

**GLYCINE UPTAKE IN CLUSTER AND NON-  
CLUSTER ROOTS OF *LEUCADENDRON*  
*LAUREOLUM* (PROTEACEAE)  
AND *LUPINUS ALBUS***

- AN ALTERNATIVE STRATEGY FOR ORGANIC  
NITROGEN ACQUISITION?

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BOTANY HONOURS

2001

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## ABSTRACT

To investigate the potential of cluster roots to exploit organic nitrogen (N), doubly-labelled glycine was fed to cluster and non-cluster roots of the crop plant *Lupinus albus* and *Leucadendron laureolum* (Proteaceae). From the isotopic ratio of carbon to nitrogen ( $^{13}\text{C}:^{15}\text{N}$ ) the amount of glycine taken up intact can be estimated. Cluster and non-cluster roots of *L. laureolum* and *L. albus* take up doubly-labelled glycine. Cluster roots of *L. albus* take up more  $^{13}\text{C}$  (5.7 vs 2.24  $\mu\text{mol}$  per plant) and  $^{15}\text{N}$  (2.01 vs 0.36  $\mu\text{mol}$  per plant) than non-cluster roots although this is not significant. *Leuca dendron laureolum* cluster roots take up ~~more~~<sup>less?</sup>  $^{13}\text{C}$  (23.03 vs 26.40  $\mu\text{mol}$  per plant) and  $^{15}\text{N}$  (8.17 vs 12.16  $\mu\text{mol}$  per plant) than non-cluster roots although this is not significant for either species. Of the total amount of glycine taken up by the roots, 49 $\pm$ 3.6 % and 36 $\pm$ 3.0% was taken up ~~intact~~<sup>okay</sup> by cluster and non-cluster roots of *L. albus* respectively. In *L. laureolum* 11.63 $\pm$ 1.2% was taken up ~~intact~~<sup>okay</sup> by cluster roots and 13.9 $\pm$ 0.3 % was taken up intact in the non-cluster roots. In *L. albus*,  $^{15}\text{N}$  is translocated at a greater rate from the glycine-fed roots to the rest of the root by cluster roots. In non-cluster roots of *L. albus*, more  $^{13}\text{C}$  is translocated to the bulk root. However there is little difference in the amount of  $^{13}\text{C}$  and  $^{15}\text{N}$  translocation to the shoot of cluster and non-cluster plants. In *L. laureolum*  $^{15}\text{N}$  is preferentially translocated from the glycine-fed roots to the bulk root, for both cluster and non-cluster roots. However there is little difference in the uptake of  $^{13}\text{C}$  and  $^{15}\text{N}$  by the shoot of cluster and non-cluster plants. Therefore it appears that as glycine and assimilates are translocated throughout the plant,  $^{15}\text{N}$  is retained in the root portions and that assimilates are translocated to the shoot as organic acids. This was supported by C:N ratios which indicated that carbon content was successively higher from the glycine-fed roots to the bulk root and the shoot. As there is no significant difference in the uptake rates of  $^{13}\text{C}$  and  $^{15}\text{N}$  between cluster and non-cluster roots of both species, clusters roots do not seem to be an adaptation for organic N uptake and are probably more significant for the phosphorus nutrition in a plant.

## INTRODUCTION

Purnell first described proteoid roots in 1960 from members of the Proteaceae. She described "dense clusters of rootlets of limited growth" that are found in all genera of the Proteaceae except the primitive genus *Persoonia* (Purnell, 1960). "Proteoid roots" and analogous structures have subsequently been found in a number of other families including Betulaceae, Casuarinaceae, Eleagaceae, Leguminosae, Moraceae and Myricaceae (Lamont, 1982; Watt and Evans, 1999), hence the more general term, cluster roots was proposed by Lamont (1982). Species with clusters have a global distribution, including Africa, Australia, North and South America, Asia and islands of the Pacific (Skene, 1998) and are often prevalent in nutrient deficient environments like the Fynbos in South Africa and the heathlands of Western Australia (Lamont, 1993). A cluster root is composed of longitudinal rows of tightly packed rootlets that gives a cluster its distinctive bottlebrush appearance. The rootlets originate opposite every protoxylem pole within the cluster root axis (Purnell, 1960; Johnson *et al.*, 1996). Cluster rootlets are relatively short-lived and growth is determinate (Purnell, 1960; Watt and Evans, 1999). One of the key features that distinguishes cluster roots from other roots is that within any single cluster rootlet initiation, growth and cessation takes place in a synchronized pattern (for review see Skene, 2001)

Analogous cluster-like structures also occur in the Cyperaceae (Lamont, 1974) and the Restionaceae (Lamont, 1982), families common in the Fynbos. The morphology of cluster roots varies between families in which they occur. Cluster roots may be ~~may be~~ simple or compound, smooth or densely covered with root hairs (Lamont *et al.*, 1984). In this study both *Leucadendron lauratum* (Proteaceae) and the crop plant *Lupinus albus* L. are used, therefore the general term cluster roots will be used for <sup>applied to?</sup> both species. Cluster roots can make up a large proportion of root biomass, up to 40% dry root biomass in *L. lauratum* (Lamont *et al.*, 1984) and *L. albus* (Gardner *et al.*, 1982; for review see Dinkelaker *et al.*, 1995). Morphology of cluster roots differs in these two species; clusters of *L. albus* are simple, crudely defined structures and occur basipetally at intervals along lateral roots (Fig 1). Cluster roots of *L. lauratum* occur as well defined compound and/or simple offshoots from the lateral roots (Fig 2). Thus in this study a cluster root is defined as the section of lateral root along which the cluster occurs for *L. albus* and as the separate offshoot for *L. lauratum*.

The widespread occurrence of cluster species in low nutrient environments (Lamont, 1993) prompted a large volume of work investigating the role of cluster roots in the enhanced uptake of limiting nutrients, particularly phosphorus (P) and nitrogen (N) (Lamont, 1981; Lamont *et al.*, 1984; Keerthisinghe *et al.*, 1998; Neumann *et al.*, 2000) and in some species e.g., *Casuarina glauca*, iron (Arahou and Diem, 1997).

Marschner *et al.* (1987) proposed that the internal P status of a plant elicits the formation of cluster roots as application of P to the shoot suppressed cluster root formation. The stimulation of cluster root formation in *L. albus* appears to be regulated by phosphonate (which blocks the plants detection of internal P status) and other phytohormones (auxins and cytokinins) (Gilbert *et al.*, 2000; Neumann *et al.*, 2000). The formation of clusters in *L. albus* is stimulated by low levels of P (1-10 mmol soluble phosphorus (Pi) and repressed at 25 mmol and higher and there is a negative relationship between the concentration of P and the production of clusters (Keerthisinghe *et al.*, 1998).

Proteoid roots of *Leucadendron uliginosum* have a greater uptake of Pi than non-proteoid roots (Vorster and Jooste, 1986) as do cluster roots of *L. albus* (Neumann *et al.*, 2000). This increased uptake is facilitated in part by an increase in surface area. Cluster roots of *L. laurosum* including the root hairs can have 15 times the surface area of an equivalent mass of non-cluster root as a result of higher rootlet density within a cluster and denser and longer root hairs on these rootlets (Lamont *et al.*, 1984).

However cluster roots are localised structures and therefore the facilitation of increased uptake cannot be through surface area alone as such a large surface area in a small volume of soil would have large depletion zones. The production of a number of exudates by cluster roots contributes to the increased uptake of limiting nutrients (Skene *et al.*, 1996; for review see Dinkelaker *et al.*, 1995 and Neumann *et al.*, 2000), particularly citric acid which mobilises P bound to Al, Fe or Ca (Johnson *et al.*, 1996). The concentration of Pi in the rhizosphere of cluster roots was found to be twice that of the bulk soil for *L. albus*, even though the rhizosphere surrounding the roots is where depletion due to uptake would occur (Gerke *et al.*, 1994). Organic acid efflux

varies along the axis of a cluster root, in *L. albus* the greatest citrate efflux is 1-3 cm from the root tip and efflux is approximately three times greater in plants grown without P than plants grown with P (Keerthisinghe *et al.*, 1998). In *L. albus* acid phosphatase has been identified as an important factor for the mobilization of organically bound P (Neumann and Römheld, 1999).

