The Effect of NO$_3^-$ and NH$_4^+$ Ions on Enzymes Involved in Nitrogen Assimilation in *Pisum sativum* L.

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Abstract. Nitrate reductase level in leaves of pea plants is higher than in roots despite of the lower content of endogenous nitrate. Addition of ammonium ions to nutrient solution containing nitrate decreases nitrate reductase level in leaves estimated *in vivo* while its level estimated *in vitro* is increased. Glutamine synthetase (GS) level in roots decreases during short (24 and 48 h) and long (14 d) term cultivation of seedlings in solutions containing ammonium ions. This decrease occurs in leaves only after the long term influence of ammonium ions. Level of this enzyme is higher in plants grown in the presence of nitrogen (ammonium and nitrate) as compared to those grown without the nitrogen.

Level of glutamate dehydrogenase in roots is increased after both short and long term cultivation of plants in the presence of ammonium ions.

Reduction of NO$_3^-$ ions absorbed by plants to NO$_2^-$ and subsequently to NH$_4^+$ is catalyzed by nitrate reductase (NR) and nitrite reductase, respectively. Formed NH$_4^+$ is incorporated into amino acids and other amino compounds. For many years the reductive amination of $\alpha$-ketoglutarate catalyzed by glutamate dehydrogenase (GDH) was considered to be the main way of NH$_4^+$ assimilation. Discovery of glutamate synthetase (GOGAT) activity in higher plants (Lea and Miflin 1974) showed that when present in low concentration the NH$_4^+$ ions are assimilated via a cycle catalyzed by the glutamine synthetase — glutamate synthetase (GS/GOGAT) system. Transition from the GS/GOGAT to GDH system occurs at high NH$_4^+$ levels and low charge. The GS/GOGAT is probably the main enzyme system which assimilates NH$_4^+$ formed in plants grown in media containing NO$_3^-$. In plants grown in media containing NH$_4^+$ the assimilation of NH$_4^+$ is supplemented by GDH (Givan 1979).

Short term (24 and 48 h) and long term (14 days) effect of NO$_3^-$ and NH$_4^+$ ions on GS and GDH in intact plants have been investigated in the present communication. With respect to ambiguous data about the effect of NH$_4^+$...
on NR in higher plants as compared to e.g. microorganisms, algi, cell suspension cultures and cultures of *Lemma* the effect of NH$_4^+$ on NR in pea plants was also studied.

**MATERIAL AND METHODS**

**Cultivation**

Seeds of *Pisum sativum* L. cv. Raman were germinated for three days in distilled water and seedlings were cultivated for another 14 days under controlled light and temperature conditions (60 W m$^{-2}$, 17–25 °C). Nutrient solutions were based on solution of Richter (Laštůvka and Minář 1967). They were supplemented with a series of concentrations of KNO$_3$ (1 to 10 mM) or K$_2$SO$_4$ (0.5 to 5 mM, i.e. solutions lacking nitrogen) and with the following salts (mM) KH$_2$PO$_4$ (1.15), MgSO$_4$.7H$_2$O (1.01) and CaCl$_2$ (3.00). Solutions supplemented with (NH$_4$)$_2$SO$_4$ (0.5 to 5 mM) also contained 0.98 mM K$_2$SO$_4$. All nutrient solutions were supplemented with microelements (Avron 1938, see Laštůvka and Minář 1967).

For investigation of induction of NR and changes of GS in dependence on time seedlings were grown for 13 days after the germinations in nutrient solution lacking nitrogen and then transferred for 24 h into nutrient solution containing 10 mM KNO$_3$ or 5 mM (NH$_4$)$_2$SO$_4$ (short term cultivation with NO$_3^-$ or NH$_4^+$ ions). Activities of NR and GS and NO$_3^-$–N content were estimated after 0, 2, 5, 9 and 24 h of incubation.

