Phytochemistry, Vol. 25, No. 8, pp. 1815–1818, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

ROLE OF CYTOCHROME *C* OXIDASE IN THE REGULATION OF NADH SUPPLY FOR NITRATE REDUCTION IN WHEAT LEAVES *IN VIVO*

M. S. NAIK and D. J. D. NICHOLAS

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, South Australia, Australia

(*Received* 11 *December* 1985)

Key Word Index—*Triticum aestivum*; Gramineae; cytochrome oxidase; alternative oxidase; nitrate reduction; NADH supply.

Abstract—Oxidation of cytosolic NADH by O_2 regulates the supply of reducing equivalents for nitrate reductase. Inhibition of the cytochrome *c* oxidase pathway stimulates nitrate reduction. The cyanide insensitive alternative oxidase is not involved in this reaction because inhibition by SHAM (salicylhydroxamic acid) did not promote *in vivo* nitrate reduction. The role of mitochondrial NADH dehydrogenase (which oxidizes exogenous NADH) in regulating nitrate reduction in wheat leaves *in vivo* is discussed.

INTRODUCTION

The NADH dependent nitrate reductase (EC 1.6.6.1), the first enzyme in the pathway of nitrate assimilation in green leaves is located in the cytosol, where it may be loosely associated with the outer membrane of chloroplasts [1–3]. This enzyme competes with mitochondrial respiration for NADH. The restriction of electron transfer to O_2 by either anaerobiosis or by respiratory inhibitors such as rotenone, amytal, antimycin A or CO, stimulates the *in vivo* nitrate reduction [4–7]. In plant mitochondria, two terminal oxidases participate in the reduction of O_2, namely cytochrome *c* oxidase (inhibited by KCN or CO) and the alternative oxidase (inhibited by salicylhydroxamic acid, SHAM) [8]. We now report that the alternative oxidase does not regulate the NADH supply for nitrate reductase as does cytochrome oxidase. The role of NADH dehydrogenase, involved in the oxidation of exogenous NADH in these reactions, is considered.

RESULTS

When wheat leaf discs were incubated under vigorous aeration, very little nitrite was formed in the tissues (Table 1). However, in the presence of uncouplers nitrite accumulated in the discs and in this respect *m*CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and FCCP (*p*-fluoromethoxycarbonyl cyanide phenylhydrazone) were more effective than DNP (2,4-dinitrophenol). As shown previously uncouplers stimulate nitrite accumulation by inhibiting nitrite reductase [9–11]. ATP is required for the synthesis of glucose 6-phosphate, which supplies NADPH for the ferredoxin dependent nitrite reductase in the chloroplasts in the dark [12].

The role of external NADH dehydrogenase

Plant mitochondria differ from their animal counterparts in that they oxidize exogenous NADH [13]. Since

Table 1. Effects of uncouplers of respiration on aerobic *in vivo* nitrate reduction in wheat leaf discs

DNP		*m*CCCP		FCCP	
Concentration (μm)	NO_2^-*	Concentration (μm)	NO_2^-*	Concentration (μm)	NO_2^-*
0	0.13	0	0.17	0	0.14
500	0.42	2	0.27	2	0.32
1000	0.64	5	0.39	5	0.36
1500	0.82	10	0.46	10	0.46
2000	0.93	50	0.99	25	0.59

Leaf discs (0.2 g) from 10-day-old seedlings of Halberd were incubated in the reaction mixture described in the Experimental, under aerobic conditions. Aqueous solutions of DNP and ethanolic solutions of *m*CCCP and FCCP were added as indicated. In the controls distilled water or ethanol only were added to the reaction mixtures. Nitrite produced was extracted and determined as described in the Experimental.

*μmol NO_2^- produced/hr/g fr. wt.

Table 2. Effects of EDTA, EGTA and calcium ions on nitrate reduction in wheat leaf discs

Additions	DNP (2 mM) NO_2^- (μmol NO_2^- produced/hr/g fr. wt)	*m*CCCP (2.5 μm) NO_2^- (μmol NO_2^- produced/hr/g fr. wt)
Nil	0.74	0.49
EGTA (0.1 mM)	1.02	0.59
EDTA (0.1 mM)	1.30	0.67
EDTA (0.1 mM) + $CaCl_2$ (0.2 mM)	0.82	0.52

EDTA, EGTA and Ca^{2+} ions were added to the reaction mixtures containing DNP or *m*CCCP as in Table 1. Nitrite produced during aerobic *in vivo* assay was determined as described in the Experimental.

nitrate reductase utilizes cytosolic NADH, the competition between O_2 and NO_3^- for this reductant operates at the level of the oxidation of exogenous NADH, which is dependent on Ca^{2+} ions [14–16]. We have, therefore, used the aerobic *in vivo* nitrate reductase assay in the presence of uncouplers to examine the role of the exogenous NADH oxidase. As shown in Table 2, EDTA (ethylene diamine tetraacetic acid) and EGTA (ethylene glycol bis(β-aminoethyl ether, *N,N'*-tetraacetic acid) stimulated nitrite formation, presumably because they inhibited the oxidation of cytosolic NADH by O_2. This effect was reversed by Ca^{2+} ions (Table 2).

