

Review

**Nitrate reduction and nitrogen fixation in symbiotic association
Rhizobium – legumes**

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The inhibitory effect of nitrate on nitrogenase activity in root nodules of legume plants has been known for a long time. The major factor inducing changes in nitrogenase activity is the concentration of free oxygen inside nodules. Oxygen availability in the infected zone of nodule is limited, among others, by the gas diffusion resistance in nodule cortex. The presence of nitrate may cause changes in the resistance to O₂ diffusion.

The aim of this paper is to review literature data concerning the effect of nitrate on the symbiotic association between rhizobia and legume plants, with special emphasis on nitrogenase activity. Recent advances indicate that symbiotic associations of *Rhizobium* strains characterized by a high nitrate reductase activity are less susceptible to inhibition by nitrate. A thesis may be put forward that dissimilatory nitrate reduction, catalyzed by bacteroid nitrate reductase, significantly facilitates the symbiotic function of bacteroids.

Nitrogen (N) is the major limiting nutrient for most plant species (Greenwood, 1982). Plants require N from soil or from atmosphere, by symbiotic N₂ fixation (Vance, 1990). Soil-derived N, generally in the form of

NO₃⁻, is reduced to ammonia by a two-step process. The first step, reduction of nitrate to nitrite, is catalysed by the plant nitrate reductase (EC 1.6.6.4; NR, Solomonson & Barber, 1990), which is an inducible plant en-

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Abbreviations: N, nitrogen; NO₃⁻, nitrate; NR, nitrate reductase; cNR, constitutive NR; iNR, inducible NR.

zyme, depending on the availability of nitrate and light (Campbell, 1999). In regulation of NR activity both, gene expression and post-translational processes are involved (Kaiser & Huber, 2001), however these aspects will not be considered here. In this paper the relationship between bacteroid nitrate reductase (EC 1.7.99.4.; NR) activity and N₂ fixation will be presented.

For a long time it has been well known that nitrate inhibits nitrogenase activity in legume plant nodules. Many investigators have demonstrated the negative effect of nitrate on root infection by *Rhizobium*, and on the ratio of the nodule dry mass to the whole plant mass. The effect of nitrate on the symbiosis between legumes and rhizobia can be divided into three major areas: (1) the role of nitrate availability during root infection, (2) the relationship between nitrate availability and nitrogenase activity, and (3) the effect of nitrate on the ratio of the nodule dry mass to the whole plant mass (Streeter, 1988).

Aside from nitrogenase activity, in many symbiotic associations between legumes and rhizobia the activity of nitrate reductase has also been observed, e.g. in root nodules of soybean (Heckeman & Drevon, 1987; Hunter, 1983; Randall *et al.*, 1978) and in yellow lupin. According to our results NR activity in nodules of yellow lupine is a few dozen times higher than in other parts of the plant (Polcyn & Luciński, 2001). Moreover, 97% of nodular NR activity is localized in bacteroids (Polcyn & Luciński, 2001). Taking this into consideration, two hypotheses concerning direct relationship between NR activity and inhibition of nitrogenase have been put forward. The first explains the decrease in the efficiency of N₂ fixation as the effect of competition for reductive power between nitrogenase and NR. The second hypothesis proclaims that inhibition of nitrogenase by nitrite is generated by bacteroid NR activity (Riguard, 1976; Streeter, 1988; Becana *et al.*, 1989; Sprent *et al.*, 1987; Giannakis *et al.*, 1988; Becana & Sprent, 1987; Arrese-Igor *et al.*, 1997).

