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INFLUENCE OF AMMONIUM AND NITRATE NITROGEN ON NITROGENASE ACTIVITY OF PEA PLANTS AS AFFECTED BY LIGHT INTENSITY AND SUGAR ADDITION

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KEY WORDS

Acetylene reduction Ammonium Bacteroids Carbohydrate supply Nitrate Nitrogenase Pea *Pisum sativum* *Rhizobium leguminosarum*

SUMMARY

Addition of ammonium chloride or potassium nitrate to nodulated pea plants resulted in a decrease in acetylene-reducing activity. Both nodule growth and specific activity of the nodules were diminished. Acetylene-reducing activity of isolated bacteroids, treated with EDTA-toluene and supplied with ATP and dithionite, had not decreased after a 3-day treatment of the plants with NH_4Cl or KNO_3. The effect of combined nitrogen could be counteracted by raising the light intensity or by the addition of sucrose to the growth medium. The latter treatment reduced the nitrogen uptake by the plants. It is concluded that combined nitrogen affects symbiotic nitrogen fixation via the carbohydrate supply to the bacteroids.

INTRODUCTION

Dinitrogen fixation by nodulated legumes is counteracted by the presence of combined nitrogen in the growth medium, like soil or a synthetic nutrient solution. As demonstrated more than a century ago, nodulation is inhibited by ammonium salts and by nitrates[21]. The effect of nitrogenous compounds on the establishment of an effective symbiosis of leguminous plants with root nodule bacteria has been studied frequently[20,22,27]. Many stages in the process of infection and nodule development are adversely affected by combined nitrogen, as is shown particularly with nitrate[5,18,27].

On the other hand, the nitrogen-fixing activity of an already established symbiosis is also affected by added nitrogenous compounds. Apart from the effect on further nodule development, a more general and non-specific effect can be assumed to arise from so-called photosynthate deprivation[19]. This deprivation –

a diminished translocation of carbohydrates to the root nodule when combined nitrogen is absorbed by the plant – would result from nitrogen assimilation and concomitant carbohydrate consumption in the roots and the shoot[19]. Experimental evidence for this theory has come from investigations with $^{14}CO_2$ on the translocation of photosynthates and their distribution among the different parts of the plant. With both peas[24] and soybeans[13] it was shown that in plants with added nitrogenous compounds less carbohydrates were transported to the nodules than in plants without this addition. The hypothesis of photosynthate deprivation is founded upon the generally accepted idea that carbohydrate supply is the natural regulator of nitrogenase activity in symbiotic systems[8,19]. This idea is supported, for instance, by the diurnal fluctuations in nitrogenase activity[3,9], by the effect of removal of flowers or pods[14,15] and by grafting experiments with two shoots on a single root[26]. Another indication for the role of photosynthates has been discussed by Lambers[12]. In nitrate-grown pea plants a cyanide-resistant, non-phosphorylative respiration has been demonstrated which may be involved in the oxidation of excess sugars. However, plants growing on N_2 do not show this 'alternative respiration'; the absence of excess sugars in these plants suggests a regulation of nitrogenase activity by photosynthate supply.

Furthermore, the value of the photosynthate deprivation theory can be demonstrated with more or less indirect tests. For instance, the reversibility of the nitrogenase inhibition by nitrate and the period of time during which it is effectuated are in accordance with the assumption and show that no irreversible damage is done[7]. The fact that nitrogenase *in vitro* is not inhibited by ammonium ions or by amino acids[11] suggests that feedback inhibition does not occur in the *in vivo* system. Finally, it has been demonstrated that the decrease in the nitrogen-fixing activity of pea plants upon the addition of ammonium chloride was not accompanied by a decrease in the *in vitro* activity of isolated bacteroids supplied with ATP and reductants[10].

In the present communication the arguments mentioned above are substantiated by experiments concerning the interaction of the effect of ammonium chloride with other physiological factors which may influence the carbohydrate supply of the nodule.