Effect of NH$_4^+$ ions on the course of reduction of NO$_3^-$ and level of GS in presence of NO$_3^-$ was investigated in another set of experiments. After germination the seedlings were cultivated for 12 d in solution lacking nitrogen and then transferred for 48 h into nutrient solution containing 10 meq NO$_3^-$ (4 mM Ca(NO$_3$)$_2$ and 2 mM KNO$_3$), (NH$_4$)$_2$SO$_4$ (10 to 30 mM), 1.15 mM KH$_2$PO$_4$, 1.01 mM MgSO$_4$.7H$_2$O and 3 mM CaCl$_2$. Levels of NR, GS and GDH and content of NO$_3^-$–N were estimated after 48 h of incubation. Controls were without (NH$_4$)$_2$SO$_4$.

The third part of the experiments dealt with the effect of long term cultivation of plants in presence of NO$_3^-$ or NH$_4^+$ ions on investigated enzymes. The germinated seedlings were cultivated for 14 d in nutrient solution supplemented with KNO$_3$ (1 to 10 mM) or (NH$_4$)$_2$SO$_4$ (0.5 to 5 mM) or in control nitrogen-lacking solution supplemented with K$_2$SO$_4$ (0.5 to 5 mM). Nutrient solutions were changed each second day. Levels of NR, GS and GDH and content of NO$_3^-$–N were estimated after 14 d of cultivation.

**Sampling of Plant Material**

Three samples were prepared from the compound pea leaves. One half of leaflets was used for estimation of NR level *in vivo*. The remaining leaflets were cut longitudinally in two parts. From one part the enzyme extracts for estimation of enzymatic activities were prepared. The second part was dried out and used for estimation of NO$_3^-$–N content.

**Estimation of NR Level *in vivo***

Level of NR was estimated on the basis of anaerobic production of nitrite without a supply of exogenous nitrate (Skrdleťa et al. 1979). Formed NO$_2^-$-
was estimated colorimetrically after addition of 1 ml of 1% sulphanilamide in 1 M HCl and 0.02% water solution of N-1-(naphtyl)ethylenediamine di-HCl. Activity is expressed in nmol NO\(_2^-\) h\(^{-1}\) g\(^{-1}\) (fresh matter).

**Homogenization and Extraction of Enzymes for in vitro Experiments**

One half of leaflets separated from the compound leaves of pea was homogenized with sand in cold mortars in 0.05 M Tris-HCl buffer, pH 7.5 (1 : 5 m/v). Roots were homogenized in the same way; the extraction ratio was, however, changed to 1 : 2.5 (m/v). Homogenates were centrifuged at 15 000 g for 20 min at \(-4\) °C. Supernatants of extracts from leaves and roots were immediately used for estimation of NR and GS \textit{in vitro} and those from roots also for estimation of GDH.

**Estimation of NR \textit{in vitro}**

Level of NADH-dependent NR was estimated \textit{in vitro} according to \textsc{Wray} and \textsc{Filenček} (1970). Formed NO\(_2^-\) was stained using the same reagents as in the case of estimation of NR \textit{in vivo}. Level of enzyme is expressed in nmol NO\(_2^-\) min\(^{-1}\) g\(^{-1}\) (fresh matter).

**Estimation of GS**

Level of GS was estimated as \(\gamma\)-glutamylhydroxamate (GHA) transferase (\textsc{Shapiro} and \textsc{Stadtman} 1970). Concentration of hydroxylamine was changed to 30 mM. The level was expressed in \(\mu\)mol \(\gamma\)-GHA min\(^{-1}\) g\(^{-1}\) (fresh matter).

**Estimation of GDH**

Level of NADH-dependent GDH was estimated by measurement of absorbance decrease at 340 nm during three min interval (\textsc{Sahulka et al.} 1975). Values of absorbance of the reaction mixture containing substrate (\(\alpha\)-ketoglutarate) were corrected by subtraction of absorbance of the same mixture without substrate. Level of GDH was expressed as the change in absorbance at 340 nm -- \(\Delta A\) min\(^{-1}\) g\(^{-1}\) (fresh matter).

**Estimation of Nitrate Nitrogen (NO\(_3^-\)-N)**

Content of NO\(_3^-\)-N was estimated using selective electrode Corning (cat. No. 467134) after extraction of dry matter with distilled water and addition of Al- and Ag-Dowex 50 WX 8 (\textsc{Paul} and \textsc{Carlson} 1968). Content of NO\(_3^-\)-N was expressed in \(\mu\)g NO\(_3^-\)-N g\(^{-1}\) (dry matter).

