Assessment of the below ground contribution of field grown pea (Pisum sativum L.) to the soil N pool

Stéphanie Mahieu

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Stéphanie MAHIEU

Devant le jury ci-dessous :

Alain GOJON, Directeur de recherches INRA, Montpellier	**Rapporteur**
Joëlle FUSTEC, Enseignante-chercheuse HDR, ESA Angers	**Examinateur**
Erik Steen JENSEN, Professeur, Risoe National Lab., Danemark	**Examinateur**
Eric JUSTES, Ingénieur de recherches HDR, INRA Toulouse	**Examinateur**
Frédérik LE DILY, Professeur, Université de Caen	**Examinateur**
Jean-Claude MAUGET, Professeur, INH Angers	**Examinateur**
Christophe ROBIN, Chargé de recherches HDR, INRA, Nancy	**Rapporteur**

et préparée au Laboratoire d'Ecophysiologie Végétale et Agroécologie
du Groupe Ecole Supérieure d'Agriculture d'Angers (ESA)

Abbreviations

BGN : Below ground Nitrogen
BNF : Biological N_2 fixation
Cdfr : carbon derived from rhizodeposition
HI : Harvest Index
N : Nitrogen
Ndfr : Nitrogen derived from rhizodéposition
NHI : Nitrogen Harvest Index
NH_4^+ : Ammonium
NO_3^- : Nitrate
PNdfr : Proportion of Nitrogen derived from rhizodeposition

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INTRODUCTION

After the first and second world wars, N fertiliser use was developed to meet world demand for food and feed. Crop yields were more than doubled (Peoples *et al.*, 1995) and the boosted world production was able to sustain food needs for a world population that increased from 2.519 billion in 1950 to 6 billion in 2000. The industrial Haber-Bosh process used to synthesize N fertilizers has been described as the most important invention of the 20th century, since 40% of all people alive depend on it as a major source of N for the synthesis of the proteins, DNA and other N molecules in their bodies (Smil, 2001 in Crews and Peoples, 2004). By 2050, Crews and Peoples (2004) reported from Smil (2001) and United Nations population projections (UN, 2000) that as many as 5.5 billion people may owe their existence to synthetic N fertilisers.

Widespread adoption of synthetic N fertiliser was firstly due to the increased possibility it gave farmers to face meet crop demand for N throughout a growing season and substantially increase yield. Secondly, it eliminated the fertility generating stage of crop rotation sequences which produce relatively few technical and economical commodities (Crews and Peoples, 2004). When comparing the environmental impact of using synthetic N fertiliser to biological N_2 fixation (BNF), Crews and Peoples (2004) concluded that legume-based agroecosystems were marginally less harmful than fertiliser-based systems and that the difference between the two N sources was not nearly as marked as many advocates of sustainable/ organic farming believed. However, the substantial difference in terms of sustainability was observed when comparing the energetic cost associated with each N source. BNF is ultimately derived from 'free and environmentally friendly' solar energy while N from the Haber-Bosch process consumes a substantial amount of fossil fuel energy. The production of 1kg of N fertiliser requires between 55 to 80 MJ or around 1.2 l of fuel and greatly contributes to the net global warming contribution of farming systems (Schlesinger, 2000).

The amount of N introduced into agrosystem by the BNF is estimated to be around 40-48 million tonnes year^{-1} while 83 million tonnes year^{-1} are fixed industrially by the Haber-Bosch process (Jenkinson, 2001; Jensen and Hauggard-Nielsen, 2003). With the improvement of crop yield using N fertiliser, an intensive agriculture based on sole crop systems has been

developed. In the long term, this system has generated a decrease in soil fertility and biodiversity favouring an increase in pests and diseases and associated use of pesticides and a large consumption of energy. At the end of the last century, it appeared that agricultural policy needed to be replanned to achieve long term production with limited use of non-renewable energy sources, including fertilisers and agrochemicals, while protecting environmental and products quality (Jensen, 1997). Moreover, the use of known commercial energy resources will be limited in the next century due to global warming and resource depletion. Additionally many countries are not able to count on an uninterrupted supply of energy or N imports due to poverty or political conflicts.

In rotational farming systems, legume N inputs are likely to represent the largest N resource fed back into the ecosystem after crop harvest. In addition to N soil enrichment, legumes are known to reduce the survival of nematode populations, suppress leaf and root diseases such as take all fungi and reduce weeds when used as break crop in cereal rich rotation (Herridge and Holland, 1992; Stevenson and van Kessel, 1996; Jensen and Hauggaard Nielsen; 2003). Surprisingly, when analysing the benefits of introducing a pea into rotations with wheat in comparison with continuous wheat, it was shown that 91% of the yield advantage of wheat succeeding pea was associated with non-N benefits, mainly reduced leaf disease (Stevenson and van Kessel, 1996). Only 9 % of the advantage was therefore associated with the increase soil N level (Jensen and Hauggaard-Nielsen, 2003). However, N benefits of introducing legumes may have been largely underestimated due to a inaccurate estimations of the amount of N_2 fixed by legume crops.

Any estimate of the amount of N_2 fixed by a legume crop must take into consideration (a) the proportion of legume N derived from the atmosphere (% Ndfa) and (b) the total amount of N in legume biomass during the study period (Unkovich and Pate, 2000). The contribution of legumes to the soil N pool (N balance) can then be calculated by subtracting the amount of N removed with the grain from the amount of N_2 fixed symbiotically. In addition to the lack of reliability of the methods used to quantify BNF, legume below-ground nitrogen (BGN), including root N and N rhizodeposition, have not been taken into account by most studies of N_2 fixation. Indeed, N balance was greater after a cereal crop than after a legume crop when the estimation of the amount of N issued from fixation was only calculated from shoot N. However, inorganic soil N measurement recorded after legumes crop was greater than after

cereal crop grown in the same conditions (Crozat and Fustec, 2006). Morever, soil inorganic N measurements do not take into account changes in the organic N pool. In a recent study, McNeill and Fillery (2008) observed that a substantial proportion of legume derived BGN was still present after a wheat season as residual insoluble organic N proving that this N pool would largely contribute to the N soil enrichment.

During a season, a large amount of N may be released from roots into the soil by different mechanisms including the root exudation and the root senescence. This pool of N was defined as N rhizodeposition and remains difficult to quantify with accuracy with traditional analytical methods. Moreover, estimation of root N may be underestimated because a large part of the root system is constituted of thin roots not easily collectable from soil by hand. For assessing the N benefits of introducing grain legumes into rotations in field conditions, it is assumed that the entire amount of N released from roots over a season may be quantified if the plant is homogeneously labelled using a ^{15}N tracer. Several ^{15}N labelling methodologies have since been developed for particular purposes or according to plant morphology. Until now, most studies have been conducted in the greenhouse and have focused on methodology while few data are available on methods suitable for field studies. Results of these studies vary considerably as experiments have been undertaken using different methods, different species and varieties, in a variety of environmental conditions. Furthermore, the factors regulating the allocation of N to below ground parts are not well understood. For a given species, effects of growth conditions, sources of N nutrition (nitrogen fixation or inorganic N uptake) or genotype have not been investigated.

It may possible to improve the nitrogen providing role of grain legume by increasing the amount of N_2 fixed by the legume crop and reducing the amount of N lost (Crews and Peoples, 2004). For this, it is essential to improve the accuracy with which below ground nitrogen can be quantified (Peoples *et al.*, 1995; Rochester *et al.*, 1998; Khan *et al.*, 2003; McNeill *et al.*, 2008) to estimate N benefits from N legume farming, to understand soil N turnover (Jensen, 1996a; Mayer *et al.*, 2003) and to predict N economies for the succeeding crops in legume-based cropping systems (Russell and Fillery, 1996b).

In this context, the first objective of the researches developed in this thesis was **to compare and evaluate the reliability of ^{15}N labelling methods for measuring N rhizodeposition** and **to develop an appropriate protocol for BGN assessment of pea in**

field conditions using an in situ ^{15}N labelling method. A second objective was **to study the effects of genetic characteristics through variation in the Nitrogen Harvest Index (NHI) as well as the effects of N sources and water availability**. All the researches was carried out on pea (*Pisum sativum L.*) which is the most important grain legumes grown in EU cropping systems.

The manuscript is composed of four sections. A definition and the functions and mechanisms involved in rhizodeposition of N and C compounds are presented in a first background section along with the state of the art concerning methodology used for measuring BGN. There follows an explanation of the organisation of the results presented in two chapters: each one is composed of two articles. A synthesis and discussion of the results obtained during the thesis constitutes the final section of the manuscript including perspectives resulting from this work.

PUBLICATIONS and SCIENTIFIC COMMUNICATIONS

Results reported in this manuscript have been published or prepared for scientific papers:

- A paper has been published (Chapter I, section I of the Results,):

Mahieu S., Fustec J., Faure M.L., Hellou G., Crozat Y., 2007. Comparison of two ^{15}N labelling method for assessing nitrogen rhizodeposition of pea. ***Plant and Soil*, 295 : 193-205.**

- A paper has been subject to certain changes (Chapter II, section II of the Results; fellowship LEVA/Risoe National Lab.):

Mahieu S., Germon F., Aveline A., Hauggard-Nielsen H., Ambus P., Jensen E.S. Below-ground N partitioning during reproductive growth of pea *(Pisum sativum* L.*)* as influenced by water stress. *Soil Biology & Biochemistry*, **accepted with changes**.

- A paper will soon be submitted to an international journal (Chapter I, section II of the Results):

Mahieu S., Fustec J., Jensen E.S., Crozat Y. Effect of labelling frequency and ^{15}N root enrichment on the assessment of the N rhizodeposition using the cotton wick method. **to be submitted to** *Soil Biology & Biochemistry***.**

- A short communication in preparation to be submitted to an international journal (Chapter II, section I of the Results):

Mahieu S., Crozat Y., Fustec J. Effects of N source, N content and plant N partitioning on the below ground contribution of pea to the soil N pool. **will be submitted to** *Soil Biology & Biochemistry***.**

This work has also been presented at several international and local congresses:

- International congresses :

Mahieu S., Matifat S., Faure M-L., Sevestre C., Fustec J., Crozat Y., 2005. Comparison of split-root and cotton-wick methods for estimating pea nitrogen rhizodeposition. In 14th workshop : "N management in agrosystems in relation to the Water Framework Directive", 24-26 October. Maastricht, The Netherlands. *(Poster)*

Mahieu S., Matifat S., Faure M-L., Sevestre C., Fustec J., Crozat Y., 2005. Comparison of two methods for estimating Pea below ground nitrogen contribution. Proceedings of the 14th workshop on nitrogen : "N management in agrosystems in relation to the Water Framework Directive", 24-26 October, Maastricht, The Netherlands, 2 pp.

Mahieu S., Faure M.L., Fustec J., Crozat Y., 2006. Nitrogen rhizodeposition of different peas measured in the field and greenhouse by cotton-wick. In IX ESA Congress, 4-7 Sept., Warsaw, Poland. *(Poster)*

Mahieu S., Faure M.L., Fustec J., Crozat Y., 2006. N rhizodeposition in pea labelled by cotton-wick in different growing conditions. *Bibliotheca Fragmenta Agronomica* 11, 553-554.

Mahieu S., Faure M.L., Reynaud A., Pineau S., Oury V., Moreau V., Renaud S., Fustec J., Crozat Y., 2007. A ^{15}N stem feeding method for measuring below ground nitrogen of field grown legumes. In proceedings of the 15th workshop on nitrogen, 28-30th May 2007, Lleida, Spain, 48-50. *(Poster and short oral communication)*

Mahieu S., Reynaud A., Faure M.L., Fustec J., Crozat Y., 2007. N source, N content and plant N partitioning effects on the below ground contribution of pea to the soil N pool. In proceedings of the 15th workshop on nitrogen, 28-30th May 2007, Lleida, Spain 345-347. *(Poster and short oral communication)*

Mahieu S., Fustec J., Faure M.L., Crozat Y., 2007. Cotton wick and split-root labelling method : a tricky comparison. In 6th European conference on grain legumes, 12-16th November 2007, Lisbon, Portugal. *(Poster with price)*

Mahieu S., Germon F., Fustec J., Aveline A., Ambus P., Hauggaard-Nielsen H., Crozat Y., Jensen E.S. 2007. Effects of water stress, N source, N content and plant N partitioning on the below ground contribution of pea to the soil N pool. In 6th European conference on grain legumes, 12-16th November 2007, Lisbon, Portugal. *(Oral communication in a plenary session)*

Mahieu S., Fustec J., Faure M.L., Reynaud A., Crozat Y., 2007. Successive improvements of a 15N cotton-wick method for measuring below ground nitrogen of field grown legumes. In 6th European conference on grain legumes, 12-16th November 2007, Lisbon, Portugal. *(Poster)*

- Local congress :

Mahieu S. 2006 – Quantification de la contribution des cultures de pois (*Pisum sativum* L.) et féverole (*Vicia faba* L.) au pool azoté du sol : rôles de la fixation biologique et de l'azote des parties souterraines. In 6ème Forum des Doctorants, 15 juin, Angers, France. *(Poster)*

BACKGROUND

I. Biological N_2 fixation of legumes

Legumes (Fabaceae) are special plants which have the ability to acquire N by uptake of N both compounds from the soil solution such as nitrate (NO_3^-) and ammonium (NH_4^+) and by fixation of atmospheric nitrogen (N_2). The principal annual crop legumes ranked in the following descending order according to the amount of N_2 fixed are: soybean (*Glycine max* L.), field pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), common bean (*Phaseolus vulgaris* L.), lentil (*Lens culinaris* L.) and chickpea (*Cicer arietinum* L.) (Table 1, Unkovich and Pate, 2000).

Table 1: Ranges in N2 Fixation observed for principal annual crop legumes (Unkovich and pate, 2000)

Crop	Area harvested ($\times 10^6$ ha)	%Ndfa	N fixed in shoot (kg ha^{-1})	Selected references containing field estimates of N_2 fixation
Soybean (*Glycine max*)	67.6	0–95	0–450	Patterson and LaRue, 1983; Rennie et al., 1988; Peoples et al., 1995a; Toomsan et al., 1995
Common bean (*Phaseolus vulgaris*)	13.0	0–70	0–165	George and Singleton, 1992; Bliss and Hardarson, 1993; Hardarson et al., 1993; Herridge and Danso, 1995
Groundnut (*Arachis hypogaea*)	23.7	22–92	32–206	Bell et al., 1994; Toomsan et al., 1995; Dakora and Keya, 1997
Chickpea (*Cicer arietinum*)	11.3	0–82	0–141	Beck, 1992; Herridge et al., 1995; Marcellos et al., 1998; Schwenke et al., 1998; Carranca et al., 1999
Field pea (*Pisum sativum*)	6.5	5–95	4–244	Armstrong et al., 1994; Evans et al., 1995; Jensen, 1997
Lentil (*Lens culinaris*)	3.3	28–87	5–191	Van Kessel, 1994; McNeill et al., 1996; Kurdali et al., 1997
Faba bean (*Vicia faba*)	2.2	19–97	12–330	Duc et al., 1988; Schwenke et al., 1998
Lupin (*Lupinus angustifolius*)	1.3	20–97	19–327	Unkovich et al., 1997; Howieson et al., 1998

I.1. Mechanisms involved in the N acquisition by legumes

I.1.1. Uptake of inorganic N

As far as we know, the mechanisms involved in the uptake of inorganic N by legumes were not studied. Nevertheless, nitrate (NO_3^-) and ammonium (NH_4^+) transporters have been described for plants such as barley (*Hordeum vulgare* L.) (Vidmar *et al.*, 2000), oilseed rape (*Brassica napus* L.) (Faure-Rabasse *et al.*, 2002) or arabidopsis (*Arabidopsis thaliana* L. Heyhn) (Orsel *et al.*, 2006). In those plants, the absorption of NO_3^- and NH_4^+ is carried out by

two classes of transporters systems referred to as Low- and High-affinity transport systems (LATS and HATS, respectively) and were characterised by an inducible and a constitutive component (Krapp *et al.*, 1998; Okamoto *et al.*, 2003). The LATS are involved in NO_3^- or NH_4^+ uptake at high external concentrations of those mineral forms in the soil solution, while the HATS are saturated at low external concentrations.

I.1.2. Symbiotic N_2 fixation

Legumes access atmospheric N_2 through symbiosis with a group of soil diazotrophic prokaryotic microorganisms from the Rhizobiaceae family (Vance, 1998). In pea, the symbiosis results from a specific association between the leguminous and the species of bacterium *Rhizobium leguminosarum*. The biological reduction of nitrogen to ammonia is catalysed by the microbial enzyme nitrogenase. This enzyme is highly oxygen sensitive because one of its components, the MoFe cofactor, which is irreversibly denatured by oxygen (Shaw and Brill, 1977) and the biological reduction may only be achieved after the differentiation of the microorganism in specialized bacterial cells, in bacteroids. Upon attachment of the bacteria and then infection by root hairs, the plants develop specific nodule structures on the roots for the multiplication of the symbiont and the differentiation in bacteroid. In the nodules, a low oxygen tension is achieved through a combination of high metabolic activity by the symbiont and an oxygen diffusion barrier favouring nitrogenase activity (Witty *et al.*, 1986).

Two types of legume nodules can be distinguished by their growth pattern: the meristem of nodules can either be indeterminate and grow over an extended period of time or be determinate with a life about few weeks (Brewin, 1991). Nodules of temperate legumes such as pea or faba bean are generally under the indeterminate form (Sprent and Minchin, 1985), whereas nodules of tropical legumes are under the form determinate. For the both types, the initiation and maintain of the nodule structure and the nitrogenase activity have high requirement in ATP and photosynthetic assimilates. In the context of the whole plant, the root nodule functions as a nitrogen source and a carbon sink (Mylona *et al.*, 1995). The bacteria provide N in the form of NH_4^+. In exchange, carbon is transferred to the bacteria in the form of sucrose by the plant host to provide energy and reductant for nitrogenase activity.

I.2. Nitrogen transport

After absorption from the soil solution, nitrate is reduced in ammonium by the both enzyme nitrate and nitrite reductase (Crawford, 1995; Faure *et al.*, 1997). Then, irrespective to the original source of N, the NH4+ will be incorporated into the cycle ATP/NADPH dependant glutamine synthetase (GS)/ glutamate synthase (GOGAT) to the synthesis of glutamine and glutamate (Hirel *et al.*, 2001). This assimilation takes place in the cytoplasm of nodule-cells when the NH_4^+ results from the symbiotic fixation of N_2 (Mylona *et al.*, 1995). The form in which the nitrogen is transported depends on the plants: in temperate legumes, the amino acids will then be metabolized in other transportable forms of amides by amido and amino transferases, whereas tropical legumes export ureides. In pea, N is transported via the xylem as nitrate and a mixture of organic nitrogenous compounds mainly asparagine, glutamine homoserine and aspartate. Asparagine and glutamine seem also to be the major forms of N transport in the phloem (Urquhart *et al.*, 1982).

I.3. Regulation of N_2 fixation and soil inorganic N uptake

The inducible high-affinity transport systems (IHATS) are induced at very low external concentration of ammonium or nitrate (Krapp *et al.*, 1998). In the presence of mineral N in the soil solution, NO_3^- or NH_4^+, legumes preferentially absorb those mineral forms which are less energy expensive. Pea can absorb mineral N from the soil as efficiently as barley (Jensen, 1986a).

In low mineral N conditions, pea relies heavily on symbiotic fixation for N acquisition and the fixation begins early in the growth cycle. The maximum symbiotic fixation rate varies from the beginning of flowering to the end of seed filling according to the plant variety (Jensen 1986b & 1987). At late pod-filling, the proportion of total N derived from fixation reaches a maximum of 70-80% (Jensen, 1987). During plant growth, the number of nodules increases linearly with time and is governed by feedback regulatory mechanisms. The regulation of N_2 fixation depends on plant-N load (abundance of glutamine, glutamate, 2-oxoglutarate in the phloem) (Parsons *et al.*, 1993). However, mechanisms involved in the regulation of N_2 fixation in the presence of mineral N are not fully understood.

At the end of the growth cycle, the decrease in both nodule activity and / or senescence seems to be linked to decreases in C assimilates which are preferentially allocated to seed

filling. Moreover, the increase in the amount of N compounds in the phloem occurring during the seed filling period due to the remobilisation of N from the vegetative plant parts may also contribute to the inhibition of the symbiotic fixation. Voisin *et al.* (2003) showed that nodule senescence is not linked to nodule age but may be maintained later in the growth cycle if N is required.

I.4. Effect of N fertilisation on plant growth

I.4.1. Effect on shoot N and biomass

In legumes, optimal yield and biomass production are generally observed in field conditions regardless of N fertilisation at sowing (Sagan *et al.*, 1993; Crozat *et al.*, 1994; Voisin *et al.*, 2002). Nevertheless, the presence of mineral N in the soil has sometimes been observed to stimulate vegetative growth particularly in greenhouse conditions, resulting in a higher biomass accumulation in the shoots in fertilised treatments (Jensen 1986b; Voisin *et al.*, 2002). This difference between field and greenhouse results may be due to a transitory nitrogen deficiency at the beginning of the growth cycle before symbiotic fixation is able to sustain plant growth for strictly symbiotically grown peas (Voisin *et al.*, 2002). However, the N concentration in each plant part remains unchanged whatever the N source. Only biomass increases in all organs except the seeds. For plants grown at very high N concentrations of mineral N (400kg N ha^{-1}), Voisin *et al.* (2002) reported that the stimulation of the vegetative growth led to lodging at the end of the growth cycle and resulted in a depressed seed yield. Effect of N fertilisation may also influence seed yield according to the moment when fertiliser is applied (Jensen, 1997).

Since seed yield and nitrogen concentration remain similar when shoot and root biomass increases with the soil mineral N availability, N fertilisation generally leads to a decrease in the Nitrogen Harvest Index (NHI) and in the biomass Harvest Index (HI) (Voisin *et al.*, 2002). In field conditions, N and biomass partitioning does not appear to be affected by N fertilisation (Voisin *et al.*, 2002).

