Effect of Oxygen on the Synthesis, Activity and Breakdown of the Rhizobium Denitrification System

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(Received 3 November 1982; revised 21 March 1983)

The synthesis, activity and breakdown of the denitrifying enzymes of *Rhizobium japonicum*, *R. lupini* and *R. meliloti* were found to be regulated by O₂. Nitrogen oxide reductases were present in anaerobically grown and symbiotic *R. japonicum*, but in the case of organisms that had been grown aerobically the enzymes were induced only after a period of incubation under anaerobic conditions. Activity of the denitrification system that had been induced in aerobically grown cells was inhibited by O₂. Denitrification by anaerobically grown cells and bacteroids was stimulated by 5% O₂. Air inhibited denitrification completely. Little loss of denitrifying activity was shown by cells incubated in 5% O₂, but cells incubated at $\geq 10\%$ O₂ showed a rapid loss of denitrification activity.

INTRODUCTION

In bacterial respiration the use of ionic and gaseous nitrogen oxides as terminal electron acceptors is usually considered an anaerobic process, and in soils and sediments anaerobic microsites have been invoked to account for denitrification under apparently aerobic conditions (Drew & Lynch, 1980; Smith, 1980). It has been generally assumed that the nitrogen oxide reductases are repressed by O₂ (Knowles, 1982). Derepression of many nitrogen oxide reductases occurs within 40 min to 3 h of the removal of O₂ (Payne, 1981). However, it has been reported that derepression of dissimilatory nitrate reductase in *Pseudomonas aeruginosa* occurs at 5% O₂ (Sacks & Barker, 1949) and that the dissimilatory nitrite reductase in *Thiobacillus denitrificans* is partially derepressed at 7.8% O₂ (Justin & Kelly, 1978).

Similar variation has been reported for the effect of O₂ on the activity and breakdown of denitrifying enzymes (Payne, 1981). Generally, O₂ completely inhibits dissimilatory nitrate reduction (Knowles, 1982), but Betlach & Tiedje (1981) reported that 10% O₂ did not inhibit the reduction of nitrate to nitrous oxide (N₂O) and dinitrogen (N₂) by *Flavobacterium* sp. during a 30 min incubation. Some, but not all, of these disparities may result from inadequate control and measurement of O₂ concentrations.

Strains of rhizobia have been shown to possess denitrifying capability both in the free-living (Zablotowicz *et al.*, 1978; Daniel *et al.*, 1980*b*, 1982) and symbiotic forms (Zablotowicz & Focht, 1979). Although denitrification by rhizobia has also been shown to occur in soils (Daniel *et al.*, 1980*a*), as yet the conditions affecting the process, and hence its agricultural significance, are not known.

The present study examined the effect of O₂ on the synthesis, activity and breakdown of denitrification enzymes in three *Rhizobium* spp. grown under aerobic, anaerobic, and symbiotic conditions, to determine the conditions under which rhizobial denitrification occurs.

METHODS

Bacterial strains and growth conditions. *Rhizobium japonicum* 505 (Wisconsin) was obtained as CC 705 from the Division of Plant Industry, CSIRO, Canberra, Australia. *Rhizobium lupini* PDD 4681 and *R. meliloti* PDD 2751 were obtained from the Plant Diseases Division, DSIR, Auckland, from Mr P. Bonish, Ruakura Agricultural Research Centre, Hamilton, New Zealand.

Rhizobia were maintained on yeast extract/mannitol (Daniel & Appleby, 1972) agar slopes. Inocula were aerobically grown in the same yeast extract/mannitol medium in an orbital shaker at 26 °C.

Preparation of aerobic bacteria. Aerobic cells were grown in 200 ml yeast extract/mannitol medium, with or without 6 mM-KNO₃, contained in a 2 litre flask on an orbital shaker (160 r.p.m.) at 26 °C. Cells were harvested in early-exponential phase ($\sim 40 \mu\text{g}$ protein ml⁻¹, $A_{680} \sim 0.3$) by centrifugation (4800 g, 15 min), washed three times in sterile 0.1 M-sodium phosphate buffer (pH 6.8) and finally resuspended in buffer. The O₂ tension during growth was tested on replicate flasks using a Clark oxygen electrode and O₂ tension was maintained at >90% of air saturation ($> 230 \mu\text{M-O}_2$).