MATERIALS AND METHODS

Plants

Pea plants (*Pisum sativum* cv. Rondo) inoculated with *Rhizobium leguminosarum* PRE were grown in

gravel with a nitrogen-free nutrient solution[16]. They were cultured in a growth chamber at 18–20°C with a 16 h light-8 h dark period, light intensity on the average being 12,000 lux. Two weeks after sowing, plants were transferred from gravel to 300-ml Erlenmeyer flasks with 200-ml nutrient solution (5 plants per Erlenmeyer, sustained with a plug of cotton wool). Treatments with combined nitrogen and/or sucrose were performed by replacing the nutrient solution by a medium containing the desired compound. This medium was refreshed daily during the experiment to prevent drop in pH, usually occurring with supply of ammonium salts.

Bacteroids

Root nodules were pressed in a Bergersen-press[2] under argon. The buffer contained 50 m*M* Tris-HCl (pH 7.2), 2.5 m*M* $MgCl_2$, 4% polyvinylpyrrolidone (PVP) and 20 m*M* sodium dithionite. Bacteroids were spinned down (10 min at 5,000 × *g*), washed with buffer (same buffer without PVP), spinned down again (10 min at 5,000 × *g*) and resuspended in buffer without PVP. A quantity of bacteroids corresponding to 80 mg of fresh weight nodules was used per ml of buffer. Immediately prior to the start of the assay bacteroids were treated with EDTA and toluene[25]. Protein was determined with the Lowry method, with bovine serum albumin as a standard.

Acetylene reduction

Intact plants were incubated in closed Erlenmeyer flasks (5 plants in 1000-ml flasks) with 10% acetylene in air. After 15–20 min, ethylene produced was measured gas-chromatographically. Bacteroids were incubated in 16.5-ml Hungate tubes in a shaker bath (25°C, 200 strokes/min). The assay mixture contained 0.5 ml of EDTA-toluene-treated bacteroid suspension, 50 µmoles of Tris-HCl, 15 µmoles of $MgCl_2$, 18.4 µmoles of creatine phosphate, 5.6 µmoles of ATP, 0.03 mg of creatine phosphokinase and 20 µmoles of $Na_2S_2O_4$, in a total volume of 1 ml. The gas phase was 10% acetylene in argon and the incubation time was 10 min.

Uptake of NH_4Cl

The uptake of ammonium chloride by the plant was determined using ammonium chloride (20 m*M*) enriched with 25% ^{15}N in the nutrient solution. After the ammonium treatment, parts of the plant (roots, shoots and nodules) were harvested seperately and dried. Total nitrogen was determined after destruction of the plant material using the Kjeldahl method. Atom % ^{15}N excess was assayed using emission spectrometric analysis[1] and calculated according to Ferraris and Proksch[6]. From this figure the amount of N taken up by the plant was computed.

RESULTS

Effect of NH_4Cl and KNO_3 on acetylene reduction

Ammonium chloride or potassium nitrate was added to 4-week-old pea plants growing in culture solution. After 1 and 3 days the acetylene-reducing activities of plants with different treatments (10 m*M* salt, 20 m*M* salt or no salt addition) were compared (Table 1). At a salt concentration of 10 m*M*, potassium nitrate had a

Table 1. Influence of NH_4Cl and KNO_3 on the acetylene-reducing activity of intact pea plants*

Addition	Day 1	Day 3
None	4.0 a	4.4 a
NH_4Cl (10 m*M*)	3.1 b	3.1 b
NH_4Cl (20 m*M*)	1.5 d	1.9 d
KNO_3 (10 m*M*)	2.5 bc	1.9 d
KNO_3 (20 m*M*)	2.1 cd	1.7 d

* Activities in μmoles C_2H_4 per plant per h.
Values are averages of 5 determinations; standard deviations 0.2–0.9. Values not significantly different at the 5% level according to Tukey's test are indicated with the same letters.

more pronounced effect than ammonium chloride, as appears from the values measured after 3 days. A concentration of 20 m*M* of either of the salts gave identical results: 50–60% inhibition. In further experiments a concentration of 20 m*M* was applied to obtain significant and pronounced short-term effects.