It is known that the concentration of free oxygen inside nodules is the major factor affecting nitrogenase activity (Layzell & Hunt, 1990). Oxygen availability in the infected zone of root nodules is controlled by the plant host (Steeter, 1994; Parsons & Day, 1990; Ianetta *et al.*, 1993; James *et al.*, 1991; Witty *et al.*, 1984; Drevon *et al.*, 1995; Layzell *et al.*, 1990; Minchin, 1997). On one hand, this is due to the presence of leghemoglobin; on the other hand oxygen availability is limited by the diffusion resistance (Streeter, 1994; Parsons & Day, 1990; Appelby, 1984; Becana & Klucas, 1992; Robertson *et al.*, 1984; Chamber-Perez *et al.*, 1997). The presence of nitrate may directly or indirectly influence the efficiency of gas diffusion resistance (Minchin *et al.*, 1986a; 1986b; Schuller *et al.*, 1988; Vessey *et al.*, 1988a; Carroll *et al.*, 1987; Arrese-Igor *et al.*, 1997).

There are several publications demonstrating that enhancement of N₂ fixation is due to the presence of active NR in bacteroids. Symbiotic associations in which NR function is complementary to that of nitrogenase are possible since bacteroids of many rhizobial strains are capable of performing dissimilatory nitrate reduction (Chamber-Perez *et al.*, 1997; Serrano & Chamber, 1990). The role of denitrification might be dual. On one hand it would remove toxic nitrite, on the other hand it could supply ATP under oxygen deprivation during the first step of denitrification process named nitrate respiration (Fig. 1) (Arrese-Igor *et al.*, 1990; Garcia-Plazaola *et al.*, 1993; Daniel *et al.*, 1982; O'Hara & Daniel, 1985; Chamber-Perez & Serrano, 1991).

The data presented above suggest that N₂ fixation and denitrification, two processes that are antagonistic in the nitrogen cycle, may perform complementary functions in the cells of symbiotic rhizobia that would be important for survival of bacteria inside root nodules.

In the first part of this paper we would like to present the influence of nitrate on nodule formation, and on the ratio of their mass to the whole plant mass. In the second part, the hy-

potheses explaining the inhibition of nitrogenase activity by nitrate, both from the plant host's and the microsymbiont's side, will be discussed.

THE INFLUENCE OF NITRATE ON NODULATION

There is much information on the negative effect of nitrate on the nodulation induced by rhizobia (Champigny *et al.*, 1985; Giannakis *et al.*, 1988; Alcantar-Gonzales *et al.*, 1988). Until the end of the 80's, it was believed that the presence of nitrate in medium completely inhibited or drastically reduced the efficiency of infection in strains that possessed any NR activity (Alcantar-Gonzales *et al.*, 1988). However, it was shown that at least in some *Rhizobium* strains the presence of nitrate did not affect this process negatively. Serrano & Chamber (1990) investigated three *Bradyrhizobium* sp. (*Lupinus*) strains differing in NR activity: the first strain possessed constitutive NR (cNR), the second inducible NR (iNR), and the third did not express NR activity (NR⁻). The results demonstrated that the strains with cNR and iNR activity were capable of infecting roots sufficiently even at relatively high (12 mM) nitrate concentration, whereas the NR⁻ strain completely lost the ability to form nodules. Moreover, at nitrate concentration of 1–2 mM, the strains with NR activity (either constitutive or inducible) infected roots slightly more efficiently than in the absence of nitrate. The described "patterns" of infection ability resulted in production of durable nodules, which was observed three weeks after. These results show that there is a possibility of formation of symbiotic associations, in which the presence of nitrate does not inhibit nodulation. The effect of NR activity on the efficiency of root infection might be explained by the removal of nitrate ions from

the bacterial environment (O'Hara & Daniel, 1985).

