The Induction of Nitrate Reductase and the Preferential Assimilation of Ammonium in Germinating Rice Seedlings

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Abstract. Nitrate reductase is induced by nitrate in excised embryos and germinating intact seedlings of rice (Oryza sativa L.). The enzyme is induced 24 hr after imbibition. The rate of enzyme formation increases with the age of seedlings. There is a lag period of 30 to 40 min between the addition of substrate and the formation of nitrate reductase. Formation of the enzyme is promoted by the presence of ammonium. Chloramphenicol, actinomycin D and cycloheximide effectively inhibit the formation of nitrate reductase.

Rice seedlings can assimilate nitrate from the beginning of germination. However, the utilization of nitrate is completely suppressed by the presence of ammonium. As soon as ammonium is depleted from the medium, nitrate utilization is resumed. Ammonium inhibits the first step of nitrate reduction, i.e., \( \text{NO}_3^- \rightarrow \text{NO}_2^- \), but does not inhibit the assimilation of nitrite. This provides an example of feedback inhibition in higher plants.

Materials and Methods

Plant Material. Rice (Oryza sativa L.) seeds (1R8, supplied by the Faculty of Agriculture, University of Malaya) were dehulled, surface sterilized and germinated in sterilized petri dishes. After 24 hr at 29°, the embryos were excised with a scalpel and 10 embryos were transferred to each 150 ml Erlenmeyer flask containing 40 ml of nutrient solution. The nutrient solution contained 15 mg KCl, 45 mg CaCl\(_2\), 50 mg MgSO\(_4\)\(_7\)H\(_2\)O, 50 mg KH\(_2\)PO\(_4\), 0.4 mg MnCl\(_2\)\(_4\)H\(_2\)O, 0.3 mg H\(_2\)BO\(_3\), 0.05 mg ZnSO\(_4\)\(_7\)H\(_2\)O, 0.02 mg CuSO\(_4\)\(_5\)H\(_2\)O, 0.04 mg NaMoO\(_4\)\(_2\)H\(_2\)O, 10 mg ferric citrate and 20 g sucrose per liter. It was autoclaved at 2 kg/cm\(^2\) for 15 min. Nitrogen was added as (NH\(_4\))\(_2\)SO\(_4\), KNO\(_3\) or NH\(_4\)NO\(_3\) depending on the experimental conditions. The pH of the solution was adjusted to 6.4 with KOH before autoclaving. The flasks were shaken on a New Brunswick Gyrotory shaker at a speed of about 60 RPM. The shaker was placed in a growth chamber at 28.5 ± 1° and was continuously illuminated with white fluorescence light at an intensity of 0.75 × 10\(^4\) ergs/cm\(^2\)-sec at the flask level.

Induction of Nitrate Reductase. Nitrate reductase was induced by adding aseptically 1400 μg NO\(_3^-\)-N in 5 ml KNO\(_3\) solution into each flask. The flasks were returned to the growth chamber immediately after addition of the substrate. In most cases, 3.5-day old (2.5 days after transfer) seedlings were used for induction studies. The pH of the media during the induction period was 5.3 to 5.5.

Enzymatic Assay. Enzymatic assay was based on the method of Hageman and Flesher (10). At the end of the induction period, the seedlings were collected on a stainless steel gauze, rinsed with distilled water, blotted dry and weighed. When induc-
assimilation period was shorter than 1 hr, the average weight of controls was used for calculating enzymatic activity in treated samples. The material was homogenized in a medium containing 0.1 M tris, 0.01 M cysteine and 0.0003 M EDTA (pH 7.4), using a glass homogenizer immersed in ice-water. The homogenate was made up to 4 ml with the grinding medium and centrifuged at 8000g for 30 min in a refrigerated centrifuge. The supernatant was used as crude enzyme extract for assay. The incubation mixture contained 2.5 ml phosphate buffer of pH 7.0, 0.5 ml of 0.1 M KNO₃, 0.5 μmole reduced NAD in 1 ml solution and 0.25 to 1.0 ml enzyme extract. Incubation was for 3 hr at 29°. When the enzymatic activity in the incubation mixture was below 25 mμmole NO₂⁻/hr, the reaction rate was constant during this incubation period. The reaction was stopped by adding 1 ml of 1% (w/v) sulfanilamide in 1.5 N HCl and 1 ml of 0.02% (w/v) N-(naphthyl) ethylene diamine hydrochloride. The absorbancy was determined at 540 mμ against its own blank (complete except for reduced NAD). Enzyme activity was shown as mμmole NO₂⁻ formed during the incubation period.

**Determination of Insoluble Nitrogen Content.**
Ten seedlings were soaked and boiled in 2 changes of 10 ml 70% ethanol as described by Rijven (17). They were then digested in concentrated H₂SO₄, and NH₄⁺ determined by Kjeldahl distillation and nesslerization.

**The Determination of NH₄⁺, NO₃⁻ and NO₂⁻ in Nutrient Media.** Ammonium in nutrient media was determined with Conway's diffusion method (4) and nesslerization. Nitrate was determined by the method of Woolley et al. (26). Nitrite was determined as described above in Enzymatic assay.

