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THE INFLUENCE OF LIGHT AND NITRATE ON THE INDUCTION OF NITRATE REDUCTASE IN DARK GROWN SEEDLINGS OF RAPHANUS SATIVUS

INEKE STULEN, T. KOCH-BOSMA and A. KOSTER

Biologisch Centrum, Afd. Plantenfysiologie, Universiteit Groningen, Haren, (Gr.)

SUMMARY

In cotyledons of 4 day old etiolated, dark-induced radish seedlings a high level of nitrate reductase was found after an induction period of 24 hours. The activity declined after a prolonged induction time. It was shown that the decrease in activity was not due to a decrease in uptake of nitrate. In the dark other factors must become limiting after induction periods exceeding 24 hours.

1. INTRODUCTION

The stimulating action of light on the enzyme nitrate reductase has frequently been observed. Light may exert its influence in various ways: on the generation of hydrogen donors (KLEPPER et al. 1971) or carbon compounds (KANNANGARA & WOOLHOUSE 1967) or, more indirectly, via development of an active protein synthesising system (TRAVIS, HUFFAKER & KEY 1970, TRAVIS & KEY 1971). BEEVERS et al. (1965) attributed the effect of light to enhancement of nitrate uptake as a result of increased permeability of the tissue. They found that the uptake of nitrate, measured as the amount of nitrate in the tissue, was greater in the light than in the dark.

Measurement of the uptake of nitrate by way of determination of nitrate in the tissue is complicated by the fact that nitrate is reduced within the tissue. Consequently it is necessary to prevent the utilization of nitrate in experiments about the influence of light on the uptake and accumulation of nitrate. Functioning of nitrate reductase can be selectively inhibited by addition of tungstate during induction (HEIMER et al. 1969; WRAY & FILNER 1970).

In the experiments described here the influence of light on the uptake and accumulation of nitrate in dark grown seedlings of *Raphanus sativus* was examined both in the absence and in the presence of nitrate reductase activity.

2. MATERIAL AND METHODS

2.1. Radish seedlings

Seedlings of *Raphanus sativus* L. cv. Cherry Belle were grown as described previously (STULEN et al. 1971). In the course of the investigations the germination room was improved so that the high (98–100%) relative humidity needed for

optimal growth could be constantly maintained. When the seeds were germinated under these conditions it was found that after 4 days, when the seedlings were used for the experiments, the cotyledons no longer contained the endogenous inhibitor of nitrate reductase previously described (STULEN et al. 1971).

2.2. Induction of nitrate reductase

In most experiments induction was carried out at a final concentration of 10^{-2} mol NO_3^- (half KNO_3, half Ca $(NO_3)_2$). Exceptions are reported in the experimental section. In the experiments on the influence of tungstate, sodium tungstate was added at the start of the induction period at various concentrations. For induction in the light the seedlings were continuously illuminated with 20,000 lux at 25°C. The results of the experiment shown in *fig. 3* indicated that no further increase in nitrate reductase activity occurred at light intensities exceeding 15,000 lux. Induction in the dark was also carried out at 25°C. All experiments were set up in triplicate.

2.3. Extraction and assay of nitrate reductase activity

Extraction and assay of the enzyme was carried out according to STULEN et al. (1971). In all experiments the pellet fraction was also assayed for nitrate reductase activity. The values found for the supernatant and the pellet fraction of each experiment were summed. For batches of seedlings raised before improvement of the germination room, and therefore containing inhibitor, the activity of the enzyme in the absence of inhibitor was calculated by incubation of various amounts of enzyme preparation (STULEN et al. 1971).

2.4. Assay of nitrate content

For determination of nitrate an aqueous extract of plant material was made, usually at a ratio of 1 gram of fresh material to 10 ml of water. The extraction was carried out at 0–4°C. In some experiments the supernatant fraction of the enzyme preparation was used for assay of nitrate in the cotyledons.

Nitrate content was estimated after WOOLLEY et al. (1960).

3. RESULTS

The inducible nature of nitrate reductase in response to addition of nitrate was shown by several authors (reviewed by BEEVERS & HAGEMAN 1969). For optimum induction in different species different levels of nitrate are required.

In order to find the optimum nitrate concentration for induction in light as well as in darkness, experiments were carried out with seedlings induced at various nitrate levels in continuous light (20,000 lux) or in the dark. The seedlings were harvested after an induction period of 24 hours, after which the nitrate reductase activity in the cotyledons was assayed (*fig. 1a/b*).