INFLUENCE OF NITRATE ON LOSS IN THE ROOT NODULE DRY MASS

The majority of legume plants respond to the presence of nitrate in medium by loss in their protein content and nodule dry mass (Arrese-Igor *et al.*, 1990). The presence of active NR, however, may alter the scale of this process. Serrano & Chamber's (1990) experiments with yellow lupin (*Lupinus luteus*) infected with three *Bradyrhizobium* spp. (*Lupinus*) strains showed that the mass of nodules produced by the strains with NR activity (either inducible or constitutive) was higher than the mass of nodules produced by the NR-negative strain. This effect was positively correlated with the nitrogen content in plant shoots. Other symbiotic associations also show some degree of resistance to nitrate. Root nodules initiated by *Rhizobium meliloti* are capable of keeping a constant protein concentration in the presence of 5 mM KNO₃ (Arrese-Igor *et al.*, 1997). These examples prompted some authors to claim that nitrate alone is unable to upset both the symbiosis itself and its initiation (Serrano & Chamber, 1990).

Resistance of several *Rhizobium* species to nitrate during infection and nodulation may, to a certain degree, depend on the activity of hydrogenase, an enzyme characteristic of some diazotrophs. The strains with hydrogenase activity are more tolerant to nitrates (Serrano & Chamber, 1990). Hydrogenase is a membrane enzyme that contributes to formation of H⁺ gradient across bacteroid membrane, which enables ATP synthesis. In *E. coli* the H⁺ gradient may also be used by the membrane NR for nitrate reduction due to difference in the potentials: -0.42 V for hydrogenase and +0.42 V for NR (Uden, 1997).

INFLUENCE OF NITRATE ON NITROGENASE ACTIVITY

A decrease in the efficiency of nitrogen fixation by bacteroids in the presence of nitrate was repeatedly reported (Chamber-Perez *et al.*, 1997; Serrano & Chamber, 1990; Arrese-Igor *et al.*, 1997; Minchin *et al.*, 1986a; Riguard, 1976). Many hypotheses have been proposed to explain the mechanism of the nitrate effect, yet it still remains unclear. Most of the research concerning this problem approached it from the plant host's "viewpoint", overlooking the fact that the bacterial microsymbiont is not entirely subordinated to plant metabolism. In recent years, several papers were published showing the other side of the symbiotic associations and they reported new observations might be helpful in attempts at understanding the effect of bacteroid nitrate and nitrite reductases on nitrogenase activity. These results will be discussed in the final chapter.

Hypotheses explaining the decreased nitrogen fixation as the effect of decreased bacteroid respiration, caused by increased resistance to O₂ diffusion in the nodule cortex, appeared in the 80's (Minchin *et al.*, 1986a; 1986b). The fast changes in gas diffusion resistance have been explained by various mechanism: as changes in osmotic potential of nodule cells (Witty *et al.*, 1986), filling of intercellular spaces in nodules by H₂O molecules (Witty *et al.*, 1986; Walsh *et al.*, 1989; Ismade, 1991), or variable quantity of passing phloem sap (Layzell & Hunt, 1990).

There are numerous data confirming the presumption that the presence of nitrate rises the gas diffusion resistance (Fig. 1). In the presence of nitrate, both the energy cost of nitrogen fixation (expressed as the quantity of released CO₂ per mol of reduced C₂H₂) and the gas diffusion resistance increases, but the efficiency of bacteroid respiration (measured as $\mu\text{mol CO}_2$ per gram dry mass per min) decreases.

Some authors have suggested that the increase in gas diffusion resistance could be a

secondary response to metabolic changes, as in the case of the effects caused by water stress and low temperature (Arrese-Igor *et al.*, 1997).

INFLUENCE OF NITRATE ON GAS DIFFUSION RESISTANCE

One of possible ways in which nitrate can affect the gas diffusion resistance is its direct influence (Minchin *et al.*, 1986a; Carroll *et al.*, 1987; Vessey *et al.*, 1988a; Diaz del Castillo & Layzell, 1995; Kuzma *et al.*, 1995). About 90% of the nitrate applied onto the nodule surface accumulated in the nodule cortex (Sprent *et al.*, 1987). Vessey and Waterer (1992) suggested that high nitrate concentration could cause formation of a local pH gradient or electrochemical gradient due to differences in the concentration of nitrate or in their reduction rate between cell layers. This was believed to affect gas permeability of cortex cell layer, and consequently to cause conditions in which nitrogenase activity was limited by deficiency of NAD(P)H₂ and ATP (Minchin *et al.*, 1989; Sprent *et al.*, 1987) (Fig. 1). The intensity of lupin bacteroid respiration corresponded to as much as 83% of total respiration activity in the infected zone (Ratajczak *et al.*, 1996). In such a situation, lowered bacteroid respiration activity, caused by an increase in the resistance to O₂ diffusion, could be the reason of significant ATP deprivation.

