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DENITRIFICATION BY GUAR RHIZOBIA STRAINS IN NEW VALLEY GOVERNORATE

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Abstract

The ability of *Rhizobium leguminosarum* bv. guar cells to denitrify nitrate in soils under several conditions was tested. All the strains tested were able to remove large amounts of nitrates from soils. Both water filled pore space 40% and temperatures above 20°C greatly increased nitrogen losses. However, even under optimal conditions for denitrification and the highest *rhizobial* populations found in agricultural soils, the contribution of *Rhizobium* to the total denitrification was virtually negligible as compared to other soil microorganisms.

Key words: *Rhizobium leguminosarum* bv. guar, respiration, denitrification, anaerobic soil.

INTRODUCTION

Rhizobium is a microorganism widespread in agricultural soils. They are beneficial organisms, because of their ability to reduce atmospheric nitrogen into ammonium when symbiotically associated with legumes. On the other hand, it has been shown that free-living *Rhizobium* cells are also able to denitrify, i.e. to reduce nitrate ions into elemental nitrogen under anaerobic conditions (Zablotowicz et al 1978), resulting in the loss of ionic forms of nitrogen from soils (Daniel et al 1980b). The effect of both processes on the nitrogen balance in plants and crops should not be overlooked, since agricultural soils may contain up to 10^6 *Rhizobium* cells/g soil (Moawad et al.1984), a quantity similar to that of the whole population of denitrification organisms (Gamble et al 1977). Since applied nitrogen fertilizer as well as mineralized nitrogen is rapidly converted into nitrate in most soils and both free-living and symbiotic rhizobial population are usually high in most soils, rhizobia

seem to be potentially capable of removing large amounts of available nitrogen from agrosystems.

It has been proposed that the capability of some strains to denitrify may enhance their survival and growth of bacteria in anaerobic soils (Daniel and Appleby 1972, Daniel et al 1980a). It could also reduce the effect of nitrate inhibition of nodulation and nitrogen fixation (O'Hara and Daniel 1985), since denitnification is carried out both in the symbiotic and free-living states (Zablotowicz and Focht 1979).

The ability to denitrify is widely distributed among the slow-growing rhizobia (*Bradyrhizobium*) and more rarely within fast-growing rhizobia. Nevertheless, except for *R.leguminosarum* bv. phaseoli (Daniel et al 1982, Bourguignon 1987) fast - growing *Rhizobium* strains able to denitrify have been identified. Recently, a survey *Rhizobium meliloti* strains showed that denitrifying ability was present in most of them (Garcia Plazaola et al 1993). In fact *R.meliloti* had the greatest proportion of denitrifying strains among the fast-growing group (Chan et al., 1989, and Myshkina and Bonartseva 1990).

Although some experiments have been done in order to assess the effect of rhizobial denitrification on the nitrogen balance in soils (Daniel et al 1980b, O'Hara et al 1984, and Breitenbeck and Bremner 1989), results are still contradictory. O'Hara et al (1984) suggested that rates of nitrogen losses from soils through rhizobial denitrification were potentially similar to those of nitrogen fixation. In contrast, Breitenbeck and Bremner (1989) indicated that the population of non-rhizobial denitrifiers were large enough to deplete soil nitrate during periods of anaerobiosis, despite the presence of denitrifying rhizobia.

Oxygen, nitrate, temperature, moisture and labile organic matter availability are the main factors controlling denitrification in soils (Tiedje 1988), and seem also to control denitrification by *Rhizobium* under laboratory conditions. However, very few reports deal with the effect of environmental factors on rhizobial denitrication in soils. Since denitrification by *Rhizobium* could have an agronomical importance, it is necessary to study the conditions under which the process is stimulated.

The aim of this study was to determine and to evaluate the extent of denitrification in soils when *R.leguminosarum* bv. guar is present and the effect of some environmental factors on the rhizobial activity of denitrification.

MATERIALS AND METHODS

Bacterial strains and experimental growth conditions:

Rhizobium strains (ARC., 800G, ARC. 801 G and ARC. 802 G) were obtained from the Agric. Microbiology Res. Dept.; Soil, Water and Envir. Res. Inst, Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt. Strains were maintained on yeast extract mannitol (YEM) agar slants (Vincent 1970) and kept at 5°C. Before starting the experiments, the collected rhizobium strains were grown in a YEM liquid medium in 100 ml Erlenmeyer flasks on an orbital shaker (150 rpm) at 25°C .

Cells were collected at equal intervals of growth by centrifugation at (12000 x g) for 10 minutes. These cells were then resuspended in 10 ml of distilled water and added to the soil samples to reach the final cell density of 10^7 cells/g dry soil.

