Nitrate reductase and acid phosphatase activities as affected by inorganic phosphate in corn roots

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(Received: May 11, 1982)

Abstract

The deficiency of inorganic phosphate in nutrient solution reduces by about 50 per cent NO₃⁻ absorption in corn seedlings, it decreases both in vitro and in vivo nitrate reductase (NR) activity, as well the potential and actual NR level and has a very weak effect on NR induction. Acid phosphatases activities increase in corn roots when the plants are grown in nutrient solution without phosphorus. We suggest that inorganic phosphate is required mainly for maintenance of NR activity rather, than for induction in vivo of nitrate reductase. It is not excluded that deficiency of inorganic phosphate in root tissue may be partly supplemented as the result of enhanced acid phosphatase activity.

INTRODUCTION

The requirement of inorganic phosphate for NR activity in vitro was observed for Neurospora (Kinsky and McElroy 1958) and for wheat embryo (Spencer 1959). Hewitt and Nicholas (1964) suggested that NR in vitro is activated by inorganic phosphate. Harper and Paulsen (1969) and Sasakawa and Yamamoto (1977) studying the effect of deficient nutrients on NR found that deficiency of phosphorus in nutrient solution decreases specific NR activity in wheat leaves and rice shoots.

We examined the effect of inorganic phosphate on in vivo nitrate reductase induction and activity as well as the effect of this compound on NO₃⁻ uptake and level of acid phosphatases in corn roots.
Seeds of corn (Zea mays L. Kb-270) were treated as described previously (Buczek et al. 1981). After 5 days growth of seedlings on distilled water under a light-dark period (16 h light, 10 000 lux) at 25°C during the day, 20°C at night and 48% relative humidity, the seed remnants were removed and the seedlings transferred to the minus N nutrient solution with or without phosphorus. The $-N+P$ solution contained (in mmol$\times$dm$^{-3}$): $K_2SO_4$ — 0.8, $CaSO_4\cdot2H_2O$ — 1.2, $KH_2PO_4$ — 1.0, $MgSO_4$ — 0.4 and micronutrients (Buczek et al. 1981). In the $-N-P$ solution 0.5 mmol $K_2SO_4$ was included instead of potassium phosphate. The pH was adjusted to 6.0 and the solution was aerated continuously. The plants were grown in these solutions for 3 days under light-dark conditions as above. Then they were transferred to the nitrate solution contained (in mmol$\times$dm$^{-3}$): $KNO_3$ — 5, $Ca(NO_3)_2$ — 5, $KH_2PO_4$ — 1, $MgSO_4$ — 1 and micronutrients. In the $-P$ nitrate solution $KH_2PO_4$ was replaced by $K_2SO_4$ with maintained constant potassium concentration. Both nitrate solutions were adjusted to pH 6.0 and aerated continuously. The plants were grown in these solutions for 5 days under the conditions described above or for 1 day under continuous light. The roots were harvested for assays of enzyme activities and analysis of inorganic phosphate. For studying NR induction (Table 2) the corn seedlings were grown for 3 days in $-N+P$ or $-N-P$ solution. Then the roots were excised and incubated in media containing 0.05 M $KNO_3$ with different buffers (see measurement of NR activity in vivo).

Extracts of NR were prepared from fresh root tips (excised 4 cm below the tip) (Buczek 1976). Nitrate reductase activity in vitro was assayed in the supernatant fraction following 18 000$\times$g centrifugation of the homogenate, by measuring NADH-dependent production of NO$_2^-$ (Hageman and Flesher 1960) with the exception that instead of K-phosphate buffer, the HEPES-buffer pH 7.0 was used. The NR activity was calculated as nmoles NO$_2^-$ formed per gram fresh weight per hour. Three replicate samples were harvested at each time period and the experiment was repeated at least 3 times.

