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NITROGEN UTILIZATION IN THE SOUTH AFRICAN FYNBOS SHRUB,

*PROTEA NERIIFOLIA* R.BR. (PROTEACEAE)

by

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requirements for the degree

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**ABBREVIATIONS AND SYMBOLS**

%En	percentage enrichment
A%E	atom percent excess
ATP	adenosine triphosphate
EDTA	ethylenediaminetetra-acetic acid
NADH	nicotinamide adenine dinucleotide (reduced)
NED	N (1-naphthyl)-ethylenediamine hydrochloride
NR	nitrate reductase
NRA	nitrate reductase activity
PVP	polyvinylpyrrolidone

**ABSTRACT**

An investigation into the utilisation of nitrogen by *Protea neriifolia* seedlings was carried out by separate feeding regimes of the two nitrogen forms; nitrate and ammonium. Both the ability of the species to utilise both nitrogen sources, and the sites of nitrogen assimilation, was examined by determining nitrate reductase activity (NRA) and by estimating the bound  $^{15}\text{N}$  fraction. *In vitro* NRA assays revealed no enzyme activity despite using casein and PVP (polyvinylpyrrolidone) as protectants against enzyme deactivation. *In vivo* NRA assays revealed very low enzyme activities in both nitrate- and ammonium-fed plants of approximately  $0.14 \mu\text{mol NO}_2^- \text{ h}^{-1} (\text{gfw})^{-1}$ . The enzyme activity was restricted to the root indicating this to be the main area of nitrogen assimilation. The 26 hour  $^{15}\text{N}$  feeding experiments revealed that *P. neriifolia* is able to utilise both nitrogen sources, but rates of ammonium assimilation were twice that of nitrate. In the nitrate-fed plants only 20% of assimilated nitrogen was transported to the leaves, confirming the roots to be the site of assimilation. It appears that this slow-growing species has adapted to the acidic, nutrient poor soils of the fynbos by developing very low rates of nitrogen assimilation, the preferred nitrogen form being ammonium.

## INTRODUCTION

*Protea neriifolia* R. Br. is an indigenous, large, perennial shrub which grows in acidic soil in the fynbos region of the southern and South Western Cape, South Africa. This area is characterised by acidic, nutrient-poor soils ( $1.2 \mu\text{g N g}^{-1}$  soil of nitrate and  $2.6 \mu\text{g N g}^{-1}$  soil of ammonium) (Stock 1985). Low soil pH inhibits the activity of nitrifying bacteria, so plants rely on  $\text{NH}_4^+$  as their main source of nitrogen (Pharis et al. 1964; Gigon and Rorison 1972). Some members of the Proteaceae appear to have adapted to these conditions by evolving low nitrogen metabolism rates and restricted utilisation of excess nitrogen, especially nitrate (Lewis and Stock 1978; Stock and Lewis 1982; Stock and Lewis 1984).

Under natural conditions the main forms of nitrogen available to plants are the ions  $\text{NO}_3^-$  and  $\text{NH}_4^+$  (Haynes and Goh 1978). Although most plants are able to utilise both forms, they show great variation in their ability to absorb and utilise nitrate and ammonium. There is evidence that some calcifuge species are adapted to a predominantly ammonium nitrogen supply (Krajina et al. 1973). A large number of the Ericaceae family (also important in the fynbos) grow on acidic soils and show a preference for ammonium rather than nitrate nitrogen (Haynes and Goh 1978).

The study of the nitrogen status of the Proteaceae is still in its infancy, and contradictions exist. The purpose of this work is to determine whether seedlings of *Protea neriifolia* develop nitrate reductase, and if so, whether in the roots or the leaves. Also to examine the preference of *P. neriifolia* for nitrate or ammonium and which form it is able to utilise more effectively.

#### LITERATURE REVIEW

Nitrate is readily and rapidly extracted from the soil by the plant where it is stored in the root tissue or enters the xylem for translocation to the leaves. The uptake of nitrate across the cell membrane of the root hairs is energy dependent, and therefore relies on the presence of carbohydrates in the roots. The absorption of nitrate into the plant is inhibited by low temperatures, alkaline conditions and the presence of ammonium in the root environment (Lewis 1986). Nitrate anion uptake is thought to be coupled with the excretion of negatively charged bicarbonate ions into the soil which maintains electroneutrality within the plant (Wallace and Mueller, 1963; Ben-Zioni et al, 1970). These bicarbonate ions however, increase the pH of the rhizosphere.

Once absorbed, nitrate crosses the root cortex via the apoplastic or symplastic pathway. Once in the plant nitrate is



reduced to an organic form either in the roots or the leaves before it can be incorporated into amino acids. This process is also energy dependent, being driven by two enzymes and requiring 347 kJ to convert 1 mole of nitrate into ammonia (Lewis 1986). The first step is the reduction of nitrate to nitrite by the enzyme nitrate reductase which is substrate induced. In the second step nitrite reductase converts nitrite into ammonia. Nitrate reduction in many plants takes place mainly in the leaves, but can also occur in the root.