Samples of soil were sealed with suba-seal septa and incubated for 48h at 5,10,15,20,25 and 30°C in a hot cold refrigerated chamber. When not specified in the figures, temperature of incubation was 25°C. All the incubations were done in the presence of 10% acetylene to inhibit the reduction of nitrous oxide to nitrogen (Yoshinari and Knowles 1976).

Soil:

Soil samples used in the experiment were collected from El-Kharga Oases, New Valley Govemorate, Agricultural Research station, from the upper 30cm layer, where guar plants (*Cyamopsis tetragonolobus*) were growing.

The soil was sandy clay loam comprising 8.62% coarse sand, 48.65% fine sand, 15.65% silt and 27.08% clay with a pH of 8.4 in a soil water suspension (1.2.5), C/N ratio of 11.8, organic matter 2%., and water-holding capacity of 29%. Nitrate content was 16.5 µg No3- N/g soil. Soil was passed through a sieve (2 mm) and sterilized twice by autoclaving at 121°C, 1.3 Kpa with one day intervals as described by Boonkerd and Weaver (1982). Six g of soil were placed in 50-ml tubes and were supplemented with 72 µg No3 N/g soil, and were moistened by adding water to the dry soil to reach 20%, 40%, 80%, and 100% water-filled pore space, when not specified in the figure water-filled pore space was 80%. They were sealed with ruber stoppers. Water-filled pore space was calculated basically as described by Aulakh et al, (1991), as follows:-

Water-filled pore space = [(gravimetric water content x soil bulk density) / total soil proosity]

Bulk density of the soil was measured for 4 replicates of known volume. Total soil porosity was calculated from bulk density divided by particle density (which was assumed to be 2.70 g/cm3) substracted from one

Samples of 0.5 ml of the headspace were taken and analysed for CO2 and N2O concentration using a gas chromatography (Shimadzu GC-gA) equipped with a Ni electron capture detector, and a 1.9 m x 1/7" stainless steel column containing Porapak Q (80-100 mesh) with nitrogen as the camier gas (flow rate 30 ml/min). Operation temperatures were 40°C for the oven and 300°C for the injector. Under these conditions the detection limits for nitrous oxide were 5×10^{-12} mol, for a 0.5 mL gas sample.

Assuming the following reaction (Burford and Bremner, 1975): 4 No3 + 4 CH2O + 4H* = 4CO2 (g) + 2 N2O (g) + 6H2O the oxidation of glucose with nitrate as electron acceptor when N2O is the final product, gives a stoichometrically CO2 produced / No3 reduced ratio of 1 under completely anaerobic conditions. According to this equation, when the CO2-C/N2O-N ratio is higher than1, this is indicative of aerobic respiration.

RESULTS

As shown in Table 1, three strains of *R.leguminosarum* bv. guar were tested for their ability of denitrification in soil. All of them produced N2O from NO3 at high rates. Denitrification was enhanced by high water-filled pore space. Strain ARG. 801 G was used for further experiments because of its higher rate of denitrification under the different water-filled pore space conditions tested.

Table 1. Denitrification rates (in nmol N2O, million/cells/h) of three *Rhizobium leguminosarum* bv. guar strains in soil amended with nitrate under different moisture conditions. Each value is the average of six determinations ± SE.

Strain	Water-filled pore space		
	40 %	80 %	100 %
ARC. 800 G	25.8±7.1	33.7±2.7	32.0±2.5
ARC. 801G	105.6±8.8	127.8±5.0	127.9±15.6
ARC. 802 G	46.6±9.8	126.1±22.4	82.9±10.6

One experiment was designed in order to check whether organic matter or nitrate were limiting for denitrification in the experimental conditions. So, when non-

limiting amounts of nitrate (72 µg No3 N/g soil) and carbon (450 µg glucose-C/g dry soil) were added, denitrification rates were 362.9±17.3 and 328.3±34.6 n mol N2O/g soil, respectively. On the other hand, in glucose and nitrate-unamended soil samples, denitrification rate was 293.8±34.6 n mol N2O/g soil. As no significant differences were found, a non-limiting effect of both nitrate and carbon on denitrification in the unamended or amended soil was indicated .