Measurement of NR activity in vivo: Method A (Jaworski 1971). Root sections (0 to 4 mm from tip) were excised, weighed (250-300 mg) and placed in 25 cm$^3$ Erlenmayer flasks containing 0.05 M K-phosphate buffer (pH 7.0), 0.1 M $KNO_3$, 5% v/v propanol in total 5 cm$^3$ volume and incubated for 1 h in water bath at 32°C in darkness. After incubation, the flasks were cooled in an ice bath, and nitrite released to the medium was determined by the procedure of Sanderson and Cocking (1964). Method B was similar to method A except that K-phosphate buffer was replaced by TCGM buffer (pH 7.0) containing 0.012 M Tris, 0.008 M citrate, 0.012 M glycine and 0.012 M maleate.
(Bieleski 1974). **Method C** was a modification of the Hageman et al. (1980) procedure. The 2 mM CaSO₄ was used instead of phosphate buffer to avoid inclusion of inorganic phosphate. Potential and actual NR activity (Bar-Akiva et al. 1970) were measured by method C, but in the case of actual NR, KNO₃ was replaced by K₂SO₄ keeping the potassium concentration constant. Nitrate reductase was expressed in nmoles of NO₂⁻ formed per 1 g fresh weight per hour. Each value in the figures or tables represents a mean of 6 replications and each experiment was repeated at least three times.

Extracts of acid phosphatases were prepared from fresh corn roots and the 18,000×g supernatant fraction was used for assay of acid phosphatases activity according to the method described in the preceding paper (Buczek et al. 1981). The enzyme activity was expressed in μmoles of Pᵢ liberated from para-nitrophenyl phosphate (p-NPP) per gram fresh weight per hour. Inorganic phosphate (Pᵢ) was determined colorimetrically by the Fiske and Subbarow (1925) method.

Nitrate uptake was assayed by measuring the loss of NO₃⁻ from the nutrient solution (Cataldo et al. 1975). The content of inorganic phosphate in roots was determined in fresh material after extraction with cold 10% trichloroacetic acid, by the method of Fiske and Subbarow (1925) using ammonium molybdate and 1-amino-2-naphthol-4'-sulphonic acid sodium sulphite.

All biochemicals were of analytical grade, purchased from Sigma (NADH, p-NPP) and POCH-Gliwice.

**RESULTS**

Changes of NR activity in roots of corn seedlings growing five days either in the presence or absence of inorganic phosphate in nutrient solution are shown in Fig. 1A. The roots were excised, homogenised and NR activity was assayed *in vitro*. The lack of phosphorus in the nutrient solution caused a decrease of NR activity in roots and its complete disappearance after 4 days.

The lack of inorganic phosphate in the nutrient solution caused an enhanced acid phosphatases activity in the roots, especially at pH 4.5 (Fig. 1B). The content of inorganic phosphate in roots of -P plants was very low in comparison with that in +P ones. However, an almost complete decline of free inorganic phosphate in roots of -P plants was observed after 4 days growth (Fig. 2). As in Fig. 1A, the decline in inorganic phosphate in roots was correlated with decay of NR activity.

The absorption of NO₃⁻ (Fig. 3) by corn seedlings growing in -P solution was decreased by about 50 per cent in comparison with that
in +P plants. However, the deficiency of P in the nutrient solution did not inhibit completely NO₃⁻ uptake.

The in vivo assays of NR activity in corn roots during induction of enzyme (Fig. 4A) showed a marked reduction of the potential NR in
roots growing without phosphorus. A complete lack of P in the nutrient solution caused a lesser diminution of the actual NR level, although the differences were significant. At the same time, the activities of acid phosphatases in roots of plants growing without phospho-

![Graph 2](image2.png)

**Fig. 2.** Content of inorganic phosphate in corn roots

Plus phosphate — solid circles; minus phosphate — open circles. Vertical bars represent ± SE

![Graph 3](image3.png)

**Fig. 3.** Uptake of NO₃⁻ by corn seedlings growing in solution with or without inorganic phosphate

Plus phosphorus — solid circles; minus phosphorus — open circles. Data represent mean ± SE of 6 replicates
rus were relatively high in comparison with those in plants growing in solution containing phosphorus. The increase of acid phosphatases was found to occur after 6 h growth of plants in nitrate solution containing no inorganic phosphate (Fig. 4B), while the content of inorganic phosphate in roots during 24-h growth of plants in −P solution was indeed low but constant (Fig. 2).

