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Regulation of Symbiotic Nitrogen Fixation in Root Nodules of Alfalfa (*Medicago sativa*) Infected with *Rhizobium meliloti*

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Abstract. Symbiotic nitrogen fixation of *Rhizobium meliloti* bacteroids in *Medicago sativa* root nodules was suppressed by several inorganic nitrogen sources. Amino acids like glutamine, glutamic acid and aspartic acid, which can serve as sole nitrogen sources for the unnodulated plant did not influence nitrogenase activity of effective nodules, even at high concentrations.

Ammonia and nitrate suppressed symbiotic nitrogen fixation in vivo only at concentrations much higher than those needed for suppression of nitrogenase activity in free living nitrogen fixing bacteria. The kinetics of suppression were slow compared with that of free living nitrogen fixing bacteria. On the other hand, nitrite, which acts as a direct inhibitor of nitrogenase, suppressed very quickly and at low concentrations. Glutamic acid and glutamine enhanced the effect of ammonia dramatically, while the suppression by nitrate was enhanced only slightly.

Key words: Root nodule symbiosis – *Rhizobium meliloti* – *Medicago sativa* – Nitrogenase activity – Regulation.

Combined nitrogen in various forms is known to suppress nitrogenase activity in free living nitrogen fixing bacteria (Drozd et al., 1972; Tubb and Postgate, 1973). On the other hand early and more recently published studies on the legume-*Rhizobium* symbiotic system showed that small amounts of combined nitrogen increase nodule formation, while large quantities suppress the formation of effective nodules (Dart and Wildon, 1969; Gibson and Nutman, 1960; Munns, 1968; Schmidt, 1955; Wilson, 1940). Pate and Dart (1961) as well as Copeland and Pate (1970) found that combined nitrogen can influence symbiotic nitrogen fixation. From pulse labelling experiments with $^{15}N_2$ on seradella nodules, Kennedy (1966) concluded that ammonia was the primary product of the fixation process with glutamic acid and glutamine as the primary amino acids. In screening for ammonia assimilating enzymes, different authors (Brown and Dilworth, 1975; Kurz et al., 1975; Robertson et al., 1976; Ryan and Fortrell, 1974) found varying amounts of glutamine synthetase, glutamate dehydrogenase and L-glutamine: 2-oxoglutarate aminotransferase in the bacteroids and in the plant fraction of various legume root nodules. But, although the mechanism and the site of incorporation of ammonia from nitrogen fixation is not clear as yet, it seems that the activity of these enzymes in the bacteroids can not account for this incorporation.

This paper presents some data on the effect of various nitrogen sources, partially in combination and in different concentrations on nitrogenase activity in the root nodules of alfalfa in vivo. These measurements as well as the kinetics of suppression of nitrogenase activity by different nitrogen sources provide suggestions for the regulation of symbiotic nitrogen fixation.

MATERIALS AND METHODS

Growth of Plants

Seeds of alfalfa (*Medicago sativa*) var. *Triesdorfer* were surface sterilized with 0.1% $HgCl_2$ and germinated on nutrient agar plates as a test for sterility. After three days seedlings were planted in 9 ml glas tubes, containing washed and sterilized small quartz gravel moistened with 1.5 ml of Jensen (1942) nitrogen free nutrient solution. The plants were grown under sterile conditions with an 18 h light period at 16° – 17° C and a 6 h dark period at 14° – 15° C and were watered weekly with 0.1 ml distilled water and 0.1 ml Jensen nutrient solution. Fifteen days after inoculation with an appropriate *Rhizobium meliloti* strain the first root nodules appeared and after 30 days had a length and a diameter of 0.5 × 1.0 mm to 1.0 × 2.0 mm. The sum of the dry weights of all nodules from one single plant averaged 2.0 mg after 30 days of inoculation.

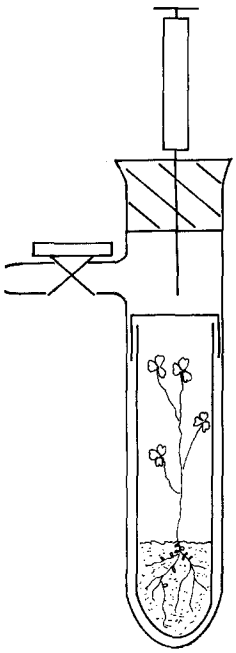

Fig. 1. Test tube for repeated measurements of acetylene reduction by intact plants. By this method the plants in the growth tubes can be kept under sterile conditions during the assay

Bacterial Inoculation

Three strains of *Rhizobium meliloti* were used as bacterial symbionts: 2011, which was obtained by Prof. Denarié and an adenine auxotroph mutant 20–26, as well as strain G1, which was isolated from alfalfa nodules by Prof. Heumann. From the stock culture on yeast mannitol broth agar plates the strains were inoculated into liquid yeast mannitol broth and grown overnight.