**RESULTS**

**Short-term (24 h) Cultivation of Plants in Presence of NO\(_3^-\) and NH\(_4^+\) Ions**

Time course (0 to 24 h) of NR induction in 13 days old pea seedlings transferred into solution supplemented with 10 mM KNO\(_3\) indicates that NR was first of all induced in roots (after 2 h). Its activity estimated \textit{in vitro} was increasing till the 9th h and not further increasing. Using the \textit{in vitro} and \textit{in vivo} methods the NR was detectable in leaves after 5 and 9 h, respectively, (Fig. 1A, B). Level of NR \textit{in vitro} was increasing during the whole 24 h period (Fig. 1A). No NR activity was detected in either roots or leaves of plants cultivated in presence of (NH\(_4\))\(_2\)SO\(_4\).
The content of NO$_3^-$—N in roots and leaves was increasing during the whole 24 h of cultivation of plants in presence of 10 mM KNO$_3$. Leaves contained 14 times more NO$_3^-$—N than roots at the end of the experiment (Fig. 1A).

Level of GS in roots and leaves was slowly increasing after the transfer of plants into the nutrient solution supplemented with 10 mM KNO$_3$ (Fig. 1B).

Transfer of plants into the nutrient solution supplemented with 5 mM (NH$_4$)$_2$SO$_4$ did not change GS level in leaves. In roots, however, GS level was decreased already after 2 h and was slowly decreasing during the whole 24 h of the experiment (Fig. 1B).

Interaction of Nitrogen Nutrients and their Influence on Enzymes Involved in Nitrogen Assimilation

There is a discrepancy in the effect of increasing concentration of (NH$_4$)$_2$SO$_4$ in presence of 10 meq NO$_3^-$ on NR level. In leaves when estimated in vitro the NR level was decreasing while in vivo estimated NR was slowly increas-
ing. Both changes were, however, statistically insignificant (Fig. 2A). The NR level in roots was not significantly affected by NH$_4^+$. The activity of GS in roots was decreased after 48 h of cultivation in presence of 10 mM, 20 mM and 30 mM (NH$_4$)$_2$SO$_4$ by 76%, 80% and 94%, respectively. Addition of (NH$_4$)$_2$SO$_4$ into the nutrient solution had no significant effect on GS activity in leaves (Fig. 2B).

The level of GDH was increased after cultivation of plants in presence of 10 mM, 20 mM and 30 mM (NH$_4$)$_2$SO$_4$ by 70%, 87% and 94%, respectively (Fig. 2B).

**Long-term (14 d) Cultivation of Plants in Presence of NO$_3^-$ and NH$_4^+$ Ions**

Level of NR was increasing with increasing concentration of KNO$_3$ in nutrient solution from 337 to 1 107 nmol NO$_2^-$ h$^{-1}$ g$^{-1}$ (fresh matter) when estimated in vivo and from 15 to 68 nmol NO$_2^-$ h$^{-1}$ g$^{-1}$ (fresh matter) in estimation in vitro. It was also increasing in roots from 5 to 11 nmol NO$_2^-$ min$^{-1}$ g$^{-1}$ (fresh matter). Content of NO$_3^-$-N in dry matter was increased from 174 to 1 369 µg NO$_3^-$-N g$^{-1}$ (dry matter) in leaves and from 7 509 to 47 950 µg NO$_3^-$-N g$^{-1}$ (dry matter) in roots. The NR level was not detectable in plants grown in nutrient solutions lacking nitrogen or containing NH$_4^+$. Nitrate concentration in nutrient solution did not affect the GS level in either leaves or roots (Fig. 3A, B). Its level was, however, decreasing with
increasing concentration of NH$_4^+$ in nutrient solution being significant in roots (Fig. 3A, B). Comparison of the effects of different nitrogen sources showed that the level of GS in leaves was higher in variants grown in the presence of KNO$_3$ as compared to those with (NH$_4$)$_2$SO$_4$. This difference is statistically significant at the highest used concentrations of KNO$_3$ (10 mM)

![Graph](image)

Fig. 3. Long term (14 d) cultivation of plants in nutrient solution lacking nitrogen (1 to 10 meq K$^+$ applied as 0.5 to 5 mM K$_2$SO$_4$) and in solution supplied either with NH$_4^+$ (1 to 10 meq NH$_4^+$ applied as 0.5 to 5 mM (NH$_4$)$_2$SO$_4$) or NO$_3^-$ (1 to 10 meq K$^+$ or NO$_3^-$ applied as 1 to 10 mM KNO$_3$).

