R. meliloti; (b) to compare *R. meliloti* with denitrifying reference strains of the species *B. japonicum* and *Pseudomonas aeruginosa*, regarding the denitrification activity; (c) to make comparisons between one *R. meliloti* strain and one *P. aeruginosa* strain regarding consumption of NO_3^- and production and consumption of NO_2^- and N_2O during nitrate respiration in a closed bioreactor; and (d) to investigate the presence of *nosZ* like genes and Nos activity in *R. meliloti*.

MATERIALS AND METHODS

Organisms

Thirteen strains of *Rhizobium meliloti* and one strain each of *Bradyrhizobium japonicum* and *Pseudomonas aeruginosa* were obtained from the collection of bacterial strains at the Department of Microbiology, Swedish University of Agricultural Sciences. Information about *R. meliloti* strains 23, 29, 50, 58, 1021, and 6963, *B. japonicum* strain 526 and *P. aeruginosa* strain 10261 is listed in Table 1. The following strains were also used: *R. meliloti* 27 (received from Denmark), 36 (received from Australia), 52, 53, 56, 57, and 59 (all strains isolated in the surroundings of Uppsala, Sweden).

Escherichia coli DH5 (Hanahan, 1983) was used as a host strain for production of plasmid DNA. The plasmid pNS220, harbouring the *nosZ* gene encoding the respiratory nitrous oxide reductase of *Pseudomonas stutzeri* strain ZoBell (ATCC14405), was a kind gift from professor Walter G. Zumft, University of Karlsruhe, Karlsruhe, FRG (Viebrock & Zumft, 1988).

Seed of lucerne, *Medicago sativa* L (cv. SW Vertus), was obtained from Svalöf Weibull (SW) AB, Sweden.

Media and culture conditions

Strains of *Rhizobium* and *Bradyrhizobium* were maintained at 4°C on yeast extract mannitol agar (Vincent, 1970), *Pseudomonas* on nutrient agar (Oxoid), and *E. coli* on Luria-Bertani (LB) agar. Cells were cultured on a rotary shaker (175 rpm) at 25°C on glutamate yeast extract (GYE) medium pH 6.8 (El Hassan *et al.*, 1985). For denitrification experiments the GYE medium was supplemented with 20 mM potassium nitrate (KNO_3) and cultivation was performed in closed bottles. To induce the production of denitrification enzymes the oxygen pressure was decreased by the bacteria themselves during aerobic respiration.

Cultivation of lucerne plants

For denitrification and nitrogen fixation experiments seeds of *M. sativa* were surface sterilised with 95% ethanol and acidic 0.1% $HgCl_2$ (Vincent, 1970), rinsed 8 times with sterile water before scattered onto water agar plates. For seed germination plates were incubated upside down for 2 days in darkness at room temperature. One seedling per tube was planted on an agar slope of 10 ml nitrogen-free Jensen medium (Vincent, 1970). The 26 ml tubes, which have an edge for sealing with rubber septa, were covered with transparent plastic caps to allow light to pass and exchange of air under plant growth and nodule development. The bacterial inoculation was made with 1 ml of a bacterial suspension (10^7 cells ml^{-1}). For nodulation tubes were kept for 7 weeks at room temperature under a greenhouse lamp (Philips HPI-T 400 W) with 16 h day.

Denitrification experiments

The acetylene (C_2H_2) inhibition technique was used for investigation of denitrification (Yoshinari, 1976). Anaerobiosis was obtained by

flushing and evacuating experimental vessels 6 times with N_2 (99.98%) through a 0.2 µm sterile filter, and venting to atmospheric pressure. C_2H_2 was injected aseptically to a content of 10% of the headspace.

Denitrification studies by free-living bacteria were performed with cells in an early stationary growth phase. Ten ml cell suspensions were transferred to a sealed bottle (118 ml) containing 10 ml fresh medium and incubated on a rotary shaker (175 rpm) at 25°C. Five gas samples were withdrawn at intervals during 4 h of incubation for *R. meliloti* and during 1 h of incubation for *B. japonicum* and *P. aeruginosa*.

Measurements of the denitrification activity in nodules were performed with intact 7-week-old plants. To induce the production of denitrification enzymes, 12 mM KNO_3 was added two days before assay. When the assay of denitrification started, the nitrate solution was replaced with fresh 20 mM KNO_3 solution, the tubes were sealed with rubber septa and the headspace was replaced with N_2 mixed with 10% C_2H_2.

During sampling, tubes with plants were incubated for 24 h at room temperature under a greenhouse lamp. Gas samples of 0.5 ml were withdrawn from the headspace with a gas-tight plastic syringe and diluted in sealed 12.5 ml glass vials with air. The glass vials were sealed with Teflon coated rubber septa. The gas standards were treated in the same way and stored until analysed for N_2O content. The Bunsen coefficient was used to take the amount of dissolved N_2O in the medium into account (Tiedje, 1982). The denitrification rate is calculated from the measurements of N_2O accumulation sampled during the first 4 h of incubation.

Assay of nitrogen fixation

The acetylene reduction assay was used to determine the nitrogenase activity (Bergersen, 1980). The sealed tubes with intact plants were supplied with 10% C_2H_2 by injection. During the 4 h incubation period five samples of 0.5 ml gas were withdrawn and transferred into sealed 3.2 ml glass vials before analysis of the ethylene (C_2H_4) formed by reduction of C_2H_2.

Nitrate respiration and growth during diminishing supply of oxygen

Growth and nitrogen transformations by *R. meliloti* strain 1021 and *P. aeruginosa* strain 10261 were investigated using a 1.3 litre bioreactor BRO.4 (Belach Biotechnology AB, Stockholm, Sweden). Bacteria were cultured at 28°C under strictly controlled conditions in a closed reactor vessel containing 600 ml of GYE-medium supplied with 20 mM KNO_3, pH 7.00±0.05, and with a stirring rate of 450 rpm. Stirring rate, temperature, and pH were kept constant and values were registered every 5 minute to a computer, which also registered the oxygen tension in the medium. Alterations in growth, concentrations of NH_4^+, NO_3^-, NO_2^-, and N_2O were followed by sampling of 3 ml liquid and 0.5 ml gas at intervals. N_2 was added to keep atmospheric pressure in the vessel when sampling. To follow the bacterial growth 1 ml of the liquid sample was immediately used for measurement of the absorbance at λ 600 nm, while remaining 2 ml was centrifuged at 10^4 rpm for 5 min at 4°C to remove cells. The supernatant was analysed for the contents of NH_4^+, NO_3^- and NO_2^- as described below. The gas samples were transferred to glass vials as described above, and analysed for the N_2O content.