Supplementation with Different Nitrogen Sources

The liquid phase in the growth tubes was held at a volume of 1.5 ml during growing, nodulation and acetylene reduction assays. This made it possible to produce a defined concentration of a fixed nitrogen source in the nutrient solution and to measure the effect on nitrogenase activity. Ammonia was added as a concentrated solution of ammonium sulfate, nitrate and nitrite as solutions of the potassium salts and glutamic acid and aspartic acid as solutions of the sodium salts.

Measurement of Nitrogenase Activity

Nitrogenase activity of the nodulated plants was determined by the acetylene reduction assay (Hardy et al., 1968, 1973) in a HP 5700 A gaschromatograph with flame ionisation detector, supplied by a HP 3380 A integrator. The acetylene peak was taken as an internal standard. For acetylene-ethylene measurements, the plants in their growth tubes were brought into a test tube as shown in Figure 1. This test tube had been especially constructed in such a way that the gas atmosphere was large enough for the plant respiration and as small as possible for a sensitive acetylene-ethylene assay. The gas mixture during the nitrogenase assay was in all experiments 80% helium, 10% oxygen and 10% acetylene.

In the first test series the plants were assayed for acetylene reduction over a period of 6 h routinely. Afterwards they were given 48 h to rest before the nitrogen source was added to the liquid phase. After an incubation time of 24 h, the treated plants were assayed for acetylene reduction again to measure the influence of a combined nitrogen source on symbiotic nitrogen fixation. This technique provided a nondestructive method for repeated measurements of nitrogen fixation in intact plants.

RESULTS

Preliminary Experiments

To test the compatibility of alfalfa plants with the tested nitrogen sources, the plants were grown without infection with *Rhizobia* on Jensen nutrient solution. Ammonium, nitrate, glutamine, glutamate and aspartate were given each as sole nitrogen source at a concentration of 30 mM. In all cases the fertilized plants grew comparably to the nodulated ones, without an additive nitrogen source on the same nutrient solution. Compatibility to nitrite was tested on nodulated plants. Nitrite concentrations of 10 mM or higher were toxic for the plants. Toxicity was indicated by fading of the leaves after three to five days, depending on nitrite concentration. Concentrations lower than 10 mM were not harmful to the nodulated plants.

For reproducible results on nitrogenase activity, it was important that the water level in the growth tubes was the same in all experiments and was not more than about one half of the sand level. Less water had little effect, but when it was the same or higher level than the sand surface acetylene dependent ethylene production dropped to zero. These results, which were not in full agreement with those of Van Straten and Schmidt (1975), may be due to impaired diffusion of gases like oxygen, acetylene and ethylene.

To test endogenous ethylene production of the plants, alfalfa seedlings were inoculated with an ineffective mutant of 20–26, which had been produced by acridine orange curing. The mutant produces white, ineffective nodules on alfalfa. After 1, 2, 3 and 4 weeks, the nodulated plants were tested for ethylene production. In no case could endogenous ethylene production be detected. Suffering from nitrogen shortage the treated plants fell behind the effectively nodulated in growth after 3 weeks and the leaves became yellow after 4–5 weeks. The specific acetylene reduction activity of different alfalfa plants under identical conditions (especially the same *Rhizobium meliloti* strain, the same age of the plants and the same light period) was 260 $\pm$ 90 nmol · mg nodule dry weight^{-1} · h^{-1}. No significant difference in acetylene reduction could be found between plants infected with one of the three *Rhizobium meliloti* strains used as symbionts.

Non-specific salt effects were tested by treatment of three nodulated plants with 50 mM K_2SO_4 or 100 mM NaCl, respectively. No significant effect on nitrogenase activity was found after 24 h.