Fig. 4. Potential and actual nitrate reductase (A) and acid phosphatase (B) activities in corn roots growing in solutions with or without inorganic phosphate. Nitrate reductase (A) was assayed in vivo (Method C): plus phosphorus — solid circles (potential activity) and solid squares (actual activity); minus phosphorus — open circles (potential activity) and open squares (actual activity). Acid phosphatases (B): explanation as for Fig. 1.
Potential and actual NR level (Table 1) were assayed in excised root tips of plants growing previously for 24 h in nitrate containing solution with or without P. NR activity was assayed in vivo by method C (see Materials and Methods). The data shows that potential NR activity in roots of plants growing without P was more decreased than the actual one, although the actual NR level in -P plants was significantly reduced after two hours of incubation.

**Table 1**

Effect of phosphorus on “potential” and “actual” nitrate reductase (nmol NO\textsubscript{2}\textsuperscript{-} x g\textsuperscript{-1} fresh weight x h\textsuperscript{-1}) in corn roots

<table>
<thead>
<tr>
<th>Nutrient solution</th>
<th>Nitrate reductase</th>
<th>Time of assay, h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>+P</td>
<td>potential</td>
<td>118.37±14.95 a</td>
</tr>
<tr>
<td>-P</td>
<td>potential</td>
<td>41.92±6.29 b</td>
</tr>
<tr>
<td>+P</td>
<td>actual</td>
<td>31.99±7.41 b</td>
</tr>
<tr>
<td>-P</td>
<td>actual</td>
<td>31.50±5.11 b</td>
</tr>
</tbody>
</table>

NR activity was assayed in vivo by method C. Mean values with similar letters did not differ significantly at 5% level. Each mean represents the average of 6 replications.

The effect of phosphorus on in vivo induction of NR is shown in Table 2. The data show that inorganic phosphate is not necessary for activation (induction) of nitrate reductase in vivo, although NR activity was lowest when the roots were incubated in medium containing no phosphate. It was found however, that the presence of inorganic phosphate in the ambient solution is necessary for maintenance of NR activity on a relatively high level.

**Table 2**

Effect of phosphorus on in vivo induction of NR (nmol NO\textsubscript{2}\textsuperscript{-} x g\textsuperscript{-1} fresh weight x h\textsuperscript{-1}) in corn root tips excised from plants growing 3 days in minus N solution with or without inorganic phosphate

<table>
<thead>
<tr>
<th>Nutrient solution</th>
<th>Incubation medium</th>
<th>Time of assay, h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>+P</td>
<td>phosphate</td>
<td>14.60±1.32 a</td>
</tr>
<tr>
<td>-P</td>
<td>buffer</td>
<td>14.07±1.42 a</td>
</tr>
<tr>
<td>+P</td>
<td>CaSO\textsubscript{4}</td>
<td>8.91±1.42 b</td>
</tr>
<tr>
<td>-P</td>
<td>phosphate</td>
<td>9.72±0.97 b</td>
</tr>
<tr>
<td></td>
<td>buffer</td>
<td>9.86±1.32 b</td>
</tr>
<tr>
<td>-P</td>
<td>TCGM buffer</td>
<td>10.50±1.21 b</td>
</tr>
</tbody>
</table>

Each mean represents the average of 6 replications.
DISCUSSION

The dependence of NR activity on inorganic phosphate was at first observed by Spencer (1959) who found that the presence of inorganic phosphate in incubation medium was required for maximum NR activity in wheat germ. Similarly Harper and Paulsen (1969) and Sasakiwa and Yamamoto (1977) found in wheat and rice seedlings growing in medium with deficient phosphorus a considerable decrease of specific NR activity and of nitrate content in plants. Our results showed that deficiency of phosphorus in the nutrient solution caused a strong decrease of NR activity, both in vitro and in vivo and reduction of nitrate uptake. Furthermore the lack of phosphorus in the nutrient solution caused an increase of acid phosphatases activities, while the level of inorganic phosphate in roots of -P plants was in fact very low but constant.