**Application of Inhibitors.** Ten seedlings originating from excised embryos were transferred aseptically to a 50 ml Erlemeyer flask containing 10 ml of phosphate buffer at pH 5.6 supplemented with chloramphenicol, actinomycin D or cycloheximide. The solutions were cold-sterilized through Millipore filters. One ml sterile NO₃⁻ solution was added 1 hr later for induction.

**Replications.** All data are the average of 4 replications except where specially indicated.

**Results**
It was found in a preliminary experiment that during the first 48 hr of germination in complete medium containing NH₄⁺ or NO₃⁻ excised embryos did not show any appreciable increase in the total 70% ethanol insoluble nitrogen content. To study the assimilation of NH₄⁺ and NO₃⁻ by germinating seedlings, excised embryos were cultured in nitrogen deficient (−N) nutrient solution. At the end of 2.5 days, 1400 μg NH₄⁺ or NO₃⁻-N was added to the cultures. The increase in the total 70% ethanol insoluble nitrogen content is shown in Fig. 1. It is evident that 3.5-day old rice seedlings can assimilate NO₃⁻, but the rate is slower than utilization of NH₄⁺.

The formation of NR in these seedlings during the first 20 hr of induction is shown in Fig. 2.

![Fig. 1. The increase in total 70% ethanol insoluble nitrogen content of rice seedlings after NH₄⁺, ○, and NO₃⁻, □, were added to the media. Δ, −N control. Vertical lines indicate standard errors.](image1)

![Fig. 2. Time course of the induction of nitrate reductase in 3.5-day old rice seedlings originating from excised embryos and previously grown in −N medium.](image2)
There was an increase in the NR activity in the first hr of induction. The enzymatic activity increased steadily from the second to the twentieth hr.

Some variability was encountered in determining the lag period between the addition of NO\textsubscript{3}\textsuperscript{-} into the medium and the beginning of enzyme formation. It was noted, however, that when the enzyme was induced in intact rice seedlings and in seedlings previously grown in the media containing NH\textsubscript{4}\textsuperscript{+}, the variation in enzymatic activity was less. The formation of NR during the first 3 hr of induction was thus studied in seedlings originating from excised embryos grown in a medium containing 2 mM NH\textsubscript{4}\textsuperscript{+} for 2.5 days. There was a sharp initial increase in NR activity between the 30 to 40 min after the addition of NO\textsubscript{3}\textsuperscript{-} (Fig. 3).

To study the rate of NR formation in embryos 1 day after imbibition, 40 embryos were excised from rice seeds at 18 to 20 hr after imbibition and were transferred to complete medium containing 2 mM NH\textsubscript{4}\textsuperscript{+}. Induction was started at 24 hr. Cultures used for studying enzyme induction in 2- and 3-day old seedlings consisted of 20 and 10 seedlings, respectively, which were excised and transferred to the same kind of medium at 24 hr after imbibition. The induction period was 4 hr. It was evident that NR activity could be induced in the 24-hr old seedlings, and that the rate of enzyme formation increased with the age (Fig. 4).

The rate of induction of NR in intact rice seedlings is shown in Table I. Dehulled seeds were germinated in petri dishes for 24 hr and were transferred aseptically to a medium complete except for nitrogen and sucrose. The induction was started 2.5 days after transfer. The results showed that the induction process was essentially the same as in the excised embryos.

The effects of NH\textsubscript{4}\textsuperscript{+} on the induction of NR in rice seedlings are summarized in Table II. Sterile (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} solution was added to the culture medium to make a final concentration of 2 and 20 mM NH\textsubscript{4}\textsuperscript{+} when the embryos had been transferred to the medium for 2 days. Induction was started in the same medium 12 hr later. The results showed that NH\textsubscript{4}\textsuperscript{+} at 2 mM in medium did not affect the rate of induction; at a 10\times higher concentration of NH\textsubscript{4}\textsuperscript{+} in the medium, the induction rate increased by 14.4 %. The results in Table II also show that when seedlings were incubated in chloramphenicol, actinomycin D or cycloheximide the formation of NR was
Table II. The Effect of NH₄⁺ and Inhibitors on the Induction of Nitrate Reductase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Promotion (+) or inhibition (−) of nitrate reductase formation¹</th>
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</thead>
<tbody>
<tr>
<td>NH₄⁺</td>
<td>%</td>
</tr>
<tr>
<td>2 mM</td>
<td>+1.8</td>
</tr>
<tr>
<td>20 mM</td>
<td>+14.4</td>
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<tr>
<td>Chloramphenicol</td>
<td></td>
</tr>
<tr>
<td>1000 μg/ml</td>
<td>−48.9</td>
</tr>
<tr>
<td>2000 μg/ml</td>
<td>−60.2</td>
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<tr>
<td>Actinomycin D</td>
<td></td>
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<tr>
<td>20 μg/ml</td>
<td>−20.1</td>
</tr>
<tr>
<td>40 μg/ml</td>
<td>−33.6</td>
</tr>
<tr>
<td>Cycloheximide²</td>
<td></td>
</tr>
<tr>
<td>0.2 μg/ml</td>
<td>−42.4</td>
</tr>
<tr>
<td>2.0 μg/ml</td>
<td>−54.6</td>
</tr>
</tbody>
</table>

¹ Controls contained no NH₄⁺ or the inhibitors.
² Induction was with 3-day old seedlings. Induction period was 3 hr.

effectively suppressed by these inhibitors of protein and RNA synthesis.