The results in Table 3 show that the calmodulin inhibitors, phenothiazine and chlorpromazine significantly inhibited the *in vitro* activity of nitrate reductase extracted from wheat leaves. Hence it was not possible to study the effects of these two inhibitors on the *in vivo* aerobic nitrate reductase. Trifluoperazine (1 mM) and compound 48/80 (30 μg/ml) did not however inhibit nitrate reductase *in vitro* but these compounds stimulated the aerobic nitrate reduction *in vivo* (Table 3).

Table 3. Effects of calmodulin inhibitors on *in vitro* and aerobic *in vivo* nitrate reductase activity in wheat leaves

Additions	Relative nitrate reductase activity *in vitro* Halberd	Bindawarra
(a) *In vitro* activity		
Phenothiazine (1 mM)	1	4
Chlorpromazine (1 mM)	57	56
Trifluoperazine (1 mM)	90	90
Compound 48/80 (50 μg/ml)	89	100
	Aerobic nitrate reductase activity (μmol NO_2^- produced/hr/g fr. wt.)	
(b) *In vivo* activity		
Control *m*CCCP (25 μm)	0.79	0.69
*m*CCCP (25 μm) + trifluoperazine (1 mM)	1.22	1.44
*m*CCCP (25 μm) + compound 48/80 (30 μg/mol)	1.25	1.27

In vitro activities in the leaf extracts were (μmol NO_2^-/15 min/ml extract) Halberd, 0.88 and Bindawarra 0.80 and with these values as 100, relative percent activities are given in the Table. Aqueous solutions of calmodulin inhibitors were added, except for phenothiazine, which was dissolved in ethanol. The method described in Table 1 was used for the aerobic *in vivo* assay of nitrate reductase in leaf discs.

Effect of alcohols

Stimulation of the *in vivo* nitrate reduction by propanol has been reported [17, 18] and it was suggested that in roots of maize and wheat NADH generated by alcohol dehydrogenase was responsible for this effect [19]. However, alcohol dehydrogenase activity is negligible in leaves of wheat [19]. The results in Table 4 show that the propanol and ethanol inhibited O_2 uptake in wheat leaf

Table 4. Effects of alcohols on the *in vivo* nitrate reduction and respiration in wheat leaves

	Halberd		Bindawarra	
	NO_2^- produced (μmol/hr/g fr. wt)	O_2 uptake (μmol/hr/g fr. wt)	NO_2^- produced (μmol/hr/g fr.wt)	O_2 uptake (μmol/hr/g fr. wt)
Control (H_2O)	Nil	9.06	Nil	8.54
Methanol (1.7 M)	Nil	8.22	Nil	8.10
Ethanol (1.7 M)	0.97	6.06	0.73	6.15
Propanol (0.85 M)	2.17	5.16	2.35	5.40

Intact leaves (1 g) of 10-day-old seedlings were placed in vials with their petioles dipping into 2 ml alcohol solutions for 4 hr at 20° in the dark. From a sample of the leaves (0.2 g), nitrite was extracted and determined as described in the Experimental. In leaf discs, O_2 uptake was monitored in an O_2 electrode as described in the Experimental.

discs while methanol had little effect. Thus stimulation of nitrate reduction by propanol and ethanol (but not by methanol) may be associated with an inhibition of NADH oxidation.

The role of the alternative oxidase

Both the wheat cultivars Halberd and Bindawarra showed some cyanide insensitive respiration which was inhibited by SHAM (Table 5). However, treatment of the leaves with SHAM did not promote nitrate reduction *in vivo* under aerobic conditions, whereas CO treatment stimulated nitrite accumulation. The amount of nitrite produced by CO treatment was equal to that produced under anaerobiosis.

DISCUSSION

When wheat leaves were treated with CO in air, the cytochrome *c* oxidase was inhibited but the alternative cyanide (or CO) insensitive oxidase would be unaffected. Under anaerobiosis both pathways would be inhibited. However, the amount of nitrite produced under anaerobiosis was equivalent to that formed after CO treatment in air (Table 5). These results indicate that the alternative oxidase is not involved in the regulation of NADH supply for nitrate reduction. Treatment with SHAM alone did not promote nitrate reduction *in vivo* in air.

