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Root exudates: a pathway for short-term N transfer from clover and ryegrass

Florence Paynel[1], Philip J Murray[2] & Jean Bernard Cliquet[1,*]
[1]*UA INRA 950 de Physiologie et Biochimie Végétales, IRBA, Université de Caen, 14032 CAEN Cedex, France.* [2]*Institute of Grassland and Environmental Research, North Wyke Research Station, Okehampton, Devon, EX20 2SB, UK* [3]*Correspondence author**

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Abstract

The short-term transfer of nitrogen (N) from legumes to grasses was investigated in two laboratory studies. One study was done in pots where the roots of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) were allowed to co-exist, and a second study was performed using a micro-lysimeter system designed to maintain nutrient flow from the clover to the grass, whilst removing direct contact between the root systems. The ^{15}N-dilution technique was used to quantify the transfer of N between species. Levels of ammonia and amino acids were measured in root exudates. The amounts of N transferred were in the same order of magnitude in both the pot and micro-lysimeter experiments. In the micro-lysimeter experiment, 0.076 mg of N were transferred per plant from clover to ryegrass during the course of the experiment. Ammonium exudation was much higher than amino acid exudation. The most abundant amino acids in both clover and ryegrass root exudates were serine and glycine. However, there was no correlation between the free amino acid profile of root extracts and exudates for both plant species: Asparagine was the major amino acid in clover roots, while glutamine, glutamate and aspartate were the major amino acids in ryegrass roots. Comparison of exudates obtained from plants grown in non-sterile or axenic conditions provides evidence of plant origin of ammonium, serine and glycine.

Introduction

One of the key factors underpinning many low-input grassland systems is the reliance on nitrogen fixing legumes to provide much of the N in the system. Such mixed cropping is a widespread practice in both temperate and tropical climates of the world (e.g. Fujita et al., 1992; Ledgard and Steele, 1992). In Europe, mixtures of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) dominate pastures allowing maximum benefit to be made of natural resources available for production. In such grassland systems, it has been shown that part of the grass nitrogen is derived from atmospheric N fixation by the clover, and subsequent transfer to the grass (Giller et al., 1991; Johansen and Jensen, 1996). Because of increasing economic and environmental pressure, study of direct interspecific nitrogen transfer has received increasing attention in the last decade (Elgersma et al., 2000; Høgh-Jensen and Schjoerring, 1997; Jones and Darrah, 1993).

The flow of N between plants occurs mainly below ground by several means. Decomposition of donor plant debris (roots, nodules or leaves) or sloughing off of cortex cells and uptake of the resulting mineralised N by the receiver plant (Dubach and Russelle, 1994; Johansen and Jensen, 1996; Russelle et al., 1994) accounts for much of the long-term transfer (year-on-year). Short-term transfer can occur directly through arbuscular-mycorrhizal fungi interconnecting the root systems of the two plant species. The extra-radical hyphae have been shown to offer a direct route for N (Bethlenfalvay et al., 1991; Haystead et al., 1988),

* FAX No: +33 0231 565 360. TEL No: +33 0231565302.
E-mail: cliquet@ibba.unicaen.fr

C (Simard et al., 1997) and P (Francis et al., 1986; Johansen et al., 1993). Another mechanism of inter-plant short-term N transfer involves exudation of N compounds into the rhizosphere by the donor plant and re-absorption of these compounds by the receiver plant.

Most exudation studies have been performed in artificial sterile media or solution culture which facilitates the collection and analysis of N release from the roots (Jones and Darrah, 1994; Shepherd and Davis, 1994a; Svenningson et al., 1990). Ammonium, amino acids, ureides, peptides and proteins have been identified in leachates of legumes (Brophy and Heichel, 1989; Ta et al., 1986), crucifers (Shepherd and Davis, 1994a, b) and grasses (Jones and Darrah, 1994; Klein et al., 1988).

The ^{15}N-dilution method has been used to quantify N transfer between plants and has given highly variable results. The amount of N transfer is dependent on plant species, plant age and the physiochemical and biological environment (Fujita et al., 1992). Published methods allow the quantification of N exudates (Jones and Darrah, 1994; Shepherd and Davis, 1994b), but none have been developed to quantify exudation and transfer of N between plants in the same experiments.