I.4.2. Effect of N fertilisation on root growth

In non-legumes, N fertilisation generally leads to an increase in shoot N concentration and biomass and to a decrease in C and N allocation to the roots. The root length and the number of root tips are reduced, and the mean root diameter increased relative to "no boost" treatments (Paterson and Sim, 2000; Darwent *et al.*, 2003; Nguyen, 2003).

In pea, contrary to the observation on non-legumes, N fertilisation generates root growth and proliferation, leading to more numerous but finer roots (Voisin *et al.*, 2002). Total root biomass and density increase but nodule biomass decreases. The maximum rooting depth and the distribution of roots with depth are unchanged (Voisin *et al.*, 2002).

I.5. Effect of some environmental factor on N_2 fixation and plant growth

The rate of N_2 fixation is positively correlated with the crop growth rate and leaf area during the vegetative and the early reproductive growth stage (Jensen, 1987) and any factor affecting plant growth such as nutrient availability, water stress, weeds or pests and diseases will influence N_2 fixation (Ravn and Jensen, 1992). The symbiotic N_2 fixation is a mechanism sensitive to numerous environmental conditions (Fig. 1). Nitrogenase activity, nodule number and mass are limited by P (Israel, 1987). The survival and persistence of nodule bacteria in soil as well as the process of nodulation itself are affected by the phenomenon of soil acidification, salinization and osmotic stress resulting from the soil degradation by intensive agricultural management practices (Graham, 1992). Nodulation and N2 fixation are also inhibited by the inorganic N fertilisation (Fransisco and Akao, 1993). However, this former does not affect either dry matter or grain yield (Jensen, 1986b; Voisin *et al.*, 2002).

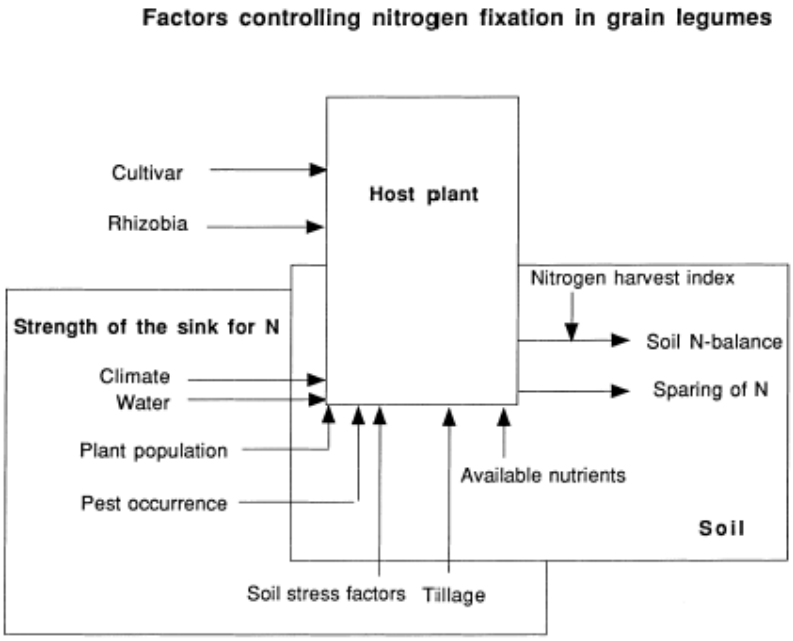


Fig.1 Conceptual model of the major factors that exercise a control on N_2 fixation of grain legumes in a cropping system. In addition to the competitiveness of the rhizobia in forming nodules and the effectiveness of the rhizobium - host plant to fix N_2, a series of edaphic, chemical and biophysical factors exert a control on N_2 fixation. Management practices like the intensity of tillage or intercropping practices will alter those edaphic, chemical and biophysical factors and therefore influence BNF indirectly. (Kessel and Hartley, 2000)

As reported above, water supply is one of the most important factors in determining growth and N_2 fixation of the pea crop in temperate cropping systems (Jensen, 1997). In dry conditions, several mechanisms contribute limiting nitrogen fixation by affecting either nodulation initiation, nodule development or functioning (Zahran and Sprent, 1986; Busse and Bottomley, 1989; Serraj *et al.*, 1999; Mnasri *et al.*, 2007). Accumulation of biomass and N is reduced and root N partitioning is modified, due to a decrease in nodule biomass. Field peas exposed to water stress have finer root systems which grow deeper into the soil and lower root dry weight compared with unstressed plants (Devries *et al.*, 1989a; Benjamin and Nielsen, 2006). Under water deficit, biomass accumulation is generally reduced due to reduced leaf area, decreased net photosynthesis and stomatal closure (Monteith, 1977; Lecoeur *et al.*, 1995; Lecoeur et Sinclair, 1996; Guilioni *et al.*, 2003; Thomas *et al.*, 2004). Both total N accumulation and N concentration are reduced compared with well-watered treatments in the field and greenhouse (Devries *et al.*, 1989b; Lecoeur and Sinclair, 2001). The effect on seed yield depends on when the water stress occurs and on the intensity and duration of the shortage. A water stress occurring during flowering or at a late vegetative stage has a more negative effect on yield than a water stress occurring during pod-filling or at early vegetative

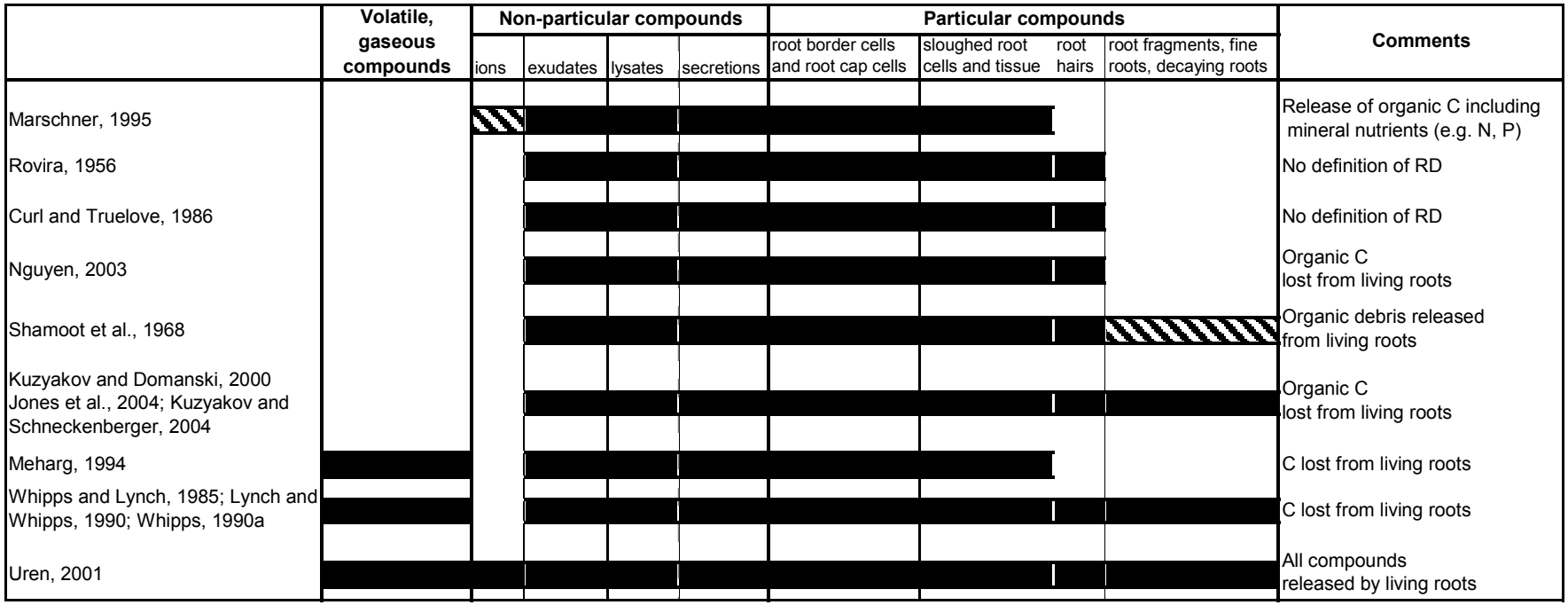
growth (Acosta-Gallegos and Kohashi-Shibata, 1989; Ney *et al.*, 1994). The effect on NHI seems to depend on the species (Chapman and Muchow, 1985; Thomas *et al.*, 2004). NHI of mungbean (*Vigna radiata* L.), cowpea (*V. unguiculata* (L.) Walp.) and lablab (*Lablab purpureus* (L.) Sweet) were not affected though NHI of soybean and blackgram (*V. mungo* L.) were reduced.

II. N rhizodeposition

II.1. Definitions of the rhizodeposition

During plant growth, numerous N and C compounds are released from roots into the soil. This phenomenon called rhizodeposition involves different pathways and facilitates i) root progression among the soil components, ii) exchanges with the soil solution and iii) a favourable soil environment for plant development. C rhizodeposition was largely studied because it represents the main pathway to fix C in the soil but less is know about N rhizodeposition. Rhizodeposition includes a high diversity of compounds which are classed either according to their origin as volatile, particular and non-particular compounds (Table 2; see Wichern *et al.*, 2008) or according to their solubility or extractability with water into water soluble exudates (sugars, amino acids, organic acids, hormones and vitamins) and water insoluble material such as decaying fine-roots, root-hairs, cell walls, sloughed cells, and mucilage (Jensen, 1996b; Merbach *et al.*, 1999). Several definitions in the literature omit to take into account one or another of these compounds (Wichern *et al.*, 2008). Therefore, including C and N compounds, Uren (2001) defined rhizodeposition as *the release of all kinds of compounds lost from living plant roots, including ions and volatile compounds*. Since at the present time all the mechanisms involved in the release of N into the soil are not fully understood and may vary according to the plant species, the definition of Uren (2001) would seem to be the most appropriate.

Table 2. Compounds released by plant roots included in the concept of rhizodeposition. (Hatching= only partly included, unclear) (Wichern *et al.*, 2008)



II.2. Composition of N rhizodeposits

Among the N compounds released into the soil during plant growth, most are in organic form. However, NH_4^+ and NO_3^- are also released during plant growth (Brophy and Heichel, 1989; Wacquant *et al.*, 1989; Merbach *et al.*, 1999). Wichern *et al.* (2008) classed the ions, exudates, lysates and secretions released from roots under the term of non-particular compounds and root border and cap cells, sloughed root cells and tissue, root hairs, root fragments, fine roots and decaying roots under the term of particular compounds (Table 2). Currently, the proportion of each kind of rhizodeposit released into the soil remains unknown. McNeill and Fillery (2008) measured a proportion of mineral N in rhizodeposits of field grown lupin of around 6-10% during vegetative growth, from 4 to 15% at peak biomass and from 10 to 17.6% at maturity. The proportion of inorganic N of the total soil-N derived from rhizodeposition (Ndfr) of pea has not been measured either in greenhouse or in field experiments. Wichern *et al.* (2007) showed that 27% of the total ^{15}N released from roots into the soil at maturity was inorganic and that 34% of the pool of inorganic N of the soil was derived from rhizodeposition.

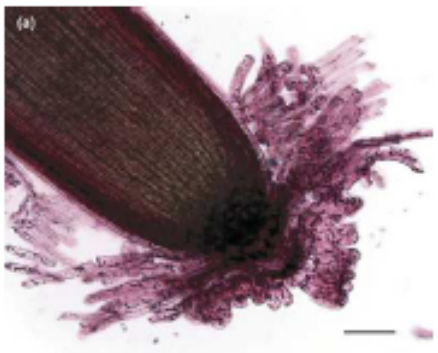
II.3. Functions and mechanisms

During vegetative growth and the beginning of the reproductive period, compounds are released into the soil due to i) slough-off of root cap and border cells, ii) secretion of mucilage, iii) senescence of root epidermis, iv) passive and controlled diffusion of root exudates, and v) senescence of roots and nodules. The production of root cap cells and mucilage or the development of hair cells are mainly involved in the facilitation of root penetration into the soil, root anchorage to the soil components and the maintenance of plant support. The passive and controlled diffusion of root exudates would likely involved in the control of the microbial environment and Nguyen (2003) calculated that 10 to 100 times more C would be released into the soil by these processes than via the release of root border cells and mucilage. Several authors also suggested that root and nodule senescence may substantially contribute to the amount of N released from below ground parts during plant growth (Jensen 1996a, Johansen and Jensen, 1996, Lory *et al.* 1992). A brief description of each one of the mechanisms contributing to rhizodeposition is given below:

i) Sloughing-off of root border cells

During plant growth, roots may exert pressures of up to 1MPa in order to penetrate hard soils (Misra *et al.*, 1986). Consequently, all plants having to grow in soil have evolved a root cap providing a protective layer in front of the meristem from abrasion by soil particles which contributes to a decrease in the frictional resistance experienced by root tips (Bengough and McKenzie, 1997). Root cap cells are continually produced by the meristem and are arranged in layers (Fig. 2). After a period, the cells are displaced from the inner zone towards the periphery of the cap where they are sloughed off to decrease the frictional resistance of root growth (Bengough and McKenzie, 1997). During their transit through the cap, the cells differentiate into secretory cells producing and secreting mucilage, proteins and other metabolites (Lynch and Staehelin, 1992; Zhu *et al.*, 1997). The properties of the mucilage seem to facilitate the adhesion of root cap cells to soil particles, and hence their detachment from the cap as the root tip moves deeper in the soil. In the soil, the cells remain viable several days after being detached from the root surface (Vermeer and McCully, 1982; Hawes *et al.*, 1990) and exhibit a gene expression differing from that of attached cells (Brigham *et al.*, 1995). The root border cells are involved in the determination root architecture (Tsugeki and Fedoroff, 1999) and in the regulation of microbial populations in the rhizosphere by attracting pathogens, preventing them from damaging the root meristem, and by promoting growth gene expression in symbiotic microorganisms (Hawes, 1990; Paterson, 2003; Bais *et al.*, 2006). At the end of the growth cycle, the production of root cap cells decreases and the entire cap itself can be lost as a result of pathogen attacks or as part of the normal development process (Varney and McCully, 1991).

Fig. 2 Sloughing off of root cap cells from oilseed rape (*Brassica napus* L.) (Driouich *et al.*, 2007)



ii) Secretion of mucilage

Mucilage is generally secreted by the cells from the outer layer of the cap. However it can also be synthetised by the rhizosphere microorganisms or derived from degradations of the epidermal cell wall (Foster, 1982). It covers the root surface and, blended with soil and living border cells forms a rhizosheath functioning like a biofilm and involved in plant nutrition. Mucilage is mainly composed of polymerised sugars and of up to 6% protein (Basic *et al.*, 1987). It has numerous acid functions which confer the ability to bind cations including those fixed to clay. This property also contributes to improve soil structure and the stability of aggregates. The hydration of mucilage can also vary widely and this property may have an important role in resistance to drought (Watt *et al.*, 1994).

iii) Senescence of root epidermis

Roots hairs are involved in root anchorage to soil particles (Fig. 3; Fig. 4; Moreno-Espindola *et al.*, 2007), in water supply and nutrient uptake. They are also indispensable to the installation of the symbiosis between rhizobia and legume since rhizobia bind to receptive root-hairs for the penetration into the root system. These cells are located just behind the root tip. Epidermal cells differentiate either into hair cells (trichoblast) or non hair cells (artrichoblast) (Schmidt, 2008). From study carried out by Dittmer (1949, in Nguyen, 2003) it has been shown that, the size of root hairs is quite constant within a given species but is very variable between species. The length can vary from 80 to 1500µm and the diameter from 5 to 20µm. Root hair density is also very variable between plants: 1 to 180 hairs mm^{-1} of root. The root hair zone is of 1 to 4 cm long on average. The lifespan of a root hair is still discussed since the cells loose the nucleus under 3 to 4 weeks though the lyse of the cytoplasm was observed within 3-4 days. It is not known whether the cytoplasm material is released into the soil or recycled within the root tissue (Nguyen, 2003).

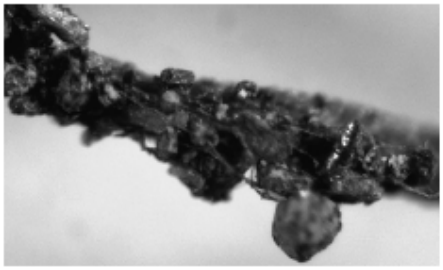


Fig. 3 Hyphae stained with cotton blue and adhering sand particles in the root system of amaranth (frame width: 1.23 mm) (Moreno-Espindola *et al.*, 2007)

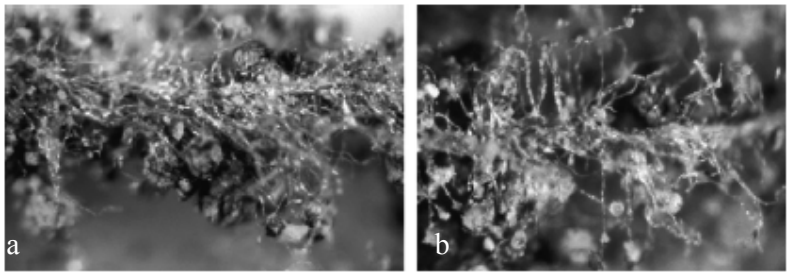


Fig. 4 Root-hair adhering sand particles. (3a) Maize (frame width: 1.33mm) and (3b) Bermuda grass (frame width: 1.27 mm). (Moreno-Espindola *et al.*, 2007)

iv) Root exudation

The rate of nutrient cycling in the soil is mediated by microbes and this process depends on the input of C from plants growing in the soil. For their growth, plants need to capture mineral nutrients from the soil solution which are limiting in temperate ecosystems. Of all the mechanisms involved in the rhizodeposition, the root exudation is most likely to influence the development of the microbial rhizosphere in favour of plant development.

Exudation was first defined as the mechanism by which low molecular weight compounds diffuse passively from intact cells to the soil solution (see Nguyen, 2003). As such, all the low molecular weight substance released from roots are classed as exudates regardless of the process by which they are released into the soil. The biochemical nature and concentration of root exudates vary according to plant species, physiological status and are influenced by environmental conditions. Compounds such as simple and complex sugars, amino acids, organic acids, phenolics, alcohols, polypeptides and proteins, hormones and enzymes, NH_4^+ or NO_3^- are reported in the literature (Grayston *et al.*, 1996; Paynel *et al.*, 2001).

Root metabolic activity varies considerably along the root axis due to a gradual maturation of root tissues and because different root classes of the same plants exploit different portions of the soil and are subjected to different external signals (see Walker *et al.*, 2003). Patterns of root exudation are therefore not homogeneous along the root axis. The main compounds released passively are sugars, amino acids and organic acids. These diffuse passively from the cytoplasm since their concentration in the cytoplasm is greater than in the soil solution and

because such compounds are rapidly taken up by microbial biomass. The spatial localisation of root exudation along the root is often linked to the concentration of the compounds inside the root (Henry *et al.*, 2005). However, release of organic anions by exudation has also been reported to follow a pattern consistent with the presence of a pH gradient from the tip to the base of the root (see Walker *et al.*, 2003). Darwent *et al.* (2003), using a bacterial biosensor method, observed that C exudation by *H. vulgare* was greater from root tip regions than from the whole root.

For various legumes the N dominated compounds exuded are NH_4^+ and the amino acids serine and glycine (Brophy and Heichel, 1989, Ofosu-Budu *et al.*, 1990; Paynel *et al.*, 2001). In older pea and oat, plants excrete material richer in amino acids compounds with an increasing proportion of specific amino acids (such as alanine in peas). This indicates that the amino acid composition of exudates changes with plant age (Rovira, 1956 in Wichern et al., 2008). In particular root exudates of legumes contain NH_4^+ and amino acids and more amino-N than the root exudates of non-legumes (Hale *et al.*, 1978).

v) Senescence of roots and nodules

In perennial legumes, the first evidence for rapid senescence and decomposition of fine alfalfa (*Medicago sativa* L.) roots was given by Jones (1943) (in Dubach and Russelle, 1994). Transient roots of alfalfa live only a few weeks. The meristem of nodules can either be indeterminate and grow over an extended period of time such is the case for alfalfa (Vance *et al.*, 1982) or be determinate with a life time of a few weeks such for birdsfoot trefoil (*Lotus corniculatus* L.). Dubach and Russelle (1994) compared the amount of N released into the soil due to root and nodule senescence by the both species and concluded that highest amounts of N were released into the soil due to senescence of the finest roots for alfalfa and due to nodule senescence for birdsfoot trefoil. They observed that specific N content in dead non-decomposed fine roots did not decline though the N content of decaying nodules in birdsfoot trefoil was significantly lower than in functioning nodules. This suggests that N was remobilised from nodules during their senescence and that root and nodule senescence may substantially contribute to the amount of N released from roots during plant growth. However quantitative data are scarce due to a lack of methodology.

In annual legumes such as pea or faba bean, nodules are indeterminate and contribution of root and nodule senescence may be higher at the end of the growth cycle since the proportion of C assimilate allocated to the roots decreases (Voisin *et al.*, 2003a) and may be to low to maintain the entire root system at this late stage.

III. Assessment of the benefits of introducing grain legume into rotations

III.1. General considerations

The study of plant below ground nitrogen (BGN) takes into account both N contained in the roots as well as the pool of N released from roots into the soil during plant growth. Though several methods were developed over the last few years, BGN is rarely measured due to the difficulty of using such methods in the field and the dubious accuracy of the results obtained. It is difficult to physically recover the root system from the soil, as most plant roots are too thin to be collected by hand (Wichern *et al.*, 2007). Several root collection protocols have been tried and this may have contributed to the variation in the results observed in the literature. Secondly, the N resulting from rhizodeposition is not distinguishable from N from other sources and small differences in the N percentage of soil are difficult to assess with accuracy using current analytical methods (Schmidtke, 2005a). Methods based on the use of ^{15}N have now been developed and seem to be more accurate than non isotopic methods for the quantification of N rhizodeposition (Jensen 1996c; Khan *et al.*, 2002a).