Production of these exudates by cluster roots would mobilise nutrients in very localised regions surrounding the root area, since cluster roots occur as concentrated structures. P-deficient *L. albus* plants have a lower  $K_m$  than P sufficient plants (8.5  $\mu\text{M}$  vs 30.7  $\mu\text{M}$ ) (Neumann *et al.*, 2000). This suggests that under conditions of P deficiency, the plants are able to adapt through the initiation of a high-affinity transport system (Schachtman *et al.*, 1998). Although cluster and non-cluster root exhibited the same  $K_m$  when grown under minus P, cluster roots had a higher  $V_{\text{max}}$ , suggesting a higher concentration of high-affinity transporters in cluster roots (Schachtman *et al.*, 1998). These results suggest that not only are cluster roots able to mobilise minerals bound in the soil matrix, but that they are also adapted for more efficient uptake of these mobilised minerals. Since clusters have a large surface area that is concentrated in a small space, their ability to excrete exudates and efficiently take them up seems an adaptation to maximally exploit nutrients that occur in localized patches.

Early work focussed on the role of cluster roots in enhanced mineral nutrient uptake. Recent work on plant nutrition has begun to investigate the role of cluster roots in organic nutrient acquisition. Purnell (1960) gave the first hint that cluster roots may be involved in the uptake of organic nutrients when she described the proliferation of cluster roots in a layer of blood and bone. For plants with mycorrhizal associations, organic nutrient uptake is facilitated by symbiotic fungi which are able to take up simple peptides (Bajwa and Read, 1985). These fungi are also able to make use of larger, more complex proteins through the production of extracellular proteinases able to break down larger molecules which are then taken up by the mycorrhizae and passed onto the host (Bajwa *et al.*, 1985; Leake and Read, 1989). Plants with mycorrhizae are dependant on the fungi maximally <sup>to</sup> exploit this supply of organic nutrients. For example growth of *Vaccinium corymbosum* grown with the ericoid

mycorrhiza *Hymenoscyphus ericae* on peptides as the sole source of N is significantly higher than plants grown without mycorrhiza (Bajwa and Read, 1985).

Plants were thought to take up nitrogen predominately in the form of  $\text{NH}_4^+$  or  $\text{NO}_3^-$ , however the concentration of amino acids in the soil can be greater than inorganic forms of N (Schmidt and Stewart, 1997) and a wide-range of growth forms (trees, shrubs and grasses) from boreal forests are able take up organic nitrogen irrespective of mycorrhizal status (Näsholm *et al.*, 1998). There is increasing evidence to show that plants are able to utilise N in organic form without a mycorrhizal association (Schmidt and Stewart, 1999). For example, the non-mycorrhizal arctic sedge *Eriophorum vaginatum* can obtain up to 60% of its nitrogen budget in organic form (Chapin *et al.*, 1993) and the alpine sedge *Kobresia myosuroides* preferentially utilises GLY before either  $\text{NO}_3^-$  or  $\text{NH}_4^+$  which is taken up intact and can account for 16% of dry mass accumulation over a four-month period (Raab *et al.*, 1996).

The majority of work demonstrating the ability of plants to take up organic N without mycorrhizae has been done on alpine (Raab *et al.*, 1996) and arctic species (Kielland, 1994) where cold temperatures inhibit microbial degradation of organic matter. However species from a wide-range of Australian environments, not necessarily limited by low mineralization rates, take up glycine, a simple amino acid (Schmidt and Stewart, 1999). Whilst many families within the Fynbos do form mycorrhizal associations, notable exceptions are the Cyperaceae, the Restionaceae and the Proteaceae (Allsopp and Stock, 1993). These families may not have mycorrhizae, but they do have cluster roots (in the Proteaceae) or analogous structures (Cyperaceae and Restionaceae). Schmidt and Stewart (1997) found that proteoid roots of *Hakea* (Proteaceae) take up  $\text{NH}_4^+$  and glycine (GLY) in equal amounts whilst non-cluster roots take up  $\text{NH}_4^+$  in greater amounts than GLY. Thus cluster roots may be an alternative strategy to exploit organic nutrients in the soil.

As yet there have been few studies on the organic N uptake capabilities of the Proteaceae in the Fynbos, the South African equivalent of the Australian Heathland in which the Schmidt and Stewart (1999) study was done. Soils of Fynbos are typically acidic with low nutrient status (Richards *et al.*, 1997) and low organic matter (*ca* 1-4%), however the ratio of organic matter to mineral nutrients is relatively high.

Patterns of mycorrhizal activity in the Fynbos are dependant on a number of interacting factors, including fire, growth-form and successional stage (Allsopp and Stock 1994) and families that do not have mycorrhizae are those which have cluster roots or analogous structures (Allsopp and Stock, 1993). Allsopp and Stock (1993) suggest that these different adaptations to nutrient acquisition facilitate the diversity of species found in the Fynbos .

Previous studies have demonstrated the ability of cluster roots to take up amino acids without mycorrhizae (Schmidt and Stewart, 1999) and that simple amino acids may be taken up intact by alpine sedges (Raab *et al.*, 1996). This study investigates if cluster roots of a representative member of the Fynbos Proteaceae, *Leucadendron laureolum*, is able to take up simple amino acids in the form of glycine. Whilst a similar study has been done by Schmidt and Stewart (1999), this study also examines whether or not the glycine is taken up intact, therefore doubly-labelled glycine is used. As a comparison, the uptake of doubly-labelled glycine is also investigated in the crop plant *L. albus*. The presence of extracellular proteinases is tested for in *L. albus*. Using cluster roots as a strategy to exploit organic nutrients will be discussed in relation to other strategies like mycorrhizal associations.



Figure 1. Simple cluster roots of *L. albus*



Figure 2. Compound cluster roots of *L. laureolum*

## METHODS

### *Plant Growth*

#### *L. albus*

Fifty seeds of *L. albus* were germinated in a glasshouse in sand and watered every second day. After three weeks they were watered every second day with 100 % Long Ashton solution minus P for one week. Thereafter they were placed in aerated hydroponics on 10% Long Ashton solution in distilled water minus P. They were kept in a growth room with a 14 hr day: 10 hr night regime at 25 °C. The light intensity in the growth room was 300-400  $\text{mmol m}^{-2} \text{s}^{-1}$ . The nutrient solution was changed weekly and the pH corrected daily to 6.5. After three weeks the plants were showing signs of severe P deficiency so 2% P was added to the Long Ashton solution. When the plants began to flower, the flowers were removed to reduce nutritional stress.

Why and how?

#### *L. laureolum*

Twenty plants of *L. laureolum* were obtained from Kirstenbosch Gardens at approximately two years of age. They were removed from the potting soil and the roots carefully washed with water, a few of the plants had old cluster roots which were removed. They were placed in the same growth room as *L. albus* for two weeks in distilled water in order to avoid nutrient shock since Proteaceae are adapted to nutrient poor environments (Lewis and Stock, 1978) and to acclimatise the roots to a hydroponic atmosphere. Thereafter they were placed in 2% Long Ashton solution minus P. However 2% P was added to the nutrient solution at the same time as *L. albus* to keep the nutrients supplied to the plants the same. The pH (5.5) for *L. laureolum* was corrected daily. Glycine-feeding period for *L. laureolum* was 16 hours therefore before commencement day/night length was changed to 18 hrs day:6 hrs night. *L. albus* plants had been harvested by this time so they were not influenced by the change in light regime.

