

Denitrification by rhizobia

A possible factor contributing to nitrogen losses from soils

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New Zealand agriculture is heavily dependent upon fixed nitrogen derived from the rhizobium-legume symbiosis, and rhizobia are widespread in soils. It has recently been established that some strains of rhizobia denitrify at high rates in liquid culture and in sterile soils under laboratory conditions. The significance of this rhizobial denitrification in agricultural ecosystems is uncertain. It may aid rhizobial survival in anaerobic conditions, and alleviate nitrate inhibition of symbiotic nitrogen fixation: but it will result in the removal of fixed nitrogen, thus contributing to the chronic nitrogen deficiency prevalent in New Zealand pastoral systems.

INTRODUCTION

The intensive pastoral farming system on which New Zealand animal production is based is almost completely dependent upon the rhizobium-legume symbiosis for the fixed nitrogen required for pasture production. The average annual fixation has been measured as 184 kg nitrogen/ha in developed lowland pastures (Hoglund *et al.*, 1979) and about 13 kg nitrogen/ha in poorly developed hill country pastures (Grant and Lambert, 1979). From these figures it can be estimated that rhizobia in New Zealand pastures fix in excess of one million tonnes of nitrogen annually. The current annual application of fertilizer nitrogen to pastures is about 12 500 tonnes (O'Connor, 1979).

Nevertheless, pastoral production throughout New Zealand is severely restricted by chronic nitrogen deficiency, and recent research has shown that the alleviation of this deficiency would result in an average increase in pasture production of about 30% (O'Connor, 1981; Steele, 1981). Recent laboratory studies (Daniel *et al.*, 1980) on free-living rhizobia grown in liquid culture, and on extracted symbiotic rhizobia (Zablotowicz and Focht, 1979), have confirmed earlier reports (Murphy and Elkan, 1965; Daniel and Appleby, 1972) that some strains of rhizobia are capable of high rates of denitrification; that is, they are able to convert nitrate to nitrous oxide or nitrogen, resulting in a loss of fixed nitrogen.

Since both free-living and symbiotic rhizobial numbers are high in New Zealand soils and since applied nitrogen fertilizer as well

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as mineralized nitrogen is rapidly converted to nitrate in most soils (Steele *et al.*, 1980), rhizobia are clearly potentially capable of removing large amounts of available nitrogen from agricultural ecosystems. The object of this paper is to discuss this possibility and its implications, and to draw the attention of other workers to the phenomenon of rhizobial denitrification.

LABORATORY STUDIES ON RHIZOBIA UNDER DEFINED CONDITIONS

Laboratory studies (Murphy and Elkan, 1965; Daniel and Appleby, 1972; Daniel and Gray, 1976; Daniel *et al.*, 1980) have shown that some strains of rhizobia are capable, in both the free-living (non-nitrogen fixing) and symbiotic (nitrogen fixing) root nodule forms, of utilizing nitrate instead of oxygen for respiration — that is, instead of using oxygen for their energy-yielding oxidation processes, they use nitrate, with the concomitant production of more reduced forms, commonly nitrite, nitrous oxide or nitrogen. This nitrate respiration only occurs when oxygen is absent or present in very low concentrations. The process is rather inefficient, yielding only about 40% of the energy gained from oxygen respiration (Rigaud *et al.*, 1973; Ratcliffe *et al.*, 1980; Daniel *et al.*, 1980). From the point of view of the organism, if nitrate respiration enhances survival its inefficiency is of little consequence, and Zablotowicz and Focht (1979) have suggested that this nitrate respiration which leads to denitrification may be an agriculturally desirable characteristic in rhizobia for this reason. In agricultural terms, however, survival of free-living rhizobia will probably not be of major importance unless rhizobial numbers in the soil are so low that a drop in numbers would result in the failure of legumes to become nodulated. This seems unlikely since nodulation can apparently be effected by as few as ten rhizobia (Dart, 1977). The advantage of enhanced survival of symbiotic rhizobia under anaerobic conditions, if it occurs, is difficult to assess. This survival is expensively bought in terms of nitrate nitrogen which might otherwise have been available to the plant (assuming it is able to survive the anaerobiosis); but rhizobial survival brings future long-term benefits to the plant in terms of symbiotically fixed nitrogen.

Nitrogen fixation is a very energy-demanding procedure for the rhizobium, and under field conditions is probably limited by the supply of carbon substrate generated by the legume from photosynthesis (Hardy and Havelka, 1975). If the nitrate used for denitrification by the rhizobium has originated from ammonia derived from symbiotic nitrogen fixation (and this is particularly likely under New Zealand conditions), then at the very most only 20% of the energy expended in fixing the nitrogen will be recovered by denitrification. In any event there are likely to be major energy losses from the ecosystem when denitrification occurs, as well as the obvious losses of fixed nitrogen.

By definition, the end products of denitrification are nitric oxide (NO), nitrous oxide (N_2O), or nitrogen, and the first is rare. In the rhizobia so far examined (Zablotowicz and Focht, 1979; Daniel *et al.*, 1980) which are capable of denitrification, the end product is N_2O. Some symbiotic rhizobia which are not capable of denitrification can nevertheless derive energy by the conversion of nitrate to nitrite. Although this may not necessarily result in a loss of fixed nitrogen, the nitrite which accumulates in the root nodule is a very powerful inhibitor of nitrogen fixation (Kennedy *et al.*, 1975).