Molecular biology methods

Restriction endonucleases and other enzymes were purchased from Boehringer Biochemicals (Mannheim, FRG) and Promega (Madison, WI, USA) and used according to the manufacturers' recommendations.

Chromosomal DNA of bacterial strains was prepared according to Chen & Kuo (1993), cleaved with *Pst*I and separated on an agarose gel. For Southern blotting, DNA fragments were transferred to nylon filters, Hybond-N 0.45 Micron (Amersham, Great Britain) using a vacuum-blot apparatus (VacuGene, Pharmacia LKB, Bromma, Sweden).

Plasmid pN220 was transformed into *E. coli* DH5 by electroporation (Dower *et al.*, 1988). Plasmid DNA from *E. coli* was then prepared by using NUCLEOBOND (Macherey-Nagel GmbH, Düren, FRG) according to the protocol supplied by the manufacturer, cleaved with *Pst*I and fractionated on an agarose gel. A 1.2 kb *Pst*I fragment located within the *nos*Z-gene was cut out, electro-eluted and labelled using the DIG (digeoxigenin) DNA-labelling and detection kit (Boehringer, Germany) according to the protocol supplied by the manufacturer.

After hybridisation at 42°C over night the filters were washed 2x5 min in 2xSSC (standard saline citrate), 0.2% SDS (sodium dodecyl sulphate) at 25°C followed by 2x15 min in 0.2xSSC, 0.1% SDS at 60°C.

Assay of nitrous oxide (Nos) reduction activity

A defined medium and a method described by Chan & Wheatcroft (1993) were used. The Nos enzyme activity was examined by measuring the reduction of about 1000 ppm N_2O initially in the

headspace of 21 ml sealed tubes with 6 ml cell suspension in a late exponential growth phase. Experimental tubes were incubated for 48 h at 25°C. One sample was taken at the start and a second at the end of the incubation period.

Analytical methods

N_2O was quantified by gas chromatography on a Chrompack, Model 9000, equipped with a Hewlett Packard headspace sampler, Model HP19395A, injecting 250 µl. Helium was used as carrier gas (4 ml min^{-1}), and N_2 as make up gas (35 ml min^{-1}). The capillary column, Poraplot Q (25 m x 0.53 mm) operated at 35°C, the injector at 125°C, and the ^{63}Ni electron capture detector worked at 350°C.

C_2H_4 was analysed by manual injection of 1 ml gas sample to a Packard gas chromatograph, Model 428, equipped with a flame ionisation detector using N_2 as carrier gas (20 ml min^{-1}). The Porapak T column (2 m x 2 mm) was operated at 125°C, and injector and detector temperatures were 150°C respectively.

NH_4^+-N was measured using a colorimetric assay based on the Berthelot reaction (Weatherburn, 1967). Colorimetric methods were used to measure the amount of NO_2^--N (Nicholas & Nason, 1957) and NO_3^--N (Cawse, 1967). The cell protein content was determined by the Lowry method according to Hanson & Phillips (1984).

RESULTS

Denitrification and nitrogen fixation by *R. meliloti* strains

Activity rates for denitrification and nitrogen fixation are presented in Table 1 for six *R. meliloti* strains. Denitrification rates by free-living cells of *B. japonicum* and *P. aeruginosa* are included for comparison.

The highest denitrification rates in *R. meliloti* were found for strains 50, 1021, 58, and 6963, which were 10-50% of the rate by *B. japonicum* and 5-25% of that found for *P. aeruginosa*. Remaining *R. meliloti* strains are equal to rhizobium strains 23 and 29, regarding the denitrification activity. The strains with a high denitrification rate as free-living cells were also high denitrifying in nodules on lucerne plants. Low denitrifying *R. meliloti* strains had activities that were ≤20 ng N_2O-N mg^{-1} protein h^{-1} in GYE medium and ≤3 ng N_2O-N g^{-1} nodule fresh wt) h^{-1}, respectively. Most low denitrifying strains showed no detectable denitrification activity in these experiments, but have shown low activity in experiments when undiluted samples were analysed (data not shown).

The Fix^- strain 50 induced many white nodules containing a great number of rod-shaped bacterial cells, but no bacteroids were present and no acetylene reduction activity was detected. The nitrogenase activity among the other *R. meliloti* strains varied from 2 to 9 µmol C_2H_4 g^{-1} nodule (fresh wt) h^{-1}. Strain 6963, an LPS mutant (Tn*5* in the *lps*B gene) defective in symbiosis (Lagares *et al.*, 1992), induced very few nodules with low nitrogenase activity.

Nitrate respiration and anaerobic growth

In the bioreactor experiments *P. aeruginosa* consumed all dissolved oxygen within 1 h compared with 5 h for *R. meliloti* strain 1021 (Fig. 1A, B). The growth rate of both strains was influenced by decreasing oxygen pressure, but to a less degree for the pseudomonad. The final absorbency at 600 nm under NO_3^--respiring conditions was 1.2 for *R. meliloti* and 1.5 for *P. aeruginosa*. The corresponding values for aerobic growth were 2.3 and 3.0, respectively. *R. meliloti* did not really stop growing during the study period, but the growth rate gradually decreased and nearly came to a standstill at the end of the experiment. The growth of *P. aeruginosa* stopped when all NO_3^- had been reduced to N_2O. The incubation time

in the experiment was 3 times longer for *R. meliloti* than for *P. aeruginosa* depending on the differences in growth rate between these two strains.

Transformation of nitrogen in the reaction system is shown in Fig. 1C, D as total amounts of NO_3^--N, NO_2^--N, N_2O-N and NH_4^+-N. A continuous reduction of NO_3^- and an accumulation of NO_2^-, N_2O as well as mineralised NH_4^+, was observed for both strains. We observed an apparent distinction between the two strains regarding the accumulation of NO_2^-, not only quantitatively but also regarding the length of the period it remained accumulated. The pseudomonad accumulated about 250 times more NO_2^- than the rhizobium and the level remained high for several hours.

