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FUNCTIONING OF NITRATE REDUCTASE IN THE LIGHT AND IN THE DARK IN SEEDLINGS OF RAPHANUS SATIVUS

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SUMMARY

The activity of the enzyme nitrate reductase in intact radish seedlings (in vivo activity) was measured in the light (at 200 and at 20,000 lux) and in the dark with ^{15}N labeled nitrate and compared with the activity of enzyme extracts (in vitro activity). Seedlings were illuminated for 24 or for 48 hours. During the first 24 hours the in vivo activity was about the same at the different light intensities whereas the in vitro activity was correlated with the light intensity. After the first 24 hours reduction of nitrate in vivo ceased when at 200 lux or in the dark; in vitro activity, however, was still detectable during the subsequent 24 hours period but at lower levels than for the first period.

At 20,000 lux, however, both in vivo activity and in vitro activity increased, although not to the same degree: the induction of the enzyme increased more than the in vivo activity in the seedlings.

1. INTRODUCTION

The activity of the enzyme nitrate reductase is generally assayed by an in vitro method in which the enzyme activity is measured by determining the amount of nitrite produced by an extract under optimal conditions of pH and of supply of nitrate and NADH. Mostly, the activity so determined is taken as a measure for the activity of the enzyme in the living tissue. Some authors, however, have questioned the reliability of such an in vitro assay in predicting the in vivo activity of the enzyme (OGHOGHORIE & PATE 1971; WALLACE & PATE 1965).

The in vivo activity of the enzyme nitrate reductase has been determined in several ways.

KLEPPER et al. (1971) developed an in vivo assay by vacuum infiltration of leaf discs or sections with nitrate medium and incubation of the infiltrated tissue and medium in the dark under anaerobic conditions. The enzyme activity was computed by determination of the nitrite production in the medium. The enzyme activity of an extract was also determined with an in vitro assay.

FERRARI & VARNER (1970) measured the in vivo activity in barley aleurone layers by mass spectroscopic analysis of $H_2{}^{18}O$ enzymically produced from $KN^{18}O_3$.

The in vivo activity can also be measured with the isotope ^{15}N. Earlier work with this method was done by DELWICHE (1951) with tobacco leaves and by MENDEL & VISSER (1951) with tomato leaves.

In both studies the reduction of ^{15}N nitrate in the presence and absence of light was studied. From the experiments of DELWICHE (1951) it was concluded that the assimilation of nitrate in the dark was the same as in the light. MENDEL & VISSER (1951) reached the conclusion that the rate of assimilation in the light was about 50% greater than in the dark. OGHOGHORIE & PATE (1971) used this method in comparison with an in vitro assay.

In radish cotyledons a high in vitro activity could be detected after an induction period of 24 hours both in the light and in darkness (STULEN et al. 1973).

In the experiments described in the present paper the functioning of the enzyme in the intact seedling was determined in the light and in the dark with ^{15}N nitrate and compared with the in vitro activity of the enzyme.

2. MATERIAL AND METHODS

2.1. Radish seedlings

Radish seedlings (*Raphanus sativus* L. cv. Cherry Belle) were grown as described previously (STULEN et al. 1971).

2.2. Induction, extraction and assay of nitrate reductase activity in the cotyledons

Induction, extraction and assay were performed in the same way as described before (STULEN et al. 1971, 1973).

2.3. Extraction and assay of nitrite reductase activity in the cotyledons

The assay of nitrite reductase activity was performed with the same extract as used for the determination of nitrate reductase activity. Nitrite reductase activity was assayed after JOY & HAGEMAN (1966) except that the reaction tubes were not evacuated but completely filled and plugged with rubber stoppers, in order to prevent non-enzymatic oxidation of reduced benzylviologen.

2.4. Estimation of reduced ^{15}N

^{15}N labeled nitrate was added to the nutrient solution during the induction period. The nitrate concentration was 10^{-2} mol; 50% was ^{15}N nitrate.

After various periods of time the seedlings were harvested and divided into roots, hypocotyls and cotyledons. The tissues were extracted with 3% HCl. Total nitrogen in the HCl soluble and insoluble fractions was determined with the Kjeldahl method (BAILEY 1962). For estimation of the enrichment of the material with ^{15}N the ammonia obtained by the steam distillation was converted to nitrogen gas by oxidation with alkaline hypobromite after which the ^{15}N content was determined spectrophotometrically after FAUST (1965) with the ^{15}N analyser.

Since free nitrate was lost during the digestion procedure all of the ^{15}N determined was in the reduced form.