In Figure 1, effects of water-filled pore space and temperature on denitnification rate are shown. Since temperature over 30°C are very unusual in flooded soils, a range from 5 to 30°C was tested. Nitrous oxide evolution increased prograssively with water-filled pore space values above 20%, although beyond 80% water-filled pore space, the increase was not significant. Denitrification is a temperature-dependent process, and the data were unable to detect nitrous oxide production below 10°C. However, denitrification increased markedly from 15 to 25°C. On the other hand, CO2 production (primarily due to aerobic respiration) showed a dramatic decrease in production between 40% and 80% water-filled pore space. In soils above 80% water-filled pore space, CO2 evolution was mainly due to anaerobic nitrate reduction, as shown by the CO2-C/N2O-N ratio which was 1.31. Respiration progressively increased with temperature and the CO2-C/N2O-N ratio decreased with increasing temperature (Table 2).

Table 2. CO2-C/N2O-N production ratios at different moisture percentages and different temperatures. **

Water-filled pore space	30°C	25°C	20°C	15°C
100 %	0.73±0.23	0.63±0.06	1.42±0.18	9.50±0.77
80 %	1.40±0.18	1.31±0.26	4.12±0.59	13.28±1.22

** Each number within the table represents the average of six determinations.

The data could not detect significant differences in N2O production at 80% water-filled pore space under atmospheres of nitrogen or air, or between these conditions and a 40% water-filled pore space with an atmosphere of nitrogen. Thus, flooding had a similar effect on denitrification due to anaerobic conditions developed.

To determine the rhizobial contribution to denitrification in soil, the five rhizobial population, (From 10^4 to 10^7 cells/g dry soil) were inoculated into sterilized

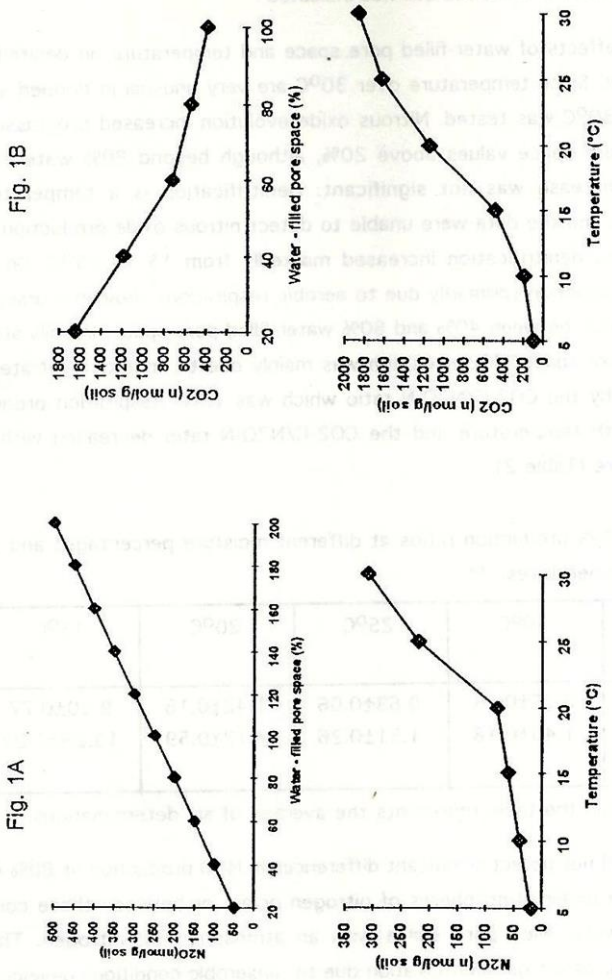

Fig. 1. N2O (A) and CO2 (B) in (n mol/g dry soil) production by strain (ARC 801 G), after 48 hours of incubation in sterilized soil under different moisture contents (expressed as water-filled pore space) and different temperatures (in ^{o}C).

and non sterilized soils. Denitrification rate increased progressively with cell density in autoclaved soils (Fig.2) reaching 13 µg No3 N/g soil with the highest inoculum of 10^7 cells/g dry soil. In non-sterilized soil, denitrification rate was 43 µg NO3 N/g soil, but this value did not increase when those soils were inoculated with *Rhizobium*.

A further experiment was made to check the time-course of N2O evolution by *R.leguminosarum* bv. guar incubated in sterile and non-sterile soils (Fig. 3). This study with non-sterilized soils showed a very rapid increase in N2O production; but after 30 hours N2O production rates were greatly decreased. In sterilized soil samples inoculated with *Rhizobium*, low rates of denitrification were detected with a progressive slight increase with time.

DISCUSSION

Soil microbial activity is strongly influenced by oxygen supply and degree of temperature (Skopp et al., 1990). Table 1 shows that denitrification rate is affected by the percent of wter-filled pore space, i.e. amount of air in the pore space. Tiedje (1988) found that the amount of oxygen supply is the major environmental regulator of denitrification in *Rhizobium* as well as in other denitrifying microorganisms.