The oxidation of exogenous NADH by plant mitochondria is mediated by a dehydrogenase located on the outer surface of the inner membrane of the mitochondria [20], which bypasses the rotenone sensitive first site of oxidative phosphorylation and donates electrons to the cytochrome pathway via ubiquinone. The alternative oxidase however is not involved in the oxidation of exogenous NADH [21, 22]. Hence the competition for cytosolic NADH between nitrate reductase and O_2 is controlled by cytochrome oxidase only.

Oxidation of exogenous NADH is dependent on Ca^{2+} ions [23] and is inhibited by EDTA and EGTA (Table 2). An involvement of calmodulin in this reaction has been suggested [24]. Recently, it was shown [25] that calmodulin was only indirectly involved in the oxidation of exogenous NADH by regulating Ca^{2+} concentration in the intermembrane space of the mitochondria. It is likely that the calmodulin inhibitors trifluoperazine and compound 48/80 stimulate NADH supply for nitrate reduction by affecting the Ca^{2+} concentration in the mitochondria (Table 3).

EXPERIMENTAL

Plant materials. Wheat (*Triticum aestivum* L.) cv. Halberd and Bindawarra supplied by the Agronomy Department of this Institute were grown in sterilized coarse sand in a phytotron (16 hr light/8 hr dark cycle). The seedlings were supplied with 0.5 strength Hoagland soln containing 10 mM KNO_3 [7]. Leaves from 8- to 12-day-old seedlings were used in the experiments.

Aerobic in vivo *assay of nitrate reductase.* Leaves were cut transversely into 5 mm segments and 0.2 g leaf discs were placed in large test tubes (2.5 × 15 cm), in 5 ml KPi buffer pH 7.0, containing in μmoles: KPi, 300 and KNO_3, 50. The tubes were incubated under dark aerobic conditions at 30° in a reciprocating water bath, ensuring vigorous aeration during incubation. After 1 hr, the reaction was terminated by placing the tubes in a boiling water bath. Nitrite produced in the leaf discs was extracted and determined in suitable aliquots as described previously [7].

Anaerobic in vivo *assay of nitrate reductase.* Leaf discs (0.2 g) were placed in test tubes (1 × 15 cm) and then closed with rubber septa. Air was removed from the tubes, via inserted needles in the septa, by rigorous evacuation with a two-stage vacuum pump. High purity argon was then sparged through the tubes for 2 min. The needles were removed and the tubes were incubated in the dark at 30° for 1 hr. Nitrite produced was extracted and determined as described in ref. [7].

In vitro *assay of nitrate reductase.* Leaf tissue (1 g) was macerated in 5 ml 0.2 M Tris–HCl buffer pH 8.5, containing

Table 5. Effects of SHAM on respiration and *in vivo* aerobic nitrate reduction in leaf discs of wheat leaves

	Halberd	Bindawarra
(a) O_2 uptake (% of control)		
Control	100	100
KCN (100 mM)	60	75
KCN (100 mM) + SHAM (5 mM)	33	38
SHAM (5 mM)	79	73
SHAM (5 mM) + KCN (100 mM)	27	23
(b) Aerobic *in vivo* nitrate reduction (μmol NO_2^-/hr/g fr. wt)		
Aerobic control	0.09	0.11
SHAM 2.5 mM	0.12	0.17
SHAM 5 mM	0.17	0.19
SHAM 10 mM	0.20	0.25
CO–air	1.41	1.13
Anaerobic control (Argon)	1.23	1.0

In leaf segments O_2 uptake was measured as described in the Experimental. Activities in the controls (μmol O_2/hr/g fr. wt) were Halberd, 8.1; Bindawarra 7.2 and with these values as 100, relative percent activities are given in the Table. Leaf discs were sparged with CO for 2 min before incubating under dark aerobic conditions for 1 hr at 30°. Anaerobic control was under Argon.

0.05 M KPi, 5 mm EDTA, 1% w/v casein, 10 μM FAD and 2 mM L-cysteine in ice-chilled mortar and pestle with acid washed sand. After thorough disintegration of leaf material, the extract was filtered through Mira cloth and the filtrate was centrifuged at 20000 *g* for 10 min at 0–4°. The supernatant soln was passed through a Sephadex G25 column (15 × 1.5 cm) and nitrate reductase eluted with 50 mM potassium phosphate buffer pH 7.5. Suitable aliquots of the enzyme preparation were used for the *in vitro* assay in 1 ml reaction mixture, containing in μmoles: KPi buffer pH 7.5, 50; KNO_3, 5 and NADH, 0.2. After incubation at 25° for 15 min, the reaction was terminated by adding 0.1 ml of 0.5 M $Zn(OAc)_2$ and then 0.1 ml PMS (phenazine methosulphate; 0.46 mg/ml) was added. The tubes were kept at room temp. for 20 min and then 1 ml of 1% (w/v) sulphanilamide (in 1.5 N HCl) followed by 1 ml of 0.01% w/v of *N*-1-naphthylethylenediamine dihydrochloride were added. The contents were centrifuged at 3000 *g* for 10 min. Nitrite was determined in the supernatant as described previously [7].