Detection of nosZ homology and Nos activity in *R. meliloti*

In the Southern blotting experiment, all but one of 9 investigated *R. meliloti* strains hybridised to the *nosZ* gene from *P. stutzeri* used as a probe (Table 2). Two different sizes of the hybridising fragments were noticed, 3 strains with a 7 kb fragment and the other 5 strains with a 5 kb fragment. This may indicate a highly conserved structural gene within each group.

Nos activity was found only in bacterial strains with a great denitrification activity (Table 2). The *P. aeruginosa* strain and the *R. meliloti* strains 58, 1021, 6963 were all phenotypically Nos^+ and reduced all or almost all added N_2O. *B. japonicum*, the Fix^- strain 50 and the other five investigated *R. meliloti* strains, did not reduce any detectable quantity of N_2O during the 2 day incubation period.

DISCUSSION

Denitrification activity rates divided the investigated *R. meliloti* strains

into two groups (Table 1). Four *R. meliloti* strains showed a rate at least 200 times higher than the other. The great variety in denitrification activity rates within the investigated strains of *R. meliloti* verifies the results presented by Garcia-Plazaola *et al.* (1993). In other experiments with undiluted samples (results not published) we have measured low activities by most of these strains not showing activity in this study. Thus all strains without detectable or with low denitrification activity are grouped together as "low denitrifying strains". Further investigations are needed to verify that some strains are completely lacking the capacity to denitrify.

The highest denitrification rate of *R. meliloti* in this study was about half that of *B. japonicum* and one-fourth compared with that of *P. aeruginosa*. The denitrification activity in *B. japonicum* strain 526 is almost equal to that in *B. japonicum* Wisc. 505 as reported by O'Hara *et al.* (1983).

Three high denitrifying *R. meliloti* strains 58, 1021 and 6963 were Nos^+ and consumed all the available N_2O (Table 2). In these *R. meliloti* strains *Pst*I fragments of approximately 5 kb, which hybridised to the *nos*Z probe from *P. stutzeri*, were detected. The low denitrifying *R. meliloti* strains were all phenotypically Nos^-, but two hybridising *Pst*I fragments of 5 and 7 kb, respectively, were detected in this group. *P. aeruginosa* was Nos^+, but did not reduce all N_2O under the incubation period. A hybridisation signal for the *nos*Z probe was found in all investigated bacterial strains except the Fix^- strain 50. This high denitrifying strain was also Nos^-. *B. japonicum* was Nos^-, but possessed a *nos*Z like gene on a 13 kb *Pst*I fragment compared with the 5 and 7 kb fragments in the *R. meliloti* strains and the 3.5 kb fragment in *P. aeruginosa*. In a previous study Chan & Wheatcroft (1993) reported that two thirds of the *R. meliloti* strains possessed a *nos*Z like gene located on 6.6 and 10.1 kb fragments when cleaved with *Pst*I and *Eco*RI, respectively.

Both *R. meliloti* and *P. aeruginosa* reduced NO_3^- to NO_2^-, and NO_2^-

further to N_2O (Fig. 1C, D). Also the Nos enzyme in *R. meliloti* seems to be made active after about 25 hours incubation. This statement is based on the fact that the reduction of NO_3^- continued, while the accumulation of N_2O ceased. The growth rate of *P. aeruginosa* was less influenced by decreasing oxygen pressure than that of *R. meliloti* (Fig 1A, B) which grew slowly anaerobically. Therefore the two criteria, anaerobic growth and emission of N_2O from NO_3^- to claim denitrification, was met (Mahne & Tiedje, 1995). The cease in growth by *P. aeruginosa* appeared when all NO_3^- was consumed, and we suggest far too short incubation time to be the reason for the coincident accumulation of N_2O, since the bacterium is able to use N_2O (Table 2). Voβwinkel *et al.* (1991) found factors such as pH, concentration of NO_2^- and length of the incubation time to influence the production and reduction of NO and N_2O by *P. aeruginosa*. They had to grow their *P. aeruginosa* strain for 3 days until the Nos activity was detected.

Casella *et al.* (1988) and Garcia-Plazaola *et al.* (1993), they proposed the role of denitrification in rhizobia, for example *R. meliloti* and *R. "hedysari"*, to be a detoxifying process. Two of the intermediates in the denitrification pathway, NO_2^- and NO, are toxic compounds and inhibitors of the nitrogenase enzyme (Trinchant & Rigaud, 1982). We did not measure the amount of NO in the bioreactor experiment, but the *R. meliloti* 1021, used in our experiment, seems to elude accumulation of NO_2^-.

The results obtained in this investigation show that strains of *R. meliloti* have different capacities to denitrify. In the denitrification study by Svensson *et al.* (1991) in cropping systems in Sweden, a greater denitrification activity in a lucerne ley than in grass and barley crops was reported. To investigate the responsibility of the inoculated *Rhizobium* strains in this respect a field experiment has to be made using inoculant strains with known denitrification capacities.

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Table 1. *Denitrification by Rhizobium meliloti with 20 mM KNO_3. GYE medium was used for experiments with free-living cells and Medicago sativa L. nodulated plants were used for symbiotic experiments. Denitrification is expressed as formed N_2O-N, using the acetylene inhibition method and nitrogen fixation in corresponding plants is expressed as formed C_2H_4, using the ARA method. Bradyrhizobium japonicum 526 and Pseudomonas aeruginosa 10261 were used as reference bacteria. Mean value ± SE (n=3)*

Bacteria		Denitrification		N-fixation
Strain	Source of information	Free-living $\mu g\ mg^{-1}$ prot h^{-1}	Symbiotic $\mu g\ g^{-1}$ nod (fw) h^{-1}	Symbiotic $\mu mol\ g^{-1}$ nod (fw) h^{-1}
R. m 23	van Schreven, 1958[c]	$\leq 2 \cdot 10^{-2}$	$\leq 3.10^{-3}$	7.3 ± 2.0
R. m 29	Mårtensson *et al.*, 1984	$\leq 2 \cdot 10^{-2}$	$\leq 3.10^{-3}$	3.3 ± 1.7
R. m 58	Swedish isolate	14 ± 4.6	20 ± 3	7.6 ± 2.8
R. m 1021[b]	Lagares *et al.*, 1992	4.6 ± 0.4	87 ± 50	7.0[a]
R. m 50	Nutman P. S [d]	4.2 ± 0.2	160 ± 25[f]	ND
R. m 6963	Lagares *et al.*, 1992	26 ± 1.8	400 ± 31	3.4[a]
B. jap 526	Brutti *et al.*, 1992[e]	50 ± 2.4	NT	NT
P. aer 10261[b]	Swedish isolate	100 ± 4.9	NT	NT

[a] n=2.
[b] Strains used in the bioreactor experiment (Fig. 1).
[c] Orig. A15, Microbiol. Lab., Kampen, the Netherlands.
[d] Orig. M5E, Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Herts, England.
[e] Orig. E110 in the culture collection from IMYZA-INTA, Argentina.
[f] No bacteroids. Only rod-shaped bacteria found inside nodules.
NT, Not tested.
ND, Not detected.