Two experiments were designed to determine N transfer, one in pots where the roots of the plants were allowed to co-exist and a second in a micro-lysimeter system which was designed to remove direct contact between root systems and enable collection of exudates. This system was used to estimate N transfer through the soil solution and to identify the main compounds involved.

Materials and methods

Pot experiment

Seeds of perennial ryegrass (*Lolium perenne* L.) cv Bravo and white clover (*Triflolium repens* L.) cv Grasslands Huia were surface sterilized with sodium hypochlorite solution, rinsed 3 times and germinated in Petri-dishes containing demineralised water. Ryegrass seeds were germinated 3 weeks later than clover to achieve comparable size plants. When the clover seedlings were transplanted, they were inoculated with *Rhizobium trifolii*. Two weeks after germination, four plants of each species were transferred together in a matrix into 600 mL pots containing 1 kg of sterilized fine sand; in addition, pots of ryegrass were grown without clover to act as controls. The plants were supplied with a nutrient solution (Laine et al., 1994) containing either 0.1, 1 or 2 mM $^{15}NH_4{}^{15}NO_3$ 5 atom%. Fifty mL of the appropriate nutrient solution was given to each pot each week. Five replicate pots for each treatment and each of the two harvest dates were established. Pots were kept in a controlled environment room at 20/15 °C day/night with a 16 h photoperiod with a PAR of 200 μmol m^{-2} s^{-1}. Plants were harvested 50 d and 69 d after ryegrass transplantation. The plants were removed from the sand, separated into roots and shoots, freeze-dried, weighed and ground. Total N and ^{15}N were determined by Isotope Ratio Mass Spectrometry (IR-MS, Europa Scientific, Crewe, UK).

Calculations

The ^{15}N isotope dilution technique allows determination of the percentage of N in the plant parts derived from the atmosphere %Ndfa (MacAuliffe et al., 1958; Zanetti et al., 1996):

$$\%\text{Ndfa} = 1 - (\%^{15}\text{N atom excess in clover} / \%^{15}\text{N atom excess in grass alone}) \times 100$$

This equation has been used to calculate the amounts of N derived from the atmosphere in both clover and grass. The proportion of N (%NT) in the grass originating from clover takes into account the ^{15}N content of the donor clover plant and was calculated as described by Ta et al. (1989) and Ledgard and Steele (1992):

$$\%\text{NT} = (\%^{15}\text{N grass in mixture} - \%^{15}\text{N grass alone}) / (\%^{15}\text{N clover} - \%^{15}\text{N grass alone}).$$

Micro-lysimeter experiment

Seedlings were prepared as in the pot experiment and were transferred to individual micro-lysimeters after 2 weeks. The micro-lysimeters were constructed from 60 mL volume syringe barrels containing 100 g of sterilized sand as the growth medium. Ten replicate sets of lysimeters were set up. The micro-lysimeter systems were kept under the same environmental conditions as in the previous pot experiment. The micro-lysimeters were connected to allow flow of nutrient solution and exudates from the lysimeter containing clover (A) to one containing ryegrass (B) (Figure 1). Ten mL of sterile (autoclaved) nutrient solution containing 0.5 mM $K^{15}NO_3$ 5 atom% was added to the clover plant in micro-lysimeter A three times per week (days