From *fig. 1* it can be concluded that the optimum concentration for induction in light and in darkness is the same: 10^{-2} mol. This concentration was also found by BEEVERS et al. (1965) for induction in radish seedlings in the light. A

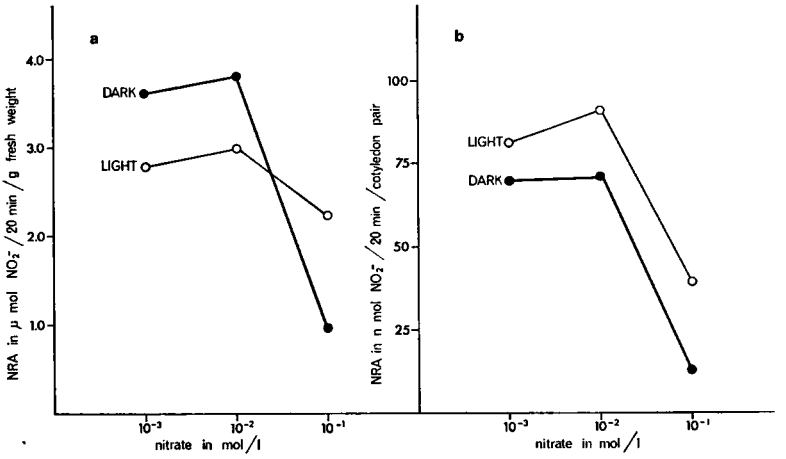

Fig. 1. Nitrate reductase activity (NRA) in cotyledons of seedlings induced at various substrate concentrations in the light (20,000 lux) and in the dark after an induction period of 24 hours, expressed on a fresh weight basis (a) and a plant basis (b).

higher concentration of nitrate suppressed the induction, as reported also by BEEVERS et al. (1965) and BOWERMAN & GOODMAN (1971). *Fig. 1* also shows that after an induction period of 24 hours the nitrate reductase activity in the dark, expressed on a cotyledon basis, was almost as high as in the light. Expressed on a fresh weight basis the activity in the dark found in this experiment was even higher than in the light.

In order to check if the nitrate reductase activity in cotyledons of dark-induced seedlings remains at the high level found after an induction period of 24 hours, seedlings were also harvested after an induction period of 48 hours (*fig. 2*). This experiment showed that the level of nitrate reductase activity in

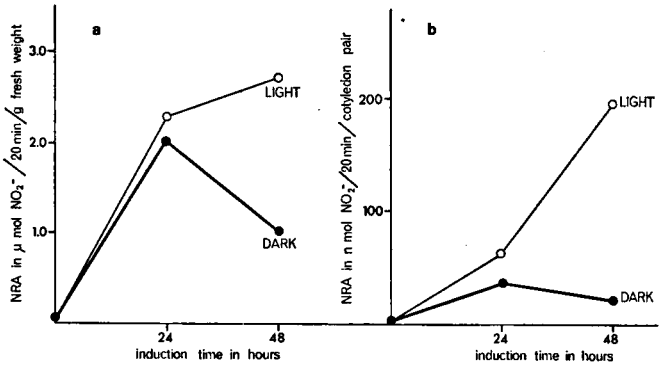

Fig. 2. Nitrate reductase activity (NRA) in cotyledons of seedlings induced for various times in the light (20,000 lux) and in the dark at a substrate concentration of 10^{-2} mol nitrate, expressed on a fresh weight basis (a) and on a plant basis (b).

dark-induced seedlings decreased after an induction period of more than 24 hours. The question arose as to whether the decrease in activity in dark-induced seedlings was due to a decrease in uptake of nitrate by the seedlings.

Comparison of the values of nitrate reductase activity found in the experiments of *fig. 1* and *fig. 2* shows that the level of induction can vary between experiments, although the seeds were germinated under standardized conditions. Since there was a slight variation in temperature in the room where induction was performed, each experiment was always done with a batch of seedlings sown on the same day and induced under the same conditions.

From preliminary experiments it was concluded that it was not possible to determine the uptake of nitrate by measuring the nitrate concentration in the nutrient solution; the total amount of nitrate taken up by the seedlings was too low in relation to the amount of nitrate given. So it was necessary to determine the amount of nitrate present in the seedlings.

In an experiment set up to determine the influence of light intensity on the induction of nitrate reductase the nitrate content of the cotyledons was determined (*fig. 3*). From this figure it can be seen that there is an inverse correlation between the amount of nitrate present in the tissue and the enzyme activity. This experiment showed that the utilization of nitrate must be prevented in experiments where the amount of nitrate present in the tissue is used for measuring the uptake of nitrate.

