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# Phytochrome regulation of nitrate reductase in wheat

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**Abstract.** In excised wheat leaves, the activity of nitrate reductase was enhanced by a brief pulse of red light and this increase was reversed by far-red light irradiation. Even under continuous far-red light, nitrate reductase activity increased by 258% after 18 h. When leaves were kept in distilled water during exposure to red light and then transferred to potassium nitrate, there was no difference in endogenous nitrate concentration. The nitrate reductase activity was the same whether leaves were floated in potassium nitrate or in distilled water during irradiation. Partial to complete inhibition of enzyme activity was observed when leaves were incubated in actinomycin-D and cycloheximide respectively, following 4 h of red light irradiation. *In vitro* irradiation of extract had no significant effect on nitrate reductase activity.

**Keywords.** Phytochrome; nitrate reductase; wheat.

## Introduction

Nitrate reductase (EC 1.6.1.1) catalyses the conversion of nitrate to nitrite, and is the first enzyme in the nitrate assimilation pathway. The involvement of phytochrome in the stimulation of nitrate reductase by light in pea was first shown by Jones and Sheard (1972), who suggested that phytochrome controls the enzyme synthesis. Johnson (1976), however, has shown a very rapid induction of nitrate reductase by red light irradiation which was cycloheximide insensitive, thus suggesting activation of preformed enzyme.

Recently, mediation of phytochrome in the regulation of nitrate reductase by light has been shown in the etiolated seedlings of *Sinapsis alba* (Johnson, 1976), maize (Duke and Duke, 1978; Rao *et al*., 1980), rice (Sasakawa and Yamamoto, 1974), and cauliflower (Whitelam *et al*., 1980).

In a previous paper, we briefly reported the reversible effects of red and far-red light on nitrate reductase activity in excised wheat leaves (Vijayaraghavan *et al*., 1979). In this paper, we present detailed experiments to show that in wheat, both low and high energy responses are operative in controlling nitrate reductase by phytochrome.

## Materials and methods

Wheat seeds (*Triticum aestivum* var. H.D. 2009) obtained from Indian Agricultural Research Institute, New Delhi, were soaked in running tap water for 24 h and then

spread on petridishes for germination in the dark. Leaves were excised from uniformly growing plants on the 7th day. Approximately 250 mg of leaves were taken for each set and floated dorsally in 20 ml of 100 mM $KNO_3$ solution for induction of enzyme activity.

For enzyme extraction, excised leaves were removed from the nitrate medium, washed with distilled water, blotted dry and ground in 2 ml of extraction buffer containing 100 mM phosphate buffer, pH 7.4 with 1 mM EDTA and 1 mM cysteine, in a mortar and pestle at 4°C, under green safe light. The extract was centrifuged at 18,000 *g* in Janetzki K-24 centrifuge for 15 min. The supernatant was employed for the enzyme assay according to the procedure of Hageman and Hucklesby (1971) with modifications (Sihag *et al.*, 1979). Protein content was estimated following the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard. Nitrate was estimated according to Wooley *et al.* (1960). Green safe light was obtained through 8 layers of green cellophane paper from a white fluorescent tube light. The intensity of green light at the plant level was never more than $\mu W\ Cm^{-2}$. CBS (Carolina Biological Supply, U.S.A.) filters were used to get red ($\lambda_{max}$ 650) and far-red ($\lambda_{max}$ 750) light as described earlier (Sharma *et al.*, 1979). Blue light ($\lambda_{max}$ 450, 140 $\mu W\ Cm^{-2}$) was also obtained through a CBS filter.

## Results

Leaves (7 days old) were excised (250 mg) and incubated in 20 ml of 100 mM $KNO_3$. One set of leaves was kept in continuous white light and two other sets were exposed to 5 min of red and blue light, respectively. The enzyme was assayed after 18 h. Compared to the activity of the enzyme in the leaves kept in the dark, the enzyme activity in the leaves exposed to continuous white light was 494%, in 5 min blue light 55%, and in 5 min red light over 200% (table 1).

**Table 1.** Effect of red, blue and white light on nitrate reductase induction in 7-day old excised wheat leaves.

| Treatment | Specific activity* | Relative activity (%) |
|---|---|---|
| Dark 18 h | 43 | 100 |
| 5 min red + 18 h dark | 94 | 219 |
| 5 min blue + 18 h dark | 67 | 156 |
| 5 min white + 18 h dark | 94 | 216 |
| White 18 h | 265 | 594 |

Red (500 $\mu W\ Cm^{-2}$); Blue (140 $\mu W\ Cm^{-2}$), White light (1200 $\mu W\ Cm^{-2}$).