How?  
And why?

### *Glycine uptake*

To investigate the uptake of amino acids, doubly labelled glycine ( $^{15}\text{N}$ - $^{13}\text{C}_2$ -GLY) was used (Sigma) (99% atom excess). The glycine was labelled on only one carbon therefore the ratio of carbon to nitrogen is 1:1. The purpose of using doubly-labelled glycine was to see if glycine (GLY) was taken up intact. If the GLY was taken up intact then the ratio of  $^{13}\text{C}$ : $^{15}\text{N}$  in the root would be 1:1. Plants are manipulated into a respiration chamber and sections of either root or cluster root are placed in 2 mM doubly-labelled GLY in 10% Long Ashton solution, with 2% P.

The details are as follows; individual plants were placed in 5 L plastic bottles as per Figure 3 containing Long Ashton solution in the same composition as the hydroponic nutrient solution. A hole was cut in the centre of the bottle top through which the plant was inserted. The plant was then fixed in place with closed cell Neoprene rubber. There were two smaller holes in the sides of the bottle through which aeration was supplied and any respired gas collected. The plants were then left for an hour in order to acclimatise the plants and to alleviate any stress resulting from manipulation into the experimental chamber. Since there was only one respiration chamber the experiment was run on consecutive days for each species. For *L. albus* there were five replicates for the cluster root feeding experiments and four for the non-cluster root (a total of nine *L. albus* plants). For *L. lauroleum* three replicates each for cluster and non-cluster root feeding experiments were done (a total of six plants). Before the uptake experiment, three leaves were taken in order to get a measurement of  $^{15}\text{N}$  and  $^{13}\text{C}$  natural abundance in the plant. The cluster roots of *L. albus* do not occur as distinct portions. They are distributed along the main root axis, therefore in order to measure doubly-labelled glycine uptake sections of roots with cluster roots were placed in two 30 ml bottles containing 2 mM doubly-labelled glycine in Long Ashton solution at the same concentration as in the hydroponics. This concentration of GLY was chosen in order to get an enrichment of about 2% which is within the range measured by the mass spectrometer. Cluster roots of *L. lauroleum* are distinct and therefore it is easy to place these clusters in the 30 ml bottles. Plants of *L. albus* were left for 4 hours and *L. lauroleum* for 16 hours as past studies have shown that the uptake rates for mineral N are low for Fynbos Proteaceae species,

4.8-5.9  $\mu\text{g } ^{15}\text{N g}^{-1}$  fresh mass  $\text{d}^{-1}$  (Stock and Lewis, 1984). Once the cluster roots have been placed in the containers, the bottle top is carefully screwed closed and sealed as described under respiration rates. The results from the uptake experiment are expressed as a rate of uptake ( $\mu\text{mol } ^{13}\text{C}/^{15}\text{N g}^{-1} \text{h}^{-1}$ ). This is calculated from the atom % excess which has been corrected against a known nasturtium standard. The natural abundance obtained from the leaf samples before the uptake experiments was subtracted from the final atom % excess for each plant. This was done to increase accuracy of measurement of atom % enrichment for each individual plant.

At harvesting the roots in the glycine containers were washed with 2 mM unlabelled glycine three times for 15 seconds to flush out labelled glycine that may be contained in extracellular spaces. The clusters were then washed in distilled water three times for 15 seconds. The plants were then harvested as shoot, cluster/non-cluster roots in the labelled glycine and the rest of the root, which is termed bulk root. They were dried at 60 °C, coarsely milled and then ground by hand with a mortar and pestle ~~and in~~ liquid nitrogen. They were then analysed using combustion carbon and nitrogen method on a mass spectrometer (Finnigan MAT 252, Germany).

To express the results, plants were divided into shoot, root (which is the doubly-labelled GLY fed cluster or non-cluster root) and bulk root (the rest of the root). Where the label "shoot before" appears it refers to the leaves that were sampled before the feeding experiments. The leaves were removed to get natural abundance values for each plant. Results are expressed as a rate of  $^{13}\text{C}$  and  $^{15}\text{N}$  uptake ( $\mu\text{mol g}^{-1} \text{dry wt h}^{-1}$ ). To obtain total uptake ( $\mu\text{mol per plant}$ ) the uptake in root, bulk root and shoot is added. This was then averaged for cluster and non-cluster plants. Final results were calculated from atom % excess, % N and C, and dry weights.

### *Respiration rates*

Respiration rates of root and shoot were measured separately in order to quantify possible glycine loss through respiration. The hole in the 5L bottle was first sealed with closed cell Neoprene rubber, then with paraffin wax and a layer of Vaseline®. Aeration tubing was sealed with silicone and prestik. The air from the root and shoot

was collected in 50 ml 0.3 M NaOH in order to trap the CO<sub>2</sub> for respiration measurements and isotope analysis. A control beaker was also set-up to correct for the amount of CO<sub>2</sub> in the atmosphere. For respiration calculations, the NaOH for the root, shoot and control was titrated with HCl three times each and an average obtained. The CO<sub>2</sub> given off by the plant is absorbed by NaOH to form sodium carbonate. Barium chloride is then added to the solution which precipitates the carbonate. This is then titrated with HCl ~~with~~ <sup>to</sup> reacts ~~with~~ the excess NaOH that has not combined with CO<sub>2</sub>. The amount of CO<sub>2</sub> respired can then be calculated from the difference between the control and the root/shoot. For some plants there were problems with the bubbling of air through the NaOH due to the tubing slipping too deep into the NaOH resulting in too much back-pressure which stopped bubbling. The plants were checked periodically and if the bubbling had stopped, the positioning of the tubing was corrected and the air allowed to flow through. However in some cases the titration showed no difference between the control and the root or shoot, in these instances the sample was left out of the calculations due to experimental error. The NaOH collected was stored in the fridge after being tightly sealed with parafilm to prevent escape of captured air. There does not seem <sup>to</sup> ~~be~~ have been any losses of CO<sub>2</sub> during storage as titration of control beakers showed little difference between those titrated immediately and those titrated up to two weeks after. Titrations used 30 ml of the 50 ml, the 20 ml left over was air-dried and run through the mass spectrometer for carbon analysis.

### <sup>13</sup>C:<sup>15</sup>N ratio

This was worked out using atom % corrected against nasturium. The ratio was calculated for the bulk root, shoot, and doubly-labelled GLY-fed root (cluster and non cluster). The ratio of <sup>13</sup>C to <sup>15</sup>N in the doubly-labelled GLY is 1:1. The amount of doubly-labelled GLY taken up intact can be calculated from the difference in the <sup>13</sup>C:<sup>15</sup>N ratio. If the ratio is more than 1, there is more <sup>13</sup>C, conversely if the ratio is less than 1, there is more <sup>15</sup>N.

### *Protease assay*

Possible activity of extracellular enzymes was measured with Micro BCA Protein Assay (Pierce) using Bovine Serum (BSA). Only *L. albus* plants were used in this assay as there were insufficient plants of *L. laureolum* that had formed cluster roots. Possible enzyme activity was measured at four pH's; 2, 3, 4 and 5 as an earlier experiment by Leake and Read (1989) showed the optimal activity of proteases produced by the ericoid mycorrhiza *Hymenoscyphus ericae* to be at pH 2.7. For each pH, there were four replicate plants, except for pH 5 for which there were only 3 plants, resulting in 15 plants total. Plants were placed in 5 L plastic bottles containing 4.5 L Long Ashton as described for the hydroponic solution. To each plastic bottle 2 g Bovine Serum Albumin (BSA) was added. Aeration was supplied via plastic tubing, this caused considerable bubbling of the BSA which possibly resulted in denaturing of the protein. The pH was adjusted using 0.0048 M citric acid and 1 M NaOH. Before the plants were placed in the bottles, 3 samples of 5 ml were taken from each of the 4 replicates for each pH (60 samples). The plants were left for eight hours after which 3 samples of 5 ml was again taken from each replicate (120 samples total). The plants were then divided into root and shoot, weighed, dried at 60 °C for 72 hrs and reweighed.