Preparation of anaerobic bacteria. Anaerobic cells were grown in 15 ml Pyrex conical centrifuge tubes containing 12 ml yeast extract/mannitol medium supplemented with separately sterilized 6 mM-KNO₃ (Daniel *et al.*, 1982). The tubes were sealed with a sterile rubber seal and cultures were grown to late-exponential phase (7–10 d) at 26 °C. The headspace of each tube was tested for the presence of N₂O, and cells were harvested by centrifugation as described above.

Preparation of symbiotic organisms. Bacteroids were isolated and purified from N₂-fixing root nodules of: soybean (*Glycine max* cv. Lincoln) inoculated with *R. japonicum* CC705; lotus (*Lotus pedunculatus* cv. Marku) inoculated with *R. lupini* PDD 4681; and lucerne (*Medicago sativa* cv. Wairau) inoculated with *R. meliloti* PDD 2751. Lotus and lucerne seeds were surface-sterilized by soaking in dry 18 M-H₂SO₄ (5 min) followed by repeated washing in sterile distilled water. Soybean seeds were washed in 95% ethanol (30 s), soaked in 10% (w/v) NaOCl (15 min) and washed in sterile distilled water. Seeds were planted directly into quartz sand, previously purified with steam-heated 18% (w/v) HCl containing 1% (w/v) oxalic acid in a 'Keebush' sand-purification unit (Hewitt, 1966) and washed with deionized water. Pots (15 cm diameter) were soaked in 10% (w/v) NaOCl for 24 h prior to addition of 1 kg of sand. Each pot was inoculated with the required *Rhizobium* strain immediately after planting, and again 2 weeks after emergence of shoots. A 2 cm layer of wax-coated sand [1 g paraffin wax (kg sand)⁻¹] was applied to the top of each pot to act as a dust filter. Pots were watered daily to water-holding capacity with a N-free nutrient solution (Smith *et al.*, 1980), the solution being introduced below the wax-coated sand.

Bacteroids were prepared from root nodules by methods used by previous workers (Appleby, 1969; Rigaud *et al.*, 1973; Kennedy *et al.*, 1975). At harvest, roots were removed from pots, shaken free of adhering sand and washed in cold (4 °C) sterile distilled water. Root nodules were rapidly picked off, rinsed in 70% (v/v) ethanol (30 s), then washed in cold 0.1 M-sodium phosphate buffer (pH 6.8) and finally suspended in 3–4 vol. of N₂-flushed, O₂-free 0.1 M-sodium phosphate buffer containing 2 mM-MgSO₄, 0.3 M-sucrose, 0.2 M-sodium ascorbate and 10% (w/v) polyvinylpyrrolidone. All subsequent manipulations were carried out under N₂ at 4 °C in a glove box. Nodules were disrupted in a sealed Waring blender for 1 min and the homogenate was twice filtered through Miracloth (Calbiochem) to remove nodule cortex tissue. The filtrate was centrifuged (200 g; 10 min) to remove starch granules and remaining cortex, then bacteroids were sedimented at 6000 g for 30 min and washed twice with anaerobic 0.1 M-sodium phosphate buffer (pH 6.8) containing 2 mM-MgSO₄ and 0.3 M-sucrose. Finally, the bacteroids were suspended in the same medium in the ratio of 1:3 (packed cells/liquid) and used immediately or stored at -196 °C.

Denitrification reactions. Denitrification by aerobically, anaerobically and symbiotically grown *Rhizobium* spp. was determined using cell suspensions of 0.1 M-sodium phosphate buffer (pH 6.8) contained in a stirred O₂ electrode (Rank Bros, Bottisham, Cambridge) maintained at 24 ± 0.5 °C. The reaction mixture consisted of 2 ml cell suspension, 6 mM-KNO₃, 2 mM-sodium succinate and 60 mM-glucose in a final volume of 3 ml, with a 0.5 ml gas headspace above the suspension. A modified perspex plug for the oxygen electrode was fitted with a 21 G hypodermic needle (Becton-Dickinson Co., N.J., U.S.A.) inserted into the reaction mixture. The dissolved O₂ concentration of the reaction mixture was monitored using the electrode and controlled by the supply of Ar/O₂ mixtures being bubbled through the needle into the reaction mixture at about 1.5 ml min⁻¹ using a peristaltic pump (Cole-Palmer, Chicago, U.S.A.). Using standard N₂O solutions, the equilibration time for the reaction mixture with the gas headspace was shown to be less than 2 min and with gas mixture flowing through the reaction suspension the half-time for removal of N₂O from solution was 80 s. Samples of the effluent gas and reaction mixture were analysed for NO, N₂O and nitrite. Replicate reactions were run with and without acetylene (C₂H₂; 10%, v/v) in the gas mixture. The synthesis of nitrogen oxide reductases was examined with and without 200 μg chloramphenicol ml⁻¹ present in the reaction mixture. Controls were done in the absence of nitrate and also with N₂O solutions in the absence of bacterial cells.