2.5. Assay of nitrate content

The nitrate content was assayed as described previously (STULEN et al. 1973).

3. RESULTS

In the experiments described below the in vivo activity of the enzyme nitrate reductase in the intact seedling was determined by measuring the amount of heavy nitrogen present. The reduction products of nitrate reductase are measured as ammonia, except any free nitrite, since with the method used free nitrate and nitrite are lost during the digestion procedure. To estimate any free nitrite, nitrite determinations were carried out on water extracts of radish seedlings. In none of the material checked, however, could free nitrite be detected. Consequently all of the nitrite formed by reduction of nitrate must be removed immediately by the enzyme nitrite reductase in the intact seedling. Comparison of the in vitro activities of nitrate reductase and nitrite reductase showed that the level of nitrite reductase exceeded the level of nitrate reductase after various induction periods in the light as well as in the dark (*table 1*).

Table 1. Level of nitrate reductase and nitrite reductase activity after various induction periods in the light and in the dark.

light intensity in lux	induction period in hours	nitrate reductase activity in μmol nitrite/hr/g.fresh weight	nitrite reductase activity in μmol nitrite/hr/g.fresh weight
20,000	24	7.0	18.9
20,000	48	8.2	12.8
0	24	6.1	16.5
0	48	3.1	14.9

In the previous experiments nitrate reductase was assayed only in the cotyledons of the seedlings. Since the objective of the present study was to measure the in vivo activity of the enzyme in the intact seedling in comparison to the in vitro activity found, it was necessary to determine whether nitrate reductase activity could also be found in extracts of other organs. According to WALLACE & PATE (1967) some Angiosperms (e.g. *Pisum*) reduce nitrate in their roots, others (e.g. *Xanthium*) do not. In order to check whether nitrate reductase activity could be detected in extracts of roots of radish seedlings various extraction and assay procedures were tried using a range of cysteine levels (*table 2*). The attempts were made with roots of light- as well as of dark-induced seedlings.

It turned out that only the method described by MIFLIN (1967) gave detectable amounts of nitrate reductase activity. In the roots of seedlings induced at 20,000 lux nitrate reductase activity, assayed by this method, was not found until after induction periods of 48 hours or more; in the roots of dark-induced seedlings no nitrate reductase activity could be detected up to 96 hours. The activity found in

Table 2. Detection of nitrate reductase activity in the roots of radish seedlings after an induction period of 48 hours.

extraction and assay after:	nitrate reductase activity
WALLACE & PATE (1965)	
pH 7.5; cysteine conc. 10^{-2}–10^{-6} mol	–
STULEN et al. (1971, 1973)	
pH 8.2; cysteine conc. 10^{-2}–10^{-6} mol	–
pH 7.5; cysteine conc. 10^{-2}–10^{-6} mol	–
MIFLIN (1967)	
pH 7.8; glutathione 10^{-3} mol	+

the roots of light-induced seedlings was very low with respect to the activity found in the cotyledons of the same seedlings; after an induction period of 48 hours the activity found in the root extracts was but 5% of the activity found in the cotyledon extracts (*fig. 1*).

Taking into account the fact that the roots of seedlings induced for 48 hours in the light have a fresh weight of about half that of the corrresponding cotyledons, the total activity found in the roots is only 2–3% of the activity found in the cotyledons.

Since the in vitro activity found in root extracts was very low compared with the in vitro activity found in cotyledon extracts, in the following experiment only the in vitro activity in the cotyledons was determined. In this experiment the in vitro activity of the enzyme extract was compared with the in vivo activity of the intact seedling after induction periods of 24 and 48 hours in the light (at 200 lux and at 20,000 lux) and in the dark. From the values found in the in vitro assay it was calculated how much nitrate could have been reduced in the intact seedling during the induction period (assuming that the enzyme would reduce nitrate at

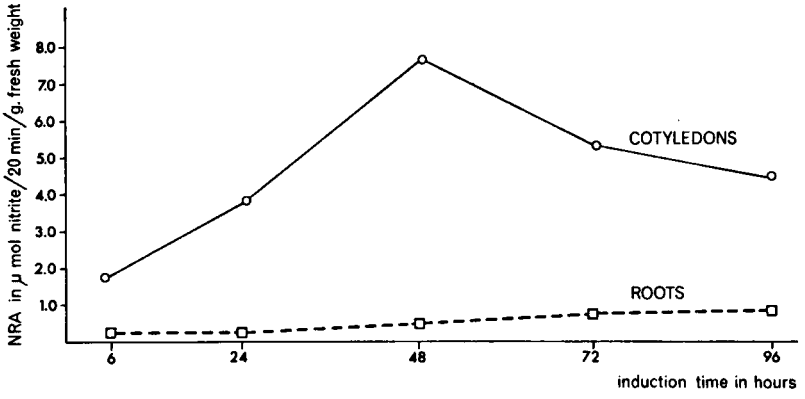

Fig. 1. Level of nitrate reductase activity (NRA) in cotyledons and roots of seedlings induced at 20,000 lux.