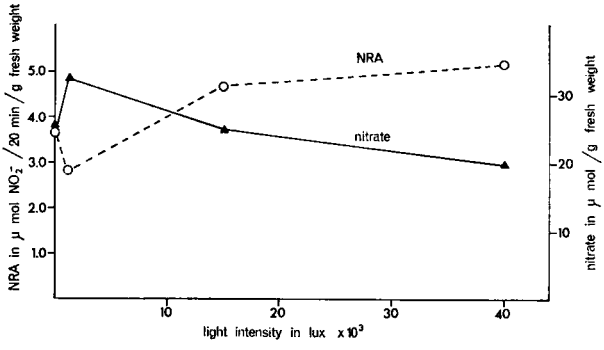

Fig. 3. The influence of light intensity on nitrate reductase activity and nitrate content in cotyledons of seedlings induced for a period of 24 hours at a substrate concentration of 10^{-2} mol nitrate.

Action of nitrate reductase can be prevented by tungstate (HEIMER et al. 1969; WRAY & FILNER 1970). It appeared that addition of 10^{-4} mol sodium tungstate during induction fully inhibited formation of nitrate reductase activity in the light as well as in darkness (*fig. 4a*). HEIMER et al. (1969) and WRAY & FILNER (1970) also found this concentration to be fully inhibitory for induction in tobacco XD cells and barley shoots. In the experiment described here, the modified A–Z solution (STULEN et al. 1971) was omitted from the nutrient solution

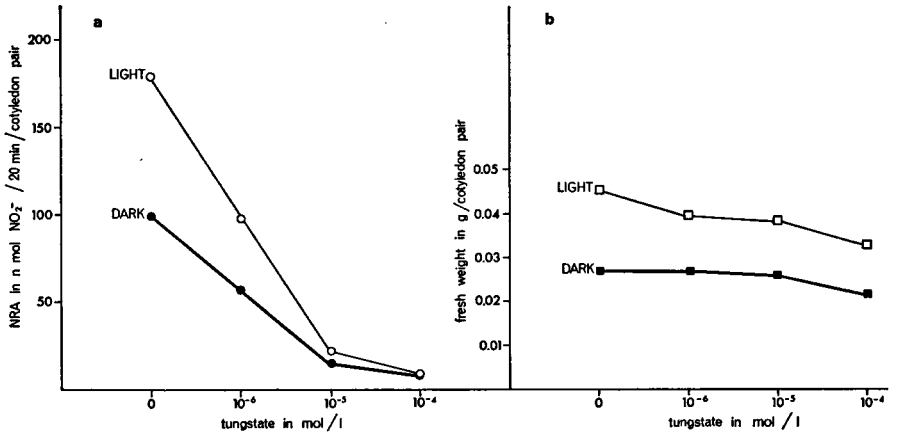

Fig. 4. The influence of tungstate concentration on nitrate reductase activity (a) and fresh weight (b) of cotyledons of seedlings induced for a period of 24 hours in the light (20,000) lux and in the dark at a substrate concentration of 10^{-2} mol nitrate.

during germination and induction since the amount of molybdate present in the nutrient solution (0,23 μmol) may cause reversal of the inhibition by tungstate (WRAY & FILNER 1970). Treatment with tungstate decreased the fresh weight of the seedlings. For the cotyledons this decrease was greater in the light than in the dark (*fig. 4b*).

In order to investigate if light may exert some influence on the uptake of nitrate, the amount of nitrate present in tungstate treated seedlings was determined in light and in darkness. In the same experiment a comparison was made with seedlings where the nitrate reductase activity was not inhibited (*fig. 5*). This experiment showed that in the absence of nitrate reductase activity the amount of nitrate present in the tissue, expressed on a fresh weight basis, was the same in light-treated and in dark-treated seedlings. When expressed on a plant basis (*table 1*), the total amount of nitrate found in the seedlings was about equal; owing to stronger growth of the cotyledons in the light the amount of nitrate present in cotyledons of light-treated seedlings was higher. In the dark the greatest amount of nitrate was found in the hypocotyl. Nitrate content continued to increase after the first period of 24 hours, in the light as well as in darkness. In the control experiment, where tungstate was omitted, the level of nitrate in the cotyledons was inversely related to the nitrate reductase activity. The amount of nitrate present in tungstate-treated plants was lower than in untreated plants. This is not in agreement with the findings of WRAY & FILNER (1970) who found that tungstate-treated plants contained a higher level of nitrate than untreated plants. In the experiment described here tungstate had an inhibitory effect on the uptake of nitrate. However, in light and in darkness the same influence was found.