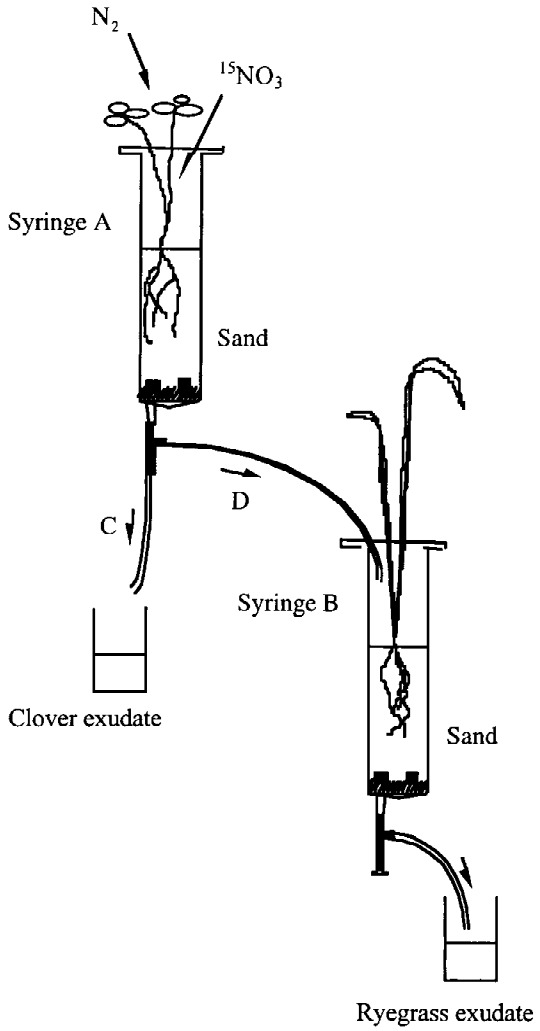

Figure 1. The micro-lysimeter system which was designed to allow quantification of compounds released and transferred by the donor plant (white clover, Syringe A) to the receiver plant (ryegrass, Syringe B). Ten mL of nutrient solution are supplied to the white clover, one half of white clover exudate is collected for analysis (C), and one half is supplied to the receiver ryegrass (D).

1, 3 and 5). Half of the leachate containing nutrient solution and exudates from micro-lysimeter A went to micro-lysimeter B and half was collected for analysis. Exudates were collected from micro-lysimeter B twice a week by adding 10 mL of sterilised ultrapure water. Six control ryegrass plants were fed three times per week with 10 mL of the same nutrient solution to measure ^{15}N uptake by ryegrass alone, and the leachate were collected for exudate analysis.

Exudate samples from two independent plants and from the different collection of the same week were bulked together before analysis. The exudate samples were frozen immediately after collection, freeze dried, redissolved in 1.5 mL of distilled water and filtered through a 0.45 μm filter. The amino acids were quantified by HPLC as *o*-phthaldialdehyde derivatives on a C-18 column using Gold System 8.0 (Beckmann Instruments, San Ramon, Ca, USA) as described in Murray et al. (1996). Ammonium content was measured using a continuous-flow analyser (Bran+Luebbe, Noderstedt, Germany). The buffered solution containing ammonium was reacted with salycilate 8% (w/v) and dichloro-isocyanurate 0.18% (w/v). Plants were removed from the sand 46 d after ryegrass germination and analysed for ^{15}N as described in the previous section.

A second micro-lysimeter experiment was designed to determine the effect of micro-organisms on ammonium and amino acid collection in the micro-lysimeters. The method for growing ryegrass and nodulated clover was based on procedures described by Benizri et al. (1995) with some modifications. The plant tops were allowed to grow in normal atmosphere and the roots were grown in a sterile sand culture. Seeds were surface sterilised by soaking them for 30 s in 95% H_2SO_4, 5 min in 95% ethanol and 30 min in 10% H_2O_2, and then rinsed thoroughly in sterile distilled water. After 2 weeks of germination in Petri dishes, they were aseptically introduced in sterile micro-lysimeters. These lysimeters containing the sand were autoclaved before use, and a sterile cotton strip was placed on the sand to separate the root and shoot compartments (Benizri et al., 1995), allowing shoot development without any constraint. Exudates were collected in tube A as described above, except that the sterile nutrient solution was added through a polyethylene tube, in a laminar air-flow cabinet.

An aliquot (200 μl) of the exudate was used to obtained a visual check of the bacterial population present in the micro-lysimeters by culturing them on a nutrient agar 1.5% plate (diameter = 9 cm). The Petri dishes were examined after 4 days, no bacteria were found in the sterile exudate, while the number of Colony Forming Units reached 10^4 . mL^{-1} exudate for the non-sterile treatment.

Statistical analysis

Levels of significance of differences between different treatments were determined by using the *F*-test of the analysis of variance and standard errors are shown

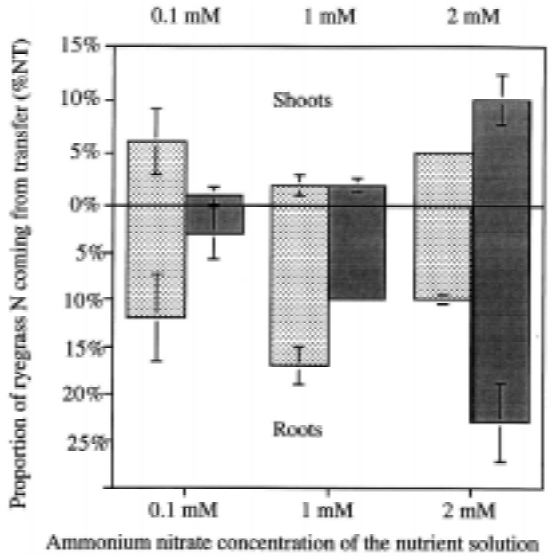

Figure 2. Effect of nitrogen content of the nutrient solution on proportion of ryegrass N originating from white clover in the pot experiment (cf 'Materials and methods'). (□), 50 days after ryegrass transplantation, (▩) 69 days after ryegrass transplantation. (± se, *n*=5).