III.2. Problems of root-N estimation

Several authors, such as Uren (2001), have suggested that around 90% of rhizodeposition may result from root fragments not collected by hand. In field experiments, root systems are generally extracted by immersion of soil blocks in saturated salted water for 24h in order to facilitate clay dispersion and improve root recovery (Voisin *et al.*, 2002; Corre-Hellou *et al.*, 2007). These root collection techniques enable most of the root system to be collected in a very short time. However when measuring N rhizodeposition, roots must be collected from

dry soil and the protocol of root collection may largely influence the results, since it have direct consequences on the ^{15}N enrichment values of soil. Some authors specify that roots were separated into subsamples such as fine and coarse roots (Rochester *et al.*, 1998; Khan *et al.*, 2002a) or that all visible roots and root fragments are carefully collected (Jensen, 1996c; Mayer *et al.*, 2003; Schmidtke, 2005b). Additionally, Mayer *et al.* (2003) sieved a sample of root-free soil over a 200µm sieve to determine the amount of fine roots remaining in the soil. He measured that fine roots represented 7% of the total N rhizodeposition in lupin, 16% in faba bean and 31% for pea. After root collection, Jensen (1996c) gently washed roots with 50ml of distilled water for 1 min, adding the dry slurry obtained to the bulk soil before analysis. Schmidtke (2005b) used a similar protocol to Jensen (1996c) and rinsed the roots in 100ml of de-ionised water for 90s, adding the slurry to the rest of the soil. Mayer *et al.* (2003) shook the roots in 50ml of de-ionised water for 5min and washed with an additional 200ml over a 2mm sieve. In contrast, Janzen and Bruinsma (1989) did not wash roots to avoid N leaching from roots and Yasmin *et al.* (2005) froze dried roots with adhering soil collected on a 2 mm sieve at - 40°C for 2 days. The soil detached from roots was added to the soil removed from roots when brushed carefully to obtain a clean root fraction. Since no comparative studies have been undertaken, we do not know how these protocols and rinsing with distilled water may influence the assessment of N rhizodeposition. Nguyen (2003) has pointed out the fact that that some data obtained from root residues not normally distributed may result from the root sampling procedure.

III.3. Methodology for assessing root rhizodeposition

To estimate total root production, Crawford *et al.* (1997) used a sequential coring and summation technique. The principle of this technique is to estimate total root production from repeated, simultaneous measurements of living roots, dead organic material, and decomposition rates of dead roots and old organic material. Using this method, BGN of faba bean accounted for 24% of total plant biomass in the dry season and for 20% in the wet season. However, Crawford *et al.* (1997) conceded that this estimation was underestimated though BGN < 15% of the total plant N was previously reported when simply measured by physically removing of roots from soil (Kumar and Goh, 2000; Voisin *et al.*, 2002; Khan *et al.*, 2003). Moreover, release of N from root exudates, mucilage and sloughed off cap and border cells was not taken into account. It was assumed that the whole amount of N released

from roots over a season and associated below ground turnover N processes may be studied with the held of the ^{15}N isotope. The ^{15}N isotope tracer is rapidly distributed throughout the plant and rhizodeposition leads to a ^{15}N enrichment of the surrounding soil. Therefore, the proportion of total soil N derived from rhizodeposition (*P*Ndfr), including thin roots not collected by hand, is calculated by dividing ^{15}N excess in the soil by the ^{15}N excess of the recovered coarse root.

III.3.1. Choice of a ^{15}N labelling tracer

The used of ^{15}N labelling methodology to measure N rhizodeposition requires that the whole plant be enriched with ^{15}N with relative uniformity and that the soil root zone not be directly labelled (Khan *et al.*, 2002b). Hertenberger and Wanek (2004) reported that labelling with $^{15}NO_3^-$ or $^{15}NH_4^+$ was realised by stem injection or foliar application on large plants like tree species or by immersion of leaves for smaller plants. ^{15}N urea was also used to label individual plants by different shoot labelling methods (Russell and Fillery, 1996a). Compared to $^{15}NO_3^-$ or $^{15}NH_4^+$, urea is an interesting N carrier because it is a non polar highly mobile molecule with a high N content which is rapidly hydrolysed in NH_4^+ and CO_2 by the ubiquitus urease enzyme (Russell and Fillery, 1996a; Rochester *et al.*, 1998; Hertenberger and Wanek, 2004). The labelled ammonium is readily assimilated into amino acids which are translocated throughout the plant. However, a high concentration of urea has been shown to induce leaf necrosis and the interest of this molecule is discussed by Hertenberger and Wanek (2004) in comparison with the application ammonium nitrate which represents a less expensive alternative. Indeed, intermediate concentrations of ammonium nitrate (10mM) are unlikely to disturb internal leaf metabolism severely as nitrate is a common vacuolar compounds of leaves in many plant species and ammonium is produced and assimilated in leaves at high rates during photorespiration. Ammonium is also readily absorbed by plant foliage and transferred to other plant parts. Finally, no differences were observed in the ^{15}N enrichment, ^{15}N recovery, uptake and translocations to shoots and roots of plants fed either with nitrate or ammonium in soybean. Labelling with ^{15}N amino acids such as glutamine or arginine would also be conceivable. However, such labelled amino acids are more expensive than ^{15}N urea and so are not used in such experiments at the present time.

III.3.2. ^{15}N labelling methods

i) Gazeous labelling methods

Uniform ^{15}N enrichment in the different plant parts of legumes was achieved using $^{15}N_2$ atmospheric labelling methods (Ross et al., 1964; Rushel *et al.*, 1979; McNeill *et al.*, 1994) or by $^{15}NH_3$ fumigation for non legumes (Janzen and Bruinsma, 1989; Bazot *et al.*, 2008). In legumes, $^{15}N_2$ is fixed in nodules, metabolised and distributed in the host indifferently from fixed atmospheric N_2. Such methods requires that plants be enclosed in a atmospherically controlled chamber. Plants are generally exposed to a ^{15}N enriched gas during a short time since long exposure would be too expensive. However, severe impacts on cellular acid-base relations were reported with high $^{15}NH_3$ concentrations or long exposure. In any case, these techniques are too sophisticated and laborious for wide-scale field use (Russell and Fillery, 1996a).

ii) ^{15}N dilution technique

Only for legumes which have the ability to acquire N by biological N_2 fixation, the rhizodeposition can be measured with the ^{15}N dilution technique (Fig. 2; Khan *et al.*, 2003). In a first time, the soil is labelled with a ^{15}N fertiliser and the N rhizodeposition is then estimated by measuring the dilution of the soil enrichment by the ^{14}N released from plant root grown in the soil. This method is also used for measuring N_2 fixation by legumes (Unkovich and Pate, 2000). The reliability of the measure requires that the soil is homogeneously labelled. However, small variations in the spatial and temporal distribution of soil ^{15}N were reported to influence strongly N rhizodeposition and N_2 fixation estimations (Hétier *et al.*, 1986; Unkovich and Pate, 2000; Khan *et al.*, 2002b).

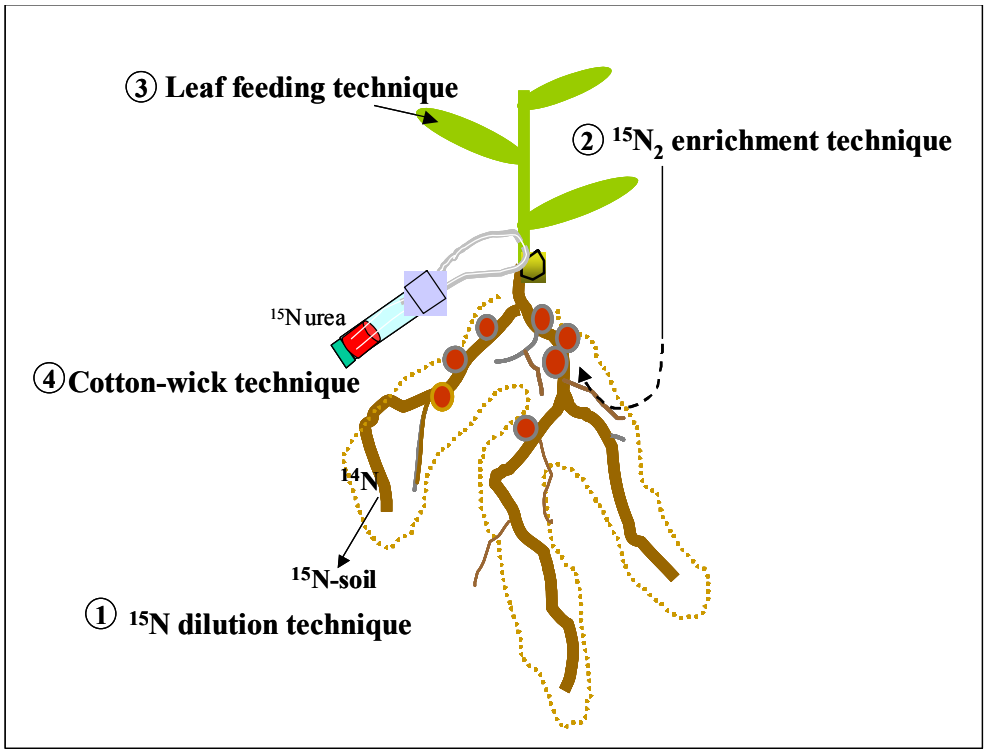


Fig. 2 Some techniques used to assess below-ground contribution of legumes to the soil N-pool in the field. (1) – in the ^{15}N dilution technique, ^{15}N is provided directly to the soil, but the differences in ^{15}N natural abundance between air and soil can also be used. (2) – $^{15}N_2$ enrichment technique : nodulated roots are exposed to $^{15}N_2$. (3) and (4) – in the leaf feeding techniques and in the cotton-wick technique, ^{15}N is provided as urea, nitrate or ammonium to the above-ground parts.

iii) ^{15}N shoot labelling method

Several ^{15}N shoot labelling techniques have been developed applying ^{15}N either by the leaves, the petiole or the stem (Fig. 2). These various approaches have been developed for particular purposes or plant morphologies (Khan *et al.*, 2002b). Plants are most often labelled by exposure to a concentrated solution of ^{15}N-enriched urea. Almost all of the ^{15}N solution is generally taken up within one day and authors carried out one or several applications during plant growth. Khan *et al.* (2002a) compared different shoot labelling methods to the soil ^{15}N dilution technique (Lory *et al.*, 1992 ; Høgh-Jensen and Schjoerring, 2000) and obtained similar results. He concluded that shoot labelling was simple, apparently accurate and, unlike the ^{15}N dilution method, it did not require pre-treatment of the soil with ^{15}N enriched material.

Several leaf feeding methods have been tested, ^{15}N was either applied as a foliar spray of ^{15}N urea or by immersion of intact or cut tip leaves in a ^{15}N urea solution (leaf-flap feeding method) (Jensen, 1996b; McNeill *et al.*, 1997; Khan *et al.*, 2002a). ^{15}N urea has also been

applied as a fine mist or as liquid on the leaves (Schmidt and Scrimgeaour, 2001). Applying ^{15}N urea as a foliar spray or as a fine mist are not possible in field experiments since losses of $^{15}NH_3$ may occur after hydrolysis of urea and N displaced from leaf surfaces or dew. This is not the case when labelling by immersion in a ^{15}N urea solution of intact or cut leaf tips. Such techniques are performed within a confined system which facilitates a greater uptake of ^{15}N and eliminates external losses. However, labelling is always achieved by short term applications as long term applications generate leaf senescence or tissue alteration. Moreover, the ability of these labelling methods to provide uniform ^{15}N root enrichment has been discussed by several authors (Zebarth *et al.*, 1991; Khan *et al.*, 2002a & b).

^{15}N petiole feeding was developed by Rochester *et al.* (1998) for labelling soybean. ^{15}N urea is placed in a plastic vial (4ml) and attached to a petiole from which the leaflet is severed. The tip of the petiole is positioned at the base of the vial, which is removed 48h when almost all the urea solution was drunk. Using this method, Rochester *et al.* (1998) were able to achieve uniform ^{15}N enrichment by multiplying applications of ^{15}N urea (Russell and Fillery, 1996a). Therefore plants tolerate much higher ^{15}N root enrichment by this method compared to foliar feeding.

Among the ^{15}N stem feeding methods, ^{15}N stem injection has mainly been used to label plants such tree species and has also been used by Rochester *et al.* (1998) to label faba bean. However this method is not adapted to plants having a thin stem such as pea.

Multiple applications of ^{15}N urea have also been successful using the ^{15}N cotton-wick method on several legumes including pea (Russell and Fillery, 1996a; Mayer *et al.*, 2003; Wichern *et al.*, 2007). Plants are labelled *via* a cotton wick inserted into a hole in the stem and connected to a vial in which is provided the ^{15}N urea solution. This system is totally water-tight since the wick is protected by two silicon tubes sealed against the stem with Terostat® putty. The labelling solution is supplied through a hole in the top of the vial with the help of a syringe and ^{15}N urea uptake by the cotton wick is driven solely by the transpiration stream. ^{15}N urea is then directly fed into the plant vascular tissue which probably results in a more uniform ^{15}N enrichment of above and below ground parts than leaf and petiole feeding. Moreover the system does not need to be removed after ^{15}N absorption which enables rapid further applications of ^{15}N urea solution. This is important when many plants have to be

labelled in field experiments. In addition, continuous labelling is possible with this method (Russell and Fillery, 1996a).

iv) Split root method

One last approach is the split root method applied with success by several authors on pea and which enables continuous labelling from very young stage to maturity (Sawatsky and Soper, 1991; Jensen, 1996c; Schmidtke, 2005a & b). Plants are previously grown in light expended clay aggregates to remove one part of the radicle and favour the development of a lateral root system. At the 4 leaf stage, the root system is split in two compartments. The labelling solution is provided continually in a first labelling compartment while the amount of ^{15}N released into the soil can be measured in a second transfert compartment. This method presents the disadvantage to substantially disturb the root-system, particularly for species with a taproot and require that plants were previously grown in light expended clay aggregates. Therefore it cannot be used for field experiments. Moreover, assessment of N rhizodeposition only results from one part of the root system.

III.3.3. BGN and Ndfr estimation

The proportions of pea and faba bean Ndfr : BGN (%), BGN : Plant-N (%) and root-N : plant-N (%) from the literature are summarised in (Table 3). In controlled conditions, BGN measured for pea was relatively stable and was around 15% of plant-N when using ^{15}N labelling methods though it varied from 15.2 to 39% for faba bean. For pea the proportion of Ndfr varied from 48 to 82% of the total BGN and between 60 and 73% for faba bean. Only one measure of BGN has been made for each species in field conditions and the results were largely higher than in the greenhouse. A BGN of 30% of the total plant-N was measured for pea and 41% for faba bean. Using ^{15}N labelling methods, BGN varyied by around 68% for chickpea (Khan *et al.*, 2003) and between 38 and 49% for lupin (McNeill and Fillery, 2008) also in the field.

Table 3 N rhizodeposited (Ndfr) expressed as a % of the total below ground N (BGN), BGN expressed as a % of the total plant-N and root expressed as a % of the total plant-N by different varieties of pea (*Pisum sativum* L.) and fababean (*Vicia faba* L.) in controlled and field conditions.

Varieties	Ndfr / BGN (%)	BGN / Plant-N (%)	Root-N / Plant-N (%)	Methods	Growth conditions	References
Pea, controlled conditions						
cv. Baccara***	-	-	6	Physically recovery of roots	Greenhouse, 4 plant pot (5l)	Voisin *et al.*, (2003a)
cv. Century (*P. avense* L.)	46.0	-	20	Split-root, continuous labelling	Growth chamber, 1 plants. Pot^{-1} (2 l)	Sawatsky and Soper, (1991)
cv. *Bodil*	48.0	14.4	7.4	Split-root, continuous labelling	Growth chamber, 1 plants. Pot^{-1} (2 l)	Jensen (1996c)
cv. *Bohatyr*	63.7	16.2	4.7	Split-root, continuous labelling	Greenhouse1 plants. Pot^{-1} (2 l)	Schmidtke (2005b)
cv. *Duel*	82.1	13.8	2.7	Cotton-wick, intermittent pulse	Tunnel, 5 plants. Pot^{-1} (8.5 l),	Mayer *et al.* (2003)
Pea, Field						
cv. *Baccara*	-	-	<2%	Physically recovery of roots	Fertilised pea 0N, 100N, 200N or 400N	Voisin *et al.*, (2002)
(not specify)		12	12	Physically recovery of roots	Rain-fed field	Kumar and Goh (2000)
cv. *Santana*	89.5	30	3.2	Cotton-wick, intermittent pulse	5 plants in PVC $columns^{-1}$ excavated from field (∅:20 cm, L:55cm)	Wichern *et al.* (2007)
Faba bean, controlled conditions						
cv. *Scirocco*	78	15.2	3.8	Cotton-wick, intermittent pulse	Tunnel, 4 plants pot^{-1} (8.5 l),	Mayer*et al.* (2003)
cv. *Fiord**	-	13	13	Physically recovery of roots	Glasshouse, 6 plants 23 l free-draining-pot^{-1}	Khan *et al.* (2002a)
cv. *Fiord**	60.7	39	19.5	Leaf-flap feeding, intermittent pulse	Glasshouse, 6 plants 23 l free-draining-pot^{-1}	Khan *et al.* (2002a)
cv. *Fiord**	12.5	11	10.4	15N dilution technique (continuous)	Glasshouse, 6 plants 23 l free-draining-pot^{-1}	Khan *et al.* (2002a)
Faba bean, Field						
(not specify)**	-	41.0	-	Single feeding events by stem feeding	5 plants 0.5m² area delimiting by Steel frames enclosing (0.5m row *20cm depth)	Rochester *et al.*, 1998

* Collected at late pod-filling
**Collected at peak biomass
*** Collected at seed filling stage

III.3.4. Methodological factors affecting BGN and Ndfr estimation

i) Calculation of the PNdfr : choice of the background values A and B

The use of ^{15}N labelling methodology to measure N rhizodeposition requires that the whole plant be enriched with ^{15}N with relative uniformity (Khan *et al.*, 2002b). For the calculations of net rhizodeposition, the following assumptions are made: 1) uptake and use of ^{14}N and ^{15}N by the plant is identical, 2) N deposited has the same enrichment as the roots. The proportion of total soil N derived from rhizodeposition (*P*Ndfr) is calculated by dividing the ^{15}N excess in the soil at harvest by the ^{15}N excess of the root grown in the soil (Eq. 1; Janzen and Bruinsma, 1989) :

(Eq. 1): *p*NdfR = atom % ^{15}N excess soil / atom % ^{15}N excess roots

(Eq. 2): *p*NdfR = (at. % ^{15}N soil – at. % ^{15}N background A) / (at. % ^{15}N roots – at. % ^{15}N background A)

The atom % ^{15}N excess is more often obtained by correcting the ^{15}N abundance in the soil and roots by the ^{15}N abundance of the background abundances for the soil and roots, respectively (Eq. 2). Using the continuous ^{15}N labelling split-root method, Schmidtke (2005a) showed that the reliability of the results may be greatly altered by the choice of the background values. The most reliable estimations were obtained when i) a non-nodulated reference plant or a legume grown on soil without labelling was used for the root background value and ii) a high ^{15}N root abundance was achieved. For example, using the natural ^{15}N abundance of atmospheric N_2 for correction, when the ^{15}N root abundance was of 0.5 at.%^{15}N resulted in a substantial overestimation of the Ndfr though it had no effect when the ^{15}N root abundance was of 2 at.%^{15}N.

ii) Homogeneity of the ^{15}N enrichment

When calculating the proportion of total soil N derived from rhizodeposition (*P*Ndfr), it is assumed that the average ^{15}N abundance of the rhizodeposits matches the average ^{15}N abundance of the roots. However, variations in the ^{15}N root enrichment along the longitudinal root axis (Russell and Fillery, 1996a; Khan *et al.*, 2002b) or between root classes (Walker *et al.*, 2003) have been observed regardless of the method used. These variations may largely account for inaccurate estimations since several mechanisms are involved in rhizodeposition

and because i) rhizodeposits are not released in similar proportions, ii) patterns of root exudation vary along the longitudinal axis (Uren, 2001; Nguyen, 2003; Walker *et al.*, 2003) and iii) proportions of N released by the different processes vary with root age. Several authors have also observed that nodules are less often enriched than roots (Russel and Fillery, 1996a; Khan *et al.*, 2002b) and to a large extent, we are still unable to determine how this uneven root enrichment may influence the assessment of N rhizodeposition.

Moreover, in the different experiments conducted to refine the ^{15}N methodology, it has been observed that ^{15}N root enrichment is influenced by the ^{15}N labelling technique used (Khan *et al.*, 2002b; Yasmin *et al.*, 2006), the frequency of labelling (Russell and Fillery, 1996a) and the concentration of the labelling solution (Khan *et al.*, 2002b).

iii) Effect of labelling frequency

Except by the split root method which enables to label the plant continuously from a very young stage to maturity, it remains difficult to continuously apply a labelling solution via the shoot. An alternative solution is to multiply applications of the labelling solution. Russell and Fillery (1996a) compared weekly and fortnightly pulse labelling and observed that the distribution of ^{15}N within the different plants parts was reproducible with the both frequencies. Nevertheless, more applied ^{15}N was retrieved in root biomass when plants were labelled fortnightly and the authors concluded that intermittent labelling of lupin was therefore preferable to maximise ^{15}N enrichment of below ground biomass.

Though a number of differences in the distributions of ^{15}N have been observed, no effects of labelling frequency on the estimation of N rhizodeposition have been reported to date.

IV. Objectives

Although the agronomic benefit of introducing a legume into a crop rotation is well known, the assessment of its nitrogen contribution to the soil N pool remains unclear, mainly because of difficulties in BGN, especially N rhizodeposition. An accurate assessment of the amount of N released into the soil by legumes is necessary i) to understand legume effects on soil N dynamics and its involvement in the positive yield effect generally observed on subsequent cereal crops ii) to enhance the use of legumes as an alternative to the application of N fertiliser for farming systems to be sustainable and remain productive in long term.