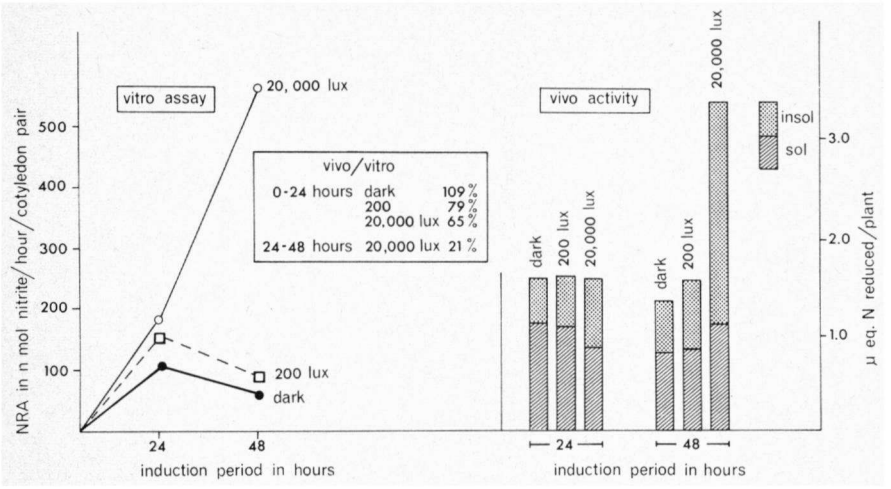

Fig. 2. Comparison of in vitro and in vivo activities of seedlings induced at different light intensities for 24 and 48 hours.

the same rate as during the in vitro incubation period) by determination of the areas under the curve between 0–24 and 24–48 hours. This action was justified by preliminary experiments from which could be concluded that the maximum values for seedlings induced at 200 lux and in the dark were reached after 24 hours. The in vivo activity was calculated from the enrichment of the plant material and expressed as the total amount of nitrate reduced during the induction period in the whole seedling. From the values calculated in this way the ratio vivo/vitro was determined (*fig. 2*).

The in vitro activity of extracts of dark-induced seedlings decreased after the first period of 24 hours, whereas the activity of the seedlings induced at 20,000 lux continued to increase. It appeared that the in vitro activity of the seedlings induced at 200 lux showed the same picture as the dark-induced seedlings.

The data derived for the in vivo activity of the seedlings induced in the dark and at 200 lux were also identical: after the first period of 24 hours no more nitrate was reduced. In the seedlings induced at 20,000 lux in vivo reduction did take place during the whole induction-period. Most of the increase of labeled nitrogen during the second period of 24 hours was found in the protein fraction. The distribution of the labeled nitrogen in the seedling at the moment of sampling is shown in *fig. 3*. It appeared that during the second induction period at 200 lux no further reduction of nitrate occurred and the labeled nitrogen was redistributed from the cotyledons to the roots. In the dark there was a slight decrease in the level of labeled nitrogen. At 20,000 lux after 48 hours the greatest part of labeled nitrogen was found in the cotyledons.

Comparison of the vivo/vitro ratios (*fig. 2*) found for the first induction period of 24 hours showed that the values were inversely correlated with the light intensity.

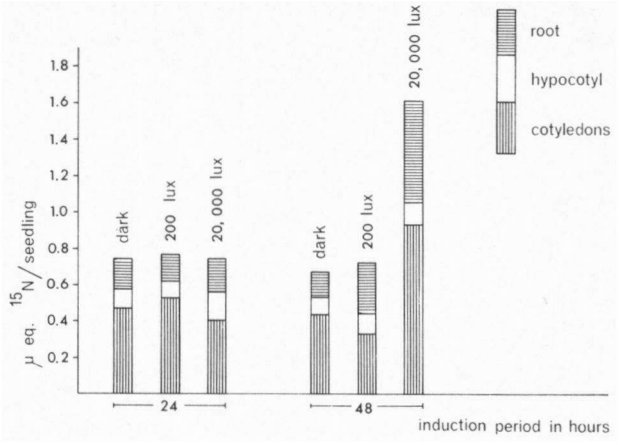

Fig. 3. Distribution of labeled nitrogen in seedlings induced at different light intensities for 24 and 48 hours.