Ammonium, on the other hand, can be taken up by the roots both actively and passively (Haynes and Goh 1978). The uptake of ammonium into the cell is facilitated by the electrochemical potential gradient (Salsac *et al.* 1987). The proton of the ammonium molecule is liberated (Salsac *et al.* 1987) when ammonium is incorporated into glutamine via the GS-GOGAT pathway (Lewis 1986). It is thought that this proton is eliminated from the cytoplasm (Raven and Smith 1976) and into the rhizosphere by means of membrane ATPases, thus acidifying the surrounding soil. An important consequence of this process is that exogenous ammonium assimilation should occur in the roots to avoid endocellular acidification (Raven and Smith 1976).

It is expected that this energetically economical supply of ammonium ions would be the preferred nitrogen source of many

plants, however, decreases in productivity are often encountered (Salsac et al. 1987). High levels of ammonium in the plant can be toxic. Ammonium, therefore, cannot be stored and must be assimilated into organic amino molecules as soon as possible. The mechanism of ammonium toxicity is not fully understood but it is thought to uncouple photophosphorylation, restricting ATP production in the leaves (Reisenauer 1978). The resulting acidification of the root environment by ammonium absorption also retards growth and impairs further nutrient uptake. Large quantities of carbohydrate are therefore required in the roots and this drain can restrict material available for growth (Lewis et al. 1986).

A preliminary investigation into the nitrogen status of the Proteaceae by Lewis and Stock (1978) revealed that *Leucadendron xanthoconus* exhibited low intensity nitrogen metabolism and that it was unable to utilise levels of nitrate above those which occur naturally. In an amino compound analysis from naturally growing populations of *Protea lepidocarpodendron*, *Protea laurifolia*, *Leucadendron xanthoconus* and *Brabejum stellatifolium*, they found that the free amino compound and amide levels of the leaves were three orders of magnitude lower than that of a non-fynbos species (*Datura stramonium* L.). Nitrate and ammonium feeding of detached shoots of *L. xanthoconus* revealed that ammonium levels greater than what occurs naturally can be incorporated into shoot metabolism. The

nitrate reducing potential of the leaves of *L. xanthoconus* was investigated using  $^{15}\text{N}$  enrichment, which revealed a limited capacity for the reduction of nitrate.

Stock and Lewis (1982) extracted nitrate reductase from *Protea repens* and *Protea cynaroides* which showed greater activity in the leaves than in the roots. Up until Stock and Lewis's study, *in vitro* techniques and conventional extraction media had been unable to demonstrate the presence of nitrate reductase in leaves or roots of Proteaceous plants. The presence of phytic acids, tannins or hydrolytic and oxidative enzymes (Loomis and Battaile 1966) are thought to be inhibitors of nitrate reductase activity. Some authors have shown that using casein in the extracting medium prevents the breakdown of nitrate reductase by proteolytic enzymes (Sherrard and Dalling 1978; Lewis, Watson and Hewitt 1982), while the addition of polyvinylpyrrolidone (PVP) successfully adsorbs polyphenolic inhibitors (Loomis and Battaile 1966). This was corroborated in Stock and Lewis's (1982) study which also showed that the NR inhibitor in *P. repens* was probably not a proteolytic enzyme but a polyphenolic constituent, which is prevalent in proteas.

The uptake and assimilation of nitrate and ammonium in *Protea repens* L. was compared by Stock and Lewis (1984) to determine the preferred nitrogen source and the sites of assimilation by  $^{15}\text{N}$  analysis. They confirmed that *P. repens* had low rates of

uptake and assimilation and utilised ammonium as the main N source, but the site of assimilation in the present study was found to be mainly in the root.

## MATERIALS AND METHODS

Nitrate reductase activity can be estimated by measuring the amount of nitrite formed from nitrate. If nitrite is allowed to react with sulphanilamide, a diazonium salt is formed, which if reacted with N-(1-naphtyl)ethylenediamine, a pink colour complex is formed which has an absorption maximum of 540 nm (method of Snell and Snell 1949, in Guerrero 1985).

### Plant Material and Feeding Programme

Year old seedlings of *Protea neriifolia* were obtained from the nursery of the Kirstenbosch Botanical Gardens in late summer. They were growing individually in a mixture of sand and humus (Figure 1). Plants were housed in a greenhouse and water was given thrice weekly and a 20% Long Ashton (Hewitt 1966) nutrient solution was fed once a week. The plants were divided into two groups, one being fed a <sup>the 2 plants containing</sup> 0.8 mM NH<sub>4</sub>Cl nutrient solution and the other a 0.8 mM KNO<sub>3</sub> nutrient solution. The pH of the nutrient solutions was adjusted to between 5.0 and 5.5. The nutrient solutions were made up according to Tables 1 and 2. Nitrapyrin (DOW Chemical, U.S.A.) of concentration 2 ppm was added to inhibit nitrification in the soil (Laskowski and Bidlack 1977).