Until now, most studies have been conducted in the greenhouse and have focused on methodology though few data are available on methods suitable for field studies. Results of these studies vary considerably as experiments have been undertaken using different methods, different species and varieties, in a variety of environmental conditions. Several ^{15}N labelling methods have been developed for assessing nitrogen rhizodeposition. However, large differences remain between values of BGN measured in the field and in the greenhouse. Morever no precise protocol exists detailing how ^{15}N-based experiments should be designed to achieve a fine degree of accuracy in measuring the amount of N derived from rhizodeposition in the field.

Furthermore, factors regulating the allocation of N to below ground parts are not well understood. For a given species, effects of growth conditions (such as water or N availability), sources of N nutrition (nitrogen fixation or inorganic N uptake) or genotype (NHI) have not yet been investigated.

The present study focuses on different aspects of BGN research:

1) Methodology: the comparison and refining of ^{15}N labelling methods for the BGN assessment of field grown pea
2) Effect of plant genetic characteristics through NHI as well as the effects of N sources and abiotic factor such as N fertilisation level and water availability.

The results presented in the manuscript were obtained from eight experiments. The first three experiments were conducted in Angers (LEVA) on pea cv. Frisson and cv. Cameor to compare the split root and the cotton-wick labelling method in measuring the N

rhizodeposition in the greenhouse (2004 & 2005) and in the field (2005). In 2006, the effect of N fertilisation levels on N rhizodeposition was investigated in the field and greenhouse on Frisson, P2 (isoline of Frisson unable to fix N_2) and cv. Baccara peas. In the greenhouse, all treatments were doubled and N rhizodeposition was measured after either continuous or fortnight labelling using the cotton-wick method. Effects of water stress on pea cv. Solara were investigated in a growth chamber in Risoe National Laboratory (GLIP fellowship Roskilde, Danemark). In 2007, a trial experiment was conducted to test the effect of the concentration of the labelling solution on Frisson.

RESULTS

Chapter I. Methodological issues

The first step of my Phd work was to compare two labelling methods in terms of their ability to assess the amount of N derived from rhizodeposition. The ^{15}N stem feeding method developed by several authors (Russell et Fillery, 1996a; Rochester *et al*., 1998; Mayer *et al.,* 2003) was reported to be suitable for field studies and the split root method was one of the most commonly used ^{15}N labelling techniques (Jensen, 1996c; Schmidtke, 2005a & b): both methods were tested in the field and in the greenhouse.

I. Comparison of two ^{15}N labelling methods for assessing nitrogen rhizodeposition of pea

Stéphanie Mahieu, Joëlle Fustec*, Marie-Line Faure, Guénaëlle Corre-Hellou and Yves Crozat

Laboratory of Plant Ecophysiology and Agroecology, ESA, 55 rue Rabelais, BP 30748, 49007 Angers cedex 01, France – j.fustec@groupe-esa.com

Short running title: 15N labelling method comparison

*Corresponding author:
Joëlle Fustec,
Lab. of Plant Ecophysiology and Agroecology,
ESA, 55 rue Rabelais, BP 30748,
49007 Angers cedex 01, France
Tel 33 2 41 23 55 55
Fax 33 2 41 23 55 03
j.fustec@groupe-esa.com

Abstract

Two ^{15}N labelling methods for assessing net rhizodeposition of nitrogen (N) in pea crop (*Pisum sativum* L.) were compared in the greenhouse and in the field: the cotton-wick (CW) and the split-root (SR) methods. Rhizodeposition is defined as the organic material lost from roots during their growth through the soil. CW is a method in which ^{15}N urea was supplied to the plant in pulses *via* a wick threaded through the stem. In SR, the root system was divided between a hydroponic labelling compartment (LC) containing the labelling nutrient solution (1 *mM* or 5 *mM* $15NO_3$-$15NH_4$) and a compartment filled with soil in which the amount of ^{15}N rhizodeposition was assessed. The percentage of N derived from rhizodeposition (%Ndfr), was used to calculate the amount of N rhizodeposition which was obtained from the ratio of atom % ^{15}N excess of the soil : atom % ^{15}N excess of the roots. Above ground parts in the field accumulated markedly more dry matter and N than in the greenhouse, regardless of the labelling method. ^{15}N enrichments of above ground parts were higher than those of roots recovered from the soil. Results indicated that amount of ^{15}N applied to plants were lower in SR than in CW. Additionally, LC roots of SR tended to retain large amounts of ^{15}N. As a consequence, atom % ^{15}N excess of roots was less than 1% in SR, whereas most values varied from 1 to 4% in CW. However, relationships between enrichments of the soil and of the roots were different in SR and CW. It was not possible to compare the Ndfr : root-N ratio between the two methods, but the ratio of Ndfr : plant-N was found to be 10% higher in SR than in CW. Finally, relative to total plant-N, the total contribution of below ground parts to the N pool of the soil reached 22-25% at maturity for the two methods. From our experiments, we could not conclude that one method is better than the other for estimating either net rhizodeposition of N or the contribution of a pea plant to the soil N pool. However, CW is easier to adapt and monitor under field conditions than SR.

Key words: Cotton-wick, Isotopic methods, Legumes, Nitrogen, *Pisum sativum* L., Split root

I.1. **Introduction**

The nitrogen (N) contribution of the below ground parts of grain legumes to the soil N pool (BGN) is rarely measured, though it is necessary for assessing the contribution of a legume crop to soil N balance in different agronomic situations. The BGN is defined as the sum of root N and N rhizodeposition (Mayer *et al.* 2003). Rhizodeposition is defined as the organic material lost from roots during their growth through the soil: root debris, decomposed root material, cell lysates and mucilages (Lynch and Whipps 1990). Moreover, when estimated, ratios of BGN to total plant-N are obtained by a variety of methods, and results reported in the literature therefore vary widely (Jensen 1997; Kumar and Goh 2000; Corre-Hellou and Crozat 2003). As a consequence, conclusions drawn about the relative role of a given species in legume crop rotation may be greatly influenced by the methodology used for BGN estimation.

For pea (*Pisum sativum* L.), values of BGN to total plant-N reported in the literature may appear similar between studies (from 12 % to 16 %; Jensen 1997; Kumar and Goh 2000; Mayer *et al.* 2003). However, such values are in fact calculated from either N rhizodeposition plus root-N, or from recovered root-N alone (Crozat and Fustec 2006). Furthermore, quantification of N rhizodeposition by legumes remains a critical point in BGN calculation. Several methods using ^{15}N labelling techniques have been used for determining the fate of N within the plant-soil system after applying ^{15}N to part of the plants. Split-root ^{15}N labelling has thus been used in the greenhouse (Reining *et al.* 1995; Jensen 1996b; Schmidke 2005a, b), $^{15}NH_3$ fumigation in the laboratory (Janzen and Bruinsma 1989), ^{15}N cotton-wick stem labelling in a greenhouse or plastic tunnel (Russel and Fillery 1996; Mayer *et al.* 2003), ^{15}N foliar feeding either in the greenhouse or in the field (Vasilas *et al.* 1980; McNeill *et al.* 1997; Khan *et al.* 2002), ^{15}N petiole feeding and physical root recovery in the field (Rochester *et al.* 1998; Kumar and Goh 2000; Khan *et al.* 2003). The method selected has often depended on the morphology of the studied plant species and on constraints induced by growing conditions (greenhouse, plastic tunnel or field).

Comparison of methods helps to understand differences obtained when quantifying N rhizodeposition and BGN contribution to soil-N. In order to improve management of crop successions that include legumes it would also be interesting to adapt ^{15}N labelling techniques for the quantification of N rhizodeposition in the field. $^{15}NH_3$ fumigation leads to a good distribution of ^{15}N in plant parts, but would be too complicated to use *in situ* (Janzen and

Bruinsma 1989; Russel and Fillery 1996). Yasmin *et al.* (2006) recently compared cotton-wick stem injection with leaf and petiole labelling and observed that ^{15}N enrichment with petiole feeding was low compared with both other methods. Leaf feeding techniques are not considered suitable for use in the field as the quantity of ^{15}N recovered in roots and soil may be invalidated because of displacements of ^{15}N from the leaf surface to the soil during rainfall, and risks of NH_3 losses (Vasilas *et al.* 1980). Russel and Fillery (1996) and Mayer *et al.* (2003) applied cotton-wick stem-feeding method to pea, lupin (*Lupinus albus* L.) and faba bean (*Vicia faba* var *minor* L.). They used ^{15}N urea which presents two advantages: it is a non-polar highly mobile molecule of high N content compared to $^{15}NO3^-$ and $^{15}NH4+$, and it is rapidly taken up and metabolized by urease (Khan *et al.* 2002; Hertenberger and Wanek 2004). This method seems suitable for a great number of legume species and is easier and more efficient *in situ* than leaf feeding. However, ^{15}N enrichment is always lower in roots than in above ground parts of the plant (Mayer *et al.* 2003; Yasmin *et al.* 2006). Schmidtke (2005a) concluded that ^{15}N enrichment should be much higher using a root-feeding than a shoot-feeding technique. At the moment, no comparison between above ground and below ground feeding methods has been reported.

The aim of this study was to compare the ^{15}N cotton-wick stem feeding technique and the split-root ^{15}N labelling method in the greenhouse in terms of their ability to assess the amount of N derived from rhizodeposition (micro-roots and exudates). Both methods were also adapted and tested in the field.

I.2. **Materials and methods**

I.2.1. Labelling methods

The cotton-wick method (CW; Fig. 1A) consisted in labelling the plants with a solution of ^{15}N urea (99 atom% ^{15}N) held in a 2-mL reservoir and absorbed by the plant *via* a cotton wick threaded through a 0.5-mm hole in the internode located just below the first true leaf (Russel and Fillery 1996; Mayer *et al.* 2003). To prevent solution losses, the wick was protected from dessication by two silicon tubes sealed against the stem with Terostat® putty.

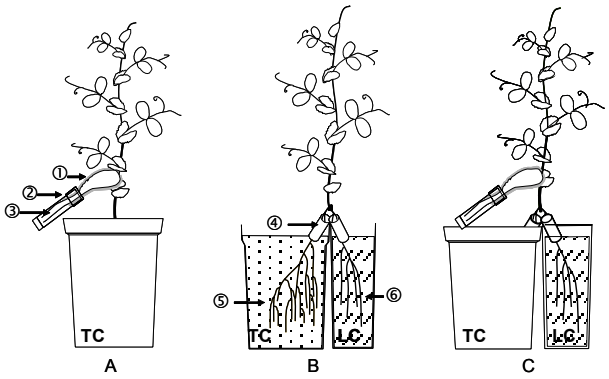


Fig. I.1: Cotton-wick (CW) and split-root (SR) methods used to label pea plants. A – ^{15}N stem feeding by CW. B – ^{15}N root feeding by SR. C – Pea treated by SR and CW and labelled either by CW or by SR (sealing putty not shown). (TC) transfer compartment filled with sandy soil in which rhizodeposition was measured, (LC) labelling compartment containing clay marbles and filled with ^{15}N labelling solution, (1) silicone tube protecting the cotton wick from desiccation, (2) vial filled with ^{15}N urea solution, (3) wick, (4) rubber tube protecting roots from desiccation, (5) roots of the TC, (6) roots of the LC.

For the split-root technique (SR; Fig. I.1B), part of the root system was bathed in ^{15}N labelling solution (Jensen 1996b; Schmidke 2005a, b). First, seeds were sown in vermiculite and the radicle was partly cut (upper 1 cm left intact) to stimulate the development of lateral roots. At the 4 leaf stage, each plant was removed from the vermiculite, and half of the root system was placed into a compartment containing soil, while the other half was placed into a 0.33-L hydroponic compartment filled with clay marbles and a ^{15}N nutrient solution (hereafter called the labelling compartment, LC). The plant roots were protected from dessication by means of rubber tubes. The stem base was fixed to the tube with Terostat® putty. Hereafter, the pot filled with soil will be called the transfer compartment (TC), since ^{15}N rhizodeposition released by ^{15}N enriched roots was measured in the soil. A plastic straw was plunged into the glass in order to remove the nutrient solution before renewing it. Before labelling, LCs were filled with a N-free solution to prevent differential growth between the treatments. The N-free solution contained: 0.2 *mM* K_2HPO_4, 0.26 *mM* K_2SO_4, 0.2 *mM* $MgSO_4$ $7H_2O$ and 0.67 *mM* $CaCl_2$ $6H_2O$ (called SR0 treatment). In labelled plants the N-free solution was replaced by another containing either 1 *mM* $^{15}NH_4$-$^{15}NO_3$ (10 atom % ^{15}N; treatment called SR1), or 5 *mM* $^{15}NH_4$-$^{15}NO_3$ (10 atom % ^{15}N; treatment called SR1+), and 0.^{15} *mM* K_2HPO_4, 0.^{15} *mM* K_2SO_4, 0.3 *mM* $MgSO_4$ $7H_2O$, 0.6 *mM* $CaCl_2$ $6H_2O$. In all the nutrient solutions, microelements were supplied using a 5% diluted commercial solution (Pokon®).

I.2.2. Greenhouse experiments

Using pea (*Pisum sativum* cv. Frisson), two successive greenhouse experiments were conducted in autumn 2004 and in spring 2005 in Angers (France, 47°37'N, 0°39'W). Plants were grown in 2-L plastic pots (TC) holding 1.8 kg of dry sand-soil mixture and randomly distributed on culture tables (one plant per pot). They were watered with a syringe to avoid N

losses by percolation. Plants were considered to have reached pod-filling stage when one seed was 6-mm wide (Ney and Turc 1993), and maturity when all above ground parts had turned yellow. Throughout the experiments, shed organs were collected in a mesh fixed around the pot.

2004 greenhouse – This experiment was carried out from mid-October 2004 to the beginning of February 2005. The mean temperature during the growing period was 17°C and the relative humidity 65%. The TC was filled with a mixture of 1/3 sand and 2/3 soil. The soil used was a clayey sand with a pH_{H2O} of 7 and containing 1% organic matter, 13% clay, ^{15}% silt and 72% sand, 0.6% total C, 0.07% of total N, 67 mg P kg^{-1} (Olsen), 80 mg K kg^{-1}, 116 mg Mg kg^{-1} (extractant: ammonium acetate). Plants were treated using either CW, or SR, or both methods combined on the same individual (Fig. 1). In CW, pulses of labelled urea (0.5 mL) were supplied either fortnightly (CW1R), or at precise stages (CW1S) from the 8 leaf stage onwards: 'flowering', 'pod-filling' and 'seed maturity'. CW reservoirs were filled with either 99 atom % ^{15}N urea solution (CW1R and CW1S) or deionised water (CW0). The labelled urea solution concentration was calculated based on N demand curves of pea to keep a 2.5% ^{15}N excess in the plant, and ranged from 0.36 to 0.82% labelled urea (w/v) (Mayer *et al.* 2003). After absorption of the labelled solution of a pulse, 0.3 mL of deionised water was supplied to rinse the CW reservoirs and remove remaining labelled urea; this water was absorbed by the plant before the next urea pulse. From the 8 leaf stage onwards, SR labelling compartments were filled with a nutrient solution containing either 1 *mM* $^{15}NO_3$-$^{15}NH_4$ (SR1) or no nitrogen (SR0). We thus obtained seven treatments with eight replicates each: (1) CW1R, (2) CW1R/SR0, (3) CW1S, (4) CW1S/SR0, (5) SR1, (6) SR1/CW0, and (7) SR0/CW0 (control plants). In each treatment, four plants were harvested at pod-filling and four at maturity.

2005 greenhouse – A second experiment was carried out from April 2005 to the middle of June 2005. The mean temperature during the growing period was 21.5°C and the relative humidity 60%. TCs were filled with a mixture of 1/3 sand and 2/3 soil. The soil was characterized by a pH_{H2O} of 7 and contained 1.4 % organic matter, 17.3% clay, 16.7% silt and 66.1% sand, 0.83% total C, 0.10% of total N, 80 mg P kg^{-1} (Olsen), 340 mg K kg^{-1}, 126 mg Mg kg^{-1}. Plants were treated using either CW or SR (Fig. 1A, B). Labelling started at the 6 leaf stage. For CW, 0.5-mL pulses of 0.6% (w/v) labelled urea solution (99 atom % ^{15}N) were supplied fortnightly (CW1R) and control CW reservoirs were filled with deionised water (CW0). SR labelling compartments were filled with either 1 *mM* $^{15}NO_3$-$^{15}NH_4$ solution (SR1)

or 5 *mM* $^{15}NO_3$-$^{15}NH_4$ (SR1+), or N-free solution (control plants, SR0). We thus obtained five treatments with eight replicates each: (1) CW0 (2) CW1R, (3) SR0, (4) SR1, (5) SR1+. In each treatment, four plants were harvested at pod-filling, and four at maturity.

I.2.3. Field experiment

A field experiment was carried out from April 2005 to July 2005 at the "Fédération Nationale des Agriculteurs Multiplicateurs de Semences (FNAMS)" experimental station near Angers. As the soil used for the 2005 greenhouse assay was removed from the upper 0-25 cm layer of this field, characteristics were the same as described above. Both labelling methods were tested on two pea cultivars: cv. Frisson (Fr) and cv. Cameor (Ca). The experiment was designed in three blocks, each containing a plot of six rows for each variety of pea (10-m long, 0.35-m spaced rows, 80 plant.m^{-2}). In order to test CW1R and SR1+ labelling methods in the field, three microplots (0.40 m × 0.20 m) were marked within each plot with four plastic sheets pushed into the soil up to a depth of 30 cm to prevent lateral losses of applied ^{15}N (Khan *et al.* 2003). Each microplot contained four neighbouring plants from a row. To test SR1+, split-rooted peas were prepared in the greenhouse until the 4 leaf stage, as it was not possible to prepare them directly in the field. Next to each microplot used to test SR1+, four glass labelling compartments were driven into the soil, against the outer side of one of the lateral plastic sheets. Plants previously sown in SR1+ microplots were removed and replaced by peas that had been split-rooted in the greenhouse. Root systems were divided between the inside of the microplot and one of the four LCs driven into the soil. LCs were filled with clay marbles and a nutrient solution without N. They were protected from rainfall and irrigation by a plastic cap. Shed organs were collected in a mesh fixed around the plant. At the 6 leaf stage, nutrient solution without N was replaced by a 5 *mM* $^{15}NO_3$-$^{15}NH_4$ solution (SR1+) and labelling of CW1R started (fortnightly 0.5 mL pulses of 0.6% (w/v) labelled urea solution 99 atom % ^{15}N). All plants were harvested at maturity. At the same time, in each plot, non labelled plants were harvested from a 1-m² area and considered as controls.

I.2.4. Sampling and measurements

After harvest, for all experiments, plants were sorted into different samples: *(i)* 'TC roots' (i.e. all recovered visible roots), *(ii)* 'seeds', *(iii)* 'leaves and stems' (i.e. other above ground parts pooled with pods and collected shed organs), and *(iv)* 'LC roots' for SR plants only. The

roots with adhering soil were shaken for several minutes with 50 mL deionised water in a closed dish before being rinsed with 200 mL water over a sieve (2 mm; Mayer *et al.* 2003). In the greenhouse assays, the soil in the TCs was sampled. In the field microplots, taproots were removed from the soil. Four soil cores (5 cm diameter) at 0-35 cm and 35-45 cm depths were collected between the pea row and the plastic sheet. In these soil samples, visible root fragments were collected by hand, washed, and pooled together with the macro root sample. In all cases, the water used to rinse the roots was added to the soil samples. Plant and soil samples were dried at 60°C and 30°C respectively, weighed, and ground to a fine powder before total N and ^{15}N analyses. Total N concentration was measured according to the Dumas method and ^{15}N : ^{14}N ratio was determined by mass spectrometry at the INRA of Laon, France (spectrometer Isochron and N analyser NA $^{15}00$, Fisons, Manchester, UK).

I.2.5. Calculations and statistics

The proportion of the total N derived from rhizodeposition (Ndfr) at harvest was calculated using Janzen and Bruinsma's equation (1989):

$$\%\ Ndfr = (at.\%^{15}N\ soil - at.\%^{15}N\ background\ A) / (at.\%^{15}N\ roots - at.\%^{15}N\ background\ B) \times 100$$

The soil and the roots (from TC) of the non ^{15}N-fed control plants were chosen as backgrounds A and B, respectively (Schmidtke 2005b). The amount of N rhizodeposition was assessed from %Ndfr value and the amount of total soil-N. The amount of rhizodeposition was used to calculate the ratio of Ndfr : 'TC roots'-N, the ratio of Ndfr : plant-N and the percentage of total BGN (visible root N + N rhizodeposition) to total plant-N. The following assumptions were made: *(i)* N deposited and roots grown in the soil had the same ^{15}N enrichment, and *(ii)* the micro roots collected by hand and by wet sieving had the same ^{15}N abundance as the macro roots.

As it was not possible to test the assumptions of normality and standard deviation equality for such small samples, data were first compared with Kruskal-Wallis and Mann-Whitney tests based on median equality (GraphPad Prism 5 Software 2007). Therefore, for each plant sample and for each harvest stage, differences in dry weights, N concentrations, N contents, N and ^{15}N distribution, atom % ^{15}N excess in the different plant parts were compared between treatments, across years and experiments. In the field, Fr and Ca were compared for all variables. Second, greenhouse data were pooled by labelling method for all harvest stages and years: SR (SR1, SR1+), CW (CW1S and CW1R), CW1SR0 (CW1S/SR0 and CW1R/SR0)

and SR1/CW0. As they did not meet the assumption of normality (Agostino-Pearson test), data were log-transformed to investigate linearity and similarity of relationships between 'TC roots' and soil ^{15}N enrichments, between Ndfr amount and plant-N, and between BGN and plant-N (GraphPad Prism 5 Software 2007).

I.3. **Results**

I.3.1. Dry weights

In the greenhouse, dry weights of CW1R and SR1 were similar at pod-filling (Fig. I.2A). At maturity, dry weights of CW1R and SR1 were significantly lower in 2005 than in 2004 (Fig. I.2B). Total dry weights were lower in peas treated solely by CW than in split-rooted plants (Fig. I.2A, B). The proportion of root biomass was 14.48 ± 0.68% in plants treated solely by CW and 11.22 ± 0.45% in split-rooted peas (p = 0.0005).