The influence of nitrogenous salts on bacteroid activity itself (when the salts were added to the intact plant) was determined with the acetylene reduction test with EDTA-toluene-treated bacteroids (Table 2). When plants were treated with NH_4Cl or KNO_3, nitrogenase activity of isolated bacteroids supplied with ATP and dithionite did not decrease, whereas the specific activity of the intact plant was inhibited by about 50% after 3 days. This result suggests that the decrease in nitrogenase activity does not originate from a drop in the amount of potentially active enzyme.

Table 2. Influence of NH_4Cl and KNO_3 on the *in vivo* and the *in vitro* nitrogenase activity of pea bacteroids

Treatment	Act./plant*	Spec. act.**	*In vitro* act.***
Control	4.1	47.6	15.6
NH_4Cl (20 m*M*, 3 days)	2.4	25.2	18.1
KNO_3 (20 m*M*, 3 days)	2.4	24.9	16.0

* μmoles C_2H_4/plant per h; standard deviations 0.3–0.9.
** μmoles C_2H_4/g fresh weight nodule per h; standard deviations 3.2–13.6.
*** nmoles C_2H_4/mg protein per min; standard deviations 0.4–1.3.
Values are averages of three determinations.

Effect of NH_4Cl on plant growth

In a 6-day experiment the influence of ammonium chloride (at a 20 m*M* concentration) on the growth of roots, shoots and nodules of pea plants was studied,

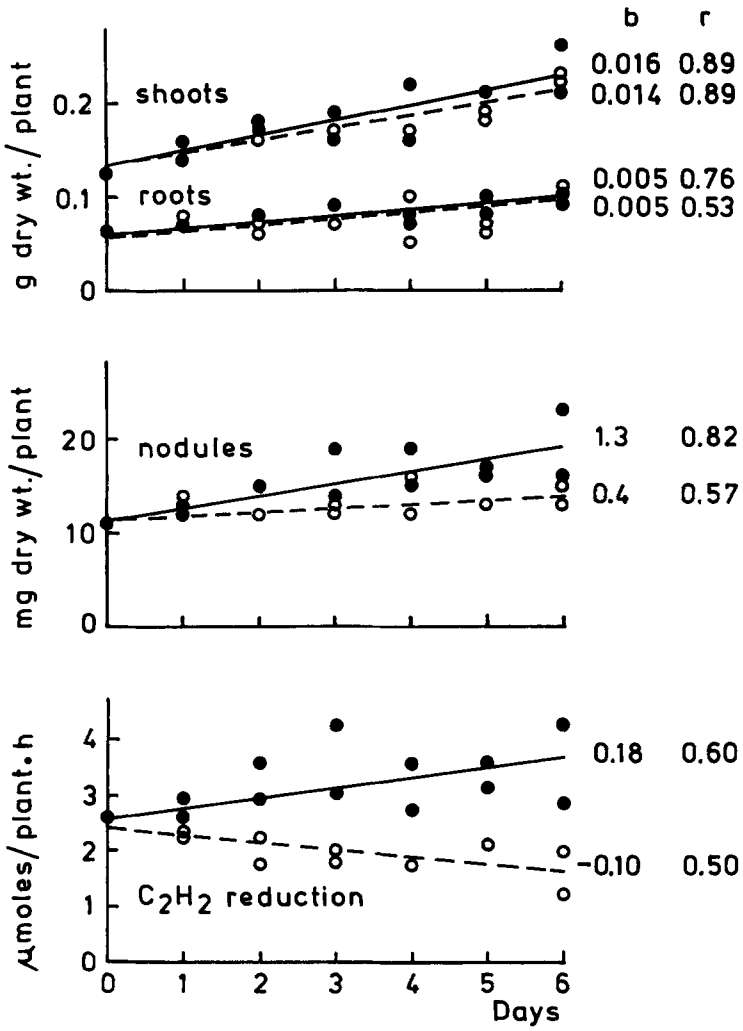

Fig. 1. Influence of NH_4Cl on the growth of roots, shoots and root nodules of pea plants, and on the nitrogenase activity of the intact plants. Each mark represents the average of 3 plants, treated with 20 m*M* KCl (●——●) or 20 m*M* NH_4Cl (○- - -○). Linear regression coefficients (b) and correlation coefficients (r) are given, upper figure applies to KCl, lower figure to NH_4Cl treatment.