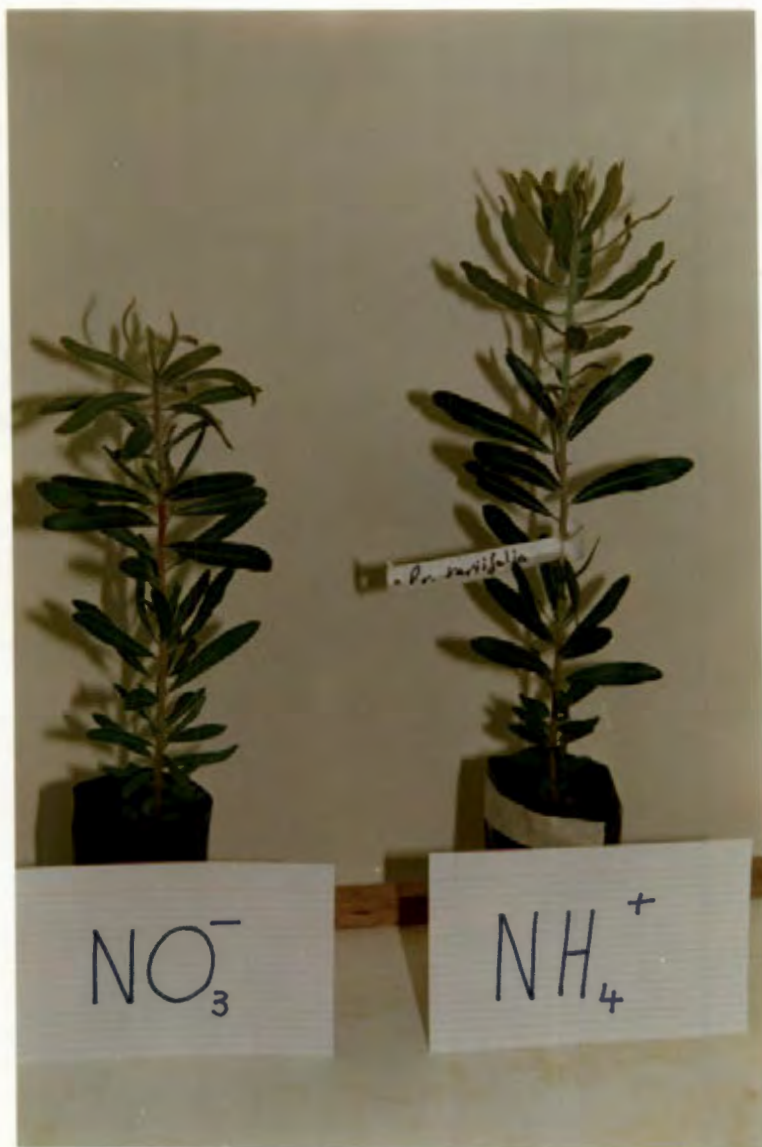


Figure 1.

Year old seedlings of *Protea neriifolia* undergoing separate feeding regimes of nitrate or ammonium.

(Photo: Dr. J. Kaiser)

	AMMONIUM	NITRATE
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.368	0.368
K <sub>2</sub> SO <sub>4</sub>	0.348	0.348
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	0.478	0.478
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	0.208	0.208
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.588	0.588
FeEDTA	0.033	0.033
KNO <sub>3</sub>	-	0.101
NH <sub>4</sub> Cl	0.107	-

Table 1. Macronutrients (in g 5 l<sup>-1</sup> deionized water) for the two feeding programmes.

Micronutrients	Mass
HBO <sub>3</sub>	4.29
MnSO <sub>4</sub> ·4H <sub>2</sub> O	2.32
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.33
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.12
Na <sub>2</sub> MOO <sub>4</sub> ·2H <sub>2</sub> O	0.03

Table 2. Micronutrients (in mg 5 l<sup>-1</sup>), used in each nutrient solution.

### In Vitro Nitrate Reductase Activity Assays

The experimental procedure for nitrate reductase activity according to (Lewis et al. (1982), was initially tested on maize seedlings which had been growing for ten days in vermiculite and fed 20% Long Ashton nutrient solution. The plants were harvested, the soil washed off with deionised water, and the roots blotted dry. Plant material was separated into shoots and roots and 1 g of each was cut into 2 mm segments and thoroughly ground in a chilled mortar and pestle with 8 ml of chilled extraction medium and about 0.2 g of acid washed sand. The extraction medium consisted of 0.1 M potassium phosphate buffer (pH 7.5), 2% casein, 0.5 mM EDTA, and 1.0 mM dithiothreitol. The extract was squeezed through a double layer of muslin to remove coarse debris. It was then centrifuged in a Beckman Model J2-21 centrifuge for 5 minutes at 2000 r.p.m. at 3°C to remove fine debris. Test tubes were prepared in triplicate with 0.1 ml potassium phosphate buffer, pH 7.5; 0.4 ml NADH (1 mg ml<sup>-1</sup>); 0.2 ml 0.1 M KNO<sub>3</sub> and made up to 1.8 ml with distilled water. The test tubes were incubated in a 30°C water bath for 5 minutes for the solutions to temperature equilibriate. With the tubes still in the water bath 0.2 ml of the extract was pipetted into each tube and the reaction allowed to run for 15 minutes. The reaction was stopped by adding 1 ml of sulphaniamide (w/v) (1% in 1 M HCl) to each tube, followed immediately by the colour reagent NED (0.01% N (1-naphthyl)-