Other analyses. Nitrite accumulation was estimated as previously described (Daniel *et al.*, 1982).

Gas mixtures were analysed using a Varian 3700 gas chromatograph equipped with a 3 m $\times$ 2 mm stainless steel column containing Porapak Q (80–100 mesh) at 35 °C, with Ar/CH₄ (95:5) as carrier gas (flow rate 25 ml min⁻¹). A ⁶³Ni-electron-capture detector at 300 °C (Kaspar & Tiedje, 1980) was used to detect very low concentrations of NO and N₂O. Under the conditions used, the detection limits for a 0.5 ml gas sample were 2×10^{-12} mol for N₂O and 2×10^{-11} mol for NO.

Protein content of whole cells was determined colorimetrically by the procedure of Goa (1953), using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Products of rhizobial denitrification

No accumulation of nitrite, NO or N_2O was detected in cell suspensions incubated in the absence of nitrate, with or without C_2H_2 in the inflowing gas. In the presence of nitrate, *R. japonicum* bacteroids accumulated nitrite [$60 \mu\text{mol h}^{-1} (\text{g protein})^{-1}$] in the reaction mixture during anaerobic denitrification, but cells grown aerobically or anaerobically did not (Table 1). Both *R. lupini* and *R. meliloti* showed very variable nitrite accumulation.

Low concentrations of NO appeared transiently in denitrifying suspensions of all three species tested, and disappeared from the headspace when all the nitrate and nitrite had been used. In *Flavobacterium* sp. and *Pseudomonas fluorescens* this has been taken to show that NO is an intermediate in the denitrification pathway of these organisms (Betlach & Tiedje, 1981).

Small amounts of N_2O were produced by *R. meliloti* in the absence of C_2H_2 . In the presence of C_2H_2 , however, high concentrations of N_2O were produced, confirming that N_2 is the major end product of denitrification by this strain (Daniel *et al.*, 1982). High levels of N_2O were produced by *R. japonicum* and *R. lupini*, with no significant increase in N_2O concentration when C_2H_2 was present, confirming N_2O as the major end product of these strains (Daniel *et al.*, 1982). *Rhizobium japonicum* was the most active denitrifier under the growth conditions used in the present study.

Effect of oxygen on the synthesis of the rhizobial denitrification system

Anaerobic and symbiotic *R. japonicum* cells were capable of immediate denitrification and the presence of the protein synthesis inhibitor chloramphenicol did not affect denitrification. However, aerobically grown cells required approximately 60 min anaerobic incubation before denitrification would occur, and these cells did not denitrify if chloramphenicol was present during this incubation. If any O_2 was present ($> 0.26 \mu\text{M-}O_2$) during this incubation, no denitrification occurred. The presence or absence of nitrate had no effect on the synthesis of these enzymes. This 60 min anaerobic incubation period is similar to the time lag necessary for the induction of denitrifying enzymes in other organisms (Payne, 1981; Knowles, 1982). Nitrite accumulated at a rate of about $1 \mu\text{mol min}^{-1} (\text{g protein})^{-1}$ in anaerobic suspensions of aerobically grown cells containing $200 \mu\text{g chloramphenicol ml}^{-1}$, showing the presence of a nitrate reductase in aerobically grown *R. japonicum*. The 60 min delay before detection of N_2O was presumably necessary for the synthesis of nitrite reductase and possibly NO reductase.