The simplest explanation consistent with our observation is that the decrease of nitrate absorption in the absence of inorganic phosphate, limits the level of NR activity. It seems also possible to assume that NO$_3^-$ and HPO$_4^{2-}$ are correlativey absorbed. Gericke (1943) found that higher plants require an increased supply of phosphorus when nitrate is being utilised. Recently Thien and McFee (1972), Taber and McFee (1972) and Barta (1977) have shown that NO$_3^-$ pretreatment stimulates the HPO$_4^{2-}$ uptake.

It seems that phosphorus deficiency has direct effect on induction or activation of nitrate reductase. However, the fact that the lack of inorganic phosphate in both nutrient solution and incubation medium decreased not only the potential but also the actual NR level, may suggest that inorganic phosphate is required not only for maintenance of NR activity, but also for its activation. Some evidence of this view has been given by Kinsky and McElroy (1958) and Hewitt and Nicholas (1964). These authors suggested the requirement of inorganic phosphate for activation of hexavalent molybdenum in molybdo-doflavoprotein. However, this supposition has not been confirmed.

It appears that the deficiency in inorganic phosphate in roots of -P plants may be partly and perhaps for a short time supplemented by an increment of acid phosphatases. It appears that our results confirm this supposition because the level of acid phosphatases increased markedly (Fig. 1B) and the level of inorganic phosphate, although very low, was almost constant (Fig. 2). It is not excluded that this fact finds reflection in the relatively low diminution of actual NR activity in -P roots in comparison with the potential one. However, when the activity of NR is high, as in the case of potential NR, the deficiency of inorganic phosphate cannot be compensated by the action of phosphatase. Thus, the increase in acid phosphatase found in our experiments results
perhaps from the well known fact that a lack of P in the medium may stimulate a number of acid phosphatases in plants (Kubicz 1973). It seems, thus, that there is a very weak correlation between NR and acid phosphatases in corn roots.

REFERENCES


Aktywność reduktazy azotanowej i kwaśnych fosfataz w korzeniach kukurydzy w zależności od obecności lub braku nieorganicznego fosforanu w pożywce

Streszczenie

Badano wpływ braku fosforu w pożywce na poziom aktywności reduktazy azotanowej (NR) i kwaśnych fosfataz oraz na pobieranie $\text{NO}_3^-$ i zawartość nieorganicznych fosforanów w korzeniach kukurydzy. Stwierdzono, że brak fosforu w pożywce załamał w 50 procentach pobieranie azotanów oraz obniżył aktywność NR mierzoną zarówno in vitro jak i in vivo. Brak fosforu wpływał przede wszystkim na obniżenie tzw. „potencjalnej” aktywności NR i w mniejszym stopniu, aczkolwiek istotnie, na tzw. „aktualną” aktywność enzymu. Aktywność badanych kwaśnych fosfataz zwiększała się w korzeniach roślin rosnących przy niedostatku fosforu w pożywce. Wyniki sugerują, że nieorganiczne fosforany niezbędne są głównie do utrzymania aktywności NR na względnie wysokim poziomie, a w mniejszym stopniu wpływają na indukcję, względnie aktywację NR. Nie jest wykluczone, że podwyższona aktywność kwaśnych fosfataz spowodowana brakiem fosforu w pożywce uzupełnia, przynajmniej częściowo, w tkankach niedostatek nieorganicznych fosforanów.