and compared with the acetylene-reducing activity of the intact plants (Fig. 1). A linear regression of dry weight against time (regression coefficient b, correlation coefficient r) was assumed and straight lines were traced to get a surveyable view. Growth of root nodules was retarded when ammonium chloride was added, whereas the growth of roots and shoots was not affected. In the same experiment the acetylene reduction was measured and plotted in the same way. A decrease in the activity per plant was found, arising in part from the diminished increase of the nodule mass and in part from the reduced specific nitrogenase activity (see the following section).

Uptake of nitrogen and nitrogenase inhibition

Plants which had developed an active nitrogenase system were fed with ammonium chloride enriched with ^{15}N. The distribution of the absorbed nitrogen was determined after 1, 2 and 3 days of treatment, in an attempt to find any correlation between the uptake of ammonia and the decrease in nitrogenase activity. Figure 2 gives the enrichment in ^{15}N of the different parts of the plant

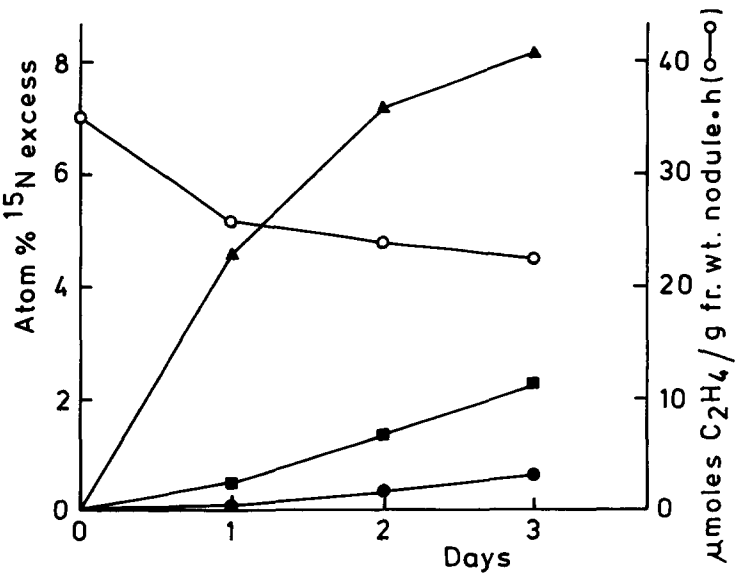

Fig. 2. Uptake of ^{15}N-enriched ammonium nitrogen and distribution of the absorbed nitrogen among roots (▲), shoots (■) and nodules (●) of pea plants, and the influence of NH_4Cl on the specific acetylene-reducing activity of the nodules (○). Each mark represents the average of duplicate determinations.

and the specific acetylene-reducing activity of the nodules. In the roots the concentration of newly absorbed nitrogen seemed to be built up to a maximum, whereas the concentration in the shoot and in the nodules increased almost linearly, but at a low rate, during the experiment. In the nodules, the concentration of absorbed nitrogen was low compared to that in the root and in the shoot. The decrease in specific acetylene-reducing activity was most pronounced during the first day of the treatment and less steeply afterwards, suggesting an inverse proportionality with the nitrogen uptake by the roots.

Effect of light intensity

The interaction between the effect of combined nitrogen and that of light intensity was investigated with two groups of four-week old plants which were placed under light intensities of 13,000 lux and 26,000 lux respectively. After 3 days the effect of added ammonium chloride (20 m*M*) was traced in both groups. Table 3 shows, that doubling the light intensity increased the nitrogenase activity by about 45% and weakened the inhibitory effect of ammonium chloride. After 2 days the nitrogenase activity of ammonium-treated plants at 13,000 lux was significantly lower than that of the control plants. At 26,000 lux this claim was not true. When the decrease in the acetylene-reducing activity after NH_4Cl application was assumed to be a linear regression with time, the regression coefficients at the two light intensities differred significantly from each other at the 5% level. Therefore, it is concluded that the inhibition of nitrogenase activity by ammonium chloride can be counteracted by raising the light intensity.