Usually, anaerobic conditions are found in flooded soils due to lack of oxygen. These conditions are found also in non-saturated soils due to an oxygen gradient with anaerobic centers in soil aggregates (Sexstone et al., 1985a and 1985b). Anaerobic conditions can be found also in hot spots where anaerobiosis is caused by the high respiratory activity of decomposing organic matter. (Christensen et al., 1990).

As shown in Figure 1A, denitrification by *R.leguminosarum* bv. guar ARC. 801 G occurred slowly at 20% water - filled pore space. However, quite high rates were measured at 40% water-filled pore space, a moisture level below soil field capacity; this would be attributed to anaerobic zones in aggregates in soils at this moisture level. Under these conditions, oxygen controls denitrification more than the availability and diffusion of nitrate and labile organic matter.

Denitrificantion was highly temperature dependent (Fig. 1A). Since N2O production rates were extremely low (almost undetectable) below 15°C. The occcurrence of low temperatures in the soil could be a major factor controlling rhizobial denitrification activity in soils even under flooded conditions. However, denitrification activity by other microorganisms in other soils has been detected at tempera-

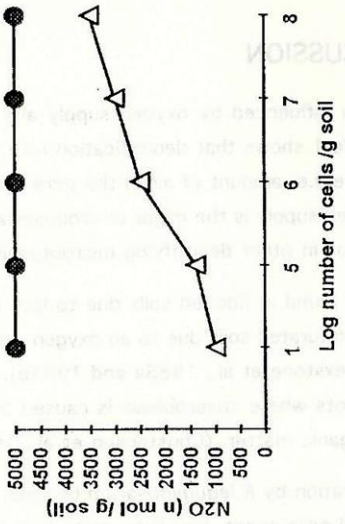

Fig. 2. N2O production in (n mol./g dry soil) after an incubation period of 48 hours with different *R. Leguminosarum* bv. gurar strain (ARC. 801 G). Populations added to sterile (—Δ—Δ—) and non-sterile (●—●—●) soils. Date are mean of six replicates.

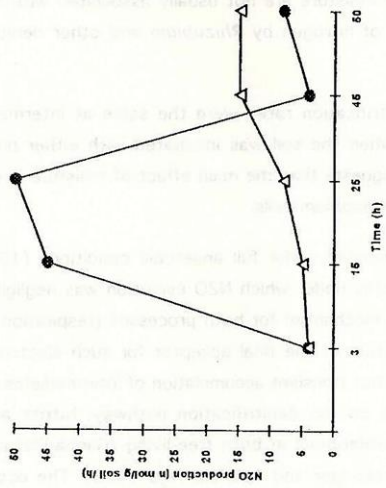

Fig. 3. Time course effect on N2O production rate in (n mol/g dry soil/h) during an incubation period of 56 hours by 10^7 cells/g dry soil of *R. leguminosarum* bv. guar strain (ARC. 801 G) inoculated to sterile (—— Δ—— Δ ——), and non-sterile (●——●——●)

tures as low as 0-10°C (knowles, 1982).

Respiration steadily increased with temperature (Fig. 1B). However, the data were able to detect CO2 production at temperature where denitrification had stopped, indicating that temperature and moistue had a differential effect on denitrification and respiration. In *Rhizobium*, both moisture and temperature have a great influence on survival rates and other characteristics (Boonkerd and Weaver 1982). Parsons et al. (1991) showed that denitrification and temperature were inversely related due to the relationship found with both water-filled pore space and temperature in soils under natural environmental conditions. On the contrary, results in Figure 1 showed positive correlation of denitrification for both water-filled pore space and temperature. Nevertheless, it is probably difficult to find high temperatures associated with high moisture content in the field. Since those parameters are strongly seasonal, higher levels of moisture are not usually associated with higher temperatures, limiting the losses of nitrogen by *Rhizobium* and other denitrifying microorganisms.

The fact that denitrification rates were the same at intermediate values of water-filled pore space when the soil was incubated with either nitrogen or air in the gas phase strongly suggests that the main effect of moisture is on the shortage of oxygen availability to *Rhizobium* cells.

CO_2 evolution proceeded under full anaerobic conditions (100% water-filled pore space) at temperatures under which N2O evolution was negligible. This clearly shows a different control mechanism for both processes (respiration and denitrification) and raises the question of the final acceptor for such electrons. Betlach and Tiedje (1981) suggested that transient accumulation of intermediates could be due to different enzyme kinetics on the denitrification pathway. Nitrite accumulations is commonly observed for *Rhizobium* in both free-living (Garcia-Plazaola et al 1993) and symbiotic forms (Arrese-Igor and Aparicio-Tejo 1992). The occurrence of respiration when N2O evolution is supperessed could mean that nitrate respiration to nitrite is favoured at low temperatures, whilst the operation of the whole denitrification chain is promoted at higher temperatures.