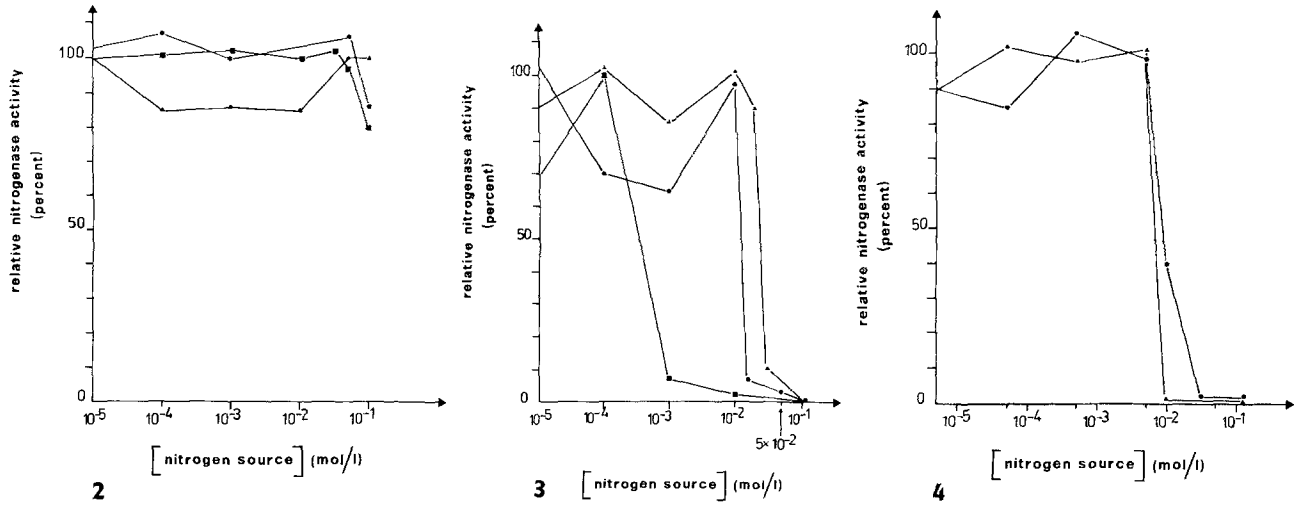

Fig. 2. Effect of amino acids on nitrogenase activity in *Medicago sativa* root nodules. The plants were assayed for acetylene reduction over a period of 6 h routinely. (See "Materials and Methods" for details of the acetylene-ethylene assay.) Afterwards they were given a 48 h rest before the nitrogen source (glutamine ●, glutamate ■, or aspartate ▲) was added to the liquid phase. After an incubation time of 24 h the plants were assayed for acetylene reduction again to measure the influence of a combined nitrogen source on symbiotic nitrogen fixation. The graph shows the relative nitrogenase activity in percent of the initial activity after treatment with the amino acids. Each point in the graph refers to the measurement of four effectively nodulated plants

Fig. 3. Effect of ammonium [$(NH_4)_2SO_4$] (▲), nitrate (KNO_3) (●), and nitrite (KNO_2) (■) on nitrogenase activity in *Medicago sativa* root nodules. The experimental procedures were the same as those described in Figure 2

Fig. 4. Effect of ammonium [$(NH_4)_2SO_4$] (▲) and nitrate (KNO_3) (●) together with 50 mM glutamate on nitrogenase activity in *Medicago sativa* root nodules. The experimental procedures were the same as those described in Figure 2

Effect of Different Concentrations of Several Nitrogen Sources on Nitrogenase Activity

In order to investigate the regulation of symbiotic nitrogen fixation three types of experiments were made, which are shown in Figures 2–5.

In the first series, the effect of three amino acids, glutamic acid, glutamine and aspartic acid, on acetylene reduction was tested. It has previously been shown that glutamate and several other amino acids do not suppress nitrogen fixation in *Klebsiella pneumoniae* (Tubb and Postgate, 1973), *Azotobacter chroococcum* (Drozd et al., 1972) and in the free living nitrogen fixing *Rhizobium* strain *32H1* (Keister, 1975). Figure 2 shows that symbiotic nitrogen fixation was not influenced significantly by the three amino acids tested.

An increase in the concentration of ammonia (Fig. 3) in the medium supplied to the nitrogen fixing nodulated plants had no effect up to a concentration of 30 mM. A further increase in ammonia concentration resulted in a rapid decrease of symbiotic nitrogen fixation to 10% at 50 mM compared to the untreated plant. At 100 mM nitrogenase activity in the nodule was fully suppressed. Nitrate had about the same effect but suppression was stronger (Fig. 3). The rapid decrease started at a concentration higher than 10 mM and at 30 mM activity dropped to 8%. Quite different from these two nitrogen sources was the effect of nitrite, which is a direct inhibitor of *Rhizobium* nitrogenase (Rigaud et al., 1973). When 1 mM of nitrite was added to the nodulated test plants, nitrogenase activity was inhibited up to 92%. Further increase in nitrite concentration inhibited nitrogenase activity completely (Fig. 3).