Table 3. Inhibition of nitrogenase activity by NH_4Cl as affected by light intensity*

Light intensity (lux)	Salt added (20 m*M*)	Day			
		1	2	3	4
13,000	KCl	2.3 ab	2.1 ab	2.2 a	2.5 a
13,000	NH_4Cl	2.5 a	1.7 abc	1.3 bc	1.0 c
26,000	KCl	3.4 de	3.5 de	3.6 d	3.8 d
26,000	NH_4Cl	3.5 de	3.1 de	2.9 de	2.4 e

* Activities in μmoles C_2H_4 produced per plant per h.
Values are averages of 8 determinations; standard deviations 0.6–1.0. Values not significantly different at the 5% level according to Tukey's test (within any of the light intensities) are indicated with the same letters.

Effect of sucrose

In another attempt to relieve a possible shortage in photosynthates, sucrose was added to the nutrient solution of the plants. To oppose bacterial growth, 12 mg/l of chloramphenicol was applied in these experiments; with this precaution acidification of the solution owing to bacterial activity could be prevented (Table 5). Sucrose was added 2 days before the start of the NH_4Cl treatment; this addition resulted in an increase in nitrogenase activity by about 30% (Fig. 3). It was shown that the inhibitory effect of NH_4Cl on the nitrogenase activity was less pronounced when sucrose was present. Linear regression coefficients of the acetylene-reducing activity against time were 0.54, 0.27 and 0.11 with 0, 0.5 and 2% of sucrose, respectively; they were significantly lower in the presence of sucrose than without sucrose addition.

However, the presence of sucrose also affected the uptake of nitrogen by the plant, as was shown with ^{15}N-enriched NH_4Cl (Table 4). After 1 day of ammonium chloride treatment less newly absorbed nitrogen was found in the shoot when 0.5% sucrose was added, whereas the concentration of absorbed nitrogen in the root was unaffected. This effect was even more pronounced when 2% sucrose was added (not shown in the Table). These results suggest a decreased transport

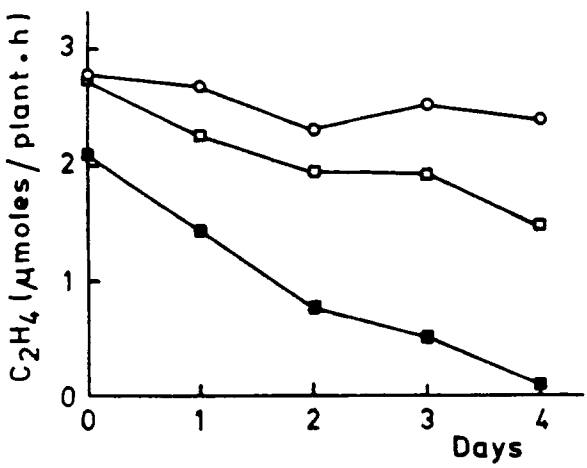

Fig. 3. Influence of NH_4Cl on the acetylene-reducing activity of intact pea plants, as affected by sucrose: no sucrose (■), 0.5% (□) and 2% sucrose (○). Each mark represents the average of 6 determinations; standard deviations 0.2–0.6.

Table 4. Uptake of ^{15}N-enriched NH_4Cl by pea plants as affected by sucrose*

Plant part	Atom % ^{15}N excess		N taken up/plant (mg)	
	no sucrose	0.5% sucrose	no sucrose	0.5% sucrose
Roots	5.07	5.48	0.64	0.75
Shoots	0.81	0.42	0.26	0.17
Root nodules	0.27	0.25	0.02	0.02

* Plants were treated with a NH_4Cl solution (20 m*M*) with a ^{15}N-enrichment of 24% for 24 h.

stream of N-compounds from the root to the shoot as a consequence of the presence of sucrose in the medium. The same conclusion can be drawn from results of transpiration measurements (Table 5): when sucrose was present in the nutrient solution less medium was taken up by the plant as compared to the control.