The reduction of nitrate to N_2O by *R. japonicum* and *R. lupini* bacteroids and to N_2 by *R. meliloti* bacteroids demonstrates the existence of complete denitrification systems in rhizobia grown symbiotically in the absence of external combined nitrogen. The presence of nitrate reductase activity in bacteroids isolated from legume nodules grown without combined nitrogen is well established (e.g. Cheniae & Evans, 1960; Daniel & Appleby, 1972; Kennedy *et al.*, 1975). Our results show that synthesis of denitrification enzymes in the three *Rhizobium* spp. examined is not controlled by nitrate but by O_2 tension, as was found by Daniel & Gray (1976) for nitrate reductase in *R. japonicum*. Furthermore, activities of nitrogen oxide reductases were similar in anaerobically grown cells and in bacteroids, presumably because the O_2 concentration within the root nodule is very low ($\approx 11 \text{ nM}$; Appleby, 1974).

Effect of oxygen on rhizobium denitrification activity

Denitrification activity in the three *Rhizobium* spp. tested was controlled by the oxygen concentration in the reaction mixture (Table 2). Aerobic rhizobia did not denitrify in the presence of O_2 , whereas the rate of denitrification was faster at 5% O_2 than at 0% O_2 for cells grown anaerobically and symbiotically. No activity was shown by cells incubated above 8% O_2 . When the O_2 tension was shifted between 0%, 5% and above 8% O_2 , in the same cell suspension, the change in denitrification rate was immediate and reversible. The enhanced rates of N_2O production at 5% O_2 were observed to continue for $> 2 \text{ h}$.

A possible increase in nitrate and nitrite reduction by *Rhizobium* spp. cultured under micro-aerobic conditions, as compared with anaerobic cultures, was suggested by the results of

Table 1. *Products of rhizobial denitrification*

Accumulation of intermediates and end products of denitrification were measured after a 1 h anaerobic incubation of suspensions (3 ml) of three *Rhizobium* spp. grown aerobically or anaerobically or symbiotically. Substrate concentrations were: succinate, 2 mM; glucose, 60 mM; KNO₃, 6 mM. Reactions were carried out with 0.7 to 2.0 mg bacterial protein. All assays were conducted after an initial 60 min anaerobic pre-incubation in the absence of nitrate. Mean values $\pm$ S.D. are given; the numbers of experiments done are shown in parentheses.

Strain	Growth condition (No. of cultures used)	Denitrification products [μ mol (g protein) ⁻¹]			
		NO ₂ ⁻	NO	N ₂ O†	
<i>R. japonicum</i> CC 705	Aerobic $\pm$ KNO ₃ * (9)	0 (21)	0.3 $\pm$ 0.1 (21)	minus C ₂ H ₂	plus C ₂ H ₂
	Anaerobic $\pm$ KNO ₃ (12)	0 (19)	0.3 $\pm$ 0.1 (19)	532 $\pm$ 35 (21)	570 $\pm$ 35 (21)
	Symbiotic (2)	60.0 $\pm$ 2.0 (4)	0.3 $\pm$ 0.1 (4)	2400 $\pm$ 200 (19)	2205 $\pm$ 210 (19)
<i>R. lupini</i> PDD 4681	Aerobic $\pm$ KNO ₃ * (4)	280.0 $\pm$ 140.0 (12)	0.01 $\pm$ 0.005 (12)	1980 $\pm$ 150 (4)	1850 $\pm$ 160 (4)
	Anaerobic $\pm$ KNO ₃ (6)	1.5 $\pm$ 1.0 (12)	0.1 $\pm$ 0.03 (12)	30 $\pm$ 5 (12)	28 $\pm$ 5 (12)
	Symbiotic (2)	2.5 $\pm$ 2.0 (4)	0.1 $\pm$ 0.05 (4)	48 $\pm$ 7 (12)	55 $\pm$ 6 (12)
<i>R. meliloti</i> PDD 2751	Aerobic $\pm$ KNO ₃ * (4)	2.0 $\pm$ 1.6 (12)	0.1 $\pm$ 0.05 (12)	145 $\pm$ 15 (4)	126 $\pm$ 16 (4)
	Anaerobic $\pm$ KNO ₃ (7)	2.0 $\pm$ 1.6 (12)	0.3 $\pm$ 0.07 (12)	0.3 $\pm$ 0.1 (12)	84 $\pm$ 4 (12)
	Symbiotic (2)	0 (4)	0.3 $\pm$ 0.09 (4)	3.0 $\pm$ 0.4 (4)	84 $\pm$ 4 (12)

* No difference was found between cells grown in the presence and absence of nitrate.

† Assays in the presence and absence of C₂H₂ in the gas phase.

