# EVIDENCE FOR A COMPLEX CONTROL SYSTEM FOR NITRATE REDUCTASE IN WHEAT LEAVES

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## 1. Introduction

Nitrate reductase (NR, EC 1.6.6.1) is induced by nitrate in higher plants [1] and the role of light in the induction process also appears to be important [2–4].

Recently, we obtained evidence suggesting the operation of a complex control system. The following kinds of variation of enzyme activity have been observed in leaf tissue:

1) Oscillation of a Circadian type [5] occurred when seedlings were subjected to continous light after initial growth in the dark.

2) An exponential decrease occurred after transfer to the dark of seedlings containing enzyme induced in the light.

3) The nitrate content of leaf tissue and the level of NR activity which was induced when seedlings were grown in the dark seemed to be characteristic of each of the varieties examined.

## 2. Materials and methods

### 2.1. *Materials*

Seeds of hexaploid wheat *Triticum aestivum* L. cv. Timgalen, Hope, Chinese Spring, Bluebird and Penjamo were obtained from the Department of Agricultural Botany, University of Sydney.

The seeds were soaked in distilled water and then placed on nylon mesh frames (approx. 140 seeds per 100 $cm^2$) suspended over liquid nutrient medium. The nutrient medium contained 10 mM $KNO_3$, 0.16 mM ferric citrate, 1.62 mM $MgSO_4 \cdot 7H_2O$, 1.28 mM $NaH_2PO_4 \cdot 2H_2O$, 4.33 mM $CaCl_2 \cdot 2H_2O$ and 1 ml per l of micronutrient solution which contained 9.14 mM $MnSO_4 \cdot 5H_2O$, 0.96 mM $CuSO_4 \cdot 5H_2O$, 1.01 mM $ZnSO_4 \cdot 7H_2O$, 2.75 mM $H_3BO_3$, and 0.026 mM $NaMoO_4$. When nitrate was excluded from the nutrient medium, K was supplied as KCl (10 mM) and N as $NH_4Cl$ (1 mM). The nutrient medium was changed at least twice daily.

The seedlings were grown at 25°, 20 cm below a bank of 6 fluorescent 20 W lamps (Plant Lights, General Electric). Approx. 20 seedlings were removed at random from the frame for each sample.

### 2.2. *Methods of analysis*

Leaf tissue (1 g) was frozen in crushed dry-ice and homogenized in a glass mortar in 4 ml buffer (0.1 M Tris-HCl, 1 mM cysteine, pH 7.5). The homogenate was squeezed through 2 layers of cheesecloth and centrifuged at 45,000*g* for 30 min. All the above steps were carried out at 0–4°.

NR was estimated by the following modification of the methods of Sanderson and Cocking [6] and Spencer [7]. The assay mixture contained 0.4 ml potassium phosphate buffer (0.1 M, pH 7.5), 0.1 ml each of the following: alcohol dehydrogenase (Sigma, 0.75 mg per ml potassium phosphate buffer, 0.1 M, pH 7.5), NADH (Sigma, 2.7 mM in 0.005 M Tris), $KNO_3$ (0.1 M), ethanol (3 M in potassium phosphate buffer, 0.1 M, pH 7.5) and 0.2 ml of the enzyme extract.

The assay tubes were incubated at 30° for 30 min and the reaction was stopped by the addition of 0.2 ml of zinc acetate (1.0 M), and 2.5 ml of ethanol (95% v/v). The tubes were centrifuged at 1,500*g* for

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10 min. To 2.0 ml of the supernatant were added 0.5 ml amounts, respectively, of sulphanilamide (1.0%, w/v, in N HCl) and naphthyl reagent (*N*-1-naphthyl-ethylene-diamine dihydrochloride, 0.01% w/v). The absorbance was measured at 540 nm.

Trichloracetic acid (TCA, 10% w/v) was added to extracts and the protein precipitated was estimated by a Biuret method [8].

Nitrate was estimated in the supernatant obtained after the TCA treatment. 0.1 ml of the supernatant was made up to 2.0 ml with water and 0.5 ml each of the sulphanilamide and naphthyl reagents were added. The nitrate was reduced to nitrite by the addition of an excess of zinc filings (Hopkin and Williams Ltd). The zinc filings had been washed thoroughly with 10% (v/v) acetic acid, dried at 120° and kept in a desiccator with silica gel. The mixture was stirred constantly for 10 min after which the filings were removed by decantation and the absorbance was measured at 540 nm.

## 3. Results and discussion

It was found in early studies that the time course of induced enzyme activity in leaves of Timgalen wheat contained apparent irregularities (fig. 1), whereas no such fluctuations occurred during the periods of decreasing activity following transfer to the dark of seedlings containing enzyme induced in light (fig. 1). Indeed, in the latter case the decrease of activity follows the first order rate law quite closely, with $t_{½}$ = 22 hr. It was found later (see fig. 2) that at certain times in the light activity could decrease more rapidly ($t_{½}$ = approx. 11 hr). Half life values of 4.3 hr and 6.5 hr, respectively, have been reported [9, 10] when cultured tobacco cells were transferred to nitrate-free medium and the value of 4 hr was obtained when corn seedlings shoots excised at ground level from seedlings fed nitrate were deprived of nitrate [11].