ethylenediamine hydrochloride). The colour was allowed to develop for 15 minutes and the tubes again centrifuged on a desktop centrifuge (Nedtex Co. Taiwan) at 500G to remove any flocculate. The optical densities were read on a Beckman Model 42 spectrophotometer with absorbance wavelength set at 540 nm. The nitrate reductase activity was calculated from a standard curve (Figure 2) prepared from a range of known concentrations of potassium nitrite ranging from 10 to 100 nmoles. The amount of  $\text{NO}_2^-$  produced was expressed in  $\mu\text{mols NO}_2^- \text{ h}^{-1} \text{ g fresh weight}^{-1}$ .

The above experimental procedure was repeated on the maize with 1.5 g PVP as well as 2% casein added to 12 ml of the extraction medium. The procedure was again repeated using seedlings of *Aulax umbellata* (Thunb.) R.Br. (Proteaceae) and leaves only of *P. neriifolia* to avoid harvesting a whole plant. The results showed no indication of nitrate reductase activity in the plant so an alternative assay, the *in vivo* nitrate reductase assay was used.

#### **In Vivo Nitrate Reductase Assay**

Duplicate samples of nitrate-fed and ammonium-fed leaves and roots from *P. neriifolia* were assayed for their nitrate reductase activity according the method of Scott and Neyra (1979). The incubation medium used in the *in vivo* assay

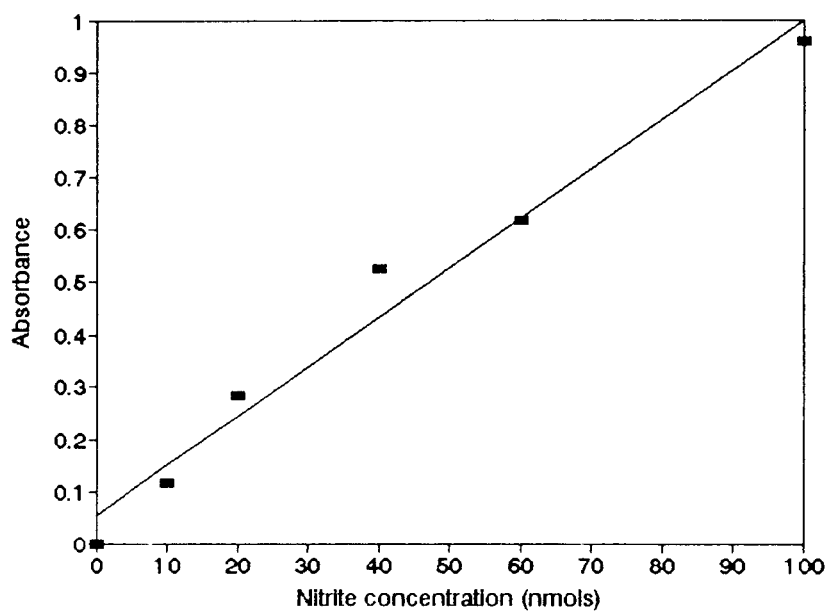


Figure 2.

Standard curve for the colorimetric determination of nitrite concentration using the colour reagent NED with the spectrophotometer absorbance wavelength set at 540 nm. The straight line represents the best fit ( $r = 0,987$ ).

consisted of a 0.1 M potassium phosphate buffer (pH 7.5) and 0.1 M potassium nitrate. Plant material (1 g) from each plant part from each treatment was cut into 1 mm segments and submerged in test tubes containing 10 ml of the incubation medium. The test tubes were stoppered with glass wool to ensure that all the plant material remained under the surface of the extraction medium. The test tubes were allowed to infiltrate under a vacuum applied by a vacuum pump (Edwards High Vacuum, Crawley, England) for two minutes. The tubes were immediately wrapped in aluminium foil to exclude light and incubated at 30°C in a shaking water bath for 3 hours. After incubation, 0.2 ml of the incubation medium from each tube was pipetted into test tubes containing 2 ml distilled water. Sulphanilamide (1 ml) and NED (1 ml) were then added and the amount of nitrate formed was calculated according to the *in vitro* assay. With all the assays, plant material was collected during the time that plants received direct solar radiation. This was to prevent severe diurnal fluctuations in nitrate reductase activity as found in barley plants by Lewis, Watson and Hewitt (1982).