*n mol of $NO_2$/mg protein/h.

To check for the involvement of phytochrome, leaves incubated in 100 mM $KNO_3$ solution were exposed to 5 min of red and far-red light. The enzyme activity increased by 115% on exposure to red light, which was decreased to 43% by far-red light (table 2). However, a second exposure to red light after an initial exposure to

**Table 2.** Red–far-red reversion of the induction of nitrate reductase activity.

| Treatment | Specific activity* | Relative activity (%) |
|---|---|---|
| Dark 18 h | 59 | 100 |
| 5 min red + 18 h dark | 127 | 216 |
| 5 min red + 5 min far-red + 18 h dark | 84 | 144 |
| 5 min red + 5 min far-red + 5 min red + 18 h dark | 150 | 254 |

Far-red light (140 μW $Cm^{-2}$). * n mol $NO_2$/mg protein/h.

far-red light increased the nitrate reductase activity to 154%. The reciprocal effects of red and far-red light suggest the possible involvement of phytochrome in the regulation of nitrate reductase in wheat leaves.

To study the kinetics of the enhancement of active red light, leaves exposed to 5 min of red light were transferred to darkness and the enzyme was assayed at different intervals of time upto 24 h. The nitrate reductase activity in dark grown leaves, as shown in figure 1, was detected only after 2 h and after that it increased

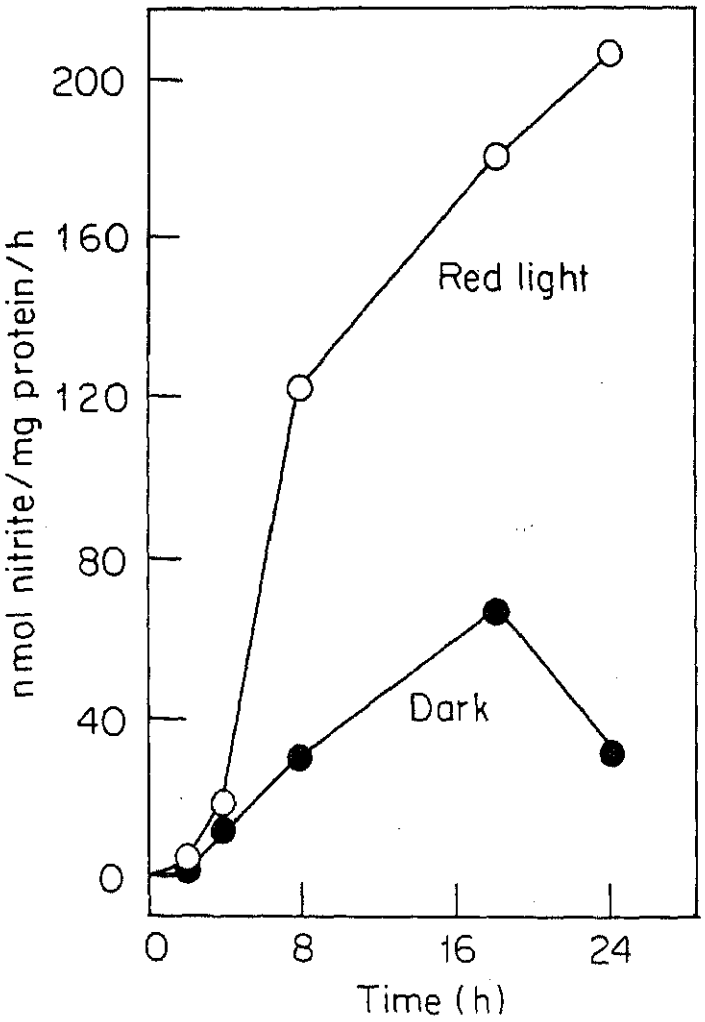


**Figure 1.** Kinetics of induction of nitrate reductase activity after irradiating the plants with red light for 5 min.

sharply till 18 h, later the activity decreased. In red light irradiated leaves, the lag period was the same as above, but the enzyme activity continued to increase until 24 h.

Even under continuous exposure to far-red light, the enzyme activity increased after a lag of 2 h and reached a maximum value at 18 h, and declined as in the dark control. However the activity was at a higher level (figure 2). This experiment shows that 'high irradiated response' is also involved lending further support to the view that phytochrome may have a role in the control of nitrate reductase.