### *Statistical analysis*

Data was analysed using students t-test for unequal variances. Results are significantly different at  $p=0.05$ .

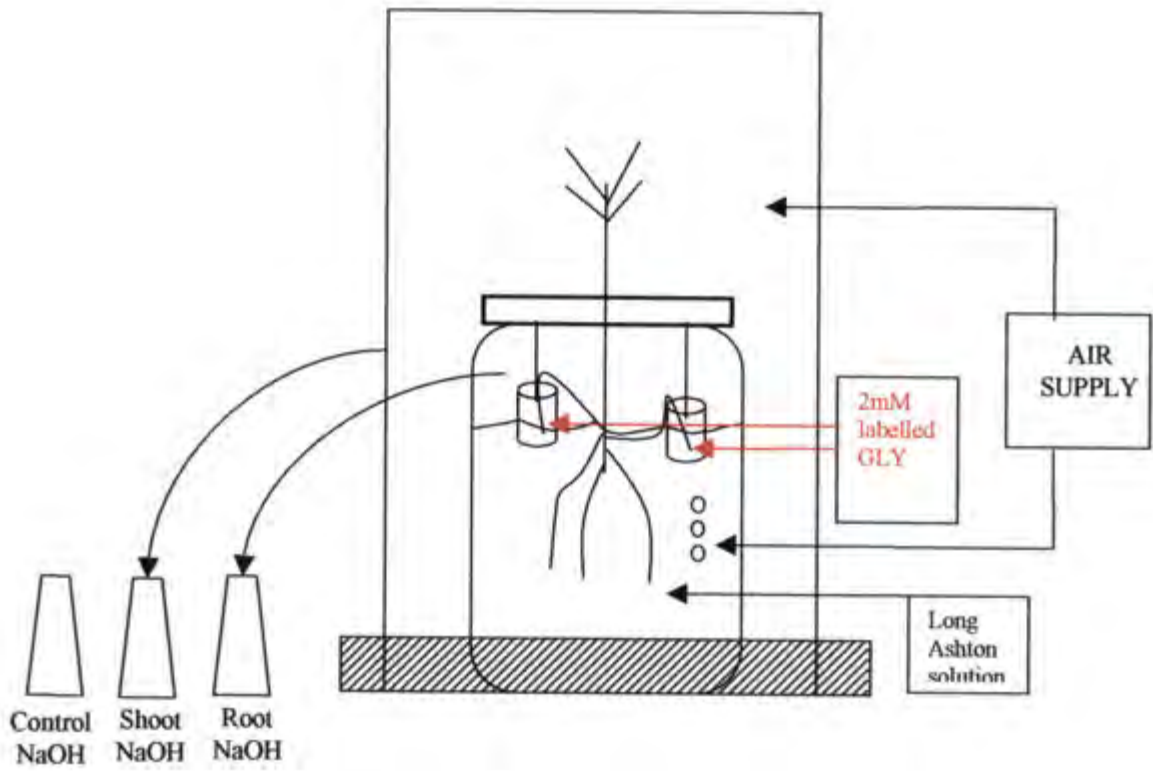


Figure 3. Schematic of respiration chamber in which feeding experiments were conducted.

## RESULTS

Cluster roots were observed to develop on lateral roots of *L. albus* within three weeks of transfer to hydroponics (six weeks old in total) and new clusters were observed to form basipetally. Cluster roots of *L. laureolum* took longer to form and were abundant on some plants within the same culture tank and not on others (Fig 1, 2).

### *Uptake of doubly-labelled GLY*

Figures 3 and 4 are representative schematics of the uptake and translocation of the doubly labelled GLY in *L. albus*; Figs 5 and 6 refer to *L. laureolum*. The larger black shaded arrow indicates that GLY was fed through cluster roots and the clear large arrow indicates non-cluster feeding.

Cluster and non-cluster roots of *L. laureolum* and *L. albus* take up doubly-labelled GLY. Cluster roots of *L. albus* and *L. laureolum* take up more  $^{13}\text{C}$  and  $^{15}\text{N}$  than non-cluster roots although this is not significant for *L. albus* ( $p < 0.5$  for  $^{15}\text{N}$  and  $p < 0.08$  for  $^{13}\text{C}$ ) or for *L. laureolum* ( $p < 0.7$  for  $^{13}\text{C}$  and  $p < 0.4$  for  $^{15}\text{N}$ ).

In cluster roots of *L. albus*  $49 \pm 3.6\%$  of the doubly-labelled GLY was taken up intact, and  $36 \pm 3.0\%$  was taken up intact in the non-cluster roots. In *L. laureolum*  $11.63 \pm 1.2\%$  of GLY was taken up intact in the cluster roots and  $13.9 \pm 0.3\%$  was taken up intact in the non-cluster roots. These percentages are calculated from the  $^{13}\text{C} : ^{15}\text{N}$  ratios (Fig 7). For example in *L. albus* cluster roots the  $^{13}\text{C} : ^{15}\text{N}$  is *ca* 0.5. This means that for every one C atom, there are 2 N atoms, therefore *ca* 50% of the doubly-labelled GLY was taken up intact.

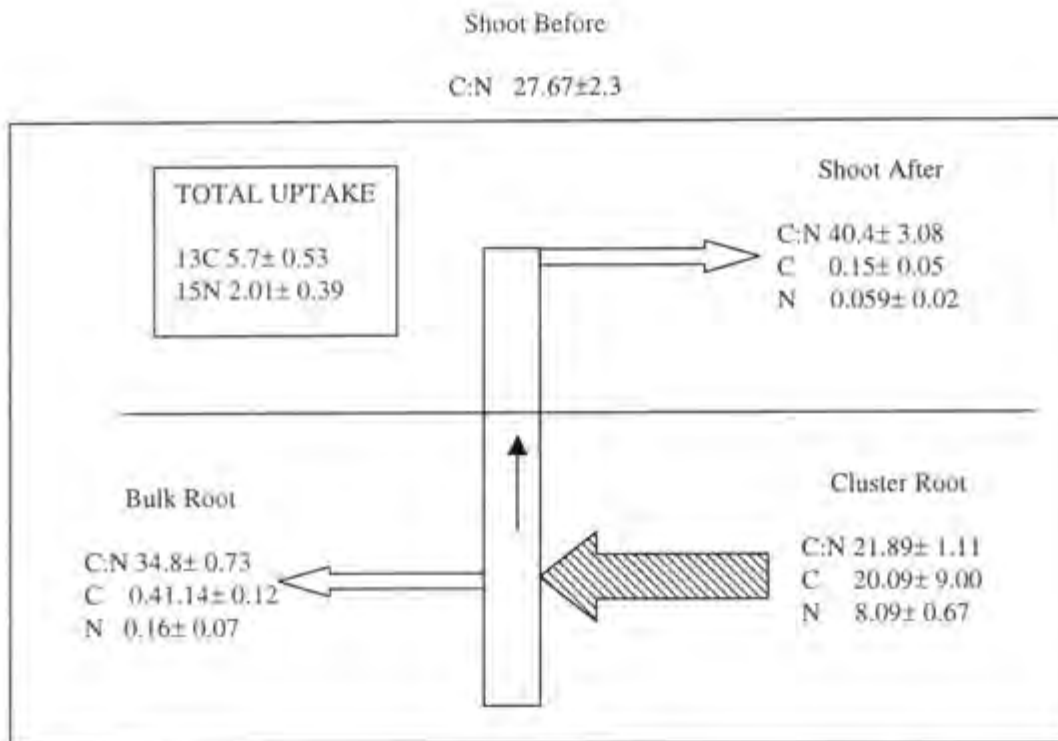


Figure 3. Representative summary of  $^{13}\text{C}$  and  $^{15}\text{N}$  uptake for cluster roots of *L. albus*. C= uptake of  $^{13}\text{C}$  in  $\mu\text{mol g}^{-1}$  dry wt  $\text{h}^{-1}$ . N= uptake of  $^{15}\text{N}$  in  $\mu\text{mol g}^{-1}$  dry wt  $\text{h}^{-1}$ . C:N= C:N ratio in plant portions. Total uptake is expressed as  $\mu\text{mol}$  per plant, n= 5. SE indicated.