Although populations up to 10^7 cells/g dry soil were used in the experiments, rhizobial densities in soils are usually below 10^5 cells/g dry soil (Moawad et al 1984). At populations of 10^5 cells/g dry soil, nitrate losses due to rhizobial denitrification were very small during short episodes of flooded soil, and with higher con-

centrations (10^7 cells/g dry soil), was as much as 13 ug No3 N/g soil were lost through denitrification in 48 hours (14% of the total nitrate) (Fig.2).

Daniel et al (1980b) suggested that because *Rhizobium* was able to remove substantial amounts of nitrogen from soils, non-denitriying strains should be selected. Although it is clear that *R.leguminosarum* bv. guar denitrified in soils, when high populations of cells were added to nonsterilized soil, total denitrifying activity in the soil samples was not increased. Regularly, 46 ug No3 N/g soil were lost after 48 hours regardless of the rhizobial population present in the soil. Under these conditions. Figure 3 indicated that N2O is produced during the first 30 hours. This quantity was within the range of potential denitrification rates of other soils (0.5-5 ug NO3 N/g soil/h) (Bijay-Singh et al 1988, De Klein and Van Logtestijn 1994). The limited rates of substrate diffusion into solution (Skopp et al 1990, El - Sayed 1995) should control total denitrifying activity more than the content of enzymes in soil. These results are in agreement with those of Breitenbeck and Bremner (1989), but the data were unable to detect any statistically significant increase in soil denitrification rate with the addition of *Rhizobium*. Time course studies indicated that non-rhizobial denitrification occurred before denitrifying activity of *Rhizobium* was detected, suggesting that soil bacteria denitrify nitrate reserves of soils very quickly in short periods of anaerobiosis (Fig.3). The data can conclude that regardless of the denitrifying ability of free-living *Rhizobium* cells, the losses of nitrate due to this microorganism were not significant in determining the nitrogen balance of soils. The activity of other denitrifying soil microorganisms such as *Alcaligenes* sp. and *Pseudomonas* sp. (El-Sayed 1976, Gamble et al., 1977) are probably the main cause of nitrate removal from soils.

The rhizobial population is not uniformly distributed through the soil and the highest density is to be found in the legume rhizospheres, usually about 10 to 200 times higher, and in exceptional cases 10000 times higher than the surrounding soil of legume rhizosphere (Robert and Schmidt 1983, Estavillo et al 1995). Steinberg et al (1989) found a great stimuulation of denitrification and growth of *Bradyrhizobium japonicum* by the presence of legume roots. Therefore, despite the small contribution to the overall nitrate losses in soils by rhizobial denitrification in the studies, it may be important for nitrate losses in the rhizosphere and thus influence the nitrogen balance with legume crops.

In conclusion, *Rhizobium* has the potential to remove high amounts of nitrate from soils through denitrification. The conditions for such a process to occur are

very low oxygen availability (or anaerobic conditions) such as under flooding, or when respiratory activity is very high. High temperatures and the presence of combined nitrogen are also necessary. However, these conditions are very rarely found together in soils under natural environmental conditions, and even under such conditions other soil microorganisms are stronger competitors and more effective in carrying out denitrification. In that view, rhizobial denitrification has a limited agricultural impact.

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عكس التأزوت بواسطة سلالات ريزوبيا الجوار بمحافظة الوادي الجديد

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تم إختبار مقدرة بكتيريا ريزوبيا الجوار علي إنطلاق الأزوت من النترات في الاراضي تحت الظروف المختلفة. جميع السلالات التي تم اختبارها قادرة علي إزالة كميات من النيتروجين الموجود في صورة نترات من التربة.

يحدث فقد كبير في النيتروجين عند ٤٠٪ من إمتلاء مسام التربة بالماء، وعندما تزيد درجة الحرارة عن ٢٠ه م . ومن ناحية أخري فعند حدوث الظروف المثلي لإنطلاق الأزوت فانه توجد اعداد كبيرة من بكتريا ريزوبيا الجوار في التربة الزراعية.

أوضحت التجربة أنه يمكن إهمال كمية الأزوت المنطلقة بواسطة ريزوبيا الجوار اذا ما قورنت بالكائنات الحية الدقيقة الأخري الموجودة في التربة الزراعية.