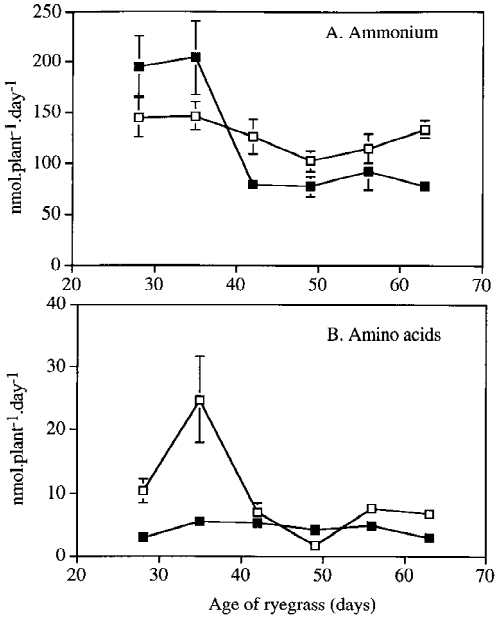

Figure 3. Time-course of ammonium (A) and total amino acids (B) exudation from white clover (□) and control ryegrass (■). White clover plants were 2 weeks older than ryegrass plants (cf 'Materials and methods'). Exudates were collected three times a week and the results are expressed as nmols per plant per day (± se, *n*=5).

on graphs. Only significant differences ($P < 0.05$) are discussed.

Results

At the first harvest of the pot experiment, 64 days after ryegrass germination (i.e. 50 days after transplantation), the proportion of N in ryegrass which was transferred from white clover was not influenced by the nitrogen content of the nutrient solution (Figure 2). However, at the second harvest, this proportion increased with the nitrogen content of the nutrient solution, in both roots and shoots: from 3% to 23% of N in the ryegrass roots originated from transfer from the legume for 0.1 mM and 2 mM NH_4NO_3 treatments, respectively. Although the %N derived from the atmosphere in clover roots declined from 92% for the 0.1 mM treatment to 33% for the 2 mM treatment, the amount of N in grass comming from transfer increased from 0.01 mg for the 0.1 mM treatment to 0.10 mg for the 1 mM treatment and 1.58 mg for the 2 mM treatment. In both harvests, the proportion of N coming from transfer was found to be higher in ryegrass roots than shoots (Figure 2).

In the micro-lysimeter experiment, 76 μg of N were transferred per plant from clover to ryegrass during the course of the experiment. Figure 3 shows the amount of ammonium (A) and amino acids (B) exuded by clover and control ryegrass fed with a nutrient solution containing 0.5 mM $K^{15}NO_3$. Clover plants were 2 weeks older than ryegrass (cf 'Materials and methods'). Exudation of ammonium expressed on a plant and daily basis by clover showed only small variation during the course of the experiment and averaged 129 nmol plant^{-1} d^{-1}. Ammonium exudation by ryegrass decreased between 35 and 42 days after germination from 204 to 78 nmol plant^{-1} d^{-1}. This exudation was similar in the sterile micro-lysimeters and averaged 135 nmol plant^{-1} d^{-1}for clover and 131 for ryegrass. Amino acid exudation was at much lower levels, between 3 and 5.5 nmol plant^{-1} d^{-1} for ryegrass and peaking at 25 nmol plant^{-1} d^{-1} for white clover at the second harvest, i.e. for white clover 35 d old. This peak of exudation was mainly due to a peak of GLY exudation.

