Extraction of nitrate reductase from members of the South African Proteaceae

W.D. Stock and O.A.M. Lewis
Botany Department, University of Cape Town, Rondebosch

The inhibition of *in vitro* nitrate reductase activity in extracts of *Protea* spp. is shown to be due to polyphenolic constituents of the roots and shoots of the plants (which can be adsorbed by insoluble polyvinylpyrrolidone) rather than to the activity of endogenous proteases. The *in vitro* nitrate reductase activity in shoots of *Protea repens* and *Protea cynaroides* fed 2 mmol $dm^{-3} NO_3^-$ for 24 h prior to nitrate reductase extraction show a nitrate reductase activity of 2 – 4 μmol NO_2^- h^{-1} while the roots of *P. repens* show an *in vitro* nitrate reductase activity of 0,2 μmol NO_2^- h^{-1} (g fresh mass)$^{-1}$. The low nitrate reductase activity of these plants possibly reflects their adaptation to growth under the low nutrient condition of the soils of the South Western Cape, South Africa.

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Die remming van *in vitro* nitraat reduktase aktiwiteit in ekstrakte van *Protea* spp. word toegeskryf aan die polifenoliese bestanddele van die wortels en lote van die plante (wat deur middel van polivinielpirrolidoon geabsorbeer kan word) eerder as aan die aktiwiteit van endogene proteases. Die *in vitro* nitraatreduktase aktiwiteit in die blare van *Protea repens* en *Protea cynaroides* wat vir 24 h met 2 mmol dm^{-3} NO_3^- gevoer is voor nitraat reduktase ekstrahering, is 2 – 4 μmol NO_2^- h^{-1} (g vars massa)$^{-1}$, terwyl die wortels van *P. repens* 'n *in vitro* nitraat reduktase aktiwiteit van 0,2 μmol NO_2^- h^{-1} (g vars massa)$^{-1}$ getoon het. Die lae nitraat reduktase aktiwiteit van hierdie plante is moontlik 'n weerspieëling van hul aanpassing by die lae voedingswaarde van die grondsoorte van die Suidwes-Kaap, Suid-Afrika.

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W.D. Stock and O.A.M. Lewis*
Botany Department, University of Cape Town, Rondebosch 7700, Republic of South Africa
*To whom correspondence should be addressed

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Introduction

Although it has been shown in ^{15}N studies by Lewis & Stock (1978) that nitrate can be used as a nitrogen source in the nutrition of the shoots of Proteaceous plants, it has not been possible to demonstrate the presence of nitrate reductase (NR), which is NADH-dependent, in the leaves and roots of the plants using *in vitro* techniques and conventional extracting media. The inability to obtain an active extract of nitrate reductase could be owing to a number of factors, e.g. the presence in the plant of phytic acids, tannins or hydrolytic and oxidative enzymes (Loomis & Battaile 1966) or simply low levels of nitrate reductase activity (NRA). Problems in extracting active NR from leaves of members of the Ericaceae (*Vaccinium angustifolium* and *V. macrocarpon*) has resulted in proposals that the enzyme is entirely absent from these plants (Townsend & Blatt 1966; Greidanus *et al.* 1972). It has subsequently been found in other members of the Ericaceae (*Leucothoe catesbaei* and *Rhododendron catawbiense*) that inhibition of NR activity in extracts was due to the presence in the plant of a galloyl ester-like compound similar in nature to tannic acid (Dirr & Barker 1973). Other plants such as barley and maize are thought to contain a proteolytic enzyme capable of inactivating the enzyme complex by acting on the NADH (NO_3^-) c R component (Wallace 1974; Lewis *et al.* 1982).

The present study investigates the protective effects of agents added against polyphenolic inhibition and proteolytic enzyme inactivation on *in vitro* NRA of extracts of shoots and roots of *Protea repens* and *Protea cynaroides*. Polyphenolic inhibitors of enzyme extracts have successfully been adsorbed by insoluble polyvinyl-pyrrolidone (PVP) (POLYCLAR AT, BDH) (Loomis & Battaile 1966) while others (Schrader *et al.* 1974; Sherrard & Dalling 1978; Lewis *et al.* 1982) have shown that casein in the extraction medium prevents proteolytic enzyme degradation of active nitrate reductase.

Materials and Methods

Plant material

Plants of *Protea repens* (L.) L. and *Protea cynaroides* (L.) L. which had been cultivated for eighteen months in pots in a sand peat mixture with no supplementary nitrogen fertilization were used in the experimentation. Twenty-four hours prior to nitrate reductase extraction the plants were

fed 200 cm^3 Long Ashton nutrient solution containing 2 mmol dm^{-3} NO_3^- (Hewitt 1966). Barley (*Hordeum vulgare* L. cv. Clipper) leaves were obtained from plants germinated and grown in a nutrient film technique trough using a Long Ashton nutrient solution containing 2 mmol dm^{-3} NO_3^-. Leaves from twenty-day old barley plants were used for all barley assays.