The uptake by rice seedlings of NH₄⁺ and NO₃⁻ was studied by transferring 10 excised embryos into 40 ml medium containing 350 μg NH₄⁺-N and 350 μg NO₃⁻-N. The decrease in the amount of the 2 forms of nitrogen in the medium was followed until both were about depleted. The results (Fig. 5) showed that young rice seedlings did not assimilate NO₃⁻-N until the NH₄⁺ in the medium was almost depleted. In another experiment, excised embryos were transferred to 40 ml complete medium containing 350 μg NO₃⁻-N, after 2 days, 350 μg NH₄⁺-N was added. Fig. 6 shows that 24-hr old seedlings started to assimilate NO₃⁻ but at a slower rate than they utilized NH₄⁺. The assimilation of NO₃⁻ was completely stopped by the addition of NH₄⁺, and was resumed when the added NH₄⁺ was used up.

The effect of NH₄⁺ on the assimilation of NO₃⁻ by rice seedlings was studied by growing the excised embryos in −N medium for 2 days. Then, 350 μg NO₃⁻-N in 5 ml of cold-sterilized NaNO₃ solution was added to the medium; 350 μg NH₄⁺-N was added 1 day later. Fig. 7 shows that the assimilation of NO₃⁻ was not inhibited but was promoted by the presence of NH₄⁺ in the medium. In this experiment, blanks consisted of complete nutrient solution containing 350 μg NO₃⁻-N at a pH of 4.8 under the same environmental conditions. No appreciable loss of NO₃ from the blanks was found.

Discussion

Rice plants may have, as other higher plants do (20), 2 distinct enzymes catalyzing the reduction of nitrate to nitrite, 1 NADH specific and 1 NADPH specific. Reduced NADP was found to be 38 % as effective as reduced NAD in enzymatic assay when the enzyme activity in the incubation mixture was about 20 μmole NO₃⁻/hr. In this experiment, only the NADH specific form was studied.

A low NR activity was found in rice seedlings grown in media containing no added nitrate. It did not seem to be induced by a trace amount of NO₃⁻.

Fig. 5. The utilization of NH₄⁺ and NO₃⁻ by rice seedlings originating from excised embryos. NH₄⁺, NO₃⁻, Δ pH. Average of 3 or 4 replications.

Fig. 6. The utilization of NH₄⁺ and NO₃⁻ by rice seedlings originating from excised embryos. 350 μg NH₄⁺-N was added into the medium on the second day. NH₄⁺, NO₃⁻, Δ nitrate reductase activity. Average of 3 replications.
which was present in the medium as impurity. Analytical grade chemicals were used throughout the experiment though some did contain trace of nitrate. The original NR activity increased in rice seedlings with their age (Fig. 4) and persisted for at least 3 days in nitrogen starved seedlings developed from excised embryos. Enzyme activity increased when the seedlings were grown in NH₄⁺-media (Fig. 2 and 3). Four-day old intact rice seedlings grown in distilled water had a NR activity of 76 μmole NO₃⁻/g fresh wt/hr. Therefore, NR may be present at low concentration in rice seedlings as a constitutive enzyme. Farkas-Himsley and Artman found that NR of Escherichia coli was constitutive (7).

The lag period between the addition of substrate and enzyme formation and the initial increase in the enzyme activity was very similar to the kinetics of NR formation in Aspergillus nidulans (5) which was suggested to be an evidence of de novo synthesis of the enzyme. The reduced variability in NR activity when induction was made in NH₄⁺-medium and the effects of protein- and RNA-synthesis inhibitors on the induction also suggested that the formation of NR was a process of protein synthesis.

The phenomenon of NH₄⁺ suppressed NO₃⁻ assimilation in rice seedlings was not apparently due to the effect of NH₄⁺ repressing the formation of NR. It was also shown that NH₄⁺ did not prevent the entry of NO₃⁻ into the cells. These evidences suggest that ammonium inhibits the activity of NR, the first enzyme in the biochemical pathway of NO₃⁻ reduction and the effect was immediate. This provides a perfect example of feedback or end product inhibition (25) in higher plants. Syrett and Morris (23) reported that NH₄⁺ assimilation completely inhibited NO₃⁻ assimilation but only partially inhibited the NO₃⁻ utilization of Chlorella. In the case of rice seedlings, the assimilation of NH₄⁺ on NH₄⁺ assimilation may be a secondary one assimilation of NH₄⁺. The promoting effect of NH₄⁺ on NH₄⁺ assimilation may be a secondary one owing to its effects on the metabolism of the seedlings as a suitable form of nitrogen nutrient.

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Literature Cited