Table 2. *Nitrous oxide reductase (Nos) activity and hybridisation of the P. stutzeri nosZ probe with a PstI-fragment to genomic DNAs in strains of Rhizobium meliloti (R. m.), Bradyrhizobium japonicum (B. j.) and Pseudomonas aeruginosa (P. a.)*

Bacterial strain	Nos activity	Size (kb) of hybridising restriction fragment
R. m. 23	-	7.0
R. m. 27	-	7.0
R. m. 29	-	7.0
R. m. 50	-	ND
R. m. 56	-	5.0
R. m. 57	-	5.0
R. m. 58	+	5.0
R. m. 1021	+	5.0
R. m. 6963	+	5.0
B. j. 526	-	13
P. a. 10261	+	3.5

ND, Not detected.
- not proved activity.
+ proved activity.

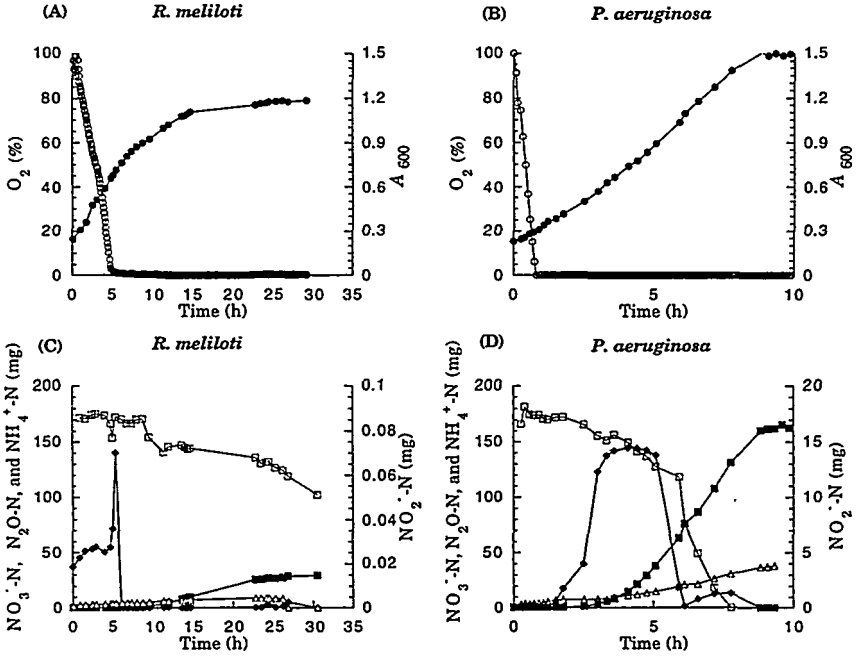

Figure 1. Growth, oxygen consumption (A, B) and nitrogen transformations (C, D) by *R. meliloti* strain 1021 (A, C) and *P. aeruginosa* strain 10261 (B, D) in a closed bioreactor, which originally contained air in the headspace and an aerated GYE medium supplemented with 20 mM KNO_3. The percentage of O_2 (O) dissolved in the medium is expressed in a relative scale and bacterial growth (●) as A_{600}. The amounts of NO_3^- (□), NO_2^- (◆), N_2O (■), and NH_4^+ (Δ) are all expressed in mg N. The incubation time was 3 times longer for *R. meliloti* than for *P. aeruginosa*.

II

Denitrification by *Rhizobium meliloti*

2. Field and laboratory studies with soil

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ABSTRACT

Four *Rhizobium meliloti* strains, two of each with "high" and "low" denitrifying capacities, respectively, were used for inoculation of lucerne (*Medicago sativa* L.) in a field plot experiment. The actual denitrification rate was independent of inoculation and approximately four times higher in soil planted with lucerne compared to ryegrass (*Lolium perenne* L.) or unplanted soil. The potential denitrification activity, on the other hand, was great in all planted soils. The significance of rhizodeposition from lucerne roots on the denitrification activity was investigated in laboratory experiments using both non-sterilised and γ-sterilised soils. The rhizobial denitrification activity was more increased than the activity of *Pseudomonas aeruginosa* by the supply of rhizodeposition. Our results indicate a demand of a more specific carbon source in *R. meliloti* compared to *P. aeruginosa*. Also in non-sterilised soil supplied with rhizodeposition the denitrification rate increased more by inoculation with *R. meliloti* than with *P. aeruginosa*. Rhizodeposition was utilised as an energy source for the denitrification activity also by the indigenous micro-organism population.

Key words: *Rhizobium meliloti*, N_2O, actual denitrification, potential denitrification, nitrification, field plot experiment, rhizodeposition, *Medicago sativa*, lucerne, γ-sterilised soil, *Pseudomonas aeruginosa*, acetylene inhibition.

INTRODUCTION

Some species of root-nodule bacteria, including *Rhizobium meliloti* and *Bradyrhizobium japonicum*, have the capacity to both fix atmospheric nitrogen and to denitrify, two contrasting processes in the nitrogen cycle. Nitrogen fixation is an energy demanding N_2-reduction process performed by genera of Rhizobiaceae in symbiosis with leguminous host plants. Denitrification, on the other hand, is a dissimilatory reduction of nitrate (NO_3^-) and nitrite (NO_2^-) via nitric (NO) and nitrous oxide (N_2O) to dinitrogen (N_2). The nitrogen oxides act as terminal electron acceptors for energy generation in the absence of oxygen or at low oxygen pressure (Tiedje, 1982).