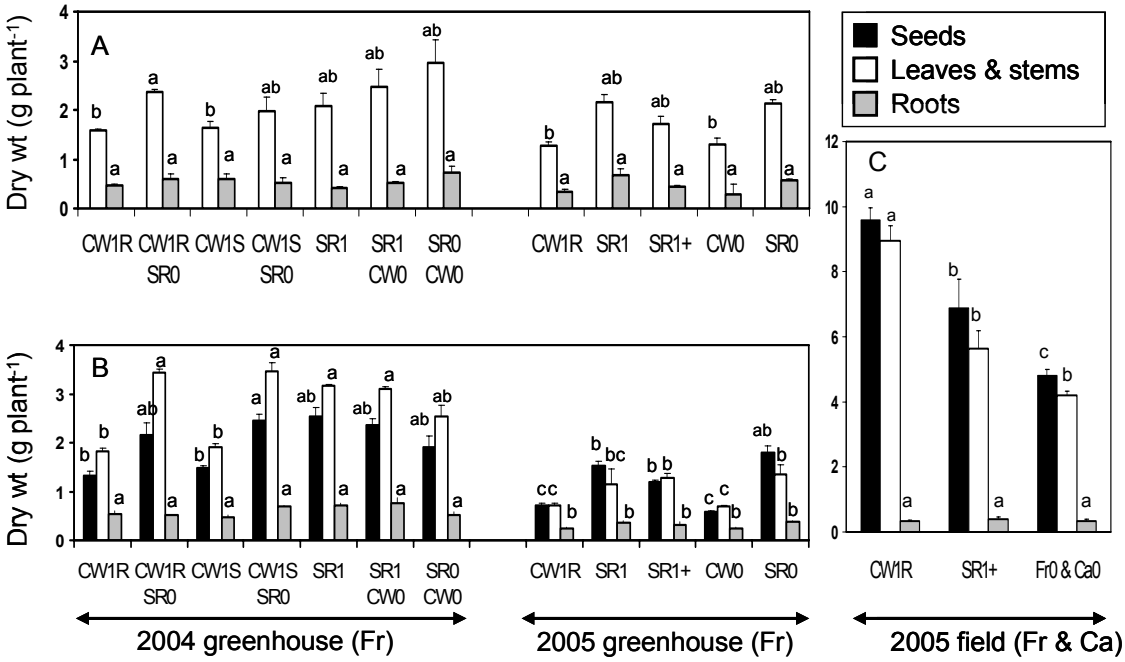


Fig. I.2: Mean dry weights of plant organs. A – At pod-filling in the 2004 and 2005 greenhouse experiments with pea Frisson. B - At maturity in the 2004 and 2005 greenhouse experiments. C – At maturity in the field with Frisson and Cameor cultivars (pooled data). Plants were labelled by cotton wick every fortnight (CW1R) or at given stages (CW1S), or by split-root 1 mM $^{15}NO3$-$^{15}NH4$ (SR1) or 5 mM $^{15}NO3$-$^{15}NH4$ (SR1+). CW0, SR0, Fr0 and Ca0 are unlabelled controls. Bars are S.E. (n = 3 to 4). Different letters (a, b, c) indicate significant differences between samples.

I.3.2. Concentrations, contents, partitioning and remobilization of N

Similar plant-N concentrations were obtained in both greenhouse assays: on average, at maturity, were encountered 23.7 ± 0.5 mg N kg DM^{-1} in 'TC roots', 17.1 ± 0.7 mg N kg DM^{-1} in 'leaves and stems' and 34.5 ± 0.5 mg N kg DM^{-1} in 'seeds'. In the field, N concentrations of 'TC roots' (18.7 ± 0.9 mg N kg DM^{-1}) and of 'leaves and stems' compartments (14.3 ± 0.9 mg N kg DM^{-1}) were lower than in the greenhouse ($p < 0.05$). In contrast, N concentration in 'seeds' was higher in the field (38.9 ± 0.7 mg N kg DM^{-1}; $p < 0.0001$). In split-rooted peas, N concentration of 'LC roots' was 30% higher than that of 'TC roots' ($p < 0.0001$).

In the greenhouse, the proportion of N allocated to roots reached about 10-15% of the plant-N ($p > 0.05$; Fig. I.3A, B). In the field, plants allocated less than 5% of plant-N to the roots (Fig. I.3C). N allocated to 'seeds' represented 40-50% of the plant-N in 2004, and 60-75% in 2005 (greenhouse and field).

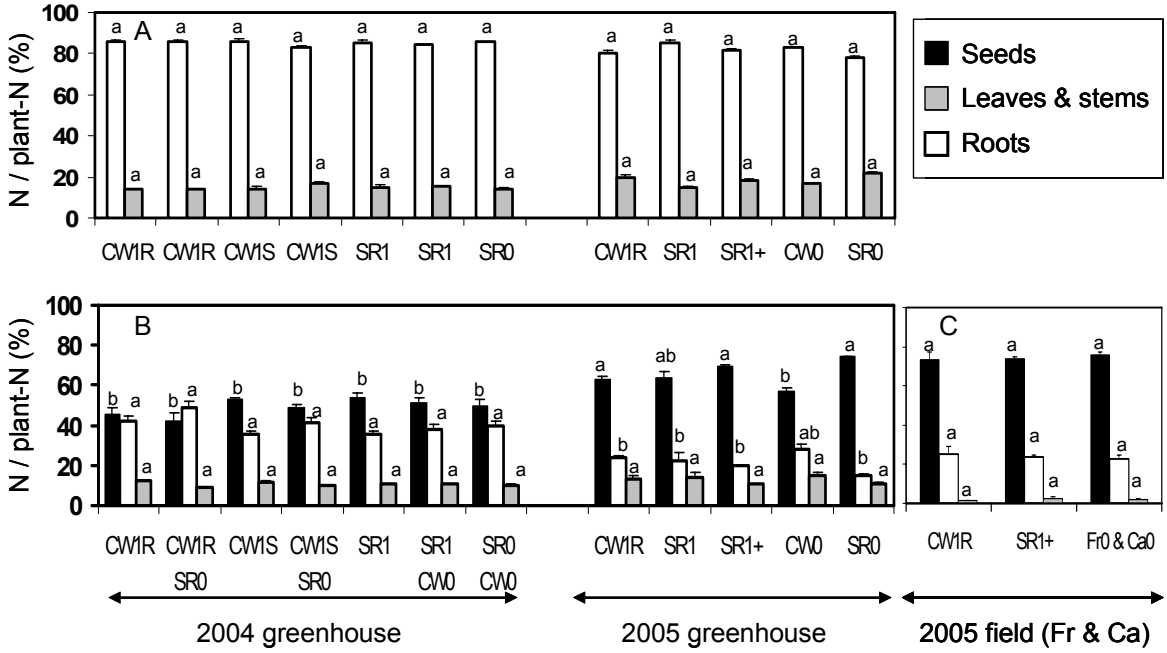


Fig. I.3: N partitioning (%) in pea at maturity. A - At pod-filling in the 2004 and 2005 greenhouse experiments with pea Frisson. B - At maturity in the 2004 and 2005 greenhouse experiments with Frisson. C - At maturity in the field with Frisson and Cameor (pooled data). Plants were labelled by cotton wick every fortnight (CW1R) or at given stages (CW1S), or by split-root 1 mM $^{15}NO3$-$^{15}NH4$ (SR1) or 5 mM $^{15}NO3$-$^{15}NH4$ (SR1+). CW0, SR0, Fr0 and Ca0 are unlabelled controls. Bars are S.E. (n = 3 to 4). Different letters (a, b, c) indicate significant differences between samples.

In the greenhouse, from pod-filling to maturity, the 'leaves and stems' compartment lost less N in CW1S and CW1R than in split-rooted plants: in 2004, about 12 mg N were lost in

CW1R and CW1S against 20 mg N in split-rooted plants (Fig. I.4A, B). The amount of N in 'TC roots' did not differ between the two stages ($p > 0.05$).

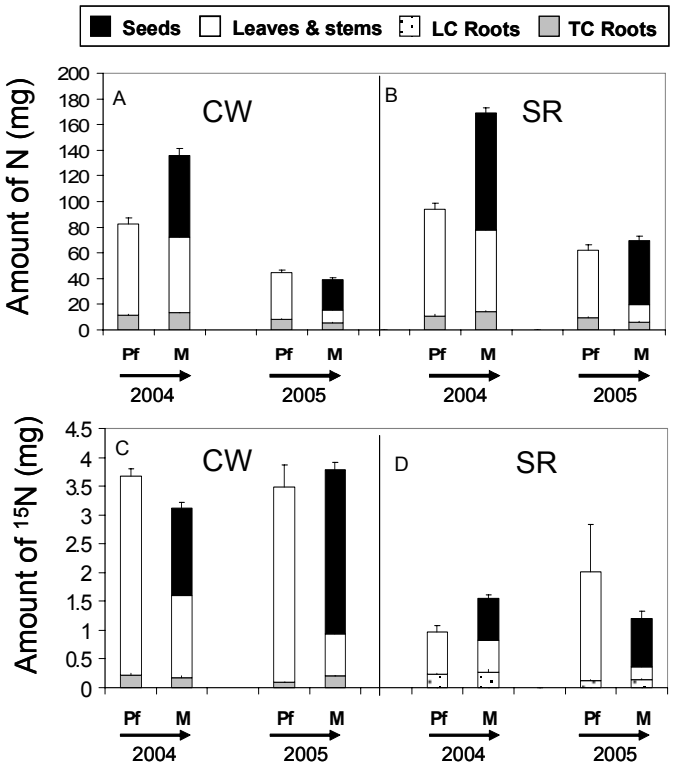


Fig. I.4: Amount of total N and ^{15}N in plant organs at pod-filling (Pf) and at maturity (M) in pea Frisson labelled either by cotton-wick (CW: CW1S and CW1R) or by split-root (SR: SR1, SR1+ and SR1/CW0) in the 2004 and 2005 greenhouse experiments. A – Amount of N in plants labelled by CW. B – Amount of N in plants labelled by SR. C- Amount of ^{15}N in plants labelled by CW. D - Amount of ^{15}N in plants labelled by SR. Bars are S.E. (n = 6 to 8).

I.3.3. ^{15}N distribution and enrichment

In the greenhouse, the amount of ^{15}N recovered from plants was more than 50% higher in CW than in SR. About 6% of the plant-^{15}N was located in 'TC roots' except in split-rooted plants in which it reached 10 to 20% of the plant-^{15}N (mainly located in 'LC roots'; Fig. I.4C, D). At maturity, 50 to 70% of the plant-^{15}N was allocated to seeds. From pod-filling to maturity, 'leaves and stems' lost 1.7 to 2.5 mg ^{15}N per plant, except split-rooted plants in 2004 for which a small amount of ^{15}N was found in 'leaves and stems' at both stages. There was no significant difference between pod-filling and maturity in the amount of ^{15}N in 'TC roots'.

The atom % ^{15}N excess of above ground parts and 'TC roots' from CW1R plants were higher in 2005 than in 2004 (Table I.1). Such a difference in N isotope enrichment was not observed in SR1. The atom % ^{15}N excess values were generally lower in the field than in the greenhouse. In all assays, at pod-filling and at maturity, atom % ^{15}N excess of 'leaves and stems' tended to be substantially higher in CW than in SR plants ($p < 0.0001$; Table I.1). As a consequence, 'TC roots' in the CW treatment had higher enrichment than in SR ($p < 0.001$).

Table I.1: Atom ^{15}N % excess of plant parts and soil.

	Seeds	Leaves & stems	TC roots	LC roots	Soil (×100)
Pod-filling					
2004 Greenhouse [a]					
CW1R	-	6.05 ± 0.06 b	2.67 ± 0.05 b	-	1.56 ± 0.14 a
CW1R SR0	-	4.50 ± 0.10 c	1.87 ± 0.02 c	4.07 ± 0.31 b *	0.62 ± 0.03 b
CW1S	-	5.15 ± 0.32 c	1.98 ± 0.06 abc	-	1.33 ± 0.11 a
CW1S SR0	-	3.55 ± 0.29 d	1.83 ± 0.12 cd	2.77 ± 0.17 c *	0.55 ± 0.04 b
SR1	-	0.87 ± 0.21 e	0.25 ± 0.06 e	6.20 ± 0.04 a *	0.12 ± 0.06 b
SR1 CW0	-	0.83 ± 0.09 e	0.26 ± 0.03 e	6.63 ± 0.23 a *	0.19 ± 0.05 b
2005 Greenhouse [a]					
CW1R	-	8.48 ± 0.57 a	3.47 ± 0.36 a	-	1.45 ± 0.18 a
SR1	-	0.80 ± 0.06 e	0.31 ± 0.06 e	5.37 ± 0.57 ab *	0.32 ± 0.02 b
SR1+	-	0.86 ± 0.12 e	0.64 ± 0.06 d	5.39 ± 0.57 ab *	0.50 ± 0.04 b
p		****	***	**	***
Maturity					
2004 Greenhouse [a]					
CW1R	3.75 ± 0.18 b	4.50 ± 0.38 b	2.02 ± 0.09 b	-	1.52 ± 0.13 b
CW1R SR0	1.39 ± 0.06 c	3.23 ± 0.03 c	1.60 ± 0.11 c	3.37 ± 0.35 ac *	1.07 ± 0.03 c
CW1S	2.17 ± 0.09 b	2.60 ± 0.06 c	1.11 ± 0.06 ce	-	0.87 ± 0.10 de
CW1S SR0	1.12 ± 0.07 d	1.82 ± 0.11 d	0.98 ± 0.11 cg	1.57 ± 0.08 d *	0.62 ± 0.07 d
SR1	0.91 ± 0.05 de	1.00 ± 0.11 e	0.34 ± 0.06 df	6.83 ± 0.14 ab *	0.40 ± 0.01 de
SR1 CW0	0.67 ± 0.03 ef	0.73 ± 0.03 e	0.27 ± 0.01 df	6.82 ± 0.17 ab *	0.45 ± 0.05 de
2005 Greenhouse [a]					
CW1R	11.01 ± 0.74 a	7.39 ± 0.47 a	3.60 ± 0.27 a	-	1.72 ± 0.17 b
SR1	1.15 ± 0.07 cd	1.00 ± 0.11 e	0.26 ± 0.03 d	6.52 ± 0.27 b *	0.35 ± 0.03 e
SR1+	2.81 ± 0.07 b	2.32 ± 0.12 c	0.69 ± 0.04 g	6.51 ± 0.27 b *	0.70 ± 0.07 d
2005 Field [a]					
CW1R	0.69 ± 0.06 f	0.96 ± 0.07 e	1.05 ± 0.34 bceg	-	1.62 ± 0.46 bc
SR1+	0.18 ± 0.04 f	0.24 ± 0.04 f	0.24 ± 0.05 df	5.35 ± 0.36 c *	2.75 ± 0.50 a
p [b]	****	****	****	**	***

Presented values are Mean ± S.E

[a] Data are for Frisson in the greenhouse and for Frisson and Cameor in the field

[b] *p* indicates a statistical significance across assays and treatments within a harvest stage.

** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$. Different letters (a, b, c, d, e, f, g) indicate significant differences between two treatments.

In the greenhouse, atom % ^{15}N excess of the soil was higher in CW1R than in SR1 and SR1+ ($p < 0.001$; Table I.1). Such a difference was not significant in CW1S (2004 greenhouse) and in the field because the results varied widely. In 2004, labelling by CW gave better results for soil ^{15}N enrichment with fortnightly urea pulses (CW1R and CW1R/SR0) than with pulses applied at given plant stages (CW1S and CW1S/SR0; $p < 0.05$; Table I.1). Enrichment of plant organs was lower in CW1R/SR0 and CW1S/SR0 than in CW1R and CW1S, while there was no difference between SR1/CW0 and SR1. In SR1, SR1+ and SR1/CW0, atom % ^{15}N excess of LC roots was always markedly higher than that of TC roots ($p < 0.05$; Table I.1). In CW1R/SR0 and CW1S/SR0, atom % ^{15}N excess of LC roots was also significantly higher than that of TC roots, although ^{15}N was not supplied by the SR reservoir, but by the CW vial ($p < 0.05$). Nevertheless, in these treatments, ^{15}N proportion in root parts was greater in TC than in LC.

In plants labelled by CW, recovery of the ^{15}N label was 65% (S.E. 1.7 %) and 85.6% (S.E. 3.1%) for Frisson grown in the greenhouse in 2004 and 2005, respectively (data not shown). There was no difference between plants with taproots and plants with split roots. In the field, ^{15}N recovery reached 70% for Frisson and Cameor.

I.3.4. Analysis of % Ndfr values and contribution of the plant to the soil N pool

Significant relationships were obtained between atom % ^{15}N excess of 'TC roots' (x) and atom % ^{15}N excess of the soil (y) in plants labelled either by CW (CW1R and CW1S), or by SR (SR1, SR1+ and SR1/CW0), or in split-rooted plants labelled by CW (CW1R/SR0 and CW1S/SR0; Fig. 5A). The best-fit equation for both datasets was the hyperbola $y = 0.011x / (0.56+x)$ ($r^2 = 0.677$; Fig. I.5A) and analysis of transformed data did not show any statistical difference between the curves. However, the relationship between enrichment of 'TC roots' and of the soil differed significantly between plants labelled by CW1R and CW1S and that of split-rooted peas (best-fit equation: $y = 0.024x / (1.25+x)$; $r^2 = 0.421$). Analysis of the transformed data indicated that the slopes were similar ($F_{1, 45} = 0.101$, $p = 0.752$, pooled slope 0.429), while the intercepts were not ($F_{1, 46} = 40.7$, $p < 0.0001$). Furthermore, peas labelled by CW were located on the plateau of the hyperbola, while plants labelled by SR were located on the steeper slope. Such results have consequences on the value of %Ndfr and on the calculated amount of N rhizodeposit released by plants in TC. The amount of Ndfr varied from 5 to 30 mg $N.plant^{-1}$, but values cannot be compared between the different methods (Table I.2).

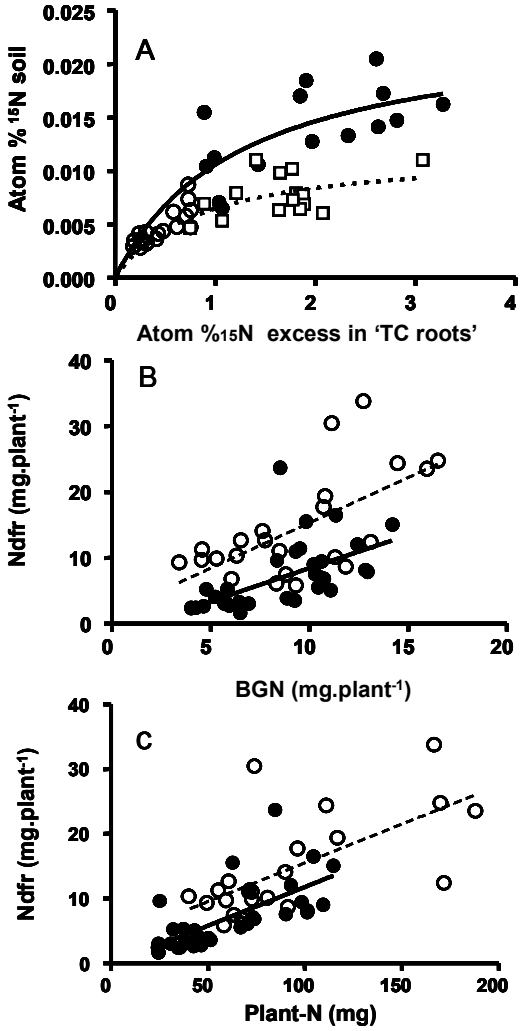


Fig. I.5: Relationships between enrichments of 'TC roots' and of the soil, and between N derived from rhizodeposition (Ndfr) and the plant-N, in pea Frisson grown in the greenhouse in 2004 and 2005. (A) Increase of atom % ^{15}N excess of the soil as a function of atom % ^{15}N excess of TC roots. (B) Increase of Ndfr (mg.plant-1) as a function BGN (mg.plant-1). (C) Increase of Ndfr as a function of the total plant-N (mg.plant-1) - (CW) non split-rooted and labelled by cotton-wick, (SR) all plants labelled by split-root, (CW1/SR0) split-rooted but labelled by CW; not shown in B and C.

In our greenhouse experiments, the ratio of Ndfr : BGN was found to be around 45% in CW and 65% in SR (Table I.2). Moreover, a linear relationship between the amount of Ndfr per plant and BGN could be established for plants labelled by CW (r^2 = 0.489) and for plants labelled by SR (r^2 = 0.308). As slopes of the lines differed significantly ($F_{1, 53}$ = 4.23, p < 0.044; Fig. I.5B), the ratios of NdfR : BGN obtained with the two methods could not be compared. Another linear relationship was found between the amount of Ndfr per plant and plant-N for peas labelled by CW (r^2 = 0.571), peas labelled by SR (r^2 = 0.430), and split-rooted peas labelled by CW (r^2 = 0.414; Fig. I.5C). Lines had similar slopes ($F_{1, 44}$ = 1.789, p = 0.189, pooled slopes 0.951), but intercepts were significantly different ($F_{1, 45}$ = 13.13, p = 0.0007). As a consequence, for a given plant-N content, Ndfr would be approximately 10%

higher in plants labelled by split-root than in plants labelled by CW. In plants labelled by CW, Ndfr would be lower in split-rooted peas (CW1/SR0) than in CW1R and CW1S.

Finally, for both labelling methods, BGN (i.e. TC root-N + Ndfr) was about 25% of the total plant-N, at pod filling and at maturity ($p > 0.05$). Similar results were obtained in the field (Table I.2). In split-rooted plants labelled by CW, BGN was only 12% of the plant-N.

Table I.2: Amount of N rhizodeposition released per plant (mg N plant^{-1}), Ndfr to total below ground part N (%), and contribution of below ground parts to the soil N relative to the plant-N (%).