Throughout the experiment, the amount of ammonium exuded by the ryegrass fed with white clover exudates (receiver ryegrass) exceeded that of the ammonium provided by these exudates (Figure 4A). This amount was highest at the first harvest and decreased with time. No differences were seen between ryegrass fed directly with the nutrient solution (Figure 3) and

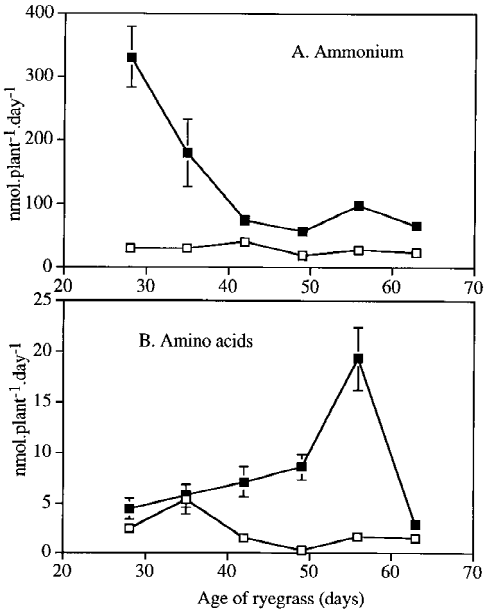

Figure 4. Comparison of amounts of ammonium (A) and total amino acids (B) collected from white clover exudate and fed to receiver ryegrass (□) and amounts exuded from receiver ryegrass (■). White clover plants were 2 weeks older than ryegrass plants (cf 'Materials and methods'). Exudates were collected three times a week and results expressed as nmols per plant per day (± se, *n*=5).

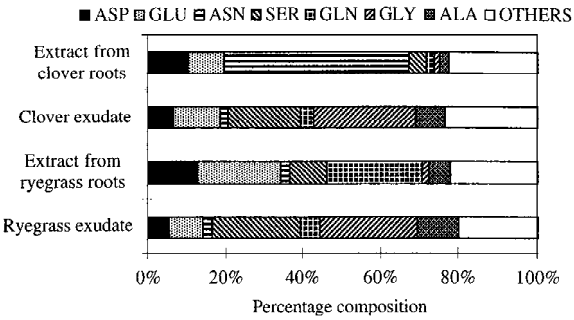

Figure 5. Amino acid profiles of root extracts and exudate of white clover and ryegrass. Extracts were analysed from 63 day old ryegrass and 77 day old white clover and the exudate represents the average of exudates collected all along the experiment from five independent replicates.

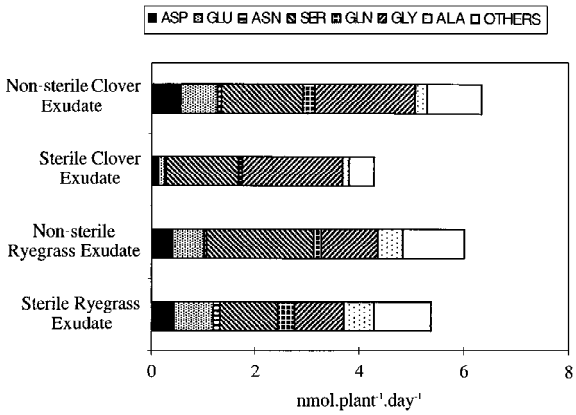

Figure 6. Comparison of amino acid composition of exudate of white clover and ryegrass grown in axenic conditions or not. Exudates were analysed from 39 day old ryegrass and 67 day old white clover and the exudate represents the average of exudates throughout the experiment from five independent replicates.

receiver ryegrass (Figure 4), except at the first harvest. The amount of amino acids exuded by receiver ryegrass increased up to 19.3 nmol plant^{-1} d^{-1} 56 d after germination and is similar to ryegrass supplied with the nutrient solution on the other dates.

The most abundant amino acids in both clover and ryegrass root exudates were SER and GLY, and there was no correlation between the amino acid profile of root extracts and exudates for both plant species (Figure 5). ASN were the major amino acids in clover roots, while GLN and GLU were the major amino acids in ryegrass roots. The same amounts and profiles of amino acids were recovered from non-sterile and sterile micro-lysimeters containing ryegrass (Figure 6). In contrast, slightly more amino acids were analysed in exudates obtained from clover grown in non-sterile conditions compared to clover grown in axenic conditions (6.3 and 4.3 nmol plant^{-1}. d^{-1}, respectively, $p < 0.01$). This was due to higher amounts of GLU and ASP in non-sterile micro-lysimeters. Exudation of SER and GLY was similar in both axenic and non-sterile treatments. Amino acids in the nutrient solution were also analysed to check for any chemical or biological contamination and were too low to be quantified.