The second possibility is the indirect influence of nitrate by limiting supply of carbohydrates into the nodule. It was suggested that the inhibitory effect of nitrate on nitrogenase activity could be caused by decreased supply of carbohydrates to root nodules (Small & Leonard, 1969). When a large part of the host plant mass is involved in nitrate assimilation, the burden of the demand for reductive power and carbon frameworks is shifted from nodules to other organs. Therefore, intense nitrate metabolism in roots and leaves might lead to decrease a supply of carbohydrates to

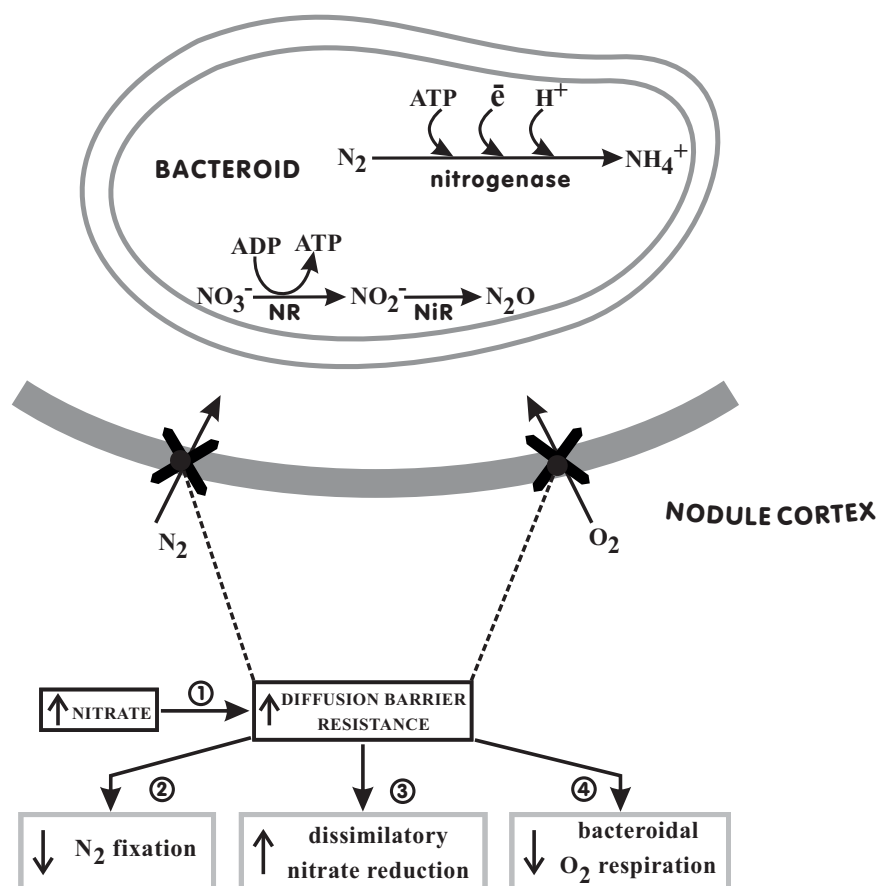


Figure 1. Effect of the gas diffusion barrier in nodule cortex on bacteroid nitrogen fixation and dissimilatory nitrate reduction.