	NdfR (mg N plant^{-1})	NdfR / BGN (%)	BGN / plant-N (%)
Greenhouse [a]			
Pod-filling			
CW1R	6.3 ± 1.2	38.1 ± 3.6	27.3 ± 2.0
CW1R SR0	5.9 ± 0.6	34.8 ± 3.2	16.6 ± 0.4
CW1S	9.1 ± 3.2	50.4 ± 6.7	32.1 ± 6.0
CW1S SR0	5.2 ± 0.6	32.2 ± 2.9	25.2 ± 4.3
SR1	15.9 ± 4.1	61.3 ± 5.5	39.6 ± 6.0
SR1 CW0	21.6 ± 3.3	61.8 ± 6.8	32.7 ± 0.7
SR1+	6.5 ± 0.5	43.4 ± 4.0	31.2 ± 5.3
p			n.s
Maturity			
CW1R	8.0 ± 1.8	45.2 ± 2.8	26.0 ± 1.7 a
CW1R SR0	10.5 ± 1.7	46.7 ± 0.0	13.2 ± 0.2 ac
CW1S	8.9 ± 0.4	44.5 ± 4.9	21.9 ± 0.7 a
CW1S SR0	8.4 ± 1.2	37.8 ± 2.7	11.7 ± 1.1 bc
SR1	14.2 ± 1.0	62.6 ± 1.9	22.9 ± 3.6 ab
SR1 CW0	29.3 ± 4.5	65.4 ± 1.7	26.5 ± 3.6 ab
SR1+	9.3 ± 0.9	64.6 ± 1.7	25.5 ± 3.6 a
p			*
Field [b]			
CW1R	283.5 ± 64.2	97.6 ± 0.6	35.1 ± 5.5
SR1+	131.5 ± 15.4	92.9 ± 2.1	29.6 ± 1.2

Presented values are Mean ± S.E

* $p < 0.05$, n.s. non significant.

Different letters (a, b, c) indicate significant differences between two treatments for a given harvest stage (for BGN/plant-N, when $n \geq 3$).

[a] Data are for Frisson grown in the greenhouse in 2004 and 2005

[b] In the field, Frisson and Cameor (pooled data)

I.4. **Discussion**

I.4.1. Dry weights and N partitioning induced by growing conditions

The 2004 greenhouse experiment was conducted from the end of autumn until mid-winter i.e. during short days (< 12 h) with low light intensity. In contrast, the 2005 experiment was carried out in the spring, during long days (> 15 h) with good light intensity. Leaf appearance rate is known to depend on temperature and photoperiod (Truong and Duthion 1993). As a consequence, in 2005, it took only 70 days (1414 cumulative degree-days; base temperature

0°C) for peas to reach physiological maturity, compared with 1998 cumulative degree-days in 2004. This difference may explain why in 2004, peas accumulated more dry matter than in 2005, but did not remobilize as much N from the 'leaves and stems' to the seeds.

Plants grown in the field were clearly different from peas grown in the greenhouse because they accumulated markedly more dry weight. Second, at maturity, in the greenhouse, roots represented at least 10-20% of the total plant weight, compared with less than 5% in the field. However, such values may be underestimated as, in the field, part of the roots might not have been recovered (Voisin *et al.* 2002).

Within each experiment, no difference was observed between SR and the other treatments either in N distribution between below and above ground parts or in N remobilization from pod-filling to maturity. However, split-rooting induced a dissymmetry in plant roots. Even though the root system was divided into two equal parts between LC and TC, roots collected from LC always accumulated less dry matter and more N than roots from TC. Such a result is not in agreement with that of Schmidtke (2005b), who used labelling split-root method in pea, and did not find a significant difference in dry matter yield and N uptake between the two sections of the root system. As Schmidtke (2005b) used vermiculite instead of clay marble as the rooting medium in the LC and K-$^{15}NO_3$ instead of $^{15}NO_3$-$^{15}NH_4$, either one or both of these factors could possibly have induced the dissymmetry we observed. In the greenhouse, as reported by Jensen (1996a), split-rooted plants had higher dry weights than non split-rooted ones. Such a difference may be due to the LC, which is a continuous source of water and nutrients throughout the experiment for part of the roots. Surprisingly, in the field, the lowest dry weights were measured in split-rooted plants. However, necrosis observed in roots recovered from field LC suggested anoxia symptoms probably caused by the plastic cap used to protect the nutrient solution from rainfall, irrigation, evaporation, and animal damage.

I.4.2. ^{15}N enrichment and distribution in plant parts

In CW, though labelling treatments were somewhat different in our experiments, similar amounts of labelled urea were supplied to plants (1.2 mg), such that ^{15}N enrichment values were linked to plant yields. In the greenhouse and in the field, the values of ^{15}N enrichment of the roots were similar to those obtained by Mayer *et al.* (2003) in pea labelled by CW. Results were not higher or more regular with either CW1S or CW1R. Russel and Fillery (1996) showed that in lupin labelled by CW, ^{15}N excess was not allocated to plant parts in proportion to plant-N partitioning. In our experiments, the part of plant-^{15}N allocated to the roots was

substantially lower than that of plant-N, in both SR and CW. As a consequence, in agreement with results reported by Russel and Fillery (1996), Mayer *et al.* (2003) and Yasmin *et al.* (2006), enrichment of aboveground parts was markedly higher than that of roots. This result was observed in plants labelled by CW as in plants labelled by SR despite the ^{15}N supply to the roots.

Using the CW method, Russel and Fillery (1996) obtained ^{15}N recovery rates of 81% and 102% in some pots, while Mayer *et al.* (2003) obtained about 84% in pea, lupin and faba bean. In the 2005 greenhouse experiment, the CW method resulted in similar recoveries of about 85%. The amount of unrecovered ^{15}N may correspond to losses of N by gaseous emission from leaves before physiological maturity and to cumulated experimental errors. In the field, recovery rates were around 70%, probably because of incomplete root sampling, leaching, denitrification of plant-N deposits into the soil and higher NH_3 losses from the wick than in the greenhouse. As a consequence, it also seems that the longer the experimentation, the lower the recovery rate, irrespective of cropping conditions (Mayer *et al.* 2003). The 2004 greenhouse experiment was conducted over four months and thus resulted in lower recovery values than in 2005.

SR was less efficient than CW for labelling plant organs. This result may be due to the ^{15}N enrichment of the labelling solution we have used in our experiments. At maturity, because the amount of ^{15}N recovered in the plant parts and in the soil were 60% lower in pea Frisson labelled by SR than in plants labelled by CW, the two methods cannot be really compared. However, in SR, the dissimilar growing conditions led to markedly higher atom % ^{15}N excess values in LC roots than in TC roots. This result is consistent to that of Schmidtke (2005b) who found that 'LC roots' contained 52% of the plant ^{15}N in split-rooted peas. Such a difference between the two kinds of roots was also obtained with CW1R/SR0 and CW1S/SR0 treatments and hence, was not due only to insufficient washing of LC roots, but also to the split-root method itself. This result suggests that LCs filled with unlabelled urea would have provided more precise controls than LCs filled with N-free solution.

I.4.3. Assessment of nitrogen derived from net rhizodeposition with split-root and cotton-wick

As we did not measure the amount of N reabsorbed by the plant, our calculation expressed relative to net rhizodeposition of N. Ndfr percentage was calculated from the ratio of atom % ^{15}N excess of the soil : atom % ^{15}N excess of the visible 'TC roots'. For a given enrichment of

'TC roots', atom % ^{15}N excess measured in the soil was higher in peas treated by CW1S and CW1R than in split-rooted peas, so was %Ndfr value. However, for an equal increase of 'TC roots' enrichment, atom % ^{15}N excess measured in the soil increased significantly more in SR than in CW. As a consequence, all comparisons between CW and SR are not meaningful. For instance, Ndfr per plant and the ratios of Ndfr : BGN obtained with the two labelling methods cannot be compared. In mature peas grown under controlled conditions, Sawatsky and Soper (1991) and Jensen (1996a) obtained with the SR method, values of atom % ^{15}N excess of 'TC roots' that were over 1%, and ratios of Ndfr to total BGN close to 46-48%. We obtained similar values of Ndfr to total BGN with the CW method in the greenhouse. However, with similar atom % ^{15}N excess of roots, Mayer *et al.* (2003) using CW in pea, found that Ndfr constituted 79-85% relative to BGN. Such values are similar to those we obtained in the field with CW, and not so different from N rhizodeposition estimated in red clover by Høgh-Jensen and Schjoerring (2001). In the Mayer *et al.* (2003) experiment, N distribution in pea plants was more similar to the values we obtained in the field than in the greenhouse where root-N represented less than 5% of plant-N. However, Mayer *et al.* (2003) worked with 19-cm diameter pots containing five peas, and the plant growth they observed may have been influenced by plant competition, as it might also have been in our microplots in the field. Plant density, plant genetics and soil characteristics that affect the root : shoot ratio may also affect the results.

The Ndfr : plant-N ratio could be compared between methods and was around 10% higher in SR than in CW. Relative to total plant-N, the total contribution of below ground parts to the N pool of the soil found in the greenhouse reached 22-25% at maturity for CW and SR. Values in the field were similar to those obtained in the greenhouse. Such values are higher than those reported for pea (12-16%) but in the range of those reported for other species (10-23%; Janzen and Bruinsma 1989; Russel and Fillery 1996; Jensen 1996b, 1997; McNeill *et al.* 1997; Mayer *et al.* 2003).

I.5. **Conclusions**

Though we obtained similar results with both methods in the ratio of BGN : plant-N, our results suggest an overestimation of Ndfr to the plant-N with SR relative to CW. Therefore, we cannot conclude that one method is better than the other for estimating the contribution of a pea plant to the soil N pool because our comparison could possibly give different results

with similar ^{15}N root enrichment ranges in peas labelled either by SR and CW. However, CW1R is easier to adapt and monitor under field conditions than SR.

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Similar ratio of BGN: plant-N (%) was obtained with both methods. However, the ratio Ndfr: plant-N (%) was higher with the split root method than with the stem feeding method. Therefore we could not conclude that one method was better than the other for estimating the contribution of pea to the soil N pool because our comparison could possibly give different results with similar ^{15}N roots enrichment ranges in peas labelled either by SR and CW. Nevertheless, it appears that the ^{15}N stem feeding technique was the easiest to adapt and monitor under field conditions. For this reason, we have chosen to refine and adapt this method to measure BGN of field grown pea and fababean. In order to establish how ^{15}N labelling should be carried out to assess the amount of N derived from rhizodeposition with as much accuracy as possible, we have tested the ^{15}N stem feeding technique (i) under different cropping conditions, (ii) using genetically different peas, and (iii) with different frequency of ^{15}N application (continuously and fortnightly) and different labelling solution concentrations.

II. Effect of the labelling frequency and the ^{15}N roots enrichment on the assessment of the N rhizodeposition using the cotton wick method

Authors: S. Mahieu[1], J. Fustec[1], E. S. Jensen[2] and Y. Crozat[1]

[1]Laboratory of Plant Ecophysiology and Agroecology, ESA, 55 rue Rabelais, BP 30748, 49007 Angers cedex 01, France.[2]Biosystems Department, Risø DTU, The Technical University of Denmark, DK-4000 Roskilde, Denmark.

Abstract

Our aim was to test and improve the reliability of the ^{15}N cotton-wick method for measuring N rhizodeposition (Ndfr) and Below ground part N (BGN = Ndfr + root N) of field grown legumes. For this, concentration of the ^{15}N labelling solution, feeding frequency and root and soil sampling were studied in two greenhouse experiments on peas cv. Frisson and P2 (isoline nod- of Frisson). In parallel, the reliability and the feasibility of the method were tested in a field experiment. In the greenhouse, neither the method nor the feeding frequency altered plant biomass and N partitioning and the method appeared well adapted for assessing below ground contribution of field grown legumes to the soil N pool. However the assessment of the Ndfr was largely influenced by the feeding frequency and the concentration of the labelling solution. At pod-filling and maturity, despite a similar root ^{15}N enrichment, the Ndfr and the proportion of Ndfr to total BGN of both Frisson and P2 was from 20 to more than 50% higher when plants were continuously labelled than when plants were fortnightly labelled. In addition, for a given plant-N content, the Ndfr of plants fortnightly labelled having a ^{15}N roots enrichment higher than 1.5% was around twice as high as that of plants having a ^{15}N root enrichment lower than 1.5%. This phenomenon appeared to be linked to the concentration of the labelling solution and was only observed for plants relying on symbiotic N_2 fixation for N acquisition. In the greenhouse, the Ndfr varied from 10 to 30% of total BGN though it represented around 90% of the total BGN in the field. Therefore, labelling conditions may substantially alter the assessment of Ndfr in the field. The effect of feeding frequency and of the concentration of the labelling solution are discussed and several labelling conditions are proposed to improve the reliability of the method for measuring N rhizodeposition.

Key words : N rhizodeposition, ^{15}N, Cotton-wick method, legumes, *Pisum sativum* L.

II.1. **Introduction**

Quantifying below ground nitrogen is important for estimating the N benefit from biological N_2 fixation by a grain legume crop (Peoples, 1995; Rochester *et al.*,1998; Khan et al, 2003; McNeill *et al.*, 2008), for understanding soil N turnover (Jensen, 1996a; Mayer *et al.*, 2003) and for predicting N economies for the succeeding crops in legume-based cropping systems (Russell and Fillery, 1996b). Studying plant below ground nitrogen (BGN) requires the consideration of N contained in the roots and the pool of N released from roots into the soil during plant growth. The latter phenomenon is called N rhizodeposition and results from several mechanisms such as the exudation of N compounds from the roots, the secretion of mucilage or the decomposition of root materials (Lynch and Whipps, 1990; Merbach *et al.*, 1999). BGN remains difficult to assess because N rhizodeposits are not contained in a well-defined structure and the root system is difficult to recover physically from the soil as many of the plants roots are too thin to be collected by hand. N derived from rhizodeposition (Ndfr) is also complex to quantify due to spatial soil problems and because it is impossible to distinguish this N pool from other N sources (Schmidtke, 2005b).

The use of ^{15}N labelling methods is more accurate than non isotopic methods for the quantification of the N rhizodeposition (Jensen, 1996a; Khan *et al.*, 2002a). When using ^{15}N methods for experiments in the soil, it is not possible to distinguish ^{15}N enrichment of N released by exudation from ^{15}N enrichment of N coming from other pathways such as secretions of mucilage or root decomposition. Therefore it is assumed that the average ^{15}N abundance of the rhizodeposits matches the average ^{15}N abundance of the roots and so the proportion of total soil N derived from rhizodeposition (*P*Ndfr) is calculated by dividing the ^{15}N excess in the soil at harvest by the ^{15}N excess of the root grown in the soil.

The atom % ^{15}N excess is more often obtained by correcting the ^{15}N abundance in the soil and roots by the ^{15}N abundance of the background abundances for the soil and roots, respectively. Using the continuous ^{15}N labelling split-root method, Schmidtke (2005a) showed that i) a non-nodulated reference plant or a legume grown on soil without labelling is required for the root background value and ii) that the lower the ^{15}N root abundance the more important the choice of adequate background values and the lower the accuracy of *P* Ndfr estimate.

Besides the split-root ^{15}N labelling technique (Schmidtke, 2005a; Mahieu *et al.*, 2007), several ^{15}N shoot labelling techniques suitable for measuring the Ndfr in field experiments, such as the ^{15}N leaf-flap feeding method (Jensen, 1996b; McNeill *et al.*, 1997), the ^{15}N petiole

feeding (Rochester *et al.*, 1998) and the ^{15}N stem feeding method (Russell and Fillery, 1996a; Mayer *et al.*, 2003) have been used. These labelling techniques have been developed for particular purposes or plant morphologies (Khan *et al.*, 2002b). Plants were labelled by exposure to a concentrated solution of ^{15}N enriched urea. Almost all of the ^{15}N urea solution was taken up within one day and either one or multiple applications were made during plant growth. Then, Ndfr value around 30% to 40% of total plant-N were measured in the field for pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.) and lupin (*Lupinus angustifolius* L.; Rochester *et al.*,1998; Khan *et al.*, 2003; Mahieu *et al.*, 2007; Wichern *et al.*, 2007; McNeill and Fillery, 2008). This approach depends on the whole plant being enriched with ^{15}N with a relatively high uniformity and on the soil in the root zone not being contaminated with the ^{15}N (Khan *et al.*, 2002b). For each of the labelling methods, it appears that nodules are always less enriched than roots. This uneven enrichment of the nodulated roots may generate an under or an overestimation of Ndfr (Russell and Fillery, 1996a; Khan *et al.*, 2002b). In the different experiments conducted to refine ^{15}N methodology, it has been observed that ^{15}N root enrichment is influenced by the ^{15}N labelling technique used (Khan *et al.*, 2002b; Yasmin *et al.*, 2006), the frequency of labelling (Russell and Fillery, 1996a) and the concentration of the labelling solution (Khan *et al.*, 2002b). Therefore, it is possible to manipulate the ^{15}N solution concentration, the volume of solution fed to plants, time of application, and frequency of ^{15}N application in order to reduce this dissymmetry and improve the accuracy of the measurement (Khan *et al.*, 2002b; Schmidtke, 2005a). It has been concluded that the best fitted method varies with the plant species.

The stem feeding (also called cotton-wick method; CW) and the split-root method have been adapted for pea (Jensen, 1996a; Mayer *et al.*, 2003) and both methods seem to produce consistent results. However, in a comparative experiment conducted on pea in the field and greenhouse Mahieu *et al.* (2007) showed that for a given plant N content, Ndfr results obtained with the continuous split-root labelling method were about 10% higher than those obtained with the fortnightly cotton-wick labelling method. Moreover, the assessment of Ndfr was influenced by root enrichment with both methods. Therefore it was not possible to conclude that one method is better than the other. This result raises two remaining questions i) does the concentration of the labelling solution influence the assessment of Ndfr ? and ii) does the labelling frequency influence the homogeneity of plant N labelling ?

Schmidtke (2005a) has proposed several experimental conditions to improve the precision of measurements of Ndfr using a split-root method in controlled conditions such as (1) more

than one legume pot per pot (2) a low amount of available plant N in the soil (3) a continuous ^{15}N fertilization or (4) to consider the extent of the disproportional distribution of N isotopes within legumes. However, split-root is not easy to adapt and monitor under field conditions. Mahieu *et al.* (2007) found that the stem feeding CW technique was more suitable for field studies. Until now, no precise protocol exists detailing how ^{15}N-based experiments should be designed to achieve a fine degree of accuracy in measuring the amount of N derived from rhizodeposition by a pea in the field.

The objective of this study was to establish how ^{15}N labelling should be carried out to assess the amount of N derived from rhizodeposition with as much accuracy as possible. We tested the CW technique (i) under different cropping conditions, (ii) using genetically different peas, and (iii) with different frequency of ^{15}N application and different labelling solution concentrations.

II.2. **Materials and methods**

II.2.1. Labelling method

Pea plants were labelled using the CW method (Russell and Fillery, 1996a; Mayer *et al.*, 2003). This method involves in labelling the plants with a solution of ^{15}N urea (99 atom % ^{15}N) injected into a 2 ml reservoir and absorbed by the plant via a cotton-wick passing into a hole through the stem of the plants. The hole is made in the middle of the internode located just below the first true leaf using a 0.5mm drill. The wick is passed through the hole with the aid of a needle and is protected from desiccation by two silicon tubes sealed against the stem with Terostat® putty to prevent solution losses. The two protected extremities are passed through two holes in the top of the reservoir and the labelling solution is supplied through a third hole in the top with the aid of a syringe.

II.2.2. Greenhouse experiments

Successive greenhouse experiments were conducted in spring 2006 and 2007 in Angers (France, 47°37'N, 0°39'W). The same soil was used for the both experiments. Peas were grown in 2 l plastic pots filled with 2 kg of a mixture of ½ sand and ½ clayey sand taken from the top 0-20 cm of the field experimental plot and passed through a 2 mm sieve. The inner wall of the pots was lined with a plastic bag to avoid N losses by percolation. Pea plants were

maintained against a tutor in the middle of the pot. Lateral ramifications from the first and second nodes were cut when they reached 1 or 2 mm to avoid a dissymmetry in the ^{15}N partitioning within the different plant parts. Each pot was inoculated with *Rhizobium leguminosarum* bv *vicieae* before the four leaf stage and plants were labelled from the 6 or 7 leaf stage. A gauze was fixed around each pot to prevent a mixing of shed organs from the different treatments. At the end of the growth cycle, shed organs were collected daily with tweezers to avoid their contact with the soil.

The 2006 greenhouse experiment was carried out from February to May on pea cv. Frisson (Fr) and P2 (isoline nod- of Frisson). The mean temperature during the growing period was 18.6°C and the relative humidity was around 50%. The experiment was designed in six blocks. Plants were watered daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution. All the nutrient solutions contained: 0.75 mM KH_2PO_4, 0.4 mM $MgSO_4$ $7H_2O$, 0.35 mM $CaCl_2$ $2H_2O$ and microelements were supplied with a 5% diluted commercial solution (Pokon®). The N0 solution was completed with 0.3 mM K_2SO_4 and 0.2 mM KCl, the N5 solution with 2.5 mM KNO_3, 1.25 mM NH_4-NO_3, 0.2 mM K_2SO_4 and 0.1 mM KCl and the N15 solution with 7.5 mM KNO_3, 3.75 mM NH_4-NO_3 and 0.15 mM K_2SO_4. The pH was adjusted to 5.7.

For each of the N treatments, individual plants of P2 and Fr were treated using the CW technique from the 7 leaf stage onwards and the labelling urea solution (99 atom% ^{15}N) was applied either fortnightly (F) or continuously (C). Regardless of labelling frequency, an equal amount of ^{15}N urea was supplied to the plants during the labelling period (3 mg $plant^{-1}$). CW reservoirs of plants treated fortnightly were provided with 0.5ml of 0.6% ^{15}N urea solution once every two weeks. After absorption, 1 ml of deionised water was supplied to rinse the CW reservoir and removed from remaining labelled urea. The labelling period lasted 6 weeks. Plants treated continuously received 3 ml of 0.2% ^{15}N urea solution supplied parsimoniously either every 3 or 4 days during the first four weeks and 1 ml of 0.3% ^{15}N urea during the last two weeks of the labelling period. We obtained six treatments for the plants labelled fortnightly: FrN0F, FrN5F, FrN15F & P2N0F, P2N5F, P2N15F and six treatments for the plants labelled continuously : FrN0C, FrN5C, FrN15C & P2N0C, P2N5C, P2N15C. Control plants (T) were fertilised with the nutrient solutions N0, N5 or N15 but were not treated using CW: FrN0T, P2N0T, FrN5T; P2N5T, FrN15T; P2N15T. For each treatment, six plants were collected at maturity when all above ground parts had turned yellow. For each N0 treatment,

six plants were also harvested at the beginning of pod filling. Plants were considered to have reached pod filling stage when one seed was 6 mm wide (Ney and Turc, 1993).