Figure 7 shows the six major amino acids provided by clover to ryegrass and exuded by receiver ryegrass. The proportion and amount of the different amino acids were highly variable with the age of the plants. Large amounts of GLY were measured in clover and ryegrass exudates from young plants, while large amounts of SER were measured in exudates from older ryegrass plants (Figure 7). The pattern of exudation of ALA and ASP mimiced that of SER and also rose until 56 d after germination and then declined sharply.

Discussion

The ^{15}N dilution method has been used by several workers to quantify N transfer from legumes to grasses

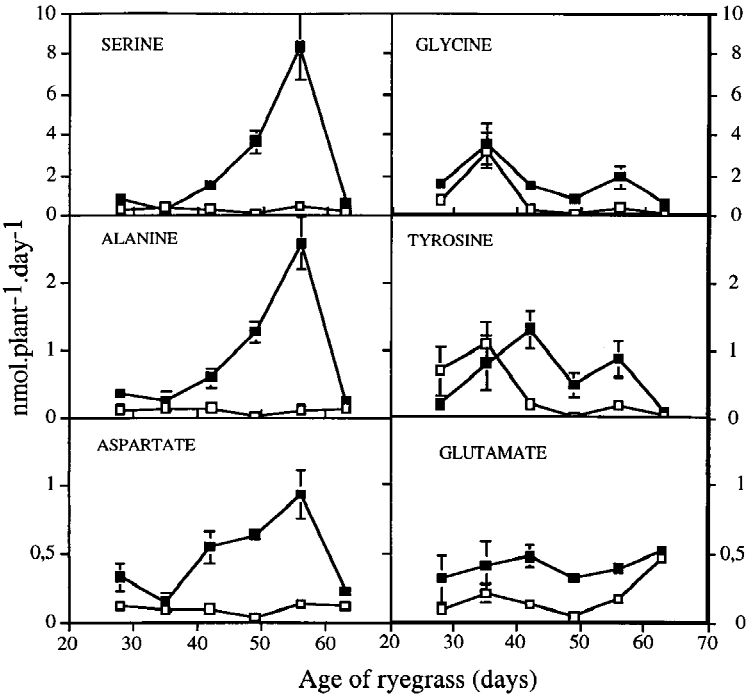

Figure 7. Comparisons on the amounts of major amino acids collected from white clover exudate and fed to receiver ryegrass (□) and exuded from receiver ryegrass (■). White clover plants were 2 weeks older than ryegrass plants (cf 'Materials and methods'). Exudates were collected three times a week and results expressed as nmols per plant per day (± se, n=5).

(for reviews see Fujita et al., 1992; Ta et al., 1989). This method allows the calculation of N transferred to be expressed in absolute amounts (in μg or mg) or relative to N in legume donor plant or in grass receiver plants (Hamel et al., 1991; Johansen and Jensen, 1996; Laidlaw et al., 1996). The estimation of the amount of N derived from the atmosphere into ryegrass takes into account the ^{15}N content of the atmosphere (Høgh-Jensen and Schjoerring, 1994, 1997; Zanetti et al., 1997), while the estimation of the amount of N derived from transfer into grass uses the ^{15}N percentage of the legume donor plant (Brophy and Heichel, 1989; Giller et al., 1991; Johansen and Jensen, 1996). We have used this last parameter to study transfer of N independently of the N fixation. The 1.58 mg N per plant in ryegrass originating from transfer in the 2 mM treatment constitutes 15% of ryegrass total N and 30% of clover total N. Long term studies in the field have produced results in the same order of magnitude for older plants (Høgh-Jensen and Schjoerring, 1997; Soussana and Hartwig, 1996).

Although, as expected (Zanetti et al., 1996; Høgh-Jensen and Schjoerring, 1994), white clover derived less N from fixation in our study for the high N treatment, we measured the highest amounts of atmospheric N (or N from transfer) in the companion ryegrass in this treatment. The high proportion observed with the 2 mM treatment could be due to higher N exudation by clover or to the stimulative effect of N on the growth of ryegrass root, bringing about a better exploration of soil volume, and a higher uptake of N released by clover. The so-called 'priming effect' i.e. the stimulative effect of N addition on the uptake of the soil N has been observed previously (Leon et al., 1995). Similar effects of N fertilisation on N transfer from clover to ryegrass have been obtained by Høgh-Jensen and Schjoerring (1997) with 1 year old plants, but not recovered with older plants, and Zanetti et al. (1997) observed with older clover and ryegrass plants that increasing N input results in a decreased N transfer.