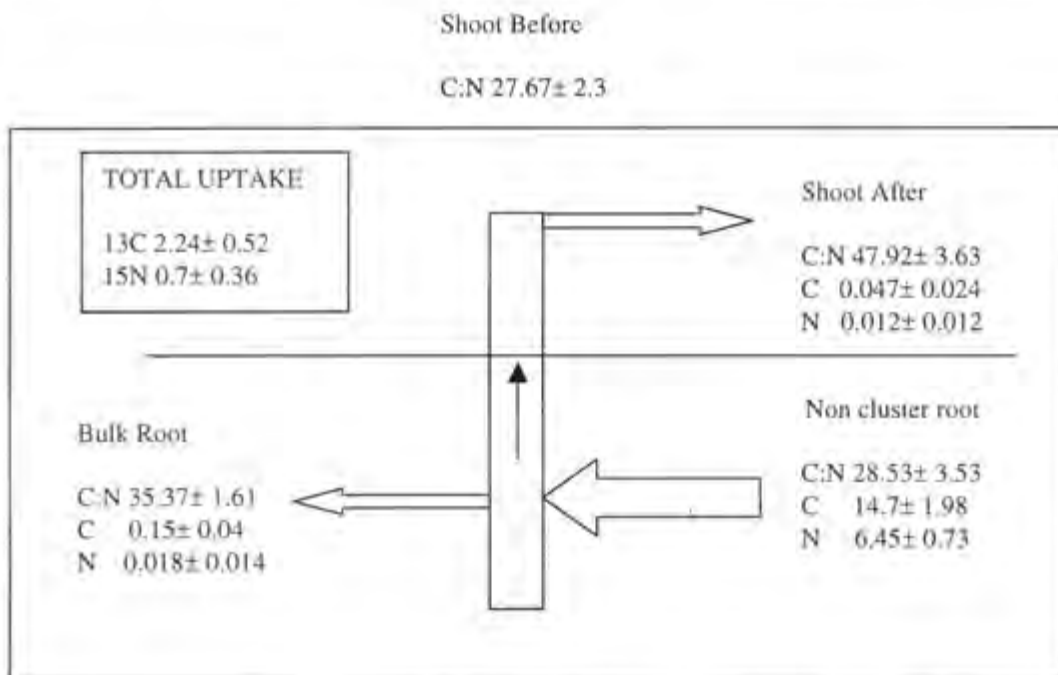


Figure 4. Representative summary of  $^{13}\text{C}$  and  $^{15}\text{N}$  uptake for non-cluster roots of *L. albus*. C= uptake of  $^{13}\text{C}$  in  $\mu\text{mol g}^{-1}$  dry wt  $\text{h}^{-1}$ . N= uptake of  $^{15}\text{N}$  in  $\mu\text{mol g}^{-1}$  dry wt  $\text{h}^{-1}$ . C:N= C to N ratio in plant portions. Total uptake is expressed as  $\mu\text{mol}$  per plant, n=4. SE indicated.

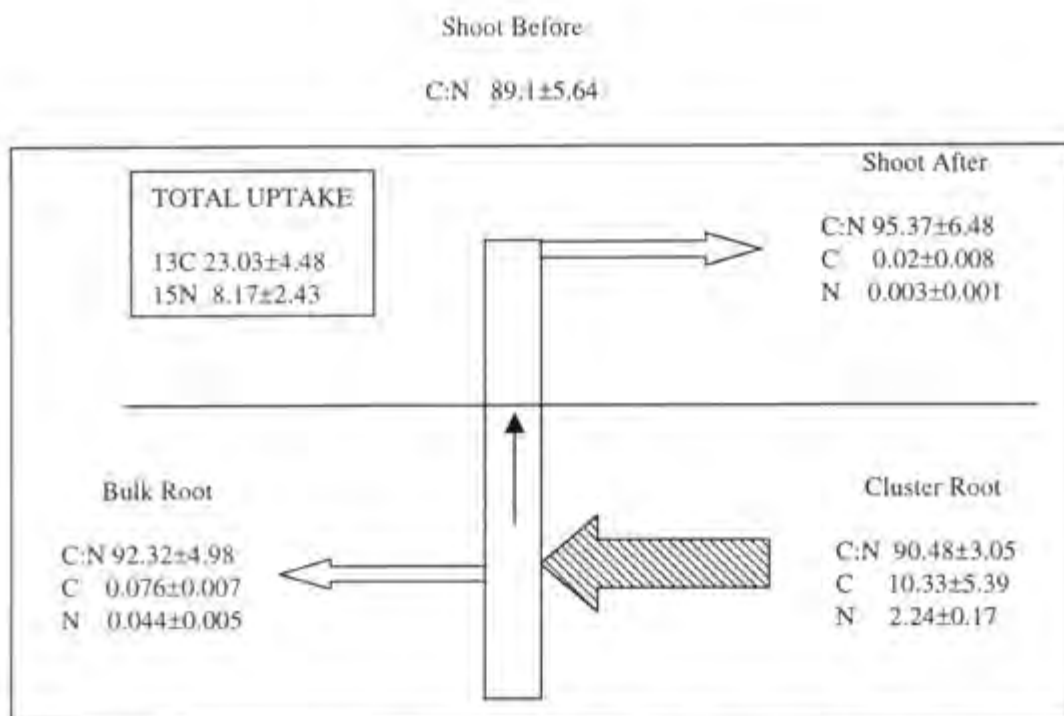


Figure 5. Representative summary of  $^{13}\text{C}$  and  $^{15}\text{N}$  uptake for cluster roots of *L. laureolum*. C= uptake of  $^{13}\text{C}$  in  $\mu\text{mol g}^{-1}$  dry wt  $\text{h}^{-1}$ . N= uptake of  $^{15}\text{N}$  in  $\mu\text{mol g}^{-1}$  dry wt  $\text{h}^{-1}$ . C:N= C to N ratio in plant portions. Total uptake is expressed as  $\mu\text{mol}$  per plant, n=3. SE indicated.