In 2007, the greenhouse experiment was carried out from April to July on unfertilised Fr pea. The mean temperature during the growing period was 21.5°C and the relative humidity was around 57.8%. Pots were randomly distributed on a culture table and were watered daily with deionised water. Individual plants of Frisson were labelled fortnightly using CW with 0.5ml of either a 0.2%, 0.4%, 0.6% or 0.8% ^{15}N urea solution (99 atom% ^{15}N). After absorption, 0.5 ml of deionised water was supplied to rinse the CW reservoir and removed from remaining labelled urea. The labelling period lasted six weeks. We thus obtained four treatments with five replicates each: F 0.2%, F 0.4%, F 0.6%, F 0.8%. Control plants were not treated by CW. For each treatment and the control, plants were collected at maturity when all pods had turned yellow.

II.2.3. Field experiment

The field experiment was carried out from March to July 2006 near Angers. The mean daily temperature ranged from 3.3 to 26.2 °C with an average of 15°C during the growing season. The total rainfall was around 147 mm and the crop was irrigated to avoid water stress. The soil from the 0-70 cm horizon was a clayey sand with 12.2% clay, 17.5% silt, 69.4% sand and 0.85% organic matter and contained 0.63% a total C, 3.5 mg inorganic N kg^{-1}, 46.7 mg P kg^{-1}, 215.7 mg K kg^{-1}, 102.5 mg Mg kg^{-1}. The pH_{H2O} varied from 6.2 to 6.6 according to the depth and the bulk density was around 1.7 g cm^{-1}. The experiment was designed in four blocks, each consisting of one plot for each pea cultivar or isoline: Fr, P2 and Baccara (Ba) and one of barley. The plots contained twelve (Fr and P2) or six 10m long rows (Ba) with a spacing of 0.35 m. The densities were 80 and 250 plants m^{-2} for peas and barley, respectively. In each plot of Fr and P2, two sub plots of 1 m² were fertilised with 250 kg $N.ha^{-1}$ provided by application of 100 kg $N.ha^{-1}$ at emergence and 150 kg $N.ha^{-1}$ at the eight leaf stage (FrN250 and P2N250 treatments). NH_4-NO_3 fertiliser was dissolved in water and sprayed on the sub-plot at a rate of 15000 l ha^{-1}. Symbiotic nitrogen fixation of plants grown on unfertilised soil was estimated with the natural abundance technique using P2 and barley as reference crop. For FrN250 and P2N250, symbiotic nitrogen fixation was estimated using the ^{15}N isotope dilution technique (Duc *et al.*, 1988). Sub plots of P2, Fr and barley were fertilised with 100 kg N ha^{-1} at emergence and 150 kg N ha^{-1} at the eight leaf stage with NH_4-NO_3 fertiliser enriched with 0.1 atom% ^{15}N (δ=300).

When plants reached the 2-leaf stage, microplots of six plants were marked out by pushing four plastic sheets into the soil to a depth of 0.30 m to prevent lateral losses of applied ^{15}N. For each N treatment (FrN0 & FrN250, P2N0& P2N250, BaN0) microplots of controls were designed like those of plants treated by cotton-wick. Their sizes were calculated according to the density of the crops and were 0.35 m in width (i.e. width of the space between the rows) and 0.23 m in length. A piece of gauze was fixed to tutor around the microplots and a plastic wire mesh was applied to the soil around the pea stem to facilitate the collection of the shed organs and avoid their contact with the soil. From the seven leaf stage, plants were treated by cotton wick. Plants were fed every ten days (F') with 0.5 ml of 1% (P2N0F') or 1.3% ^{15}N urea solution (99 atom% ^{15}N) (FrN0F', BaN0F', FrN250F' and P2N250F'). Peas received four pulses of ^{15}N urea. Microplots were harvested when plants reached the maturity and when all above ground parts had turned yellow.

II.2.4. Sampling and measurements

In the two greenhouse experiments, above ground parts were collected by cutting the stems under the first scale and were sorted into 'seeds' and 'pods, leaves and stems'. In the field, above ground parts of the six plants from each microplot were collected. For the soil, two horizons, 0-35 and 35-70 cm, were considered. A metal sheet of 0.35cm width was placed in the middle of the microplot and driven into the soil to a depth of 35 cm in order to remove a volume of soil equivalent to three plants. For the 35-70 cm horizon, three soil cores were collected with tubes 35 cm high and 5 cm wide. After harvest, the cores were roughly crumbled and dried at 30°C. All the complete soil contained in the plastic pot or collected from the 0-35 cm horizon was passed through a 2mm sieve on which visible roots were collected using tweezers (Fig I.6). The roots were shaken into a closed dish to remove most of the adhering soil and then washed with 120 ml (greenhouse) or 192 ml (field) of a solution of 0.5 mM $CaCl_2$ at 4°C. The adhering soil was added to the rest of the soil and was passed through a sampler to keep a sample of 1/8e (greenhouse) or 1/64e (field) of the total soil. The rest of the soil was thrown out and all the visible roots fragments around 1 mm long were removed from soil samples with the aid of a magnifying glass. The quantity of root fragments obtained was negligible and was thrown out. The solution used to wash the roots was shaken, centrifuged at 2500tr.min^{-1} and 1/8e (greenhouse) or 1/64e (field) of the volume was added to the corresponding soil sample.

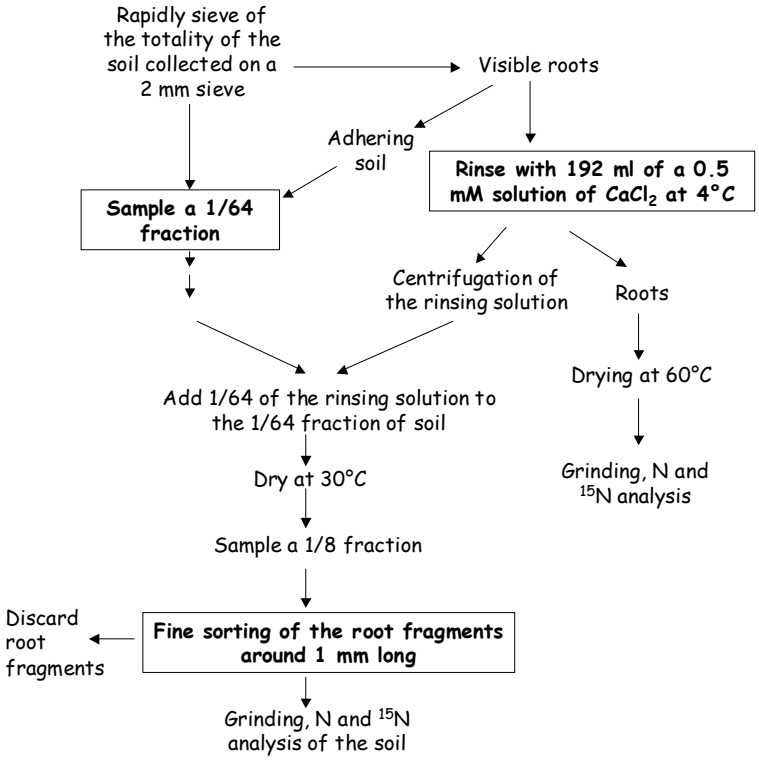

Fig. I.6: Soil/root collection and sampling from field microplots

In the two experiments, plants were sorted into samples called (i) 'seeds', (ii) 'pods, leaves and stems', (iii) 'roots' and (iv) 'soil' (greenhouse) or 'soil 0-30' & 'soil 35-70'(field). Plant and soil samples were dried at 60°C and 30°C respectively, weighed, and ground to a fine powder before total N and ^{15}N analyses. Total N concentration was measured according to the Dumas method and ^{15}N: ^{14}N ratio was determined by mass spectrometry at INRA, Laon, France (spectrometer Isochron and N analyser NA 1500, Fisons, Manchester, UK).

II.2.5. Calculations and statistics

The proportion of total N derived from rhizodeposition (NdfR) at harvest was calculated using Janzen and Bruinsma's equation (1989):

% NdfR=(at.%^{15}N soil - at.% ^{15}N background A) / (at.% ^{15}N roots - at.% ^{15}N background B)×100

The soil and the roots of the non ^{15}N-fed legumes were chosen as backgrounds A and B respectively (Schmidtke, 2005b). The amount of N rhizodeposit was assessed from the % NdfR value and the amount of total soil-N. The amount of N rhizodeposit was used for calculating the ratio of the percentage of total BGN (visible root N + N rhizodeposits) to total plant-N. The following assumptions were made: (i) N deposited and roots grown in the soil

had the same ^{15}N enrichment, and (ii) the micro roots collected by hand and by wet sieving had the same ^{15}N abundance as the macro roots.
In 2006 greenhouse and field experiment, the ^{15}N distribution ratio was calculated for each treatment. This ratio corresponds to the proportion of ^{15}N recovered in a plant part (% of total plant-^{15}N) to the proportion of recovered N (% of total plant-N) in the same plant part (Russell and Fillery, 1996). A ^{15}N distribution ratio of 1 represents uniform ^{15}N distribution and deviation from this value indicate either preference for (>1) or discrimination against (<1) ^{15}N assimilation.

Statistical analyses were carried out using Statbox Pro software. When possible, analysis of variance was used for multiple comparisons of means using Newman and Keuls test with a confidence level of 0.05. When data normality or equality of variances was not respected, comparisons of means were carried out using non parametric Kruskal-Wallis tests with a confidence level of 0.05. GraphPad Prism 4.03 Software was used for linear regression analysis and comparisons.

II.3. **Results**

II.3.1. Absorption of ^{15}N urea by the plants

To accurately compare the effect of the frequency of labelling on the assessment of N rhizodeposition (Ndfr), plants had to receive the same amount of ^{15}N urea every two weeks provided either continuously or fortnightly. The totality of the 0.5ml of ^{15}N urea solution provided to the fortnightly labelled plants (F) was generally absorbed within 3 days whatever the level of N fertilisation. Therefore, plants received the entire amount of ^{15}N urea expected every two weeks (3 mg.plant^{-1}; Table I.3). Due to the higher volume of solution absorbed by continuously labelling plants (C) (3ml during vegetative growth and 1ml at the end of the growth cycle), some tissue damage appeared around the cotton-wick after the second-stem feeding events. Morever, due to the blockage of the drilled hole, more C plants were re-drilled compared to F plants (Table I.3). At maturity, except for P2N0, the amount of ^{15}N urea absorbed every two weeks by the C plants was very similar to the amount absorbed by F plants.

Table I.3: Number of re-drilling of the stem and amount of ^{15}N urea (mg.plant^{-1}) absorbed every 2 weeks by plants of Frisson (Fr) and the isoline P2 treated continuously (C) and fortnightly (F) and either collected at pod-filling or at maturity in the 2006 greenhouse experiment. Plants were provided daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution. Amounts of ^{15}N urea (mg.plant^{-1}) absorbed by P2N0C was significantly different from the other treatments and are presented in a separated line.

			Amount of ^{15}N urea absorbed (mg.plant^{-1})			
		Number of Re-drilling	Weeks 1-2	Weeks 3-4	Weeks 5-6	Total Weeks 1-6
Pod-filling						
P2N0	C	4	2.48 ± 0.6	2.42 ± 0.5	2.00 ± 0.5	6.90 ± 1.4
	F	0	3 ± 0.0	3 ± 0.0	2.30 ± 0.7	8.30 ± 0.7
FrN0	C	3	2.65 ± 0.5	2.77 ± 0.7	1.54 ± 0.6	6.95 ± 1.1
	F	2	3 ± 0.0	3 ± 0.0	2.55 ± 0.3	8.55 ± 0.3
Maturity						
P2 $_{N0}$	C	5	2.37 ± 0.6	2.51 ± 0.7	2.52 ± 1.3	7.4 ± 2.0
P2 $_{N5 \& N15}$	C	3	2.74 ± 0.4	2.59 ± 0.5	3.23 ± 0.5	8.57 ± 1.0
P2 $_{N0, N5 \& N15}$	F	3	3 ± 0.0	3 ± 0.0	2.92 ± 0.3	8.92 ± 0.3
Fr $_{N0, N5 \& N15}$	C	11	2.64 ± 0.5	2.98 ± 0.5	3.33 ± 0.4	8.95 ± 0.2
	F	7	3 ± 0.0	3 ± 0.0	3 ± 0.0	9.00 ± 0.0

In the field, the 0.5ml of ^{15}N urea solution provided to the plants every ten days (F') was generally absorbed within 3 days whatever the level of N fertilisation (data not shown). As in the greenhouse, plants received the entire amount of ^{15}N urea expected every ten days.

II.3.2. Plant dry weight and nitrogen content

In the 2006 greenhouse and in the field experiments, whatever the level of N fertilisation, total dry weight (DW) and nitrogen (N) content as well as DW and N partitioning of plants collected at maturity were not affected by the method or by the labelling frequency compared to control (T) plants (Table I.4). Therefore values presented at maturity in Table 2 are means of T, F and C treatments for the results obtained in the greenhouse. Among all treatments, only plant-N partitioning of P2N0 treated by CW (F & C) collected at pod filling stage, were different from the control (T).

Table I.4: Pea total dry weight (g plant^{-1}), total N content (g plant^{-1}) and N and dry weight partitioning (%) in Frisson (Fr), isoline P2 or Baccara (Ba) measured at pod-filling stage and at maturity in the 2006 greenhouse and at maturity in the 2006 field experiments . In the greenhouse, controls plants (T) and plants treated by cotton-wick either fortnightly (F) or continuously (C) were provided daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution. When no significant differences between T, F and C treatments were found, data were pooled (T, F, C). In the field, controls plants (T) and plants labelled regularly every 10 days (F') were grown either on unfertilized soil (N0) or on soil fertilized with 250 kg N.ha^{-1} (N250). Values are means ± SE, n = 6 or n = 18 for pooled T, F, C (greenhouse), n=4 (field). Veg are 'pods, leaves and stems'.

		Total dry weight	Total N	Dry weight partitioning (%)			N partitioning (%)		
Treatments		(g plant^{-1})	(mg plant^{-1})	Roots	Veg	Seeds	Roots	Veg	Seeds
Greenhouse 2006 [a]									
	Pod-filling								
FrN0 (T, F, C)		2.54°	62.7°	27.6°	72.4°		19.2°	80.8°	
P2N0	T	1.56	20.3	50.1	49.9		48.9a	51.1b	
	F	1.64	21.5	49.7	50.3		39.1b	60.9a	
	C	1.56	21.1	47.9	52.1		38.9b	61.1a	
	***Maturity** (pooled T, F, C)*								
FrN0		3.43c	76.7d	20.2b	45.3	34.5c	14.7b	38.5b	46.8b
FrN5		3.99b	97.3c	19.7b	44.1	36.2bc	12.9c	38.6b	48.6ab
FrN15		4.62a	155.7a	13.2c	46.9	39.8a	10.8cd	43.4a	45.8b
P2N0		1.59d	21.7e	35.7a	45.8	18.6d	33.9a	35.0b	31.1c
P2N5		3.90bc	93.5c	18.2b	43.2	38.6ab	12.5cd	35.2b	52.3a
P2N15		4.31ab	144.4b	12.6c	46.1	41.3a	10.2d	43.4a	46.4b
P		**	**	**	ns	**	**	**	**
Field 2006 [b]									
	Maturity								
FrN0	T	5.8 b	116.9 b	5.9 b	39.3 b	54.9 ab	4.9 b	11.3 c	83.8 a
	F'	9.4	204.8	5.9	41.4	52.8	4.8	12.3	82.8
Fr N250	T	10.7 a	220.3 a	4.9 b	43.7 ab	51.4 b	4.2 b	15.0 bc	80.9 ab
	F'	12.3	251.7	5.0	42.2	52.8	4.2	13.6	82.2
P2N0	T	2.6 c	37.1 c	14.3 a	43.6 ab	42.1 d	14.3 d	18.3 a	67.3 c
	F'	2.2	37.9	11.2	42.7	46.1	10.3	18.1	71.6
P2N250	T	9.7 a	192.5 a	5.5 b	45.8 a	48.6 c	4.6 b	15.5 b	79.9 b
	F'	12.1	248.3	4.7	47.3	47.9	5.9	15.9	78.2
Ba	T	11.8 a	252.0 a	4.8 b	39.9 b	55.2 a	4.0 b	13.0 c	83.1 a
	F'	11.9	252.3	5.2	40.2	54.7	4.6	10.9	84.5
		**	***	*	*	*	*	*	*

[a] At pod-filling stage, ° indicates significant differences between treatments FrN0 and P2N0 T, R or C treatment, $P < 0.0001$.
Different letters indicate significant differences between treatments T, R and C, $P < 0.05$
for P2N0 at pod-filling stage. At maturity, they indicate significant differences between isolines and fertilization levels. *P* indicates a statistical significance across treatments within maturity stage. n.s. non-significant. * $P < 0.05$, ** $P < 0.01$.

[b] *P* indicates a statistical significance across means of T and CW of the different treatments in the field (Fr N0, Fr N250, P2 N0, P2 N250, Ba). * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. Different letters indicate significant differences between varieties and fertilization levels.

In both experiments, DW and N content of P2N0 were lower than for the other treatments and the proportion of total plant-N allocated to the roots was higher (Table I.4). For all the

peas, total plant DW and N content increased with the level of N fertilisation. DW and N content of FrN5 and P2N5 treatments in the greenhouse or BaN0, FrN250 and P2N250 in the field were similar. In the greenhouse, dry weight and N content of FrN15 and P2N15 were higher than for the other treatment.

II.3.3. Effect of the labelling frequency on the assessment of the N rhizodeposition (Ndfr)

At pod filling stage, the amount of N allocated to rhizodeposition per plant, the proportion of Ndfr to total BGN and to total plant N, were around twice higher for FrN0C as for FrN0F. However, in P2, the results obtained with the two labelling frequencies were similar.

At maturity, the amount of N allocated to rhizodeposition per plant was similar with the two labelling frequencies (C & R) for FrN0, FrN5 and P2N0. However for all the other treatments, values were significantly higher when plants were labelled continuously than fortnightly (Table I.5). Ndfr expressed as a percentage of total plant-N or as a percentage of total BGN for FrN15, P2N0, P2N5 and P2N15, was also higher when plants were labelled continuously compared to plants labelled fortnightly.

Finally, the amount of N allocated to rhizodeposition per plant represented between 10 and 30% of the total BGN depending on the level of N fertilization (Table I.5). At maturity, with the exception of P2N0, BGN was around 16% of total plant-N for all C treatments, though it varied from 12.4 to 17.5% of total plant-N for the R treatments (Table I.5).

In the field, Ndfr of P2N0 reached 38.5 mg plant^{-1} and the BGN was around 51% of the total plant-N (data not shown). For the other treatments, the amount of Ndfr varied from 83 to 106 mg plant^{-1} and the BGN varied from 29 to 37% of the total plant-N. For all treatments, the Ndfr represented around 90% of the total BGN.

Table I.5: Roots ^{15}N excess (%), Nitrogen derived from rhizodeposition (Ndfr) (mg plant^{-1}), Ndfr expressed as a % of the total below ground N (BGN) and Ndfr and BGN expressed as a % of the total plant-N measured at pod-filling stage and at maturity in the 2006 greenhouse experiment. Plants of Frisson (Fr) and the isoline P2 were provided daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution and labelled either fortnightly (F) or continuously (C) with the same amount of ^{15}N urea. Presented values are means ± SE, n = 6.

treatments			Roots ^{15}N excess (%)	Ndfr (mg plant^{-1})	Ndfr / BGN (%)	Ndfr / plant N (%)	BGN / plant N (%)
Pod-filling							
Frisson	N0	R	2.8(0.5)	1.4(0.7)	10.8(3.8)	2.4(0.9)	22.2(4.2)
		C	2.1(0.4)	2.9(0.8)	19.4(4.7)	4.3(1.4)	22.1(2.6)
			n.s.	**	****	****	n.s.
P2	N0	F	6.7(1.2)	2.0(0.6)	19.3(3.3)	8.6(2.1)	44.3(6.5)
		C	5.2(2.1)	2.1(0.8)	17.3(5.3)	8.3(2.7)	48.9(13.8)
			n.s.	n.s.	n.s.	n.s.	n.s.
Maturity							
Frisson	N0	F	2.9(0.6)	2.1(1.0)	12.8(2.6)	2.2(0.5)	17.5(2.1)
		C	2.9(0.9)	2.2(0.3)	15.3(2.1)	2.7(0.3)	17.5(1.4)
			n.s.	n.s.	n.s.	n.s.	n.s.
	N5	F	2.1(0.3)	2.6(0.5)	17.6(4.3)	2.6(0.7)	14.8(0.5)
		C	1.8(0.4)	3.1(1.0)	18.7(3.5)	3.0(0.6)	16.0(1.5)
			n.s.	n.s.	n.s.	n.s.	**
	N15	F	1.5(0.4)	5.5(1.0)b	24.8(2.9)	3.3(0.7)	13.3(2.0)
		C	1.3(0.2)	7.1(2.0)a	32.9(7.7)	5.5(1.2)	16.8(2.7)
P			n.s.	**	**	**	**
P2	N0	F	4.9(2.2)	1.2(0.4)	14.2(2.4)	4.4(1.3)	30.7(6.2)
		C	4.9(1.8)	1.8(0.6)	21.7(6.0)	8.8(3.2)	39.9(8.9)
			n.s.	n.s.	**	**	n.s.
	N5	F	1.8(0.5)	1.4(0.7)	10.1(3.7)	1.4(0.6)	13.6(1.7)
		C	2.0(0.4)	3.4(1.9)	23.2(10.1)	3.7(2.1)	15.1(2.1)
			n.s.	*	**	**	n.s.
	N15	F	1.5(0.5)	3.4(1.6)	18.9(4.7)	2.4(1.1)	12.4(3.0)
		C	1.2(0.4)	7.3(2.3)	35.0(4.2)	5.7(1.3)	16.3(3.8)
P			n.s.	***	**	**	n.s.