Release of N by the legume donor plant implies death and decay of nodules and roots (Laidlaw et al., 1996) or exudation of N compounds from legume roots (Ta et al., 1986). The micro-lysimeter system, adapted from Shepherd and Davies (1994a), was designed to measure these N-compounds. The amount of N transferred from clover to ryegrass is in the same

order of magnitude in both experiments (76 μg per plant in the micro-lysimeter system compared with 57 μg for the same nutrient solution in pots). This suggests that exudation of N compounds into the soil by legume donor plants followed uptake by companion grass could account fully for estimates of transfer in such young plants, where death of legume organs is unlikely to occur significantly.

Ammonium and amino acids were the major N compounds released by both white clover and ryegrass in this study. Proteins and peptides have been measured but were too low to be quantified accurately. This is in contrast with studies concerning nitrogen release from roots of alfalfa and soybean (Brophy and Heichel, 1989; Ofusu-Budu et al., 1990). One explanation for this could be that exudates were filtered before analysis in the present experiment, allowing determination of soluble N compounds, but not of N included in cell debris. However, a similar experiment where clover exudates were not filtered has given similar results (data not shown).

The high proportion of NH_4^+ exuded by both species, compared to amino acid exudation is in agreement with results from soybean (Ofusu-Budu et al., 1990) and alfalfa (Brophy and Heichel, 1989). However, this is in contrast to other work on alfalfa (Ta et al., 1986). On average, ammonium exudation was similar in clover and ryegrass (129$\pm$30 nmol plant^{-1} d^{-1} and 122$\pm$29 nmol plant^{-1} d^{-1}, respectively), but lower than previously observed on hydroponically grown legumes (Ta et al., 1986). Expressed per unit length of roots ammonium exudation was twice as high in clover that in ryegrass (30 vs 16 nmol m^{-1} d^{-1}). Axenic conditions were used in a complementary experiment because the nitrogenous compounds in the non-sterile medium may not accurately represent exudation from roots. The similar amounts of ammonium observed in sterile and non-sterile exudates show that this compound originates from plants. Ammonium exudation by white clover was stable throughout the experiment, and was not increased by plant growth, as it has been shown in alfalfa (Brophy and Heichel, 1989). Over the same time period, exudation of ammonium from ryegrass decreased between 28 and 42 days after germination.

The significance of amino acid exudation by plants is still unclear. It has been shown that they have a limited role in rhizosphere nutrient mobilization (Jones et al., 1994), but they could be important in governing the size of the rhizosphere microflora population (Jones and Darrah, 1994). Amino acid exudation from roots of white clover and ryegrass in the present work was low compared to that observed from plants grown in solution e.g. alfalfa (Ta et al., 1986), soybean (Ofusu-Budu et al., 1990), forage rape (Shepherd and Davies, 1994a) and maize (Jones and Darrah, 1994)), but at the same level as that observed from soybean and alfalfa growing in sand culture as in the present study (Brophy and Heichel, 1989). The observed low level could be explained by differences in the experimental procedures and differences in plant culture. Exudation of N or C compounds has been shown to be modified by chemical (Shepherd and Davies, 1994a), physical (Groleau-Renaud et al., 1998) or biotic environments (Klein et al., 1988; Murray et al., 1996; Shepherd and Davies, 1994a). Shepherd and Davies (1994a) have established that the quantity of amino acids collected is related to the concentration gradient between plant roots and rhizosphere, the reabsorption of amino acids exuded by either species or by uptake in bacterial consumption. Amino acid release from roots has been suggested to occur by passive diffusion along a concentration gradient (Shepherd and Davies, 1994a), however most of these free amino acids in the rhizosphere are recaptured by an active transport mechanism (Jones and Darrah, 1994). This is in agreement with our measurement in a previous study (Cliquet et al., 1997) of uptake rate of SER by 49-d-old ryegrass grown in micro-lysimeter which was approximately 400 nmol plant^{-1} d^{-1} compared with a net exudation rate of approximately 4 nmol pl^{-1} d^{-1} for 49-d-old ryegrass in the present study.