Nitrate directly or indirectly causes the increase of diffusion barrier resistance (1) which then led to decrease of N_2 fixation (2) and bacteroidal O_2 respiration (4) (Minchin *et al.*, 1989; Sprent *et al.*, 1987; Vessey *et al.*, 1988b; Vessey & Waterer, 1992) Lowering of nodule oxygen conditions stimulate simultaneously dissimilatory nitrate reduction (3) (Arrese-Igor *et al.*, 1997), NR, NiR, dissimilatory nitrate and nitrite reductases.

nodules (Vessey *et al.*, 1988b). Consequently, it lowers the energy state and increases the resistance to diffusion of gases, including oxygen. Another interpretation suggests that the efflux of carbohydrates from the nodule may decrease their osmotic action towards the diffusion barrier, which would lower its permeability (Vessey *et al.*, 1988b).

PHLOEM TRANSPORT CONCEPTION

Parsons *et al.* (1993) explained changes in nitrogenase activity as the result of high concentration of nitrogen containing compounds flowing in with the phloem sap from lower leaves into the surroundings of root nodules, which could inhibit both the nodule growth

and nitrogenase activity. Such an effect could be caused indirectly by changes in the resistance to O_2 diffusion induced by high concentration of nitrogen compounds containing a reduced form of N (e.g., some amino acids and amides) in the phloem sap flowing into the nodule surroundings. In the case of legume plants, which nodules export reduced nitrogen as amides, such compounds as glutamate, serine or proline may play an important role.

This hypothesis was supported by experiments indicating increased nitrogenase activity in plants that had only young leaves and decreased level of nitrogen fixation in plants with only old leaves (Malik, 1983). It can be concluded that components originating from leaves, such as compounds containing reduced nitrogen, the concentration of which is

higher in phloem sap from older leaves, can affect nitrogen fixation.

DENITRIFICATION AS A SYSTEM COMPLEMENTARY TO NITROGENASE

Aside from nitrogenase activity, high NR activity is characteristic of many symbiotic associations between legumes and rhizobia. In several species, such as pea, bean (Becana & Sprent, 1987), and vetch (Caba *et al.*, 1995), NR occurs only in nodule cytosol. In other species, e.g., soybean (Hunter, 1983), lucerne (Becana *et al.*, 1985a; 1985b), yellow lupin (Alikulow *et al.*, 1980), and narrow-leaf lupin (Manhart & Wong, 1980), NR activity has been detected also in bacteroids. Moreover, in soybean (Hunter, 1983) and lucerne (Becana *et al.*, 1985a; 1985b), the activity of bacteroid NR forms 90% of total nodule NR activity. For this reason, as early as in the 70's, it was believed that the decrease in nitrogenase activity in the presence of nitrate was caused by accumulation of toxic nitrites – products of the NR activity. This, however, would require accumulation of nitrite inside bacteroids, while several researchers have demonstrated that this process is strongly limited (Sprent *et al.*, 1987; Giannakis *et al.*, 1988; Becana *et al.*, 1989).

On the other hand, there are also data that nitrate might have access to the inside of bacteroids (Arrese-Igor, 1990) as a strong induction of nitrate reductase activity by the presence of nitrate was observed in lucerne bacteroids, independently of the type of investigated *Rhizobium* strain (Arrese-Igor, 1990).

The presence of nitrite reductase activity allows nitrite to accumulate inside bacteroids only for a short time and in low quantity (Becana *et al.*, 1989). This was confirmed by Serrano & Chamber (1990) and Becana *et al.* (1985b), who demonstrated that the addition of nitrates caused an increase in NR activity and a simultaneous high increase in nitrite reductase activity in the bacteroid fraction.

Moreover, the activity of nitrite reductase may even surpass NR activity, effectively preventing the accumulation of nitrites inside bacteroids.