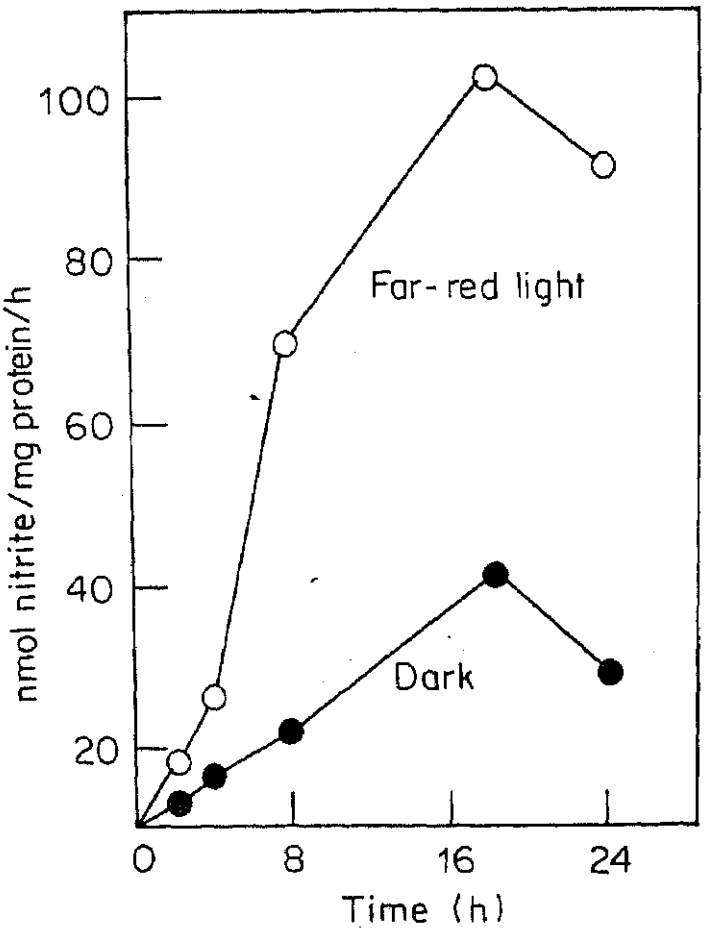


**Figure 2.** Kinetics of induction of nitrate reductase activity under continuous far-red light irradiation.

To study whether light causes changes in membrane permeability, thus leading to enhanced transport of $NO_3^-$ during illumination, leaves from 7-day old seedlings were excised and incubated either in 20 ml of 100 mM $KNO_3$, or kept in 20 ml of distilled water, and both the sets were irradiated with red light for 5 min. After irradiation, the plants from both the sets were transferred to fresh medium containing nitrate. As shown in table 3, the leaves which were kept in nitrate during irradiation had the same enzyme activity as those leaves which were irradiated in water. There was also no difference in the accumulation of nitrate in dark and red light treated plants, which was about 312 n mol/g fresh weight.

In another experiment, leaves from 7-day old seedlings were excised and incubated in 100 mM $KNO_3$ with 20 μg/ml actinamycin-D or 20 μg/ml cycloheximide to study their effect on the increase mediated by short duration of

**Table 3.** Effect of exposure to red light on the induction of nitrate reductase in leaves kept in water and nitrate medium.

| Treatment | Specific activity* | Relative activity (%) |
|---|---|---|
| $KNO_3$ 18 h dark | 60 | 100 |
| $KNO_3$ 5 min red + $KNO_3$ 18 h dark | 128 | 214 |
| $H_2O$ 5 min red + $KNO_3$ 18 h dark | 124 | 206 |

Plants were grown for 7 days in distilled water. On the 7th day leaves were excised ana one set was incubated in 20 ml of 100 mM $KNO_3$ and the other set was kept in 20 ml distilled water and illuminated with red light for 5 min after which from both sets the leaves were trasferred to 100 mM $KNO_3$ medium. The enzyme was assayed after 18 h.

* n mol $NO_2$/mg protein/h.

red light treatment. The inhibitors were removed after 4 h of red light irradiation and the leaves transferred to fresh $KNO_3$ solution. Enzyme activity was measured after 14 h. As shown in figure 3, cycloheximide treatment resulted in 91%