(A) Levels of GS in leaves of plants cultivated in nutrient solution lacking nitrogen (1) and in solution supplemented either with NH$_4^+$ (2) or NO$_3^-$ (3). LSD values ($P_{0.05}$) are LSD$_1$ 2.53, LSD$_2$ 3.52 and LSD$_3$ 2.61.

(B) Levels of GS in roots of plants cultivated in nutrient solution lacking nitrogen (1) and in solution supplemented either with NH$_4^+$ (2) or NO$_3^-$ (3) and activity of GDH (ΔA min$^{-1}$ g$^{-1}$ (fresh matter)) in roots of plants cultivated in the same solution lacking nitrogen (1) and in solution supplemented either with NH$_4^+$ (5) or NO$_3^-$ (6). LSD values ($P_{0.05}$) are LSD$_1$ 0.55, LSD$_2$ 1.04, LSD$_3$ 1.47, LSD$_4$ 0.36, LSD$_5$ 0.42 and LSD$_6$ 0.38. Levels of GS are expressed in the same way as those in Fig. 1.

and (NH$_4$)$_2$SO$_4$ (5 mM). There was no significant difference in GS level in roots. Variants with nitrogen (NO$_3^-$ and NH$_4^+$) were higher in GS in both roots and leaves (Fig. 3A, B).

Level of GDH in roots was increasing in presence of (NH$_4$)$_2$SO$_4$ in nutrient solution (Fig. 3B) and was not changed when plants were cultivated in nutrient solution containing KNO$_3$ or lacking nitrogen.

**DISCUSSION**

Concentration of NO$_3^-$ in plant tissue absorbing this anion reaches its external concentration soon after addition of NO$_3^-$ . This is evident from our results (Fig. 1A) as well as from data of Bretele r and Hä nisch Ten Cate.
Most of NO₃⁻ is reduced at the beginning of cultivation of plants in presence of nitrate (ASHLEY et al. 1975 and others) while in the later periods the uptake of NO₃⁻ exceeds its metabolic utilization. The uptake of NO₃⁻ is not inhibited under these conditions and this anion is accumulated in plants (BRETELER and HANISCH TEN CATE 1980). Induction phase of NR 2 to 5 h found in our experiments corresponds to the data of other authors (e.g. JACKSON et al. 1973). Courses of NR induction and NO₃⁻ uptake are different. This difference, however, does not exclude a functional connection of these two processes. In our experiments the level of NR in roots was increasing up to the 9th h after the application of NO₃⁻ and reached its maximum at 760 μg NO₃⁻-N g⁻¹ (dry matter) content (dry matter of roots represents 5% of their fresh matter). There is no further change in NR level after the long term cultivation (14 d) of plants in presence of 10 mM KNO₃ while NO₃⁻-N accumulation in roots continues. In spite of NO₃⁻-N content in leaves is low (156 μg NO₃⁻-N g⁻¹ (dry matter) after 24 h of cultivation in presence of KNO₃ when dry matter of leaves represents 12% of their fresh matter) their NR level is higher than that of roots. It follows from the course of NR induction that after the saturation of the metabolic and store pools of NO₃⁻ in roots the NO₃⁻ ions are translocated to leaves where they induce NR. Leaves of pea have higher capacity of NO₃⁻ reduction. Long term cultivation (14 d) of plants in presence of 10 mM KNO₃ increases NR in leaves as compared to the short term cultivation (24 h): its level was increased 50 and 7 fold when NR was estimated in vivo and in vitro, respectively. The content of NO₃⁻-N was also increased (9 fold). Content of NO₃⁻-N estimated in our experiments includes NO₃⁻ in cytosol and vacuole. To understand the dynamics of changes of NO₃⁻ in the cell it would be necessary to estimate NO₃⁻ content in the two compartments separately. Induction of NR is dependent on the level of applied NO₃⁻ in nutrient solution. When its level is substantially increased the shoots become the main site of NO₃⁻ reduction. This situation corresponds to the results of WALLACE and PATE (1965). Activity of NR is dependent not only on the size of the metabolic pool of NO₃⁻ (FERRARI et al. 1973) but also the flow of NO₃⁻ ions from roots to shoots where they act as an inductor and substrate (SHANER and BOYER 1976 and others).