### <sup>15</sup>N Isotope Feeding

Twenty four hours before feeding <sup>15</sup>N, the seedling pots were flushed with deionized water to remove all nutrients. The leachate from the ammonium-fed plants was tested for the presence of ammonium using Nessler's reagent (B.D.H. Ltd,

Poole, Dorset, U.K.) and that from the nitrate-fed plants was tested for nitrate using Szechrome NAS reagent (R & D Authority, Ben-Gurion University, Negev). A 0.5 ml sample of leachate from the ammonium-fed plants was added to 2.5 ml of Nessler's reagent when a yellow colour indicated the presence of ammonium. The same was done with the leachate from the nitrate-fed plants using Szechrome reagent when the development of a purple colour indicated the presence of nitrate. Neither ammonium nor nitrate residual nutrients were found. Nutrient supply to the plants was then withheld for 24 hours. The evening before the isotope feeding the  $^{15}\text{N}$  solutions were prepared and allowed to equilibriate overnight. The solutions contained 0.8 mM  $\text{Na}^{15}\text{NO}_3$  (BOC-Prochem, Deer Park Road, London, U.K.) and 0.8 mM  $^{15}\text{NH}_4\text{Cl}$  in 20% Long Ashton solution. The following day, 300 ml of the appropriate  $^{15}\text{N}$  solution was supplied to each of three plants from each treatment with a 45 minute interval between feeds to facilitate harvesting at precise time periods.

### Harvesting and Extraction

Twenty six hours after the start of the  $^{15}\text{N}$  feeding, the plants were harvested and the roots washed thoroughly in deionized water to remove soil and organic matter, and blotted dry. Plants were divided into root and shoot material (stems were excluded) and the fresh weight of each part recorded. The

tissue from each part was coarsely chopped, frozen in liquid nitrogen and then homogenised in 80% ethanol at 0°C (approximately 100 ml for 5 g plant material) using a Ultra-Turrax T25 homogeniser (Janke and Kunkel IKA Labortechnik). Flasks were sealed with parafilm and extraction allowed to take place for 24 hours at 0°C. The homogenate was then filtered through weighed Whatman No. 1 filter paper. The residue and the filter paper was dried in a drying oven at 80°C for 24 hours, and the mass of the residue and filter paper was recorded. The residue was further refined by milling it through a grinder (Arthur H. Thomas, Mass., U.S.A.).

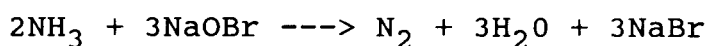
#### Kjeldahl Digestion of the Bound Fraction

The dried residue was digested by the Kjeldahl method (Chadwick 1985), to determine total bound nitrogen content and  $^{15}\text{N}$  enrichment. Duplicate samples of 0.15 g were digested in a Kjeldahl tube with a selenium catalyst tablet (B.D.H., Poole, Dorset, U.K.) and 3 ml of nitrogen-free sulphuric acid containing salicylic acid (34 g in 1 litre). The tubes were placed in a digestion block set at 200°C for 2 hours, after which the temperature was raised to 375°C until the samples were clear. The digestion continued at this temperature for a further 2 hours. The resulting digest was made up to a volume of 15 ml with distilled water, and from this, 2 ml was taken for distillation. The 2 ml aliquots were alkalised with 15 ml

of 50% sodium hydroxide. The total nitrogen was distilled off in a Markham semi-micro distillation unit, and trapped in 2 ml 0.02 M hydrochloric acid (Titrisol, E-Merck, Darmstadt, F.R.Germany). The total nitrogen content was determined by back titrating the excess acid with 0.005 M NaOH (Titrisol, E-Merck, Darmstadt, F.R.Germany), in an automatic titrator (Schott & Gerate TR85)). After titration, the sample was reacidified with approximately 2 ml 0.1 M HCl to prevent the loss of ammonia. The distillate was then evaporated on a hot plate and under an airstream to a suitable volume for  $^{15}\text{N}$  determination.

### $^{15}\text{N}$ Analysis

The samples were analysed for  $^{15}\text{N}$  by atomic emission spectroscopy using the sodium hypobromite method described by Faust (1967). By adding an oxidant of alkaline hypobromite solution, the sample was reacted under a vacuum to release nitrogen gas according to the following equation:



For each sample, 0.2 ml of the sample and 0.2 ml of hypobromite were pipetted into opposite lobes of a small Rittenburg vessel. The vessel was then sealed onto the vacuum system, as was the glass discharge tube. The sample was frozen and degassed three times to ensure the removal of all the water vapour and air.

The vessel was rotated to allow the sample and the hypobromite to react and the nitrogen gas which was liberated was collected in the discharge tube. The tube was sealed and ionized using a high voltage gun.