Nitrate reductase extraction

Nitrate reductase was extracted from the leaves and roots of fresh *Protea* spp. by grinding 1 g plant material in a chilled mortar and pestle at 4 °C with 12 cm^3 of one of the following extracting media:

(a) 0,1 mol dm^{-3} phosphate buffer pH 7,5; 1 mmol dm^{-3} EDTA and 1 mmol dm^{-3} dithiothreitol to which was added 1,5 g of insoluble polyvinylpyrrolidone (BDH, POLYCLAR AT) (Loomis & Battaile 1966)

(b) 2,5% soluble casein (BDH), 0,1 mol dm^{-3} phosphate buffer pH 7,5; 1 mmol dm^{-3} EDTA and 1 mmol dm^{-3} dithiothreitol (Lewis *et al.* 1982).

The extract was squeezed through a double layer of cheese cloth and the filtrate was centrifuged at 2000 *g* for 5 min at 3 °C. All extractions of barley material followed the method of Lewis *et al.* (1982). The efficacy of each NRA protectant was determined by measuring its ability to prevent the loss of nitrate reductase activity in barley leaf extracts when extracts of *Protea* leaf and root were added to them.

Nitrate reductase assay

The reaction mixture for the determination of NRA was as follows: 0,1 cm^3 of 1 mol dm^{-3} phosphate buffer pH 7,5; 0,1 cm^3 NADH (1 mg cm^{-3}); 0,2 cm^3 of 0,1 mol dm^{-3} KNO_3 and 0,2 cm^3 barley extract or 0,3 cm^3 *Protea* extract made up to a final volume of 2 cm^3 with distilled water.

In assays where the inhibition of barley NRA by the *Protea* extract was investigated, 0,2 cm^3 barley extract and 0,3 cm^3 *Protea* extract were added to the reaction mixture before it was made up to final volume. The samples were incubated at 27 °C for 15 min and the reaction terminated with 1 cm^3 of 1% (w/v) sulphanilamide in 1,5 mol dm^{-3} HCl and 1 cm^3 of 0,01% (w/v) N-(1-napthyl) ethylenediamine hydrochloride solution. Absorbance was read at 540 nm after 5 min. Samples containing casein required centrifugation at 2000 *g* for 5 min to remove the coagulated protein. Triplicate aliquots of extract were assayed in each experiment.

Results and Discussion

Casein as a NRA protecting agent for *Protea* extracts

It is evident from the results shown in Table 1 that casein does not protect NRA in extracts of *Protea repens* leaf and root material. No NRA could be detected in the *P. repens* extracts and, in addition, the casein protected extracts of *P. repens* inhibited nitrate reductase activity in casein protected barley leaf extracts. The leaf extracts of *P. repens* caused a greater inhibition of barley NRA than did the root extract of the same plant.

These results indicate that the inhibitor responsible for NRA inactivation in *Protea repens* extracts is not a proteolytic enzyme as is probably the case in barley, but some other factor that is distributed in greater quantities in the leaf than in the root.

Table 1 Inhibition of barley leaf nitrate reductase activity by extracts of *Protea repens* leaf and root tissue in the presence of casein. Mean ± SE

	Plant extract				
	Barley leaf	Barley leaf + *Protea* root	Barley leaf + *Protea* leaf	*Protea* leaf	*Protea* root
Protectant	Casein	Casein	Casein	Casein	Casein
NR activity in μmol NO_2^- h^{-1} (g fresh mass)$^{-1}$	6,9 ±0,23	4,8 ±0,19	2,4 ±0,08	0	0
Percentage inhibition of barley extract	0	29,6	64,6	–	–

Table 2 Nitrate reductase activity of *Protea repens* extracts protected with PVP. Mean ± SE

	Plant extract			
	Barley leaf	*P. repens* root	*P. repens* leaf	Barley leaf + *P. repens* leaf
Protectant of *Protea* extract	[a]	PVP	PVP	PVP[a]
NR activity in μmol NO_2^- h^{-1} (g fresh mass)$^{-1}$	7,7 ±0,14	0,2 ±0,01	1,3 ±0,06	8,7 ±0,30

[a] Barley always extracted with casein

PVP as a NRA protecting agent for *Protea* extracts

The activity of nitrate reductase extracted from *P. repens* leaves (protected by PVP), barley leaves (protected by casein) and a mixture of the two is shown in Table 2.