DISCUSSION

Inhibition of nitrogen-fixing activities of legume-Rhizobium symbiotic systems by ammonium and nitrate ions – as well as by other, less frequently studied nitrogenous compounds – has drawn the attention of several investigators since many years. In many cases the effect of ammonium salts is less pronounced than that of nitrates[17, 22]. This difference was also found in the present study with peas, when the salt was added at a concentration of 10 m*M* (Table 1). It could be accounted for by a better uptake mechanism for nitrates compared to that for ammonium salts. A higher concentration of ammonium ions would overcome this apparent disparity (see Tables 1 and 2, results of a 20 m*M* treatment), thus allowing an investigation on the fundamental effects of nitrogenous salts independent of concentration effects.

Table 5. Influence of sucrose on the uptake and on the pH of the nutrient solution*

Sucrose addition	Solution used/plant (ml)	pH of solution
None	5.9 ± 0.3	6.5
0.5%	5.4 ± 0.4	6.4
1.0%	4.9 ± 0.6	6.4
2.0%	3.6 ± 0.4	6.3

* Plants were treated with a 20 m*M* NH_4Cl solution, with sucrose. During 4 days the daily uptake of solution (determined by means of the decrease in weight of the nutrient medium) and the pH of the solution were measured. Average values over these 4 days are given, ± standard deviations.

The decrease in nitrogenase activity of nodulated pea plants caused by added ammonium chloride (Fig. 1) partly originated from a decreased nodule growth. Apart from this effect on nodule development, the specific activity of the nodules (C_2H_2 reduced per unit nodule weight) also diminished. This drop was not brought about by a decreased quantity of potentially active nitrogenase: the activity of isolated bacteroids, treated with EDTA and toluene and provided with ATP and reductants, had not declined (Table 2). Therefore, it is concluded that the decrease in specific nitrogenase activity of the root nodules was a result of plant influences. When the time course of nitrogenase inhibition after addition of NH_4Cl is followed and compared with the uptake of nitrogen, a correlation with the concentration of newly absorbed nitrogen in the roots was observed (Fig. 2).

As mentioned in the introduction, a theory to explain the effects of nitrogenous compounds on nitrogen fixation of symbiotic systems emphasizes the carbohydrate supply as a major regulatory factor. According to this theory an increase in photosynthate supply might counteract the inhibitory effect of nitrogen salts. Table 3 shows that variation of photosynthetic activities by changing the light intensity, affected the drop in nitrogenase activity of intact pea plants caused by NH_4Cl according to this prediction. Similar relationships were found by other investigators, using somewhat different techniques[4]. Variation of the carbohydrate supply affects both specific nitrogenase activity and growth of the nodules.

Another way to provide the plant with additional carbohydrates is the addition of sugars to the rooting medium. This treatment enhances the nodulation of legumes[23]; an increase in C_2H_2-reducing activity by added sucrose can be observed in Fig. 3. Sugars have been utilized in investigations on the nodulation process, in attempts to alter the C to N ratio; addition of sugars to intact plants or to isolated root systems was found to favour nodulation in the presence of combined nitrogen[20,27]. Figure 3 shows that the decrease in acetylene-reducing activity caused by added ammonium chloride is counteracted by sucrose. As sucrose apparently also affects the uptake and transport of nitrogen (Tables 4 and 5), influences of sugars can not merely be ascribed to an increase in the internal carbon concentration (additional supply of carbohydrates).

Summarizing the results reported here and in a former publication[10] it can be concluded that ammonium chloride (and probably potassium nitrate as well) when added to nodulated leguminous plants has no direct effect on nitrogenase activity. Its effect, when added to whole plants, may be ascribed to creating a shortage of carbohydrates in the nodules.

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