Although both sand and nutrient solutions were sterilized in this system, bacterial consumption and/or transformation of N-compounds released by plants could have occurred in our experiment. However, this process was thought to be limited as SER and GLY, which are the most abundant amino acids in both clover and ryegrass exudates and they have been shown to have the higher rate of consumption by micro-organisms (Shepherd and Davies, 1994a, b). Moreover, the comparison of exudates collected from plants grown in axenic conditions or not (Figure 6) shows that GLU and ASP are more concentrated in non sterile clover exudates, but the two major amino acids SER and GLY are identical in both treatments.

The fact that only a few of the amino acids (mainly SER and GLY and smaller amounts of GLU, ASP and ALA) were found in the exudate supports the observations by Ta et al. (1986) and Ofusu-Budu et al. (1990) on selective net exudation by the root systems of legumes. SER, GLY and GLU were also shown to

be the major amino acids released by sterile forage rape (Svenningson et al., 1990; Shepherd and Davies, 1994a), sterile alfalfa (Richter et al., 1968), while alfafa released mainly ASP, GLU and ALA (Ta et al., 1986) and steppes grasses studied by Klein et al. (1988) released mainly ARG, SER and CYS. Amides such as ASN and GLN, which are the major amino acids in clover and ryegrass roots, respectively, have never been measured in significant amounts in either sterile or non-sterile exudates. ASN is the major transport molecule for N in white clover and GLN and ASN are the major transport molecules for N in ryegrass (Bigot et al., 1991). Their low levels in root exudates could be explained by a rapid and efficient translocation of these compounds to the shoots, as previously suggested (Murray et al., 1996; Ta et al., 1986). These low levels could also be due to partitioning in the cell, root contents presented in Figure 5 reflect both cytoplasmic and vacuolar pools, and exudation is considered to be the result of the cytoplasm-soil diffusion gradient (Jones and Darrah, 1994, Shepherd and Davies, 1994a).

Comparison of amounts of the major amino acids in clover exudates and exudates of receiver ryegrass shows various profiles, and two groups of amino acids could be distinguished (Figure 7). Amounts of GLY, TYR and GLU exuded by receiver ryegrass show little variations and mirror variations in clover exudates, but SER, ALA and ASP exhibit a late peak of exudation, only in receiver ryegrass. The similarity in the concentrations of GLY in the exudates collected from the clover and those collected post the ryegrass plants could have two explanations, GLY fluxes to and from the ryegrass plants being in equilibrium, or a low absorption of this compound by the ryegrass plants. It is strongly suspected that the former is the mechanism that is operating as it has been demonstrated that GLY is readily taken up by plants (Jones and Darrah, 1994). The late peak of SER, ALA and ASP could result from the hydrolysis of proteins from the older senescing leaves. Exudation of SER increased per unit dry weight until 56 d after germination and decreased thereafter in control ryegrass and receiver ryegrass.

The comparison of N compound quantification in exudates and the quantification of transfer with ^{15}N can provide further information. From analysis of ammonia and amino acids in white clover exudates, it can be deduced than 41 μg of N were exuded by donor clover plants and fed to receiver ryegrass during the course of the experiment, while we have estimated with the ^{15}N dilution technique that 76 μg of N were actually transferred from the legume to the grass in the same period. If it is hypothesised that N transferred from legume to grass is directly issued from the atmosphere, this figure reduces to 65 μg of atmospheric N transferred between plants. Different hypothesis could account for this discrepancy between N transfer and N exudation in the micro-lysimeter system; 1. Sloughing and decomposition of epidermal clover cells resulting in release of insoluble N and 2. Exudation by clover roots and nodules, of N-compounds that were not measured in this study.

With the first mechanism, estimates of N due to decay of epidermal cell for comparison with our data are scarce (Laidlaw et al., 1996). Although this mechanism is probably low in such young clover, it cannot be neglected and should be measured in future works. With the second mechanism, the method used in this study did not allow the quantification of nitrate exuded by clover, because this ion was included in the nutrient solution, but several studies have provided evidence of nitrate efflux when this ion is fed to white clover (Macduff and Jackson, 1992). Although this efflux was not measured in our study, it can be hypothesised that nitrate transfer from clover to grass occurred through the nutrient solution. Future work should include measurements of this component and of ^{15}N-labelled ammonium and amino acids uptake by receiver plants to describe more accurately the short-term N transfer occurring through the nutrient solution between legumes and grasses, which the present work has confirmed to be a highly significant transfer pathway.

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