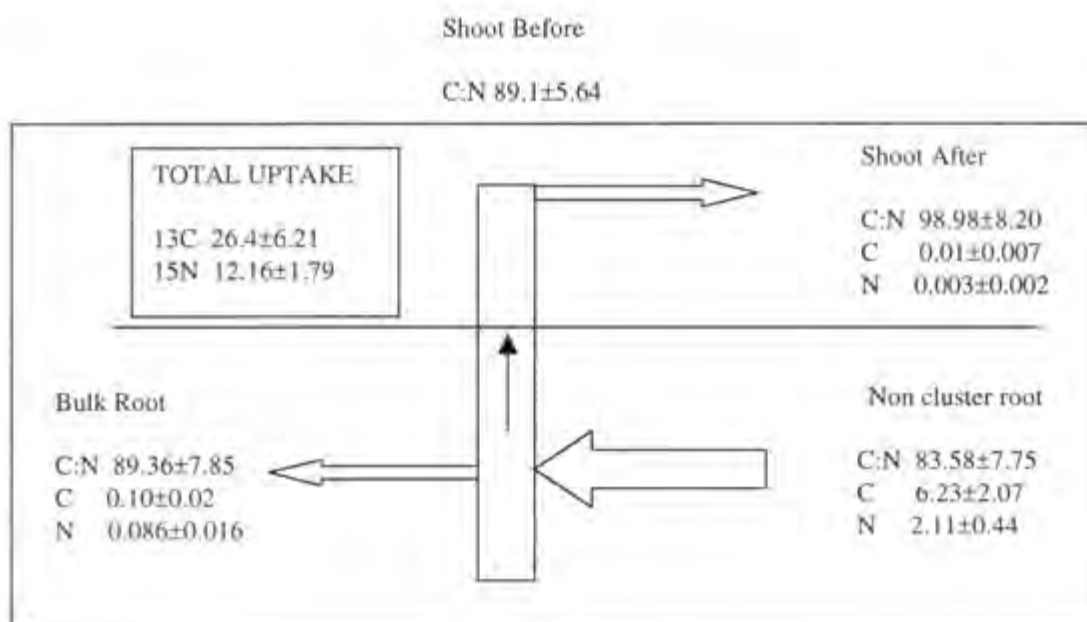


Figure 6. Representative summary of  $^{13}\text{C}$  and  $^{15}\text{N}$  uptake for non-cluster roots of *L. laureolum*. C= uptake of  $^{13}\text{C}$  in  $\mu\text{mol g}^{-1}$  dry wt  $\text{h}^{-1}$ . N= uptake of  $^{15}\text{N}$  in  $\mu\text{mol g}^{-1}$  dry wt  $\text{h}^{-1}$ . C:N= C to N ratio in plant portions. Total uptake is expressed as  $\mu\text{mol}$  per plant, n=3. SE indicated.

In *L. albus* 49±3.6 % of the glycine taken up is taken intact by cluster roots, and 36±3.0 % is taken up intact by non-cluster roots. This translates to 29.4±2.2 μmols doubly-labelled GLY taken up intact by cluster roots and 21.6± 1.8 μmols taken up intact by non-cluster roots (out of 60μmol doubly-labelled GLY supplied in the feeding experiments).

In *L. laureolum* 11.63±1.2 % of the total glycine taken up is taken up intact in cluster roots and 13.9±0.3 % by non-cluster roots. This translates to 7.0±0.72 μmol for cluster roots and 8.3±0.2 μmols for non-cluster taken up intact (out of 60μmol doubly-labelled GLY supplied in the feeding experiments).

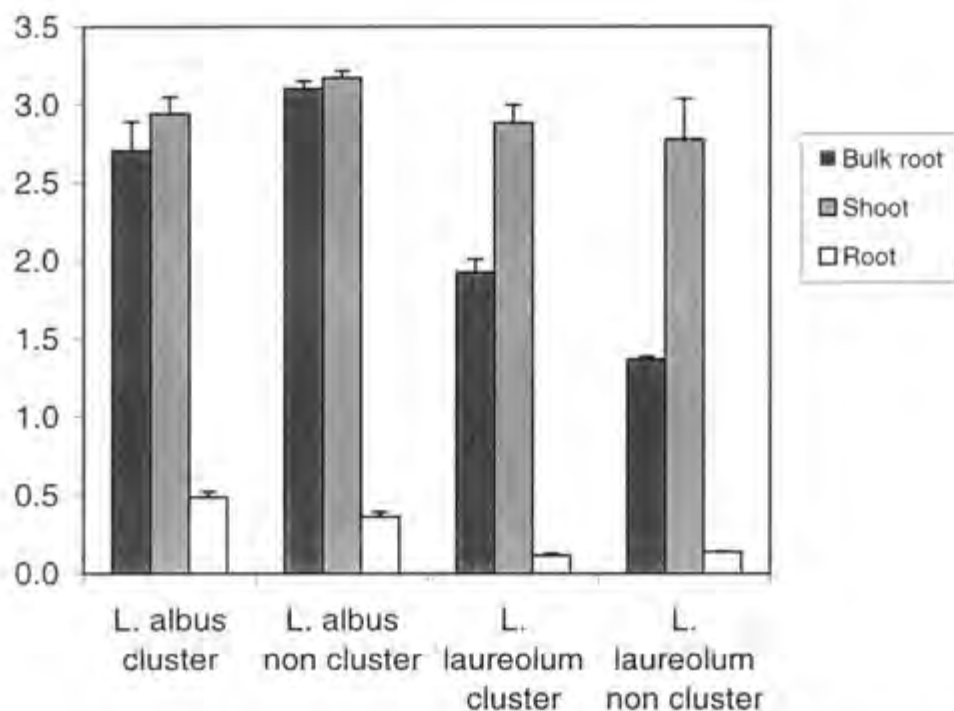
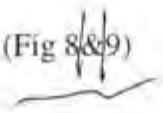


Figure 7. Average  $^{13}\text{C}$ :  $^{15}\text{N}$  ratios of bulk root, shoot and GLY-fed roots for *L. albus* and *L. laureolum*. SE indicated.

Translocation of assimilate (Fig 8&9)



In cluster roots of *L. albus* more  $^{15}\text{N}$  than  $^{13}\text{C}$  is translocated from the GLY-fed roots to the bulk root. In non-cluster roots, more  $^{13}\text{C}$  is translocated to the bulk root. There is little difference between  $^{13}\text{C}$  and  $^{15}\text{N}$  in the shoot of both cluster and non-cluster plants. Translocation of  $^{13}\text{C}$  and  $^{15}\text{N}$  to the bulk root and shoot is greater in cluster than in non-cluster roots (Fig 8).

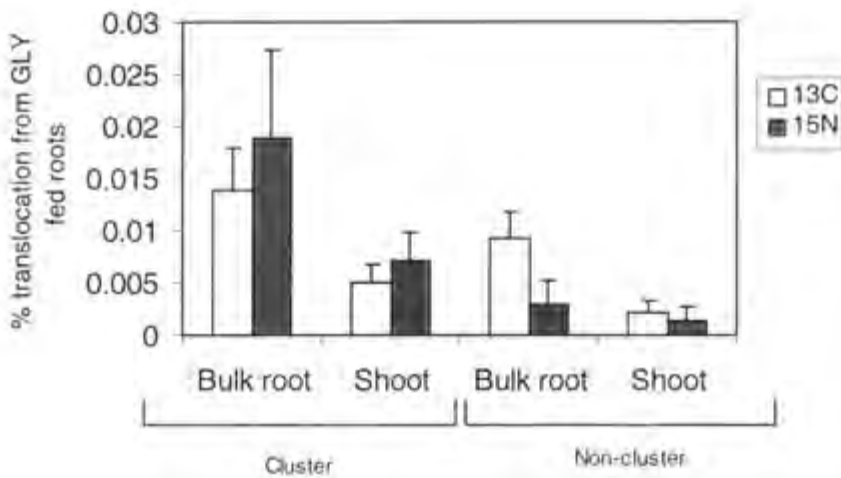


Figure 8. Translocation of  $^{13}\text{C}$  and  $^{15}\text{N}$  from GLY- fed cluster and non-cluster roots of *L. albus* to the root and shoot. Results are expressed as a % of total plant uptake rate. SE indicated.