Table 2. *Effects of oxygen on the rate of rhizobial denitrification*

Effects of oxygen on the denitrification rate were determined for three *Rhizobium* spp. grown aerobically, anaerobically or symbiotically. Substrate concentrations were: succinate, 2 mM; glucose, 60 mM; KNO₃, 6 mM. Reactions were carried out with 0.7 to 2.0 mg bacterial protein. All assays were conducted after an initial 60 min anaerobic pre-incubation in the absence of nitrate. Mean values $\pm$ S.D. are given; the numbers of experiments done are shown in parentheses. No N₂O was produced by any of the preparations when the gas phase was air.

Strain	Growth condition (No. of cultures used)	Denitrification rate [$\mu\text{mol N}_2\text{O min}^{-1}$ (g protein) $^{-1}$] with gas phase:	
		0 % O ₂	5 % O ₂
<i>R. japonicum</i> CC 705	Aerobic $\pm$ KNO ₃ * (9)	23.0 $\pm$ 4.1 (21)	0 (21)
	Anaerobic + KNO ₃ (12)	40.0 $\pm$ 5.2 (19)	80.0 $\pm$ 6.5 (19)
	Symbiotic (2)	33.0 $\pm$ 4.4 (4)	75.0 $\pm$ 7.5 (4)
<i>R. lupini</i> PDD 4681	Aerobic $\pm$ KNO ₃ * (4)	0.5 $\pm$ 0.3 (12)	0 (12)
	Anaerobic + KNO ₃ (6)	0.8 $\pm$ 0.5 (12)	2.6 $\pm$ 0.7 (12)
	Symbiotic (2)	2.1 $\pm$ 0.5 (4)	11.3 $\pm$ 1.4 (4)
<i>R. meliloti</i> † PDD2751	Aerobic $\pm$ KNO ₃ * (4)	5.7 $\pm$ 1.3 (12)	0 (12)
	Anaerobic + KNO ₃ (7)	6.2 $\pm$ 1.2 (12)	10.5 $\pm$ 2.6 (12)
	Symbiotic (2)	7.3 $\pm$ 1.7 (4)	13.2 $\pm$ 1.2 (4)

* No difference was found between cells grown in the presence and absence of nitrate.

† *Rhizobium meliloti* suspensions contained 10% (v/v) C₂H₂ in the gas phase.

Murphy & Elkan (1965), and Picci & Lepidi (1967). Increasing aeration of *R. japonicum* cultures increased nitrate reduction, and, using a mass spectrophotometer, the products of nitrate reduction were suggested to be nitrite, N₂O and possibly NO and N₂ (Murphy & Elkan, 1965). However, in these earlier studies the O₂ tension of the culture medium during growth was not measured or controlled.

Effect of oxygen on the breakdown of rhizobium denitrification enzymes

Denitrifying cells of *R. japonicum* incubated for 48 h in O₂ concentrations of 0%, 3% and 5% showed little loss of denitrification activity when assayed under anaerobic conditions. However, cells incubated at $\geq 10\%$ O₂ showed an irreversible loss of anaerobic denitrification activity, with more than half of the activity being lost after 1 h incubation. Thus O₂ affects the stability of rhizobial denitrification enzymes as well as their activity.

Ecological considerations

Denitrification allows rhizobia to survive and grow under anaerobic conditions (e.g. Zablotowicz *et al.*, 1978; Daniel *et al.*, 1980*b*). Furthermore, since nitrate and nitrite ions in the soil are known to inhibit the infection and nodule development stages of the *Rhizobium*-legume symbiosis (Dart, 1977; Gibson, 1977; Dazzo & Brill, 1978), it might well be of selective advantage to free-living rhizobia if inhibitory concentrations of nitrate and nitrite were removed from the rhizosphere by denitrification. Although the assimilation of such soil nitrate would seem to be a much 'cheaper' process than establishment of a symbiosis for N₂ fixation, the latter may be of greater long term benefit to rhizobia.