Aerobic in vivo *assay nitrate reductase with* CO. Leaf discs (2 mm thick, 0.2 g) were placed in test tubes (2.5 × 15 cm) which were covered with black plastic. High purity CO from a cylinder was sparged through the open tubes for 2 min. The tubes were incubated under dark aerobic conditions for 1 hr and nitrite produced was determined as described in ref. [7].

Respiration in leaf discs. Leaves (0.4 g) cut transversely into 2 mm thick discs with a sharp razor blade, were suspended in 0.05 M KPi buffer pH 7.0, containing 0.3 M mannitol and 0.2 mM $CaCl_2$. After washing by filtration on a fine mesh sieve, the discs were transferred into 5 ml of the same buffer in a reaction vessel of a Clark type O_2 electrode (Rank). O_2 uptake was measured at 25° [26]. All chemicals including uncouplers and calmodulin inhibitors were obtained from Sigma Chemical Co., U.S.A.

Acknowledgements—MSN, who is on leave from the Indian Agricultural Research Institute, acknowledges with thanks support from the Hannaford Bequest Fund and the Australian Research Grants Scheme for the award of a research fellowship. The skilled technical assistance of Mr. Geoffrey Clarke is gratefully acknowledged.

REFERENCES

1. Beevers, L. and Hageman, R. H. (1980) in *Biochemistry of Plants* (Miflin, B. J., ed.) Vol. 5, pp. 115–168. Academic Press, New York.
2. Abrol, Y. P., Sawhney, S. K. and Naik, M. S. (1983) *Plant, Cell Environ.* **6**, 595.
3. Naik, M. S. and Nicholas, D. J. D. (1986) *Phytochemistry* **25**, 571.
4. Mann, A. F., Hucklesby, D. P. and Hewitt, E. J. (1979) *Planta* **146**, 83.
5. Canvin, D. T. and Woo, K. C. (1979) *Can. J. Botany* **57**, 1155.
6. Sawhney, S. K., Naik, M. S. and Nicholas, D. J. D. (1978) *Biochem. Biophys. Res. Commun.* **81**, 1209.
7. Sawhney, S. K., Naik, M. S. and Nicholas, D. J. D. (1978) *Nature* **272**, 647.
8. Laties, G. G. (1982) *Annu. Rev. Plant Physiol.* **33**, 519.
9. Emes, M. J. and Fowler, M. W. (1983) *Planta* **158**, 97.
10. Dry, I. B., Wallace, W. and Nicholas, D. J. D. (1983) *Planta* **152**, 234.
11. Bourne, W. F. and Miflin, B. J. (1970) *Biochem. Biophys. Res. Commun.* **40**, 1305.
12. Prakash, S. Singh, P., Sawhney, S. K. and Naik, M. S. (1984) *Plant Sci. Letters* **34**, 25.
13. Palmer, J. M. and Møller, I. M. (1982) *Trends Biochem. Sci.* **7**, 258.
14. Moore, A. L. and Ackerman, K. E. O. (1982) *Biochem. Biophys. Res. Commun.* **109**, 513.
15. Møller, I. M., Palmer, J. M. and Johnston, S. P. (1983) *Biochim. Biophys. Acta* **725**, 289.
16. Møller, I. M., Johnston, S. P. and Palmer, S. M. (1981) *Biochem. J.* **194**, 487.
17. Jones, R. W. and Sheard, R. W. (1977) *Can. J. Botany* **55**, 896.
18. Lawrence, J. M. and Herrick, H. E. (1982) *Plant Sci. Letters* **24**, 17.
19. Aryan, A. P. and Wallace, W. (1983) *Plant Sci. Letters* **30**, 25.
20. Palmer, J. M. (1984) *Physiol. Veg.* **22**, 665.
21. Day, D. A., Arron, G. P. and Laties, G. G. (1980) in *The Biochemistry of Plants* (Davies, D. D., ed.) Vol. 2, p. 197. Academic Press, New York.
22. Day, D. A., Neuburger, M. and Douce, R. (1985) *Aust. J. Plant Physiol.* **12**, 119.
23. Dunn, P. P. J., Slabas, A. R., Cottingham, I. R. and Moore, A. L. (1984) *Arch. Biochem. Biophys.* 229, 287.
24. Roux, S. J., McEntire, K., Slocum, R. D., Cedel, T. E. and Hale, C. C. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 283.
25. Schwitzguebel, J. P., Nguen, T. D. and Siegenthaler, P. A. (1985) *Physiol. Plant.* **63**, 187.
26. Azcon-Bieto, J., Lambers, H. and Day, D. H. (1983) *Plant Physiol.* **72**, 598.