The discharge tubes were then placed between the terminals of a Jasco N-150  $^{15}\text{N}$  Analyzer, and the gas excited. On excitation the three isotopic molecules  $^{15}\text{N}^{15}\text{N}$ ,  $^{14}\text{N}^{15}\text{N}$  and  $^{14}\text{N}^{14}\text{N}$  emit a characteristic bandhead which is photoelectrically recorded. A typical trace showing the characteristic peaks for  $^{15}\text{N}$  enrichment is shown in Figure 3. The percentage enrichment of the sample is then calculated from the formula:

$$\text{En}\% = \frac{100}{2 (A/B \times C) + 1}$$

A and B represent the bandheads of the  $^{14}\text{N}^{14}\text{N}$  and  $^{14}\text{N}^{15}\text{N}$  respectively and C is the attenuation or gain setting of the spectrophotometer at which the bandheads were recorded. Enrichment figures were an average of three complete traces and were corrected using a calibration curve drawn up for the spectrophotometer. In order to obtain the percentage enrichment in excess of the natural abundance, known as the atom percent excess (A%E), the natural abundance (0.37%) was subtracted from the corrected %En. The A%E value was then multiplied by the total nitrogen content (obtained from the distillation procedure) to obtain the  $^{15}\text{N}$  content of each sample, which was expressed as  $\mu\text{g } ^{15}\text{N (g dry weight residue)}^{-1}$ .

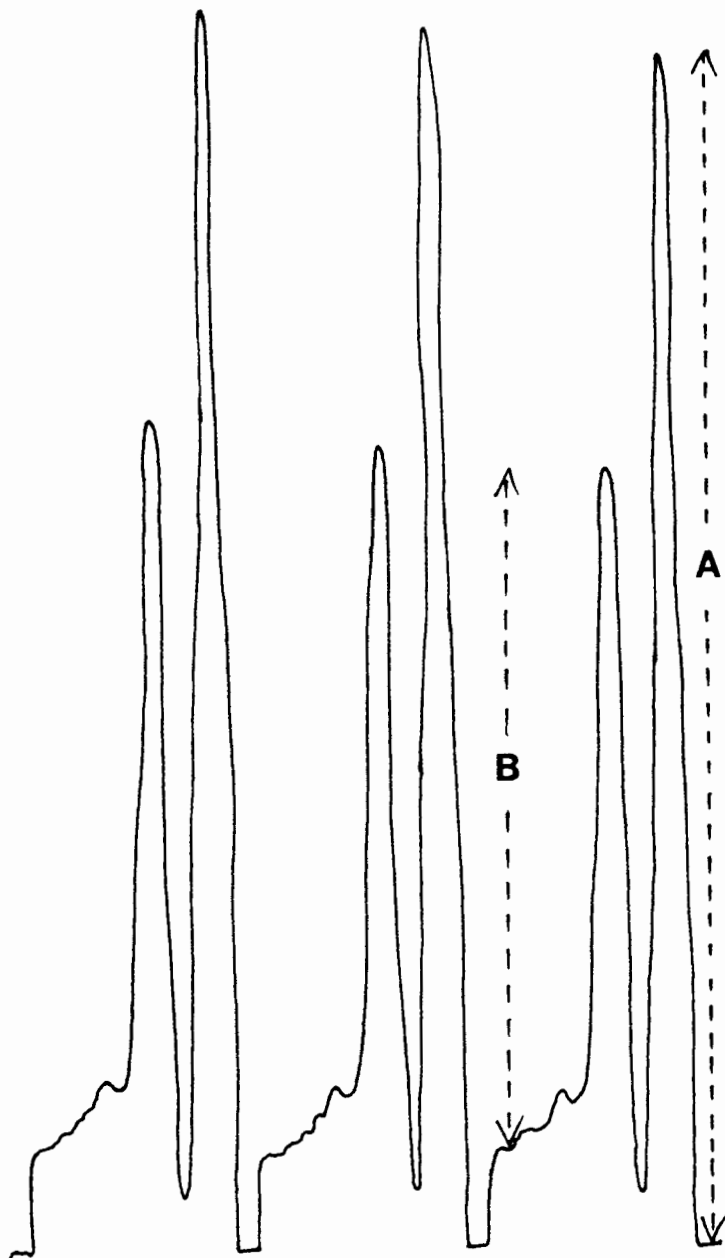


Figure 3.

Typical traces for  $^{15}\text{N}$  enrichments below 50% showing good separation of the nitrogen molecules. Lines A and B represent the peak heights of the  $^{14}\text{N}^{14}\text{N}$  and  $^{14}\text{N}^{15}\text{N}$  bandheads respectively.



## RESULTS AND DISCUSSION

### Nitrate Reductase Assays

The results of the *in vitro* nitrate reductase activity (NRA) assay for maize, *A. umbellata* and *P. neriifolia* are shown in Table 3, and were obtained from a standard curve (Figure 2). The shoot NRA of maize using only casein in the extraction medium was found to be  $14.9 \mu\text{mol NO}_2^- \text{ h}^{-1} (\text{g fresh weight})^{-1}$  which is higher than that found by Sherrard and Dalling (1978) of  $9 \mu\text{mol NO}_2^- \text{ h}^{-1} (\text{g fresh weight})^{-1}$ . The addition of PVP to the extraction medium resulted in a higher result of  $18.5 \mu\text{mol NO}_2^- \text{ h}^{-1} (\text{g fresh weight})^{-1}$ . The absence of statistical replicates in this study however, make the results inconclusive. The root NRA for maize was found to be  $1.6 \mu\text{mol NO}_2^- \text{ h}^{-1} (\text{g fresh weight})^{-1}$  in the casein only treatment and the casein and PVP treatment yielded  $1.3 \mu\text{mol NO}_2^- \text{ h}^{-1} (\text{g fresh weight})^{-1}$ , which are both lower than the shoot NRA.