Investigation of the effect of NH₄⁺ on NR published by different authors gave conflicting results. The main reason for these differences is seen in different methods and plant material used in their experiments. In our experiment the NR estimated in vivo was significantly decreasing during 48 h exposure of plants to NH₄⁺ in presence of NO₃⁻ while NR estimated in vitro was increasing (Fig. 2A). The NR in roots was not changed after the (NH₄)₂SO₄ application. Decrease of NR activity in vivo was observed after the influence of NH₄⁺ ions in vivo, e.g. in roots of cotton (RADIN 1977) and pea (PATE 1973). On the contrary OAKS et al. (1979) found an increase of NR activity in vitro induced by NH₄⁺ ions in maize roots which was accompanied by an increase of its activity in shoots. The inhibiting effect of NH₄⁺ in presence of NO₃⁻ on NR in intact plants may be an indirect one being probably connected with inhibition of NO₃⁻ translocation (BUCZEK and BURZYNSKI 1979).

The NH₄⁺ does not always inhibit NR in mature leaves (CANVIN and ATKINS 1974). Different results obtained by in vitro and in vivo methods used
for estimation of NR stress the importance of careful choice of the method for a particular experiment. This conclusion corresponds to that of Stulen (see Oaks et al. 1979).

The GS is considered to be the key enzyme in assimilation of \( \text{NH}_4^+ \) which originates from reduction of \( \text{NO}_3^- \) (Lea and Miflin 1974). However, high external \( \text{NH}_4^+ \) concentration may irreversibly inactivate GS (Stewart and Rhodes 1977). In our experiments the GS was decreased in roots but not in leaves after 5 h of incubation of plants in presence of \( (\text{NH}_4)_2\text{SO}_4 \) (Fig. 1B). This decrease was not observed when \( \text{KNO}_3 \) was used instead of \( (\text{NH}_4)_2\text{SO}_4 \) in nutrient solution (Fig. 1B). The increasing concentration of \( (\text{NH}_4)_2\text{SO}_4 \) in solution containing this compound as the only source of nitrogen had a decreasing effect on GS especially in roots (Fig. 3A, B). This seems to be the result of preferential assimilation of \( \text{NH}_4^+ \) ion in roots before it is translocated to shoots. The GS in leaves and roots does not seem to be exclusively connected with the primary \( \text{NH}_4^+ \) assimilation. It can be detected in both leaves and roots of plants grown for 14 days in absence of nitrogen (Fig. 3A, B). In addition to the primary \( \text{NH}_4^+ \) assimilation GS has also other functions in metabolism (see Keys et al. 1978). According to results of some authors the GS/GOGAT system may be functional in addition to GDH under conditions of increased \( \text{NH}_4^+ \) level (Rhodes et al. 1979). Transition from GS/GOGAT to GDH system, however, would occur at low energetic charge and increased \( \text{NH}_4^+ \) level (Miflin and Lea 1976). At high concentration of exogenous \( \text{NH}_4^+ \) in roots the assimilation of \( \text{NH}_4^+ \) proceeds predominantly via GDH which is increasing with increasing concentration of \( \text{NH}_4^+ \) (Figs. 2B, 3B) (see Joy 1971 and others).

In general, our results showed that quality of nitrogen nutrition is reflected in the level and activity of enzymes catalyzing nitrogen assimilation in plants. In contrast to \( \text{NH}_4^+ \) the \( \text{NO}_3^- \) nutrition correlates with the activity and level of NR and GS. On the contrary the GDH level is enhanced by the \( \text{NH}_4^+ \) ions. These results support the conception that GS/GOGAT system is involved in assimilation of \( \text{NH}_4^+ \) when this ion is present in low concentration while the GDH system is active in high \( \text{NH}_4^+ \) concentrations.

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REFERENCES