Further investigation of events in the induction period revealed a regular pattern in the variations of enzyme activity and tissue nitrate content.

Enzyme activity values obtained from a batch of seedlings kept for 3 days in continuous light are shown graphically in fig. 2. Peaks occur in the graph at the end of each 24 hr period. Activity increased rapidly

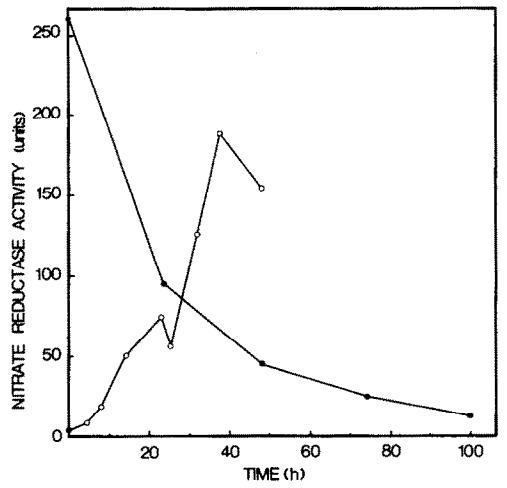


Fig. 1. NR activity (nmoles nitrite per 30 min per mg protein) in leaf tissue extracts of seedlings of *T. aestivum* cv. Timgalen. Seedlings were grown initially for 6 days at 25°, using liquid culture medium containing 10 mM $KNO_3$.
(•–•–•): NR activity following transfer to the dark after initial growth in the light. (○–○–○): NR activity following transfer to the light after initial growth in the dark.

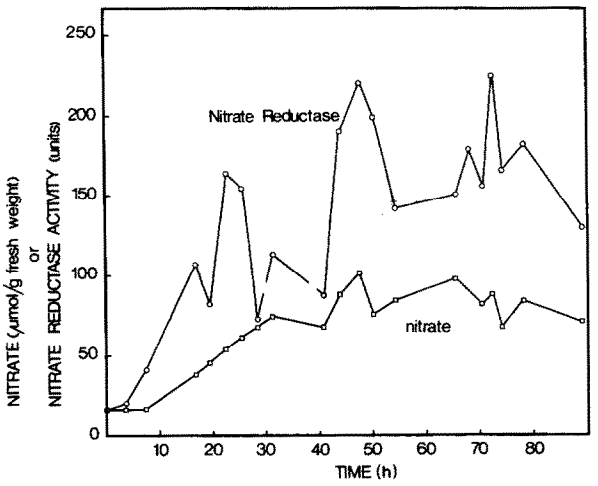


Fig. 2. The time course of NR activity (○–○–○) (nmoles nitrite per 30 min per mg protein) and nitrate content (□–□–□) (μmoles per *g* fresh tissue) of Timgalen seedlings in the light. Seedlings were grown initially for 6 days in the dark. Details of the conditions for growth are given in the text.

during the 6 hr period before and decreased rapidly in the 6 hr period after each peak. There appears to be a 12 hr period between the peaks during which the rate of change of activity is relatively low.

Nitrate accumulation by the seedlings is also shown in fig. 2. A steady rate of accumulation is observed during the initial 32 hr period of light induction, after which fluctuation in nitrate content appears to be correlated with fluctuations in NR activity.

It is likely, if enzyme level is influenced by nitrate concentration, that the operative concentration may be quite different from the net concentration in the tissues. Mellor and Tregunna [12] have shown that reduction of nitrate to nitrite occurs primarily in mesophyll cells of certain $C_4$-plants and in the case of *Gomphrena globosa* the concentration of nitrate in these cells was more than 30 times the concentration in the neighbouring cells of the bundle sheaths. Filner and Heimer [10] have provided evidence that in cultured tobacco cells 2 pools of nitrate exist, one an inducing pool and the other a substrate pool.

It is possible that hormones have a basic function in a mechanism for the rapid adjustment of nitrate concentration at an effective regulation site, which in the case of the tobacco plant cell cultures studied by Filner and Heimer [10], is represented by the inducing site of low half-life. Mechanisms for the control by hormones and regulatory substances of periodic variations of enzyme activity have been proposed [13]. Recently Rijven and Parkash [14] showed that kinetin brought about a marked increase in NR activity in isolated cotyledons of Fenugreek (*Trigonella foenum graecum* L.).

Induction of NR activity in leaves is possible in the absence of light (table 1). The varieties Timgalen and Hope, apparently, did not readily accumulate or utilize nitrate when germinated and grown in the dark, while Chinese Spring, Penjamo and Bluebrid accumulated higher concentrations of nitrate and developed higher levels of NR activity. Further investigations of enzyme activity in tissues from plants of different genotype are in progress.

Table 1
NR activity of leaf tissue from 6 day old seedlings of 5 wheat varieties.

| Variety | NR activity * | Nitrate content ** |
|---|---|---|
| Timgalen | 4.3 | 8 |
| Hope | 2.6 | 8 |
| Chinese Spring | 10.2 | 20 |
| Penjamo | 9.7 | 28 |
| Bluebird | 12.1 | 22 |

* nmoles of nitrite per 30 min per mg protein.
** $\mu$moles per *g* fresh weight.
The seedlings were grown in the dark on a nutrient medium containing 10 mM $KNO_3$.

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