IN VITRO NITRATE REDUCTASE ACTIVITY ASSAY (Values in $\mu\text{mol NO}_2^- \text{ h}^{-1} \text{ gfw}^{-1}$ )			
Species	Treatment	ROOTS	SHOOTS
Maize	Casein	1.60	14.89
Maize	Casein + PVP	1.27	18.49
<i>Aulax</i>	Casein + PVP	0	0
<i>Protea</i>	Casein + PVP	0	0

Table 3. *In vitro* NRA assays of maize, *Aulax umbellata* and *Protea neriifolia*. Mean only.

The *in vitro* NR assay using both casein and PVP on the two members of the Proteaceae showed no enzyme activity. Stock and Lewis (1982) however, found that using both casein and PVP in an *in vitro* NRA determination in *P. repens* and *P. cynaroides*, the PVP proved most successful as a protectant against polyphenolic inhibitors. They found that NRA was more prevalent in the leaves than the roots, but nevertheless low ( $2-4 \mu\text{mol NO}_2^- \text{ h}^{-1} (\text{g fresh weight})^{-1}$ ) when compared to more nitrophilous plants. The absence of any enzyme activity in *A. umbellata* and *P. neriifolia* could be that they have higher polyphenol and/or proteolytic enzyme levels than *P. repens*, which would inhibit enzyme activity. In the present study enzyme activity was found in the *in vivo* assay and not in the *in vitro* assay because in the former assay, cells are kept intact, therefore the inhibiting compounds do not come into contact with the enzymes.

The results of the *in vivo* NRA assay are shown in Table 4. NRA levels were found to be extremely low and entirely restricted to the roots. This enzyme activity is consistent with the findings of Stewart and Orebamjo (1983) for the savanna and woodland vegetation of West Africa. These climax grasslands (Stewart and Orebamjo 1983), as with the fynbos region, are characterised by low rates of nitrification.

IN VIVO NITRATE REDUCTASE ASSAY OF <i>PROTEA NERIIFOLIA</i> (Values in $\mu\text{mol NO}_2^- \text{ h}^{-1} \text{ gfw}^{-1}$ )		
	ROOTS	SHOOTS
NITRATE-FED	0.143 $\pm$ 0.13	0
AMMONIUM-FED	0.137 $\pm$ 0.05	0

Table 4. *In vivo* NRA assay of *P. neriifolia*. Mean  $\pm$  range.

Results of *in vivo* assays are expected to be lower than *in vitro* because they only reflect the actual enzyme activity whereas the *in vitro* assays reflect the potential activity. The above results are conflicting with a previous study on *P. repens* and *P. cynaroides* in which leaves were the major area of nitrate reduction (Stock and Lewis 1982). Although the NRA of the ammonium-fed plants was lower than that of the nitrate-fed plants, there was little difference between the two. This means that even trace amounts of nitrate produced by soil nitrifying processes in the ammonium-fed plants induced almost the same amount of enzyme activity as the nitrate-fed plants. This could be due to difficulties experienced in completely eliminating nitrate and nitrifying bacteria from the growth medium of the ammonium-fed plants. Blevins (1976) reported that bacterial contamination, especially with plants grown in poorly aerated media, could contribute to a proportion of nitrate reductase in roots. Results could possibly have been improved by growing plants in a sterilized medium.

### <sup>15</sup>N Analysis

Results of the <sup>15</sup>N feeding showed that *P. neriifolia* is able to utilise both sources of nitrogen (Figure 4). The ammonium-fed plants however, were able to assimilate more than double the amount of nitrogen ( $65 \mu\text{g } ^{15}\text{N g dry weight residue}^{-1}$ ) into insoluble protein than the nitrate-fed plants ( $26 \mu\text{g } ^{15}\text{N g dry weight residue}^{-1}$ ), considering both roots and leaves. The rate at which the ammonium-fed plants translocated combined nitrogen to the leaves was 8 times greater than that of nitrate-fed plants. Only 20% of the assimilated nitrogen had been exported out of the root of the nitrate-fed plants. This confirms the root as being the main site of nitrate assimilation. There was a more even distribution of bound nitrogen between the roots and the leaves of the ammonium-fed plants. Although the primary assimilation of ammonium must still be occurring within the roots (Raven and Smith 1976; Lewis 1986), the more rapid incorporation of ammonium into nitrogen metabolism resulted in faster translocation of bound nitrogen to the leaves. Although the overall results would indicate that this species "prefers" ammonium as its N source, it must be borne in mind that most higher plants absorb and assimilate ammonium at a faster rate than they do nitrate (Lewis 1986).