An investigation of denitrification activities in cropping systems in Sweden by Svensson *et al.* (1991) showed a higher activity in soil cropped with lucerne than in soil with grass or barley crops. Denitrification by *R. meliloti* was proposed as one explanation among others. In an attempt to verify this proposal, Rosén *et al.,* (submitted) found that both free living cells and cells in the nodules are able to denitrify although with a great variation among strains. Besides causing loss of nitrogen from arable land the N_2O gas contributes both to the depletion of stratospheric ozone and acts as a green-house gas (Robertson, 1995). Tropical leguminous plants were considered to contribute to N_2O fluxes both directly through denitrification by rhizobia, and indirectly, by the input of nitrogen by fixation (Anonymous, 1990). Woldendorp (1962) found a higher denitrification rate in soil cultivated with pea compared with ryegrass. He assumed the reason for this influence of legumes to be a higher excretion of amino acids, which are favourable substrates for denitrifiers. With a cylinder technique, von Rheinbaben & Trolldenier (1984) showed that denitrification mainly occurs close to the roots.
Our objective was to examine the contribution of denitrifying *R. meliloti* strains, to the total denitrification activity in a lucerne ley. The potential denitrification activity in a number of *R. meliloti* strains, reported by Rosén *et al.* (submitted), was the base for the selection

of four *R. meliloti* strains with different denitrifying capacities, to be used as inoculum in a field plot experiment. Denitrification and nitrification activities were investigated in the field soil at time for harvest the second year. The influence of lucerne roots and rhizodeposition on denitrification activities by *Rhizobium*, *Pseudomonas* and the indigenous soil bacterial population was studied in a laboratory experiment with non-sterilised and γ-sterilised soil.

MATERIALS AND METHODS

Organisms

The *Rhizobium meliloti* strains 23, 29, 58 and 1021 were used as inoculants in the field plot experiment. The *R. meliloti* strains 29, 1021 and *Pseudomonas aeruginosa* strain 10261 were also used in the laboratory experiments. Sources of information regarding bacterial strains and culture conditions are given in Rosén *et al.,* (submitted). Bacterial cells were harvested by centrifugation and rinsed in phosphate buffer before inoculated into the soil.

Seeds of lucerne, *Medicago sativa* L (cv. SW Vertus) and perennial ryegrass, *Lolium perenne* L. (cv. SW Helmer) were obtained from Svalöf Weibull (SW) AB, Sweden.

Field plot experiment

The field plot was established in a field without previous history of lucerne cultivation at Ultuna estate of Swedish University of Agricultural Sciences, Uppsala, Sweden. The topsoil consists of silty clay with a humus content in the range of 3-6% and a pH of 7.3±0.1 (in distilled water). The field site has an annual mean temperature of 5.5°C (1.5 m above soil surface) and an annual precipitation of about 530 mm. The experimental area (6 m x 10 m) was divided into 18 subplots (0.45 m x 0.45 m) distributed in a randomised blocking

design with 3 blocks and 6 treatments per block of which 5 were sown with lucerne and 1 with ryegrass.

Before sowing the presence of indigenous *R. meliloti* in the field soil was investigated with the most probable number (MPN) technique. Soil inoculum for the MPN experiment was prepared in a mixer (Multimix MX32, Braun, FRG) using 10.0 g fresh soil and 95 ml sterilised 1/4 concentration of Jensen's medium (Vincent, 1970) supplied with 0.2% sodium hexametaphosphate (Calgon). The mixer was running for 2 min at the highest rate, rested for 5 min, followed by another 1 min of high-speed mixing. The suspension was sedimented for 5 sec before sampling of 10 ml for the dilution series. Five replicates of aseptically cultivated lucerne plants in-tubes with Jensen's agar medium were supplied with 1 ml aliquots of the successive tenfold dilutions 10^{-2}, 10^{-3} and 10^{-4} of the soil suspensions. The tubes were incubated in a growth chamber for 4 weeks before scoring the nodule frequencies. The numbers of bacteria per g of soil were evaluated with the MPN-table (de Man, 1975).

In May 1993 each subplot was sown with either lucerne or perennial ryegrass in five rows with 12 cm between rows (2 kg seeds ha^{-1}). After emerging, lucerne seedlings were inoculated with 10^9 cells per subplot. Four strains of *R. meliloti* were used, strains 58 and 1021 with a high, and strains 23 and 29 with a low denitrification activity (Table 1). Strain 29 was included in the field plot experiment because no measurable denitrification activity in pure culture studies was detected, neither in liquid medium nor together with plants. The inoculum mixture used for inoculation of lucerne investigated by Svensson *et al.* (1991) included strains 23 and 29. Subplots with perennial ryegrass, uninoculated lucerne and soil without plants were used as controls. The field was fertilised with a solution of NH_4NO_3 at amounts of 31, 63 and 48 kg N $ha^{-1}yr^{-1}$ to inoculated lucerne, uninoculated lucerne and perennial ryegrass, respectively.

In the autumn of 1994, two undisturbed soil cores per subplot were

In the autumn of 1994, two undisturbed soil cores per subplot were sampled from the surface layer (0-10 cm) within plant rows using stainless steel cylinders with a diameter of 7.2 cm. Actual denitrification was measured directly on undisturbed soil cores. Soil cores of each subplot were brought together before sampled for potential denitrification and potential nitrification. The three investigations were performed within 24 hours.

Denitrification assay

The acetylene (C_2H_2) inhibition technique, as described in Rosén *et al.* (submitted), was used for investigation of denitrification activities. Actual denitrification activity was estimated on soil cores enclosed in 0.7 dm^3 sealed jars fitted with a septum for sampling of gas and incubated 1 h at 20°C. The potential denitrification activity in the soil was analysed using the method described by Tiedje (1982) and modified by Pell *et al.*, (1996) without chloramphenicol. The activity was measured on a soil slurry, consisting of 10 g fresh soil and 25 ml of a solution of 1 mM glucose and 1 mM NO_3^-, incubated on a rotary shaker (175 rpm) at 25°C during gas sampling.

Nitrification assay

Nitrification rates were determined with the ammonium oxidation method (Torstensson, 1993). A soil slurry, consisting of 25 g fresh soil and 100 ml of a solution of ammonium sulphate (76 µM NH_4) and 10 mM chlorate, was incubated on a rotary shaker (175 rpm) at 25°C. Sampling of the suspension took place during 5 h. Chlorate was used to inhibit an oxidation of NO_2^- to NO_3^-, and the accumulation of NO_2^- was followed spectrophotometrically with a flow injection analyser (FIA star 5020, Tecator AB, Höganäs, Sweden).