Although the induction of the enzyme (measured as the in vitro activity of the enzyme extracts) was not the same for the various light treatments, equal amounts of nitrate were reduced in the intact seedling during 24 hours. So the activity per enzyme unit was highest in the dark. The vivo/vitro ratio decreased during the second induction period at 20,000 lux; the level of enzyme activity, assayed in vitro, increased more than the in vivo activity.

From the data obtained in the experiment described above the relationship between nitrate uptake and reduction could be found (*table 3*).

For each treatment the values of the amount of free nitrate and the amount of reduced nitrate were added; the sum appeared to be in the same range for the different light treatments after a similar induction period. The amount of free nitrate was inversely correlated with the amount of nitrate reduced. These data give further support to the conclusion drawn from experiments described previously (STULEN et al. 1973) about the influence of light on the uptake of nitrate;

Table 3. Nitrate uptake and nitrate reduction at various light intensities. Radish seedlings were given ^{15}N-labeled nitrate at various light intensities. After 24 hours and 48 hours free nitrate and the amount of ^{15}N-labeled nitrate which had been reduced were determined.

ligth intensity	free nitrate in μmol/seedling	reduction of nitrate in μmol/seedling	uptake of nitrate in μmol/seedling
		induction period 24 hours	
0	2.1	1.6	3.7
200	2.4	1.7	4.1
20,000	1.7	1.6	3.3
		induction period 48 hours	
0	5.4	1.3	6.7
200	4.0	1.5	5.6
20,000	3.4	3.4	6.8

the uptake of nitrate is not enhanced by light, whereas during a light period of 48 hours the nitrate reduction is enhanced by light.

4. DISCUSSION

From the experiments described above the conclusion can be drawn that the activity of the enzyme nitrate reductase, measured in an in vitro assay, is not always correlated with the true rate of assimilation of nitrate in the intact seedling. After an induction period of 24 hours in the dark the in vitro activity agreed closely with the in vivo activity. In the light, however, especially at 20,000 lux, the in vivo activity was less than the in vitro activity. In the dark and at 200 lux the intact plants reduced as much nitrate in a 24 hour induction period as in 48 hours. In the second period of 24 hours, therefore, no reduction of nitrate occurred. The in vitro activity of the enzyme in extracts decreased under these circumstances during the second 24 hours.

At 20,000 lux the seedlings did reduce nitrate during the second induction period. The difference, however, between the activity of an enzyme extract and the nitrate reduction in the intact seedling became more pronounced: the in vitro activity increased more than did the activity in vivo.

In the experiments of KLEPPER et al. (1971), OGHOGHORIE & PATE (1971) and FERRARI & VARNER (1970) a similar discrepancy between in vivo activities and in vitro activities was found. From experiments with detached leaves infiltrated with nitrate KLEPPER et al. (1971) reached the conclusion that the in vitro activity of leaves, previously grown in the light, was higher than the in vivo activity. FERRARI & VARNER (1970) concluded that the rate of in vivo reduction did not increase parallel with the increased level of nitrate reductase activity of the extracts; only a small fraction of the total enzyme activity induced in response to nitrate was functioning in the tissue. OGHOGHORIE & PATE (1971), however, concluded from their experiments with intact plants that had been given ^{15}N nitrate, that the in vitro assay agreed well with the true rate of nitrate assimilation at low levels of nitrate in the induction medium; at higher levels of nitrate, however, the in vivo activity was much higher than the in vitro activity.

From the experiments described in this paper a conclusion can be drawn about the influence of light on the enzyme nitrate reductase. During the first induction period the in vivo reduction in the dark and at 200 lux reached the same value as at 20,000 lux, whereas the in vitro activity was correlated with the light intensity. So light did not stimulate the assimilation rate during the first period; light stimulated only the induction of the enzyme (measured as the in vitro activity). After the first induction period a high light intensity (20,000 lux) had a stimulating influence, whereas a light intensity of 200 lux had the same effect as the dark treatment. The high light intensity influenced both the induction of the enzyme and the activity of the enzyme in the intact seedling although not to the same degree: the induction of the enzyme increased more than the activity. Therefore other factors than induction per se become limiting for the assimilation of nitrate in the light.

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