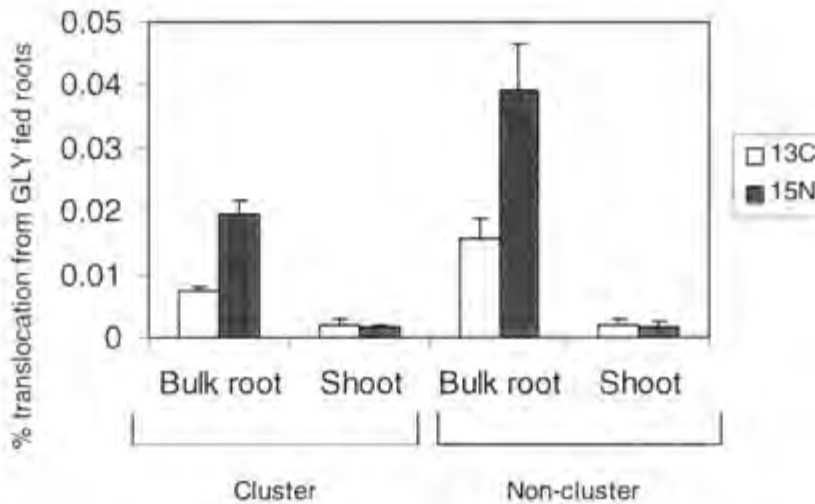


Figure 9. Translocation of  $^{13}\text{C}$  and  $^{15}\text{N}$  from GLY- fed cluster and non-cluster roots of *L. laureolum* to the root and shoot. Results are expressed as a % of total plant uptake rate. SE indicated.

There is the same general pattern of translocation in *L. laureolum* as in *L. albus* i.e.,  $^{15}\text{N}$  is translocated in greater amounts to the bulk root and there is little difference between  $^{13}\text{C}$  and  $^{15}\text{N}$  in the shoot. However in *L. laureolum* the non-cluster roots translocate greater amounts of  $^{13}\text{C}$  and  $^{15}\text{N}$  than the cluster roots. It is also interesting to note that there is a greater % difference between  $^{13}\text{C}$  and  $^{15}\text{N}$  in the bulk root of *L. albus* compared to *L. laureolum*.

#### *Protease Assay*

Results from the spectrophotometer indicated that in a number of samples there was considerably more BSA after the experiment than before, which obviously cannot be unless the solution was somehow concentrated. However the levels in the plastic bottle were not observed to decrease and the highly variable results could be a result of a number of experimental errors. Firstly there was a considerable amount of bubbling of the BSA caused by the aeration which could have caused denaturing of the BSA protein. Secondly the assay that was used is very sensitive and the results were obtained from a standard curve plotted at pH 6.5. The sensitivity of the assay to pH was not tested so it cannot be ascertained whether or not this was the reason for the variable results. The results cannot be used to infer anything about possible extracellular enzyme activity of *L. albus*, however they are presented in the index.

#### *Respiration rates (Fig 10)*

In both species average root respiration was greater than shoot respiration. In *L. albus* average root respiration ( $0.20 \pm 0.03 \text{ mmol g}^{-1} \text{ h}^{-1}$ ) is significantly higher than average shoot respiration ( $0.04 \pm 0.01 \text{ mmol g}^{-1} \text{ h}^{-1}$ ) ( $p < 0.0001$ ). Root respiration ( $0.03 \pm 0.004 \text{ mmol g}^{-1} \text{ h}^{-1}$ ) in *L. laureolum* is also significantly higher ( $p < 0.004$ ) than shoot respiration ( $0.15 \pm 0.002 \text{ mmol g}^{-1} \text{ h}^{-1}$ ).

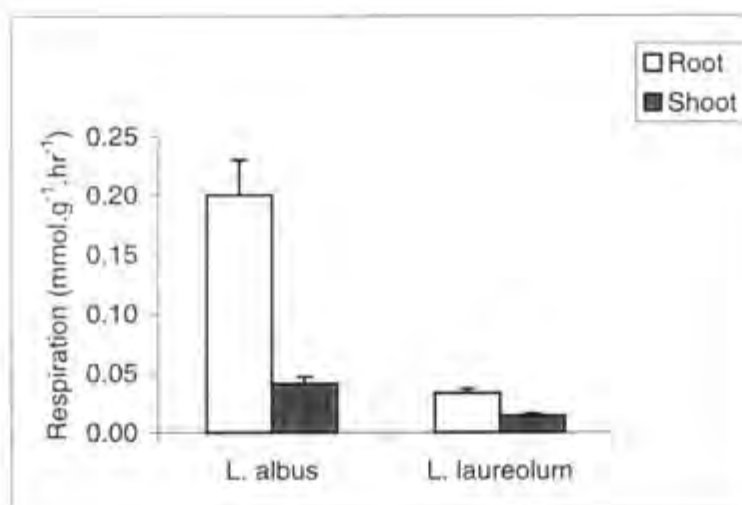


Figure 10. Average respiration rates of root and shoot of *L. albus* and *L. laureolum*. Rates are in  $\text{mmol g}^{-1} \text{h}^{-1}$ . In both species root respiration is higher than shoot respiration. SE indicated. For *L. laureolum* root and shoot  $n=5$ . *L. albus* root  $n=8$ , shoot  $n=6$ .

There was insufficient carbon collected in the NaOH loaded into the mass spec for it to obtain a signal, therefore the results are discarded. Therefore nothing can be said about the loss of GLY through respiration.

## DISCUSSION

Cluster roots occur predominately in the litter layer <sup>of soil</sup> (Purnell 1960; Lamont, 1984), where a large proportion of nutrients are present in organic form. This is not a developmental constraint as cluster roots have been stimulated to develop to depths of 25 cm if the organic layer is artificially created at lower depths (Lamont, 1993). Cluster roots are able to enhance P uptake through the production of exudates which solubilise P bound to metal ions. Recently the role of cluster roots in organic N acquisition has been investigated in *Hakea* species by Schmidt and Stewart (1999). They found that cluster roots take up more glycine than  $\text{NO}_3^-$  but that the contribution of cluster roots to amino acid uptake is small relative to uptake by the rest of the root. In the Schmidt and Stewart study, only the N atom was labelled, therefore the study did not elucidate whether or not glycine was taken up intact. In this study, doubly-labelled glycine is fed to cluster and non-cluster roots to investigate whether the glycine is taken up intact.

The results indicate that cluster and non-cluster roots of both *L. laureolum* and *L. albus* are able to take up glycine. Cluster roots for both species have slightly greater rates of  $^{13}\text{C}$  and  $^{15}\text{N}$  uptake but there is no significant difference between cluster and non-cluster uptake rates (Fig's 3-6). This suggests that cluster roots are not an adaptation of the Proteaceae to exploit organic N in the absence of mycorrhizae. Out of the total amount of glycine that was taken up by GLY-fed roots, ca 11% in cluster and 13% in non-cluster roots was taken up intact by *L. laureolum*. A higher percentage was taken up intact in cluster (49%) and non-cluster roots (36%) in *L. albus*, even though plants of *L. albus* were glycine-fed for four hours as opposed to 16 hours for *L. laureolum*. However lower uptake rates were expected in *L. laureolum* due to previously demonstrated low uptake rates of other Proteaceae species (Lewis and Stock, 1978). As there were problems with the running of the carbon samples for respiration, nothing can be said about the amount of  $^{13}\text{C}$  lost through respiration. However root respiration was significantly higher than shoot respiration for both species, therefore losses through respiration are likely to be high.

No explicit statements can be made about the form in which the rest of the GLY is translocated as no detailed studies on xylem composition were performed. However

from the percentage of total plant uptake (Figs 8 & 9), it can be seen that in *L. laureolum* the difference between  $^{13}\text{C}$  and  $^{15}\text{N}$  is greater in the root than in the shoot, for both cluster and non-cluster plants (Fig 9). This means that more  $^{15}\text{N}$  than  $^{13}\text{C}$  is translocated from the GLY-fed roots to the rest of the root. However in the shoot, there was little difference in the rate of  $^{13}\text{C}$  and  $^{15}\text{N}$  translocation. This suggests that as assimilatory compounds are translocated from the point of feeding to the rest of the root and shoot, some  $^{15}\text{N}$  is retained and that the compounds are translocated as organic acids. This is supported by the C:N ratio in the different plant parts. There was a general trend for the C:N ratio to increase from the GLY-fed roots, to the bulk roots and the shoot.