Much of the work described above has been carried out on a single strain of rhizobium, *Rhizobium japonicum* strain 505 (Wisconsin), which nodulates soybean, and which is capable of denitrification. Preliminary screening (Daniel and Smith, 1980) of various species of rhizobia for their ability to utilize nitrate as an energy source for growth, and to produce N_2O, suggests that at least 20 of the 50 strains examined are capable of denitrification. Although screening for N_2O production is convenient in that a simple gas chromatographic assay can be used, it is not a particularly good criterion for denitrification. Denitrifying rhizobia which produce N_2 as an end product are likely to be overlooked by this technique, so that a figure of 40% should be regarded as a minimum until more work is done. However, detailed studies on a few rhizobia con-

firm that some strains definitely do not denitrify, and as far as we can tell at this early stage the ability to denitrify is not well correlated with any other obvious characteristics such as strain or speed of growth.

LABORATORY WORK ON RHIZOBIA IN SOILS

The laboratory studies reported above, all carried out in liquid culture, have established that some rhizobia are capable of denitrification, but conditions experienced by bacteria in liquid culture bear little resemblance to those in the field, and care is needed in extrapolating from one to the other. The work reported below* was designed to determine whether denitrification by rhizobia would occur in soils and if it is therefore likely to occur under field conditions.

The results shown were obtained by inoculating sealed 100 ml flasks containing 5 g of sterile soil with pure cultures of *R. japonicum* 505. The soil contained 80 ppm nitrogen as KNO_3, which is comparable with soil nitrate levels in urine-affected areas or following nitrogen fertilizer application. The headspace gas was assayed for N_2O, and for N_2 where indicated, by gas chromatography.

Table 1 shows a typical set of results. Control flasks 1 and 2 show negligible activity. Experimental flasks 3 and 4 show high rates of denitrification, apparently quite unaffected by the presence of 10% oxygen in flask 4. Although this oxygen concentration is only half of the ambient concentration, this result was surprising since denitrification is generally believed to occur only in anaerobic or near anaerobic conditions. The soil layer in the flask was only about 3mm in depth and should have been well aerated.

Rhizobial numbers in soil vary from zero up to 10^8 cells/g soil (*e.g.*, Krasilnikov, 1958). They will be highest in the rhizosphere of soils where legumes are growing or have been grown, probably in the region of 10^5-10^7 cells/g soil. The results in Table 2 indicate that high rates of denitrification will occur with this number of rhizobia. We attribute the discrepancy between experiments in flask 4 to the use of *R. japonicum* at different growth stages as inocula for experiments 1 and 2. A comparison of the tables shows that denitrification rates are apparently increased by the presence of 20% oxygen. It may be that this is an effect on bacterial numbers and that denitrification occurs in anaerobic microenvironments within the soil crumb, but it suggests that high rates of denitrification can occur in well-aerated soils.

TABLE 1: N_2O PRODUCTION BY *R. JAPONICUM* STRAIN 505 IN STERILE YELLOW-BROWN EARTH

Flask No.	Conditions: % O_2 in Flask	Cells/g Soil	Added NO_3' (ppm N)	% NO_3'-N Recovered as N_2O after 7 days	% NO_3'-N Recovered as N_2O after 24 days
1	0	0	80	0	0.5
2	0	2×10^7	0*	0.5	1.5
3	0	2×10^7	80	14.8	36.9
4	10	2×10^7	80	14.3	39.3
	No N_2 was produced				

O_2 concentration in flask 4 was checked by sampling at 3-day intervals and replenished as necessary. O_2 concentration did not drop below 8%.

* No attempt was made to remove endogenous nitrate which was less than 5 ppm N.

TABLE 2: EFFECT OF CELL NUMBERS ON N_2O PRODUCTION BY *R. JAPONICUM* IN STERILE YELLOW-BROWN EARTH

Flask No.	Cells/g Soil	Added NO_3' (ppm N)	% NO_3'-N Recovered as N_2O after 6 days: Expt 1	Expt 2
1	0	80	0	0.4
2	10^7	0*	2.0	2.8
3	10^3	80	0	0
4	10^5	80	32.7	10.8
5	10^7	80	42.9	46.9
	Run under air			

O_2 concentration in all flasks was checked at 2-day intervals, and replenished as necessary. O_2 concentration did not drop below 15%.

* No attempt was made to remove endogenous nitrate, which was less than 5 ppm N.

DISCUSSION

From the organism's point of view, denitrification may enhance survival or even permit growth under anaerobic conditions if nitrate is present, but it is difficult to know if this survival is an agricultural advantage. It has

* Full details of the experimental procedures outlined in this paper are available from the authors.

been suggested (P. Bonish, pers. comm.) that in the symbiotic situation it may serve the useful purpose of removing exogenous nitrate and nitrite, both inhibitors of nitrogen fixation (and of nodulation), from the immediate vicinity of the nitrogen-fixing enzyme system. This would enable nitrogen fixation to proceed even in urine spots or in the presence of added fertilizer, while nitrate not actually diffusing into the nodule might still be available for plant use: but since rhizosphere numbers of free-living rhizobia are high in the region of nodulated legumes much of this nitrate may be lost by denitrification. Furthermore, in the absence of added nitrogenous fertilizer, and particularly in marginal nitrogen-deficient soils where rhizobia may be the main source of fixed nitrogen, the nitrogen loss caused by rhizobial denitrification may be very deleterious.

There is now conclusive evidence that some rhizobia denitrify in liquid culture under laboratory conditions, and evidence presented here shows that they also do so in soils. There is every reason to believe this will also occur under field conditions. The findings of Limmer and Steele (1980) that in all New Zealand soils examined denitrification occurred mainly in the rhizosphere, and that maize paddocks exhibited much lower rates of denitrification than adjacent paddocks in pasture, are consistent with this. Field studies are needed to determine if rhizobial denitrification is of agricultural significance, and if so under what conditions. When this is known, since only some rhizobial strains denitrify, it should be quite possible to include denitrification ability as one of the strain characteristics on which a choice of agriculturally recommended rhizobial strain is based.

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