This study corroborates the findings of two previous studies which demonstrated two species of the Proteaceae to be

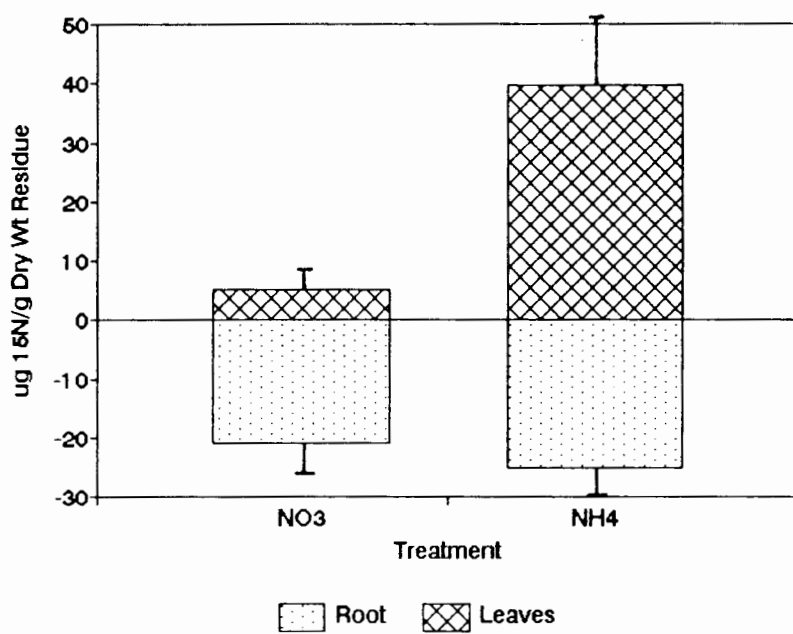


Figure 4.

Incorporation of  $^{15}\text{N}$  into protein in *Protea neriifolia*.

(Bars = S.E.)

inefficient nitrate utilisers. On feeding  $^{15}\text{N}$  to shoots of *L. xanthoconus*, Lewis and Stock (1978) found extremely low enrichments of three important amino acids, confirming the poor nitrate processing properties of this species. A later study on *P. repens*, when fed  $^{15}\text{N}$ , also revealed low rates of enrichment when compared to rapidly growing species from fertile habitats (Stock and Lewis 1984). They also demonstrated a greater absorption and assimilation rate of ammonium by *P. repens* than of nitrate.

Plants have been shown to differ widely in their ability to reduce and assimilate nitrate in the leaves or the roots. The primary area of nitrate assimilation in cocklebur and barley has been shown to be the leaves (Wallace and Pate 1967 and Lewis, Watson and Hewitt 1982 respectively), whereas that of apples and *P. repens* was found to be the roots (Grasminis and Nicholas 1967 and Stock and Lewis 1984 respectively). These differences in region of ~~and~~ assimilation can be explained not only by species, but also by rhizosphere conditions and plant age (Lewis, Watson and Hewitt 1982).

There is evidence that soils of climax vegetation are generally low in nitrate (Rice and Pancholy 1972) because of inhibition of the nitrification process partly because of high tannin levels of the vegetation (Rice and Pancholy 1973). It is well known that *Protea* spp. have high polyphenol and tannin levels

*Studies*

(Stock and Lewis 1982). A study by Stewart et al. (1988, 1990) of the occurrence and localisation of nitrate reductase in Australian rainforest plants revealed that plants of early successional stages utilised nitrate as their predominant N source and that these pioneer species assimilated nitrate predominantly in their leaves. Alternatively, closed climax vegetation had low NR levels in roots and leaves suggesting limited nitrate utilization. It would appear that some members of the Proteaceae such as *P. repens* (as found by Stock and Lewis 1984), and *P. neriifolia* (this study) are suited to the later successional stage by utilising ammonium as their primary source of nitrogen. It has been hypothesized by Stewart and Orebamjo (1983) that plants which are able to utilise ammonium more efficiently have a selective advantage in climax vegetation.

The greater ammonium utilisation by *P. neriifolia*, could be attributed to the low energetic costs of ammonium uptake (Gutschick 1981). Costs which could be incurred is the need to expand the rooting system to facilitate encountering the slow-moving ammonium ions. The Proteaceae do put out seasonal proteoid roots for just this purpose (Lamont 1982; 1983).

### CONCLUSIONS

From this study, it appears that *Protea neriifolia* is able to take up and assimilate both nitrate and ammonium forms of nitrogen effectively. The uptake and assimilation rates of ammonium was, however, found to be greater than that of nitrate. The primary area of nitrate assimilation was found to be in the roots, as shown by both NRA assays and the distribution of  $^{15}\text{N}$  within the plant. Previous research has indicated that the Proteaceae have adapted to a low-nutrient environment by developing low levels of nitrogen metabolism. Despite this, much remains to be elucidated regarding the nitrogen status of the Proteaceae.



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