In order to differentiate between the mechanism of suppression by nitrate and by ammonia, these two nitrogen sources were tested in the presence of glutamine and glutamic acid, respectively (Figs. 4 and 5). The amino acids were added at a concentration of 50 mM. In both cases a significant difference to the results of Figure 3 was obvious: Ammonia under these conditions gave a stronger suppression of symbiotic nitrogen fixation than nitrate. By addition of glutamate nitrate suppression was not significantly changed, whereas ammonia excited a complete suppression at 10 mM compared to 100 mM when given as the sole source of combined nitrogen. By glutamine the suppressive effects both of nitrate and of ammonia were increased. Ammonia suppression, however, was stronger than nitrate suppression. The curves in Figures 4

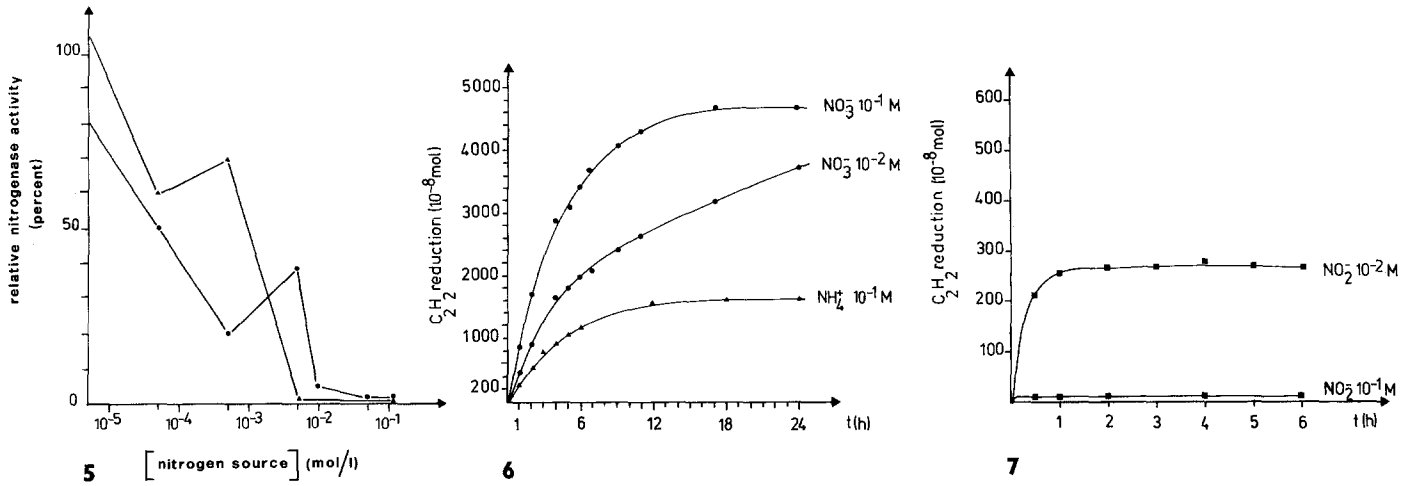

Fig. 5. Effect of ammonium $[(NH_4)_2SO_4]$ (▲) and nitrate (KNO_3) (●) together with 50 mM glutamine on nitrogenase activity in *Medicago sativa* root nodules. The experimental procedures were the same as those described in Figure 2

Fig. 6. Kinetics of suppression of nitrogenase activity in *Medicago sativa* root nodules. Nitrate (KNO_3) and ammonium $[(NH_4)_2SO_4]$ were added at time zero. The kinetics shown in the graph refere to measurements with one plant each. Therefore the specific activity at the beginning is different for all three cases

Fig. 7. Kinetics of nitrite suppression of nirogenase activity in *Medicago sativa* root nodules. Both plants used in this experiment were pretested for acetylene reduction activity. KNO_2 was added at time zero in the graph. Nitrite is an unspecific inhibitor of nitrogen fixation in this experiment and is taken as a label in estimating the speed of diffusion and/or transport of low molecular weight nitrogen sources to the interior of the nodule

and 5 also indicate that the effect of glutamine was different from that of glutamate. Glutamine caused partial suppression by ammonia and nitrate at concentrations much smaller than that giving full suppression. Compared with glutamate, it caused a stronger suppression of nitrogen fixation in the case of ammonia and of nitrate.