Influence of lucerne roots on denitrification

Soil from the field experimental area was air-dried before being sieved through a 4 mm sieve. Portions of 80 g were transferred to plastic bags. Half the number of bags was sterilised by γ-radiation (28.5 kGy) and all bags were stored at 4°C. Experiments with these soils, one performed with lucerne plants, and another without plants but with supply of rhizodeposition. In both experiments, with and without plants, 3.3 g soil (dry wt) supplemented with 0.4 mg KNO_3-N g^{-1} soil (dry wt) was used and the incubated for 76 h. Test-tubes with a volume of 26 ml were sealed with rubber stoppers. The air in the headspace was replaced with N_2 and C_2H_2 before sampling of gas. At inoculation 10^7 cells were added to each tube. The water content for planted soil was adjusted to water holding capacity, whereas unplanted soil was saturated with water.

Seedlings from surface sterilised lucerne seeds (Rosén *et al.,* submitted) were planted in soil in sterilised 5 ml plastic micro-pipette tips placed inside test-tubes equipped with plastic covers. *R. meliloti* strains 29, 1021 and *P. aeruginosa* were used for inoculation, separately or in combinations of a rhizobium strain and the pseudomonad. Tubes were incubated for 6 weeks in a growth chamber at 24°C with 16 h day for nodule development. Rhizobium inoculated plants were consequently inoculated twice, first for nodulation and the next accompanying the beginning of the denitrification experimental period. For calculation of the denitrification rate gas was sampled during the first 4 hours.

Half the numbers of tubes without plants were supplied with rhizodeposition extract (0.2 ml $tube^{-1}$) from lucerne planted soil (cf. below). Tubes with unplanted soil were inoculated once with *R. meliloti* 1021 or *P. aeruginosa* and incubated in a constant room at 25°C. After 17 h of incubation the determination of denitrification rates started and continued for 90 min.

Preparation of rhizodeposition extract

Soil from 9 tubes with lucerne plants grown in sterilised soil (as described above) was mixed with 50 ml of 0.1 M sodium phosphate buffer, pH 7.0. The soil suspension was shaken violently for 10 min, and centrifuged 20 min at 10 000 rpm before filtration of the supernatant through a 0.2 µm sterilised filter.

RESULTS

Field plot experiment

The actual denitrification activities observed in undisturbed soil cores were 3 to 4 times higher in lucerne planted soil compared with soil sampled in the fallow or the grass ley, which were nearly equal in denitrification activity (Fig. 1A). The difference was not due to the water content in the soil, which was relatively high and equal in all treatments (0.38±0.004 g H_2O g^{-1} dry soil). The actual denitrification activity was slightly higher in lucerne planted soil inoculated with the high denitrifying strain 58, compared with uninoculated lucerne and lucerne inoculated with the low denitrifying strains. Two more soil cores sampled from each subplot 1 month later (data not shown) did not confirm the differences among lucerne treatments shown in figure 1A. On the other hand did they confirm the differences among lucerne, grass, and fallow.

The potential denitrification activity was high in all planted soils compared with fallow (Fig. 1B). Thus no significant differences were found among lucerne planted soils in neither actual nor potential denitrification activities.

The potential nitrification activity was highest in the grass ley and lowest in the lucerne planted soil inoculated with strain 1021 (Fig. 1C).

The MPN technique, used for investigation of the indigenous *R. meliloti* population, showed a mean content of 24 *R. meliloti* cells g^{-1} soil (dry wt). When a 99% confidence interval is used, the limits are 0 and 160 cells g^{-1} soil (dry wt). Lucerne plants in all subplots were nodulated at harvest independently of inoculation. However the nodulation frequency could not be satisfactory established due to the high clay content of the soil. The crop yields were almost equal for all lucerne treatments both years, but the second year the grass ley produced twice as much dry matter as any of the lucerne leys (Fig. 2).

Influence of lucerne roots on denitrification

The denitrification activity was measured both as a rate (Table 2 and 4) in the early stage of the incubation period and as a total amount of accumulated N_2O (Table 3 and 5) during the 76 h incubation period.

P. aeruginosa accumulated most N_2O in the sterilised planted soil (Table 3), while highest denitrification rate in this soil was found when the plants were nodulated with *R. meliloti* 1021 (Table 2). The accumulation of N_2O by *P. aeruginosa* was lower when it was introduced into a soil previously inoculated with *Rhizobium*, and this applies particularly to the low denitrifying strain *R. meliloti* 29 (Table 3).

In the non-sterilised soil with plant the denitrification rate was higher for all inoculant strains (Table 2), however no differences were found in the total accumulation, which depended on a carbon limitation (Table 3).

Addition of rhizodeposition extract to sterilised unplanted soil, increased the denitrification activity for both species. *R. meliloti* 1021 doubled both the denitrification rate (Table 4) and the total accumulation of N_2O (Table 5). However the corresponding

accumulation by *P. aeruginosa* was only slightly increased. A non-linear production of N_2O was found in the sterilised soil, which contained lot of carbon from the killed population. The increased denitrification activity, indicates an increase in the number of bacterial cells, and is most obvious concerning the pseudomonad.

In non-sterilised soil without plant and without addition of rhizodeposition, no contribution of inoculant strains on the denitrification activity was found (Table 4 and 5). The denitrification rate was almost constant during the hole incubation period. However, when rhizodeposition extract was added it was utilised very well by the indigenous population of micro-organisms as well as by the inoculants. The *R. meliloti* 1021 was more stimulated by the added rhizodeposition extract than the pseudomonad or the indigenous population. The denitrification rate of *R. meliloti* 1021 was more than doubled (Table 4), while for the *P. aeruginosa* the rate was similar to the control.

DISCUSSION

The actual denitrification activity in soil increased in the presence of lucerne roots (Fig. 1A) whereas the potential denitrification increased in the presence of both grass- and lucerne-roots (Fig. 1B). These results agree with the observation by Scaglia *et al.* (1985) and Smith & Tiedje (1979) that the potential for denitrification usually is higher in planted compared to unplanted soil, while presence of NO_3^- is necessary for a high actual denitrification rate in the rhizosphere. In our experiment the highest potential nitrification activity was found in soil planted with grass and lucerne inoculated with strain 58 (Fig. 1C). Nitrification occurs also in heterotrophic organisms, prokaryotic as well as eukaryotic, and sometimes simultaneously with the denitrification process (Castignetti & Hollocher, 1984; Kuenen & Robertson, 1994) which could be a possible explanation for the high actual denitrification activity in lucerne ley soils (Fig. 1A).