When considering the effect of nitrate on nitrogenase activity, one should keep in mind that the presence of nitrate ions does not cause degradation of nitrogenase. The amount of enzyme protein remained at a constant level for 8 days following the addition of nitrate, and it remains so even for the next 20 days (Arrese-Igor *et al.*, 1997). This observation is in agreement with the results of other studies showing that the repression of nitrogenase activity is reversible. Thus, neither the presence of nitrate in medium nor an increase in the resistance to O₂ diffusion do cause permanent inhibition of nitrogenase: the inhibition is reversible after removal of nitrate from the medium (Arrese-Igor *et al.*, 1997; Noel *et al.*, 1982).

Legume plants differ considerably in their tolerance to nitrate. Some varieties of soybean are capable of fixing nitrogen despite the presence of relatively high nitrate concentration (Serraj *et al.*, 1992). The activity of nitrogenase in yellow lupin nodules grown in the presence of 3, 5 and 7 mM NO₃⁻ for 40 days was higher by 246%, 159% and 133%, respectively than in nodules grown in the absence of NO₃⁻ (Lang *et al.*, 1993).

A variety of narrow-leaf lupin was found in which a 40%-stimulation of the nitrogenase activity occurred despite the fact that the plant was grown on a medium containing 15 mM NO₃⁻ (Manhart & Wong, 1980).

These data suggest that after appropriate selection of a legume plant and a nitrate-resistant *Rhizobium* strain, it is possible to obtain a symbiotic association that is capable of fixing nitrogen despite the presence of nitrate in the medium.

Chamber-Perez *et al.* (1997) compared the values of several parameters influencing nitrogen fixation (dry and fresh mass, total nitrogen content in shoots, specific and whole nitrogenase activity) with NR activity in

Bradyrhizobium spp. (*Lupinus*) strains. The results indicated a high positive correlation between the presence of NR activity and the majority of the parameters related to nitrogen fixation, suggesting that NR activity may play functions complementary to these of nitrogenase (Serrano & Chamber, 1990). A positive correlation between the nitrogen content in shoots and NR activity is in agreement with the suggestion that NR activity is somehow correlated with the nitrogen metabolism of the whole plant not, however, necessarily in a direct way.

The presence of NR in bacteroids is related to their ability to dissimilatory nitrate reduction. This process can also occur in free-living rhizobia under anaerobic or microaerobic conditions (O'Hara & Daniel, 1985).

The experiments carried out on soybean nodules demonstrated that the addition of nitrate caused, apart from an increase in the resistance to O₂ diffusion, a high increase in the level of denitrification in relation to the control grown without nitrate (Arrese-Igor *et al.*, 1997) (Fig. 1). It should be emphasized that the level of nitrite reductase activity was 10 times higher than NR activity, which guaranteed that nitrite originating in the infection zone was quickly removed.

The ability to denitrify may facilitate survival of free-living rhizobia during temporary hypoxia or anoxia in the soil. It has been demonstrated that bacteroids could utilize ATP originating from nitrate respiration to fix nitrogen (O'Hara & Daniel, 1985). Such an adaptation would be especially profitable to bacteroids at times of restricted aerobic respiration, caused by, e.g. draught or flooding of the root zone (Serraj *et al.*, 1999). Denitrification may also play a role in detoxification when high activity of the cytosol NR may cause nitrite accumulation (Becana *et al.*, 1985a; 1985b; Heckman & Drevon, 1987). Denitrification can also play a protective function for nitrogen fixation system, as it has been demonstrated by Garcia-Plazaola *et al.* (1993) with 13 *Rhizobium meliloti* strains.

All these potential functions of the denitrification system in symbiotic rhizobia would be especially advantageous in construction of symbiotic associations capable of surviving under frequently occurring anaerobiosis in the soil, which takes place e.g., on areas highly polluted with phenolic compounds. Lupin, which possesses a highly developed root system, could play a considerable role in recultivation of such lands. Construction of a symbiotic association between a legume plant and a bacterial strain with an efficient denitrification system could contribute to more effective recultivation of contaminated soils (Vance, 1998).

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