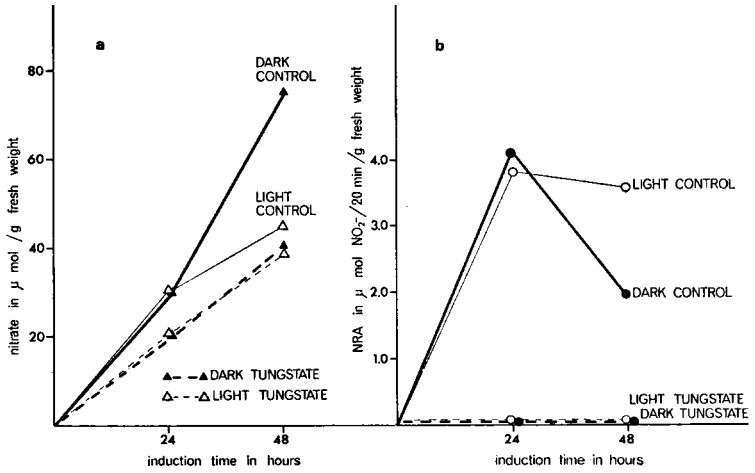

Fig. 5. The influence of light on nitrate content (a) and nitrate reductase activity (b) of cotyledons of tungstate treated and control seedlings at a light intensity of 20,000 lux and a nitrate concentration of 10^{-2} mol.

Table 1. Nitrate present in tungstate treated and control seedlings in the light (20,000 lux) and in the dark.

	nitrate content in μ mol/part			
	root	hypocotyl	cotyledon pair	whole seedling
	induction time 24 hours			
light	0.8	0.7	1.4	2.9
light, tungstate	0.5	0.6	0.7	1.8
dark	0.6	1.9	0.8	3.2
dark, tungstate	0.5	1.1	0.5	2.1
	induction time 48 hours			
light	1.1	1.2	3.1	5.4
light, tungstate	1.0	1.4	1.6	4.0
dark	1.0	4.3	2.1	7.4
dark, tungstate	0.8	2.4	1.0	4.2

4. DISCUSSION

The experiments described in this paper show that in etiolated seedlings in the dark a high level of nitrate reductase activity could be induced by the substrate. The nitrate reductase activity induced after a period of 24 hours in the dark was as high as in the light. These findings are not in agreement with those of BEEVERS et al. (1965). They found a low level of nitrate reductase activity in dark-induced etiolated seedlings. Probably this difference may be partly attributed to the fact that Beevers et al. assayed only the nitrate reductase activity in the supernatant fraction. In the experiments described here a high activity was detected in the

pellet fraction (40–50 % of the overall activity found). More detailed data will be given in a later paper. The difference between the experiments described here and those performed by Beevers et al. could also be due to the fact that they used 5 day old radish seedlings instead of the 4 day old seedlings used in the present experiments. TRAVIS & KEY (1971) showed for etiolated corn seedlings that the ability to produce an active nitrate reductase depended on the presence of an active protein synthesizing apparatus. They showed a gradual loss of protein synthetic activity from 3 to 10 day old corn seedlings.

In contrast to the findings reported by BEEVERS et al. (1965) no influence of light on nitrate uptake was found in the experiments described here. In the absence of nitrate reductase activity the amount of nitrate taken up by the seedlings in the dark was the same as in the light. After the first period of 24 hours the nitrate reductase activity in the dark decreased, whereas the nitrate content of the tissue continued to increase. It is unlikely that the nitrate taken up after the first period of 24 hours is accumulated in the vacuole, where it cannot function as an inducer. Therefore it may be concluded that the amount of nitrate reductase induced in the dark is controlled by a factor or factors other than the availability of nitrate: light must exert its stimulating influence in other ways.

Other authors reached this conclusion after somewhat different experiments. TRAVIS, JORDAN & HUFFAKER (1970) eliminated nitrate uptake by using detached *Hordeum* leaves that were given nitrate independently of light. They came to the conclusion that once sufficient nitrate is available, the extent of induction of nitrate reductase activity is regulated by light.

Studies are in progress to determine which factors other than the availability of nitrate may become limiting for induction and activity of the enzyme in the dark.

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