The advantage of rhizobial denitrification within the root nodule is less clear. Although rhizobial denitrification may remove the nitrogen oxides which also adversely affect N₂ fixation (Pate, 1977; Burris, 1979; Trinchant & Rigaud, 1981, 1982) it is not obvious how this removal benefits the bacteria or the plant, since the energetic cost of assimilating nitrogen by N₂ fixation is large compared with nitrate assimilation. The reduction of nitrate to N₂O by *R. japonicum* (Daniel *et al.*, 1980*b*) and the assimilation of ¹⁵N₂O by soybean bacteroids via nitrogenase action (Mozen & Burris, 1954) suggest that nitrate may be incorporated via this pathway, but at a considerable cost in energy. Indeed, the assimilation of ¹⁵NO₃ by pea, soybean and lucerne root nodules (Pate, 1977; Randall *et al.*, 1978; Ohya & Kumazawa, 1979; Vance & Heichel,

1981) may occur by this process, as it does in the photosynthetic, denitrifying, nitrogen-fixing bacterium, *Rhodopseudomonas sphaeroides* (Dunstan *et al.*, 1982). Under these conditions the bacteroids could be considered to be parasitic on the host legume because they provide a nitrogen assimilation system which is more energy expensive than that involving plant enzymes alone. However, the total amount of plant nitrogen derived from reduction of nitrate in root nodules is considered to be small (Vance & Heichel, 1981) and it is not known how much, if any, nitrogen is lost as N_2O and N_2 . Further studies using ^{15}N -labelled compounds will be necessary to determine whether significant losses of nitrogen occur through bacteroid denitrification.

Since the concentration of O_2 is low in root nodules, the control of rhizobial denitrification by O_2 observed in the present study probably does not actually occur in functional bacteroids. It is more likely to be important in free-living rhizobia in the rhizosphere, in immature and senescing bacteroids in root nodules, and in senescent root nodules when rapid use of available energy sources may be advantageous for survival. The rationale for the control by O_2 of the induction and breakdown of the denitrification system for rhizobia therefore seems straightforward. However, the advantage to anaerobic and symbiotic cells of an increasing denitrification rate up to an O_2 tension of 5% is not clear. Respiration is fully active in *R. japonicum* at this O_2 tension in the presence of nitrate (R. M. Daniel, unpublished observations), so that the use of nitrate as an electron acceptor is inefficient, since less energy is generated per mol of substrate consumed (Daniel *et al.*, 1980*b*). Perhaps the capacity to simultaneously respire to O_2 and nitrate is an advantage when excess carbon source is available, such as might occur during plant or nodule senescence.

Some of the uncertainties concerning the effect of nitrate reductase on symbiotic efficiency (e.g. Cheniae & Evans, 1960; Bergersen, 1961; Antoun *et al.*, 1980; Vasconcelos *et al.*, 1980) may have arisen because of the presence of other denitrification enzymes. To date, most reports of the synthesis and accumulation of nitrite in legume nodules, and the interaction of nitrate reductase and nitrogenase, have not included consideration of the influence of the other nitrogen oxide reductases, (e.g. Sik *et al.*, 1976; Gibson & Pagan, 1977; Manhart & Wong, 1980; Streeter, 1982).

The current concept of anaerobic micro-environments invoked to explain denitrification in aerobic soils (Drew & Lynch, 1980) is based largely on O_2 flux calculations (Smith, 1980), and the production of N_2O or H_2S , which are usually associated with anoxic conditions (Paul & Victoria, 1978). However, there have been few direct measurements to test models of anaerobic zones in soils (Smith, 1980), and the present results demonstrate that significant denitrification by rhizobia may occur in aerobic as well as anaerobic microsites in the soil after quite short exposures to anaerobic conditions. The bulk of soil in land use for most agricultural crops is aerobic or micro-aerobic (Drew & Lynch, 1980), and the results here, together with those of Daniel *et al.* (1980*a*), show that rhizobial denitrification may occur and persist in the rhizosphere, even under conditions where O_2 is present. Until information is available on rhizobia grown at O_2 tensions approximating those found in soils, it is difficult to comment on the significance for agricultural systems of the O_2 control of denitrification shown here.

The denitrification system in rhizobia has two potential functions. The reduction of nitrate to nitrite yields energy and enables anaerobic growth (Daniel & Appleby, 1972; Daniel *et al.*, 1980*b*), and possibly enhanced survival under anaerobic conditions. Denitrification also has a detoxification role since nitrate and nitrite prevent nodulation and inhibit nitrogen fixation. This study has shown both these functions of the denitrification system are dependent on O_2 .

We acknowledge financial support from the Ministry of Agriculture and Fisheries. We thank Mr P. Bonish of M. A. F. Ruakura Research Centre for the supply of rhizobium strains, Mr P. de Nicolo for technical assistance and Dr A. B. Cooper for helpful discussions.

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