There was a similar pattern in *L. albus*, i.e.,  $^{15}\text{N}$  is retained in the glycine-fed roots and bulk root as the glycine and assimilatory products are transported to the shoots, except in the non-cluster roots, the trend was reversed and more  $^{13}\text{C}$  was translocated to the bulk root. However there is little difference in the shoot of cluster and non-cluster plants (Fig 8). The difference between the percentage  $^{13}\text{C}$  and  $^{15}\text{N}$  translocated to the bulk root and shoot is not as marked as it is in *L. laureolum*. This suggests that there are different assimilatory products for *L. albus* and *L. laureolum*. Another illustration of this can be seen in Fig 7, the  $^{13}\text{C} : ^{15}\text{N}$  ratios. There is little difference between the ratio of  $^{13}\text{C}$  and  $^{15}\text{N}$  in the bulk root and shoot for both cluster and non-cluster roots of *L. albus*, however the <sup>ir</sup> difference is relatively greater in *L. laureolum*, supporting the suggestion that there are different assimilatory products of doubly-labelled glycine in *L. albus* and *L. laureolum*.

Voorste and Jooste (1986) found that the translocation of  $^{32}\text{P}$  to the shoots was higher in non-cluster roots than cluster roots, even though the opposite was true for the uptake in the roots i.e., cluster roots had a higher uptake of higher  $^{32}\text{P}$ . In *L. laureolum* there is no difference between  $^{13}\text{C}$  and  $^{15}\text{N}$  translocation to the shoot in cluster and non-cluster roots. However non-cluster roots have a higher translocation of  $^{13}\text{C}$  and  $^{15}\text{N}$  to the bulk root than cluster roots. Therefore it would appear that the same patterns of translocation exist for P, N and C.

Cluster roots of both *L. laurosum* and *L. albus* do not appear to be especially adapted for the uptake of amino acids as there is no significant difference between uptake of GLY in cluster and non-cluster roots. The results of this <sup>study</sup> are surprising as Proteaceae are a major family in the Fynbos and can compose the dominate vegetation. Proteaceae species are non-mycorrhizal (Allsopp and Stock, 1993) therefore their continued success in the Fynbos is perplexing if they do not have any alternative adaptation facilitating the exploitation of nutrients in an environment that is in part characterized by its severe growing environment. As the ratio of organic nutrients to mineral nutrients can be relatively high, it would be expected that Proteaceae species have some form of mechanism to exploit this supply of resources, especially since it is hypothesized that 73% of the rest of the Cape Floristic Region have some form of mycorrhizal association (Allsopp and Stock, 1993).

→ Meaning

Part of the explanation may be physiological, members of the Proteaceae are adapted to a low nutrient environment. Proteaceae species may not be able to exploit these additional resources as they typically have low concentrations of translocatory compounds (Lewis and Stock, 1978). Thus the limit to amino acid uptake may not be at the level of the cluster root but at the level of the whole plant. Plants growing in low nutrient environments usually have low uptake capacities (low  $K_m$  and  $I_{max}$ ) (Neumann *et al.*, 1999). *L. albus* plants grown in a P-deficient environment have a lower  $K_m$  than P sufficient plants (Neumann *et al.*, 1999). However in a P-deficient environment cluster roots have a higher  $V_{max}$  than non-cluster roots, suggesting a higher concentration of transporters <sup>r</sup> in cluster roots (Schachtman *et al.*, 1998) which means that as nutrients become more limiting, the cluster root adapts for more efficient uptake. If this is true for cluster roots of Proteaceae, then it is likely that the uptake of amino acids is not limited by the cluster roots, but by the internal regulation of the whole plant.

There may be other external factors that influence the uptake of organic N, namely the microbial component in the soil. Owen and Jones (2001) demonstrated that wheat roots were only able to take up 6% of added amino acids and that they were outcompeted by the microbial biomass. It is generally thought that low mineralization rates are a major factor stimulating the uptake of organic nutrients by plants. There is

increasing evidence to show that low mineralization rates are not a prerequisite for the uptake of organic N (Falkengren-Grerup *et al.*, 2000). This study demonstrates that low mineralization rates do not necessarily mean high uptake of organic N. Therefore low mineralization rates in the Fynbos (Stock *et al.*, 1998) may not be a factor in uptake of organic N in this system. However there is a negative correlation between soil acidity and uptake of amino acids (Falkengren-Grerup *et al.*, 2000). As soils of the Fynbos are typically acidic, this may be a factor influencing the potential uptake of amino acids.

To conclude, it has been demonstrated that cluster and non-cluster roots of *L. albus* and *L. lauroolum* are able to take up glycine intact. There is no significant difference between cluster and non-cluster uptake, therefore it is unlikely that cluster roots are an adaptation to exploit organic nutrients in the soil. However cluster root formation seems to be more linked with P nutritional status of the plant. The assumption of this study is that nitrogen is limiting, it could be that clusters roots are an adaptation for phosphorus nutrition.

## ACKNOWLEDGEMENTS

I am deeply indebted to Dr. Heidi Hawkins for her continual input throughout all stages of this study. I thank my supervisor Prof Stock for his valued guidance and review of the document. I am grateful to Des Barnes for his prompt help in the growth room and to Gonzalo Aguilar for keeping the computers going.

## APPENDIX

Table A1. Percentage decrease of BSA. Note that negative values indicate % increase from at the end of the experiment.

pH	% decrease	pH	% decrease	pH	% decrease	pH	% decrease
2a	73.76543	3a	-52.4852	4a	-91.7466	5a	-31.3169
2a	78.59853	3a	-250.535	4a	-1.91721	5a	33.21597
2a	-11.4683	3a	36.82021	4a	14.96036	5a	-24.4106
2b	32.29366	3b	-568.274	4b	64.98719	5b	44.21481
2b	-476.966	3b	-4071.19	4b	48.54434	5b	37.82734
2b	-337.767	3b	-142.493	4b	-57.7682	5b	-29.7634
2c	-105.205	3c	-42.6151	4c	12.76846	5c	49.60564
2c	-30.9425	3c	-326.359	4c	35.3028	5c	-115.141
2d	-48.1402	3c	-131.737	4d	-81.4259		
2d	49.24895	3d	38.30128	4d	51.91138		
2d	34.7226	3d	59.64045				
		3d	-26.841				

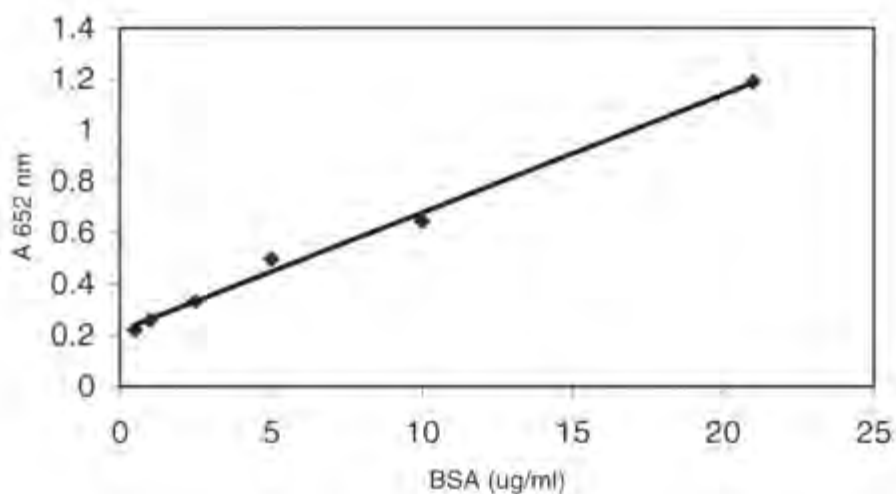


Figure A1. Standard curve for BSA assay plotted at pH 6.5

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