Svensson *et al.* (1991) reported nitrogen losses in a lucerne ley to be 4% of the input by fixation. Kilian & Werner (1996) found an increase in the denitrification activity in plots planted with N_2-fixing crops caused by an increase of the soil NO_3^-. Our soil cores were sampled after harvest in September when precipitation is large and the plant growth is falling-off. The results agree with Kilian & Werner (1996) regarding the difference between a N_2-fixing and a non-fixing crop.

Differences in root distribution in soil cores are supposed to be responsible for most of the variation among replicates. Root excretory products also differ among plant species (Bolton Jr *et al.*, 1993). Rovira (1956) found a higher and more diverse excretion of amino acids from pea roots than from oat roots. Gram negative bacteria are frequent in the rhizosphere (Rovira *et al.* 1974) and gradually increase in number towards the root surface (Clays-Josserand *et al.* 1995). Using DNA probes derived from genes encoding dissimilatory NO_2^- and N_2O reductases, von Berg & Bothe (1992) investigated the distribution of denitrifying bacteria in planted soils and found that bacteria with strong hybridisation signals for both probes were mainly associated with the roots. The numerically dominating denitrifiers in soil are species of the genera *Pseudomonas*, *Alcaligenes* and *Flavobacterium* (Gamble *et al.* 1977).

The experiment with sterilised and non-sterilised soil, indicated that the non-sterilised soil contained enough number of denitrifying bacteria to eliminate the effect of both inoculant species *R. meliloti* and *P. aeruginosa*, if not carbon simultaneously was added (Table 4 and 5). The availability of carbon was obviously the limiting factor for denitrification, since the supply of rhizodeposition increased the denitrification activity. *R. meliloti* 1021 increased the denitrification rate with 100% or more in both sterilised and non-sterilised soils when rhizodeposition was added (Table 4).

The planted soil was interwoven with roots, which influenced the whole soil volume. The decreased denitrification activity in sterilised

planted soil, inoculated with both species, compared to simple inoculation with the pseudomonad, most likely depends on an occupation by the *R. meliloti* strain in the soil (Table 3). In the sterilised soil the denitrification activity was increasing during the incubation period, while in non-sterilised soil the activity was approximately constant or declined gradually due to limited carbon availability (Table 2-5).

In the planted non-sterilised soil the increased denitrification rate was the same for both *R. meliloti* strains (Table. 2). Rhizodeposition extract added to non-sterilised soil was utilised very well by the indigenous bacterial population. However, *R. meliloti* 1021 was more stimulated by the extract than the indigenous population or the pseudomonad. Garcia-Plazaola *et al.* (1993) found a negligible contribution of denitrifying *R. meliloti* compared to the activity by other micro-organisms in the soil. They used glucose-C as the carbon source and no plants were included. They also found a low denitrification rate by *R. meliloti* in the sterilised soil. However, Garcia-Plazaola *et al.* (1993) found that *R. meliloti* cells were able to remove large amounts of NO_3^- from the soil, but not in the range of many other soil micro-organisms.

The denitrification activity rate by *R. meliloti* in sterilised media is low compared to that of the *P. aeruginosa* (Table 1). The denitrification activity in both sterilised and non-sterilised soils were doubled by simultaneous inoculation with *R. meliloti* and supply of rhizodeposition from lucerne roots (Table 4). The influence of inoculum strains with different denitrifying capacities did not appear in the field experiment, which may be explained by a high denitrification activity in the indigenous soil population, including *R. meliloti*, disguising the activity by the inoculant strains. The contribution of inoculant strains to the denitrification activity in the laboratory experiment with plants planted in non-sterilised soil was also insignificant, and no differences were found between "high" or "low" denitrifying strains.

More investigations are needed regarding the role of rhizobium and the high denitrification activity in the lucerne leys. I propose to use rhizodeposition from the host plant as the carbon source in potential denitrification experiments with rhizobium, at least for *R. meliloti* strains.

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Table 1. *Denitrification and nitrogen fixation activity in pure culture studies (Rosén et al., submitted). Results expressed in N_2O-N and C_2H_4, respectively. Mean value ± SE (n=3)*

	Denitrification		Nitrogen fixation
Bacterial strains	Free-living ($\mu g\ mg^{-1}$ prot h^{-1})	Symbiotic ($\mu g\ g^{-1}$ nod (fw) h^{-1})	Symbiotic ($\mu mol\ g^{-1}$ nod (fw) h^{-1})
R. meliloti 23 [a]	$\leq 2 \cdot 10^{-2}$	$\leq 3 \cdot 10^{-3}$	7 ± 2
R. meliloti 29 [a,b]	$\leq 2 \cdot 10^{-2}$	$\leq 3 \cdot 10^{-3}$	3 ± 2
R. meliloti 58 [a]	10 ± 5	20 ± 3	8 ± 3
R. meliloti 1021 [a,b]	5 ± 1	87 ± 50	7 [c]
P. aeruginosa 10261 [b]	100 ± 5	NT	NT

[a] Strains used as inoculum in the field experiment (Figs. 1 and 2).

[b] Strains used as inoculum in the experiments with γ-sterilised and non-sterilised soil.

[c] dublicates.

NT=Not tested.

Table 2-5. *Denitrification activity in γ-sterilised and non-sterilised soil planted (2 and 3) with Medicago sativa L. or unplanted (4 and 5) expressed both as a rate and as an accumulation of N_2O-N under the 76 h of incubation. Rhizobium meliloti (R. m.) strains 29 or 1021 and/or Pseudomonas aeruginosa 10261 (P. aer.) were used as inoculum and rhizodeposition was supplied to the unplanted soil as a carbon source. Mean±SE (planted soil n=6 , unplanted soil n=3)*

Table 2. *The denitrification rate in planted soil (ng N_2O-N g^{-1} (soil (dry wt) h^{-1})*

Bacteria	Sterilised	Non-sterilised
uninoculated	ND	38 ± 11[b]
R. m. 29	2.7 ± 0.6[a]	72 ± 12
R. m. 1021	9.8 ± 2.0	67 ± 13
P. aer.	2.7 ± 0.4	120 ± 42
P. aer.+R. m. 29	6.2 ± 2.7[b]	NT
P. aer.+R. m. 1021	15 ± 4.1[b]	NT

[a] n=4.
[b] n=5.
ND=Not detected.
NT=Not tested.