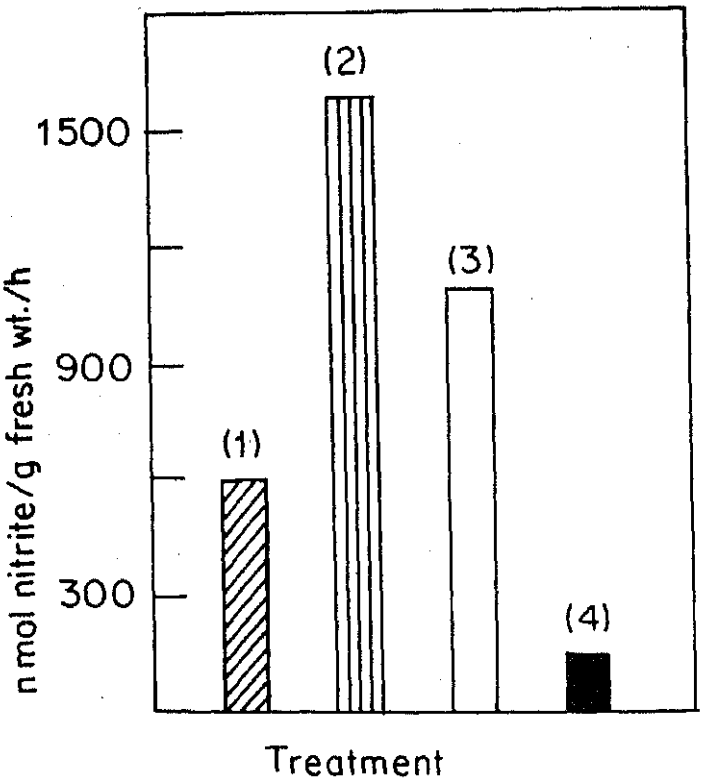


**Figure 3.** Effect of actinomycin-D and cycloheximide on nitrate reductase activity.

dark; red light for 5 min followed by incubation in the dark for 18 h; red light for 5 min+ actinomycin D; red light for 5 min + cycloheximide.

inhibition of the enzyme activity, whereas only 32% inhibition was noticed in the leaves incubated in actinomycin-D. This experiment, suggests the probable involvement of RNA and protein synthesis in red light mediated enhancement of nitrate reductase activity.

To study if the nitrate reductase is activated directly, the enzyme, after extraction, was irradiated *in vitro.* Also, the leaves (not the extract) were irradiated for 5 min just before the enzyme extraction. When the extract was exposed to red light for 5 min or when the leaves were exposed to red light for 5 min before extraction, about 10-20% increase in the activity was noticed in different experiments in both the cases. As usual, however, 5 min of red light exposure followed by dark interval for 18 h increased the nitrate reductase activity by over 100% in these experiments (data not included).

## Discussion

The role of light in the control of nitrate reductase activity has not been fully established, and it is not clear whether such effects are entirely mediated via photosynthesis in chlorophyllous tissue, or involve the phytochrome system. In this work we have not studied the role of photosynthesis but have tried to confirm whether phytochrome regulates the level of nitrate reductase in wheat leaves and to study the mechanism of phytochrome action on nitrate reductase.

The involvement of phytochrome in a system is suggested by (i) low energy response, characterised by red and far-red light reversible phenomenon, and (ii) high energy response. In the present system both these criteria are satisfied. The enzyme activity was increased by a brief pulse of red light, an effect that was reversed by far-red light. When a series of alternating red and far-red light treatments (R + FR + R) was given, the expressed activity of the enzyme was the light given last (table 2). The nitrate reductase activity increased following a lag phase of 2 h.

The involvement of phytochrome in a system is also characterised by the 'high energy reaction' (HER). Here, absolute length of the period of illumination is more important in increasing the enzyme activity. Wheat leaves when kept under continuous far-red light showed an increased enzyme activity upto 18 h following which it declined. There was again a lag period of 2 h (figure 2). Drumm *et al.* (1975) showed that the high irradiation response in anthocyanin formation is related to phytochrome by the use of continuous far-red light. A cyclic phytochrome receptor model explains the action spectrum of high irradiance. According to Schafer (1975), under far-red light treatment, phytochrome reaches a steady state with respect to phytochrome synthesis and phytochrome destruction. The involvement of high energy reaction, which was not reported earlier in wheat confirmed the involvement of phytochrome in this system.

When red light was given to leaves floated earlier on water and then transferred to nitrate or to leaves incubated in nitrate all through (table 3), no difference in the enzyme activity was observed. Moreover, there was no difference in endogenous $NO_3^-$ accumulation ruling out the possibility of increase of enzyme activity due to an increased nitrate uptake by red light.

The presence of a lag phase and the inhibition of nitrate reductase with RNA and protein synthesis inhibitors, actinomycin-D and cycloheximide (figure 3), suggests *de novo* synthesis of the enzyme in this system in response to red light treatment. This is similar to our results obtained in maize (Rao *et al.*, 1980).

Johnson (1976) however, showed that phytochrome induces rapid cycloheximide insensitive increase in nitrate reductase activity in mustard seedlings. When red light was given to the extract or to the leaves just before extraction, although no large stimulation in enzyme activity was observed, thus ruling out the possibility of enzyme activation, 10-20% increase was noticed. It is difficult to envisage the operation of both possibilities–activation and *de novo* synthesis. More work is needed to clarify this point.

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