Kinetics of Suppression of Symbiotic Nitrogen Fixation

Typical results for the effect of the length of time spent after addition of different nitrogen sources are shown in Figures 6 and 7. When ammonia and nitrate were added to the plants at a concentration of 100 mM, nitrogenase activity decreased over a period of 12 h and remained totally suppressed after this time for at least another 12 h (Fig. 6). A concentration of 10 mM nitrate resulted in only a partial suppression of nitrogen fixation in the nodulated plants. After 7 h activity had reached a reduced level, which remained constant for at least another 12 h (Fig. 6).

100 mM nitrite suppressed nitrogenase activity totally after a very short time, so that no significant amount of ethylene was produced. At a concentration of 10 mM nitrite ethylene production could be measured, but it was totally stopped after no more than 1 h (Fig. 7).

DISCUSSION

As in free living nitrogen fixing bacteria, the three amino acids, aspartate, glutamate and glutamine did not suppress nitrogen fixation of nodulated plants when it was once established. On the other hand high concentrations of these nitrogen sources interfered with the nodulation of the plants. The effect of ammonia and nitrate on symbiotic nitrogen fixation was very small compared to free living nitrogen fixing organisms as for instance *Klebsiella pneumoniae* (Tubb and Postgate, 1973) and *Azotobacter chroococcum* (Drozd et al., 1972). In *Azotobacter vinelandii* nitrogenase activity is fully suppressed by ammonia at a concentration higher than 25 μM in continuous as well as in batch cultures as Kleiner (1974) showed. The measurements presented here indicated that in the symbiotic system of *Medicago sativa* with *Rhizobium meliloti* nitrogenase activity was not affected up to an ammonia concentration of 20 mM. The suppression by nitrate was only slightly stronger.

Nitrite, which had the strongest effect on symbiotic nitrogen fixation in our system, can act on many metabolic and regulatory sites linked with nitrogenase activity. Therefore it can be regarded as a nonspecific inhibitor. In the experiments described above, however, the results with nitrite were still meaningful.

Estimating the speed of diffusion and/or transport of low molecular weight inorganic nitrogen compounds like nitrate or ammonium to the interior of the nodule, nitrite could serve as a label. A comparison of the kinetics of inhibition of nitrogenase activity with 100 mM and with 10 mM nitrite respectively indicated that a gradient of inhibitor concentration existed. For 100% inhibition a nitrite concentration lower 10 mM was necessary (Fig. 3). When 10 mM of nitrite were in the medium, the critical concentration was reached after at least 1 h and was reached after a very short time at a concentration of 100 mM of nitrite.

Nitrate can act on nitrogen fixation in three different ways: 1. It can directly inhibit nitrogenase as Sorger (1969) showed. 2. After reduction by plant enzymes it can act via ammonia. This mechanism was supported by the small enhancement of nitrate inhibition by glutamine. 3. Nitrate is reduced to nitrite within the bacteroids which have no nitrite reductase. Nitrite itself is a strong, direct inhibitor of nitrogenase (Kennedy et al., 1975).

Recent studies on *Klebsiella pneumoniae* indicated that ammonium via glutamine synthetase acts at the regulatory site of nitrogenase synthesis in this organism. The level of ammonium in the medium compatible with nitrogen fixation in nodulated *Medicago sativa* can be explained by an enzyme system and a transport system within the nodule which keep the ammonium level low in the immediate environment of the bacteroids. A high concentration of ammonium supplied over a relatively long period (12 h in our experiment) results in an excessive supply and the protection against ammonium does not work any longer. This interpretation was supported by our results presented in Figures 4 and 5 and by the fact that ammonia is assimilated by the glutamine synthetase/glutamine: 2-oxoglutarate aminotransferase oxidoreductase (GS/GOGAT) pathway in the plant cytoplasm rather than in the bacteroids by bacterial enzymes (Robertson et al. 1975, 1976; Kurz et al., 1975). The high level of these enzymes developing during nodule growth could well account for a low ammonium concentration in the immediate environment of the bacteroids. Glutamate and glutamine alone did not directly interfere with nitrogenase activity in symbiotic nitrogen fixation. If, however, ammonium (or nitrate, which can be reduced to ammonium) was given together with these two amino acids, ammonium protection via GS/GOGAT was blocked in the nodule.

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