Table 3. *The total accumulation of N_2O in planted soil (μg N_2O-N g^{-1} soil dry wt)*

Bacteria	Sterilised	Non-sterilised
uninoculated	ND	1.3 ± 0.5[b]
R. m. 29	1.1 ± 0.3[a]	1.6 ± 0.5
R. m. 1021	6.1 ± 3.1	1.2 ± 0.6
P. aer	35 ± 13	2.4 ± 0.9
P. aer.+R. m. 29	8.3 ± 2.5[b]	NT
P. aer.+R. m. 1021	28 ± 2.1[b]	NT

[a] n=4.
[b] n=5.
ND=Not detected.
NT=Not tested.

Table 4. *The denitrification rate in unplanted soil (ng N_2O-N g^{-1} soil (dry wt) h^{-1})*

	With rhizodeposition		Without rhizodeposition	
Bacteria	Sterilised	Non-sterilised	Sterilised	Non-sterilised
uninoculated	ND	370 ± 26	ND	220 ± 7.2
R. m. 1021	30 ± 4.2	650 ± 52	15 ± 7.9	240 ± 40
P. aer.	350 ± 94	350 ± 56	190 ± 31	160 ± 26

ND=Not detected.

Table 5. *The total accumulation of N_2O in unplanted soil (μg N_2O-N g^{-1} soil (dry wt)*

	With rhizodeposition		Without rhizodeposition	
Bacteria	Sterilised	Non-sterilised	Sterilised	Non-sterilised
uninoculated	ND	28 ± 2.0	ND	17 ± 0.4
R. m. 1021	11 ± 5.7	26 ± 4.9	5.4 ± 0.3	17 ± 0.6
P. aer.	60 ± 2.1	23 ± 2.2	57 ± 0.8	16 ± 0.1

ND=Not detected.

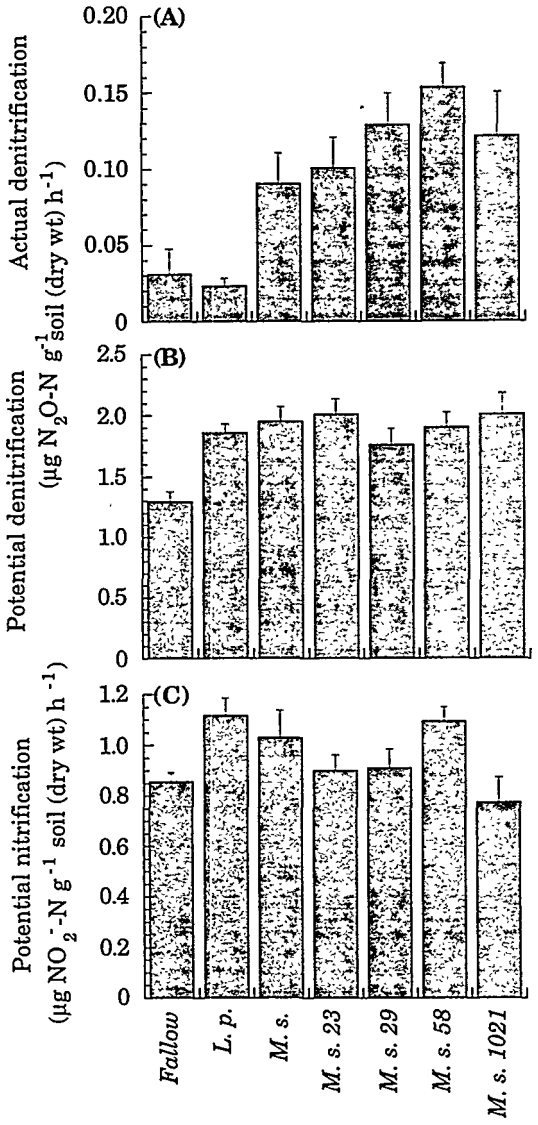

Figure 1. Actual denitrification activity in undisturbed soil cores (A) sampled within rows of *Medicago sativa* L. (*M.s*) uninoculated or inoculated with one of four different *Rhizobium meliloti* strains (Table 1), and *Lolium perenne* L. (*L.p*) cultivated soil or naked soil (fallow). Potential denitrification activity (B) measured in slurries of mixed soil core replicates, supplemented with glucose and nitrate, and potential nitrification (C) also measured in a soil slurry of mixed fresh soil supplemented with ammonium. Results are expressed in µg N g^{-1} soil (dry wt) h^{-1}. Mean value ±SE, n=6 in (A) and n=9 in both (B) and (C).

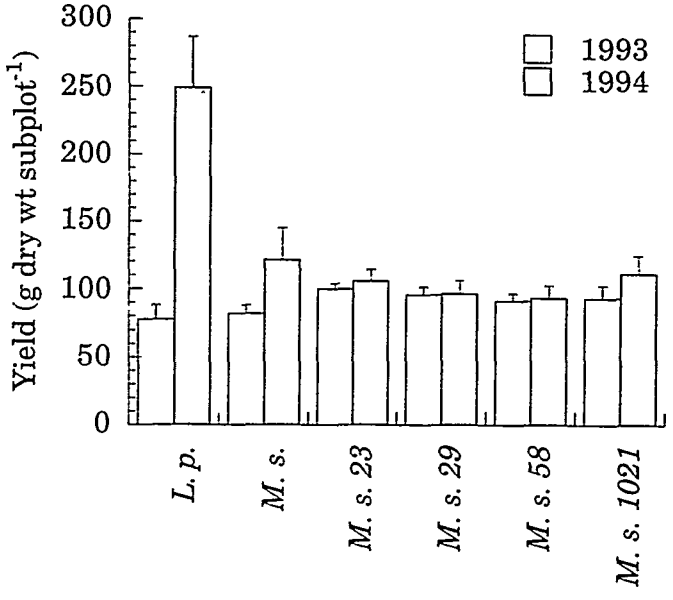

Figure 2. The total yields of lucerne (*Medicago sativa*) and ryegrass (*Lolium perenne*) in 1993 (white) and 1994 (grey), respectively, are presented as g dry matter per subplot. Mean value ± SE (n=3).

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