From these results it is apparent that when PVP is utilized as a protectant, significant NRA in *Protea* leaves can be demonstrated. In experiments where *Protea repens* extract was added to barley leaf extract virtually no inhibition of barley NRA was detected (barley leaf/*Protea* leaf mixture exhibited 96% the activity of the sum of the two extracts assayed individually). These results demonstrate the effectiveness of PVP as a protection agent for the extraction of NR from *Protea repens* and indicate that the leaves of this plant have a low (less than 15% barley leaf NRA) but detectable nitrate reductase activity. The NRA of *Protea repens* root (PVP protected) is also shown in Table 2; this is approximately 10% the activity of the leaves of this plant.

The *in vitro* NRA of the leaves of a second species of *Protea, Protea cynaroides*, was investigated using PVP protection in the extract preparation. The results are shown

Table 3 Nitrate reductase activity of *Protea repens* and *Protea cynaroides* leaf material. Mean ± SE

	Plant extract	
	Protea repens leaf	*Protea cynaroides* leaf
Protectant	PVP	PVP
NR activity in μmol NO_2^- h^{-1} (g fresh mass)$^{-1}$	2,2 ±0,05	3,7 ±0,01

in Table 3 and indicate that the shoots of this plant have very similar NRA to *Protea repens*.

The results of these experiments indicate that the inhibitor responsible for NR inactivation in *Protea* spp. is probably not a proteolytic enzyme as in the case of barley, but a polyphenolic constituent of the plant that is distributed in greater quantities in the leaf than in the root. It is well known that *Protea* spp. have a high content of polyphenolic compounds as they were once used widely in the Cape Province, South Africa, as domestic tanning agents (Wehmer 1931; Williams 1930). Shoot NRA in the Proteaceae is low (2 – 4 μmol NO_2^- h^{-1} (g fresh mass)$^{-1}$ when compared with nitrophilous plants such as *Zea mays* (9 μmol NO_2^- h^{-1} (g fresh mass)$^{-1}$, Sherrard & Dalling 1978), *Hordeum vulgare* (14,8 μmol NO_2^- h^{-1} (g fresh mass)$^{-1}$, Lewis *et al.* 1982) and *Helianthus annuus* (24,7 μmol NO_2^- h^{-1} (g fresh mass)$^{-1}$, Kaiser & Lewis 1981) and is possibly an ecophysiological characteristic of those members of the Proteaceae which are restricted to the low nutrient soils of the South Western Cape, South Africa.

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References

DIRR, M.A., BARKER, A.V. & MAYNARD, D.N. 1973. Extraction of nitrate reductase from leaves of Ericaceae. *Phytochemistry* 12: 1261 – 1264.

GREIDANAUS, T., PETERSON, L.A., SCHRADER, L.E. & DANA, M.N. 1972. Essentiality of ammonium for Cranberry nutrition. *J. Am. Soc. Hort. Sci.* 97: 272 – 277.

HEWITT, E.J. 1966. Sand and water culture methods used in the study of plant nutrition. Technical communication No. 22, 2nd edn, Commonwealth Agricultural Bureaux, England.

KAISER, J.J. & LEWIS, O.A.M. 1981. (Abstract) *In vitro* nitrate reductase and glutamine synthetase activity in the leaves of *Helianthus annuus*. *Supp. Plant Physiology* 67: 8.

LEWIS, O.A.M. & STOCK, W.D. 1978. A preliminary study of the nitrogen nutritional status of members of the South African Proteaceae. *Jl S. Afr. Bot.* 44: 143 – 151.

LEWIS, O.A.M., WATSON, E.F. & HEWITT, E.J. 1982. Determination of nitrate reductase activity in Barley leaves and roots. *Ann. Bot.* 49: 31 – 38.

LOOMIS, W.D. & BATTAILE, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5: 423 – 438.

SHERRARD, J.H. & DALLING, M.J. 1978. Effect of casein on the extractability and stability of nitrate reductase from wheat leaves. *Ann. Bot.* 42: 1421 – 1427.

SCHRADER, L.E. CATALDO, D.A. & PETERSON, D.M. 1974. Use of protein in extraction and stabilization of nitrate reductase. *Plant Physiology* 53: 688 – 690.

TOWNSEND, L.R. & BLATT, C.R. 1966. Lowbush Blueberry, evidence for the absence of a nitrate reducing system. *Plant and Soil* 25: 456 – 460.

WALLACE, W. 1974. Purification and properties of a nitrate reductase-inactivating enzyme. *Biochim. Biophys. Acta* 431: 265 – 276.

WEHMER, C. 1931. Die Pflanzenstoffe. 2nd edn, Fischer, Jena.

WILLIAMS, C.O. 1930. Union of South Africa Department of Agricultural Science, Bulletin 74.