P indicates a statistical significance across R and C labelling frequency of the different treatments. n.s. non-significant. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$

II.3.4. The effects of Root and soil ^{15}N enrichments on the assessment of the N rhizodeposition

In the 2007 greenhouse experiments, Fr peas were not affected by the concentration of labelling solution (data not shown). Root DW and N content of the different treatments were similar to control plants (2.4 ± 0.7 g plant^{-1}, 21.3 ± 3.0 mg plant^{-1}). Total plant-N content and partitioning were similar for all treatments. A range of root ^{15}N enrichments varying from

0.51 ± 0.05% to 2.21 ± 0.29% was obtained depending on the concentration of the ^{15}N urea solution used for the labelling (Table I.6, $p<0.01$).

Table I.6: Roots and soil ^{15}N excess (%), Nitrogen derived from rhizodeposition (Ndfr) (mg plant^{-1}), Ndfr expressed as a % of the total below ground N (BGN) and BGN (mg plant^{-1}) measured at maturity in the 2007 greenhouse experiment. Peas were labelled fortnightly (F) with different ^{15}N urea solution concentration 0.2%, 0.4%, 0.6% or 0.8%.Values are means ± SE, n = 5.

	^{15}N excess (%)		Ndfr		BGN
treatments	Soil (×100)	Roots	mg plant^{-1}	% of BGN	mg plant^{-1}
F 0.2%	0.23 (0.05)d	0.51 (0.05)d	4.00 (0.51)b	15.9 (1.71)b	25.2 (2.40)
F 0.4%	0.54 (0.08)c	1.08 (0.10)c	4.27 (0.48)b	16.6 (3.37)b	26.0 (2.50)
F 0.6%	0.94 (0.46)b	1.52 (0.07)b	4.11 (0.45)b	16.7 (2.14)b	23.1 (3.89)
F 0.8%	2.07 (0.52)a	2.21 (0.29)a	7.66 (2.79)a	27.8 (10.48)a	28.0 (3.16)
P	*	*	*	*	n.s.

P indicates a statistical significance across the different treatments. n.s. non-significant. * $P < 0.01$.

For the F 0.2, F 0.4 and F 0.6% treatments, Ndfr values, calculated from root and soil enrichment, as well as the proportion of the total BGN released into the soil were similar: around 4.13 mg plant^{-1} and 16.4 % of the total BGN, respectively (Table I.6). For these treatments, a single linear relationship was obtained between ^{15}N enrichment of roots and that of soil (Fig. I.7). Such a relationship was not found when F 0.8% treatments were included in the dataset. Furthermore, the proportion of Ndfr to the total BGN for this treatment was around twice as high as for the other treatments (Table I.6).

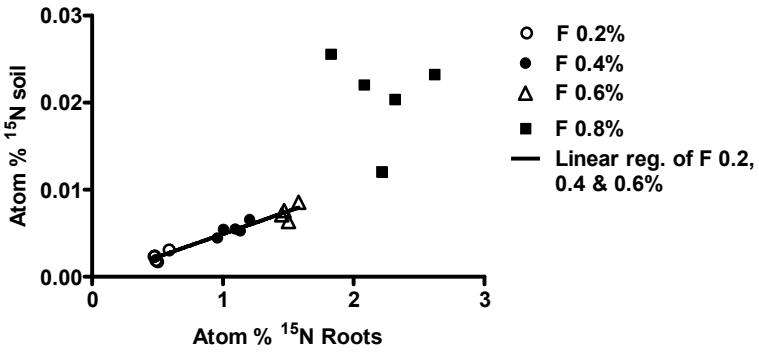

Fig. I.7: Relationships between atom %^{15}N excess of roots and atom %^{15}N excess of the soil measured at maturity in the 2007 greenhouse experiment. Peas were labelled fortnightly (F) with different ^{15}N urea solution concentration 0.2%, 0.4%, 0.6% or 0.8%. Linear reg. of F 0.2, 0.4 & 0.6% : $P<0.0001$; $r^2=0.95$.

II.3.5. Plant ^{15}N recovery and partitioning

In the greenhouse and in the field, around 80% of the total ^{15}N applied during plant growth was recovered at harvest for the different treatments (Table I.7).

In the greenhouse, for each level of N fertilisation, the distribution of ^{15}N in P2 was similar between R and C treatments. In Fr, the proportion of ^{15}N allocated to the seeds of C plants tented to be higher than in the seeds of F plants. The difference in ^{15}N distribution was substantial between the seeds or the other above ground parts of R and C treatments of FrN15 (Table I.7).

Table I.7: Percentage of the recovered applied ^{15}N and plant ^{15}N partitioning in Frisson (Fr), isoline P2 or Baccara (Ba) measured at pod-filling stage and at maturity in the 2006 greenhouse and at maturity in the 2006 field experiments. In the greenhouse, peas were provided daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution and labelled either fortnightly (F) or continuously (C) with the same amount of ^{15}N urea. In the field, peas were grown either on unfertilized soil (N0) or on soil fertilized with 250 kg $N.ha^{-1}$ (N250) and labelled regularly every 10 days. Veg are 'Pods, leaves and stem'. Values are means ± SE, n = 6 (greenhouse), n=4 (field).

treatments		Recovery of ^{15}N (%)	Distribution of recovered ^{15}N (%) Soil		Roots	Veg	Seeds
Greenhouse 2006 [a]							
	Pod-filling						
FrN0	F	87.4(4.8)	1.2(0.5)b		9.8(1.3)b	89.0(1.5)a	-
	C	86.7(5.5)	2.0(0.6)b		8.4(1.3)b	89.5(1.5)a	-
P2N0	F	81.8(8.0)	4.0(1.0)a		16.4(3.3)a	79.6(4.1)b	-
	C	82.0(3.8)	3.5(0.8)a		17.8(5.6)a	78.7(5.7)b	-
		n.s.	***		***	***	
	Maturity						
FrN0	F	83.6(1.9)	1.7(1.1)		8.6(2.4)	47.7(8.5)a	42.0(7.9)b
	C	80.4(4.1)	1.4(0.5)		8.2(1.4)	41.1(2.1)ab	49.3(3.2)b
FrN5	F	79.8(3.2)	1.5(0.4)		7.4(1.8)	47.7(6.8)a	43.4(7.8)b
	C	80.5(4.0)	1.5(0.8)		6.2(2.1)	42.4(9.1)ab	50.0(11.6)b
FrN15	F	77.0(6.6)	2.4(0.6)		7.1(1.2)	52.0(7.2)a	38.5(7.4)b
	C	73.9(6.6)	2.2(1.2)		4.2(2.1)	30.4(15.5)b	63.2(18.6)a
P		n.s.	n.s.		n.s.	*	*
P2N0	F	69.9(14.6)	2.4(1.1)		13.8(5.1)a	52.2(10.2)	31.5(15.6)
	C	80.0(2.8)	3.0(1.8)		12.0(5.1)a	40.8(5.8)	44.2(7.7)
P2N5	F	79.0(5.3)	0.8(0.5)		6.1(2.5)b	47.6(3.9)	45.6(4.4)
	C	77.1(2.3)	1.8(0.7)		6.1(1.7)b	36.6(7.2)	55.4(7.0)
P2N15	F	78.4(3.9)	1.5(0.6)		6.0(1.5)b	47.5(6.3)	45.1(6.2)
	C	77.1(6.1)	2.8(1.0)		4.9(1.2)b	45.1(6.9)	47.1(7.1)
P		n.s.	n.s.		***	n.s.	n.s.
Field 2006 [b]							
	Maturity		**Soil 35-70**	**Soil 0-30**			
FrN0		84.3(5.5)a	1.2(0.3)bc	6.3(1.0)c	0.8(0.1)b	10.1(1.9)b	81.6(1.1)a
FrN250		80.2(4.9)ab	0.9(0.3)c	6.7(4.0)bc	0.8(0.4)b	10.6(2.2)b	81.2(3.8)a
P2N0		72.9(7.0)b	2.5(0.9)a	16.4(5.1)a	2.0(0.6)a	14.3(0.8)a	68.9(9.3)b
P2N250		81.2(3.8)ab	1.2(0.3)bc	8.8(2.7)bc	1.3(0.4)b	13.4(2.8)ab	75.6(3.8)ab
BaN0		78.7(0.9)ab	1.6(0.6)b	12.6(2.3)b	2.0(0.7)a	9.5(1.5)b	74.4(3.4)ab
P		*	*	*	***	**	**

[a] *P* indicates a statistical significance across treatments within a harvest stage. n.s. non-significant. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.0001$. Different letters indicate significant differences between treatments.

[b] *P* indicates a statistical significance across treatments. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.001$. Different letters indicate significant differences between treatments.

In both set of growing conditions, except in FrN15C (Table I.7), there was no effect of N fertilisation level on plant ^{15}N partitioning in Fr. In the greenhouse around 45% of total plant-^{15}N was allocated to the seeds, 45% to the 'stem, leaves and pods' and less than 10% was retrieved in the below ground parts (Table I.7). Plant ^{15}N partitioning in P2N5 and P2N15 treatment were not different from FrN0, FrN5 and FrN15 treatments. However, in P2N0, the proportion of ^{15}N allocated in the below ground parts was twice as high as in the other treatments.

In the field, plant ^{15}N distribution was quite different from in the greenhouse. Around 80% of the total plant ^{15}N was allocated to the seeds and about 10 to 15% to the soil and roots (Table I.7). More than 80% of the total below ground ^{15}N was retrieved in the soil and less than 20% in the roots though the opposite was true in the greenhouse.

In the field and in the greenhouse, the ^{15}N distribution ratio was largely lower than 1 in the roots for each treatment and was either higher or close to 1 in the above ground parts depending of the treatment (data not shown). As a consequence, ^{15}N was preferentially allocated to the above ground part with the both labelling frequency.

II.3.6. Consistency of the Ndfr estimation according to feeding frequency : Relationship between the amount of ^{15}N in the roots and in the soil

In the 2006 greenhouse experiment, the ^{15}N root enrichments of plants labelled either continuously or fortnightly was around 5 at. $\%^{15}N$ excess for P2N0 and ranged from around 1 to 3 at. $\%^{15}N$ excess according to the plant N content for the other treatments (Table I.5). Since root and soil ^{15}N enrichment varied with soil N fertilisation and plant N content, only data from treatments for which plants received the same N fertilization were plotted. Looking at the relationship between amount of ^{15}N in the roots and in the soil seemed to be the most suitable method for evaluating the reliability of the results.

A significant linear relationship was obtained between root and soil ^{15}N content for each of the C treatments. Since there were no differences between the linear regressions of P2N0C, P2N5C and FrN5C, data were pooled (Fig. I.8A). The same was done for FrN15C and P2N15C.

For F treatments, a good linear relationship was also obtained between root and soil ^{15}N content for P2N0, P2N5 and FrN15 treatments (Fig. I.8B) but root and soil ^{15}N content of the repetitions for the treatment FrN0 and FrN5 were not correlated (Fig. I.8C).

In the field, a single good linear relationship was obtained between root and soil ^{15}N content of FrN0 and FrN250 but not for P2N250, P2N0 and BaN0 (data not shown).

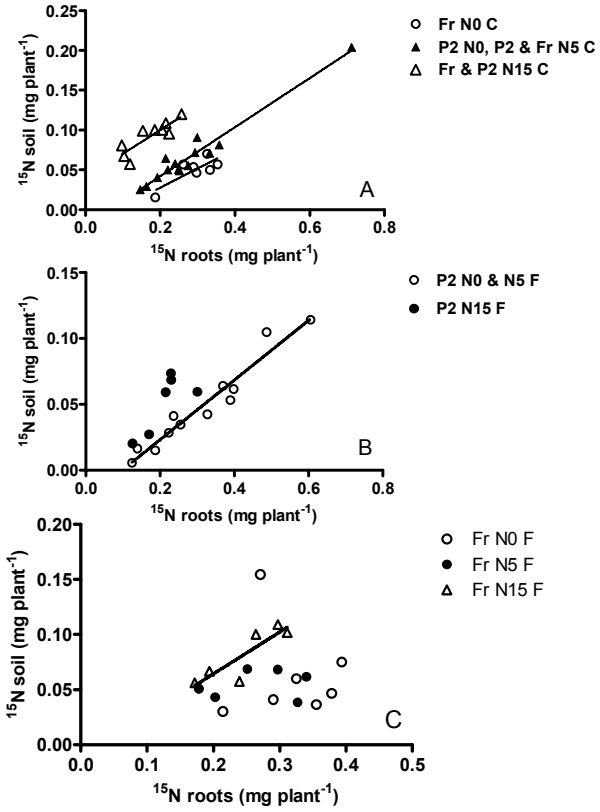

Fig. I.8: Relationship between the amount of ^{15}N in the soil and in the root of Frisson (Fr), Baccara (Ba) or P2 measured at pod-filling (Fig D) or at maturity in the 2006 greenhouse (Fig. A, B, C). In the greenhouse, peas were provided daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution and labelled either continuously (C) or fortnightly (F). **(Fig. A**: Lin. reg. of FrN0 C: R^2= 0.63, P=0.03; Lin. reg. of P2N0, P2 & FrN5 C: R^2= 0.95*, P<0.001; Lin. reg. of Fr & P2 N15 C: R^2= 0.75**, P<0.002 - **Fig. B**: Lin. reg. of P2 N0 & N5 F: R^2= 0.94*, P<0.0001 - **Fig. C**: Lin. reg. of FrN15 F: R^2= 0.75, P=0.02)

Discussion

4.1. *Distribution of N and ^{15}N and effects on the Ndfr estimation*

The cotton-wick method did not alter neither plant biomass and total N content nor N and biomass partitioning. However, in both field and greenhouse conditions, the ^{15}N isotope was preferentially allocated in the above ground parts and whatever the labelling frequency. Similar results were observed by Russell and Fillery (1996a) and Khan *et al.* (2002b). Russell and Fillery (1996a) explained that when plants are labelled by CW, the ^{15}N is fed directly in the secondary xylem and transported in the leaf which is the major sink for N in pre-reproductive growth. Therefore, the ^{15}N in the below ground parts and allocated to rhizodeposition would result in the transportation of the N assimilates from the leaf to the roots via the phloem. Those authors observed that ^{15}N enrichment of nodules was always lower than roots only. They suggested that this poor ^{15}N assimilation within nodules would explain that root tissues are less enriched than shoots (Russell and Fillery, 1996a; Khan *et al.*, 2002b). However in our experiments, for a given plant N content the root ^{15}N enrichment of P2 and Fr were similar as well as the ratios of recovered ^{15}N in the roots whatever the level of N fertilisation. According to our results, the uneven nodulated roots enrichment would not have effect on the Ndfr assessment. The lower ^{15}N enrichment in the above ground parts than in the roots may be linked to the fact that labelling started at 6 leaves, a time in which investment of C and N in the roots decreases, though allocation to the above ground parts increases (Pate et Herridge, 1978; Voisin *et al.*, 2003). As a consequence, the labelling should start very early in the vegetative period to improve the homogeneity of the ^{15}N plant enrichment.

4.2. *Effect of roots and soil ^{15}N enrichments on the Ndfr assessment*

4.2.1. Effect of the concentration of the labelling solution on the reliability of the Ndfr assessment for plants relying on the N fixation for N acquisition

In the 2007 greenhouse experiment, plants grown on non N fertilized soil were treated with a range of labelling ^{15}N urea solution concentration varying from 0.2% to 0.8% (99 atom% ^{15}N). Despite of a similar plants N content, Ndfr of plants fortnightly labelled with a

0.8% ^{15}N urea solution was around twice higher than for the other treatments. For this treatment, the ^{15}N root enrichment of the different repetitions was varying from 1.7% to 2.7% and was not correlated with the ^{15}N soil enrichment, though a single linear relationship was obtained for all the other treatments when the ^{15}N root enrichment was lower than 1.5%. It suggests that either the ^{15}N root enrichment or the concentration of the labelling solution had an influence on the assessment of the Ndfr.

Similar results were obtained for pea plants of Fr fortnightly labelled in the 2006 greenhouse experiment though plants were labelling with a 0.6% ^{15}N urea solution concentration: root and soil ^{15}N contents of the different repetitions for a given treatment were no correlated when the ^{15}N root enrichment was higher than 1.5%. However, it is not easy to conclude if whether this phenomenon is linked to the concentration of the labelling solution or to the root ^{15}N enrichment since total plant N content of pea from the 2006 greenhouse experiment was lower than for pea from the 2007 greenhouse experiment.

Conversely, for the plants continuously labelled, significant linear relationship was obtained between roots and soil ^{15}N content though ^{15}N roots enrichment of some treatments was substantially higher than 1.5%. In those cases, a diluted solution (0.2%) of ^{15}N urea was supplied from the stage 6 or 7 leaves until harvest. As a consequence, the ^{15}N root enrichment seems to have no effect on the assessment of the Ndfr when plants are continuously labelling with a diluted solution of ^{15}N urea. Therefore, the fact that results obtained for the F 0.8% (2007 greenhouse) or F 0.6% (2006 greenhouse) treatment was not reproducible and overestimated would likely come from a too high concentration of solution of ^{15}N urea for a given plant-N content. As a consequence, to obtain a reproducible assessment of the Ndfr, the concentration of the labelling solution should be calculated in order to obtain a root enrichment lower than 1.5% when plants are labelled by intermittent pulses.

4.2.3 Effect of the concentration of the labelling solution on the reliability of the Ndfr assessment for plants relying on the N uptake from the soil solution for N acquisition

Curiously, although the ^{15}N root enrichments of P2N0 and P2N5 fortnightly labelling were higher than 1.5%, a good linear relationship was obtained between roots and soil ^{15}N

content for those treatments. This difference between fixing and non fixing plants is not linked to the presence of nodule. Among rhizodeposits of legume, compound such as NH_4^+, NO_3^- or amino acid are released (Jensen, 1996a, Paynel *et al.*, 2001; McNeill and Fillery, 2008). Morever the rate of turnover of some rhizodeposits is high and re-absorption of root derived-N during the growing season was demonstrated (Jimenez *et al.*, 2002; Molina *et al.*, 2005*).* In plants of P2 unable to fix N_2, one part of the N released into the soil may have been reabsorbed creating an homogenisation between roots and rhizodeposits enrichment. Therefore, the ^{15}N retrieved in the soil would not be representative of the ^{15}N abundance of the rhizodeposits at harvest.

4.3. *Effect of the labelling frequency on the Ndfr assessment*

For each treatment for which there was a good linear relationship between root and soil ^{15}N content, the Ndfr expressed as a percentage of total plant-N or as a percentage of total BGN was substantially higher when plants were continuously labelled than when plants were fortnightly labelled. Such a difference in the measurement of the Ndfr between plants treated continuously by split root (SR) and plants fortnightly labelled by cotton wick was also observed by Mahieu *et al.* (2007): the proportion of Ndfr: plant-N was 10% higher in SR than in CW. However they cannot compare the both methods as the atom % ^{15}N excess of roots was less than 1% in SR, whereas most values varied from 1% to 4% in CW. In the present study, for each one of the treatments, the atom % ^{15}N excess of roots of C and F plants was similar. Therefore, the difference in the assessment of the Ndfr due to the labelling frequency would probably result from the fact that a fortnightly labelling do not enable an uniform enrichment of the root N content. Indeed, it was suggested that legumes species can differ in their disproportional ^{15}N distribution in spite of identical ^{15}N abundance in their total plant biomass (Schmidtke, 2005a).

The rhizodeposition results from several mechanisms which contributions vary in time and space along the root axis (Henry *et al.*, 2005; Darwent *et al.*, 2003). For the F0.8% treatments in the 2007 greenhouse experiment, the proportion of total below ground ^{15}N retrieved into the soil was close to 30% though it was around 16% for the other treatments. Therefore, the enrichment of one of the N compounds released into the soil may have been

influenced more than the others during the time of absorption of the labelling solution when plants are fortnightly labelling.

Ndfr assessment is reliable when plants were continuously labelling with a diluted solution of ^{15}N urea**.** Those results are consistent with those of Schmidtke (2005a) obtained using a continuous split-root method. He has shown that with this method the reliability of the result increased with ^{15}N root abundance. However, in pea plants continuously labelled the ^{15}N root enrichment varied between pod-filling and maturity though the ^{15}N root enrichment at harvest is used for the calculation of the Ndfr. These variations in the ^{15}N root enrichment along the growth cycle largely influence the assessment of the Ndfr and may lead either to an over or an under estimation of the Ndfr (McNeill and Fillery, 2008). As a consequence, the labelling should be realised in order to maintain a homogeneous root enrichment from the beginning to the end of the labelling period to improve the reliability of assessment of the Ndfr.

Finally, Ndfr represented from 10 to 30% of the total BGN depending of the level of N fertilization. As the labelling frequency had no effect on the proportion of total plant N allocated to the roots, differences observed in the BGN expressed as a percentage of total plant-N between R and C treatments are due to the differences in the assessment of the Ndfr. Except for P2N0, BGN was around 16% of total plant-N for all C treatments at maturity, though it varied from 12.4 to 17.5% of total plant-N for the R treatments. The variation in the assessment of the Ndfr for plants fortnightly labelled changes the interpretation about the effect of the N fertilisation on the N rhizodeposition.

4.4. *Reliability of results obtained in the field*

In the field, BGN was around 56% of the total plant-N for P2N0 and varied from 29 to 37% of the total plant-N for the other treatments. Similar Ndfr of 30 and 36% of the total plant-N were measured for field grown pea at maturity by Mahieu *et al.* (2007) and Wichern *et al.* (2007), respectively. In the greenhouse, the Ndfr represents a proportion lower than 30% of the total BGN and the influence of the labelling frequency on the Ndfr estimation did not change significantly the BGN expressed as a percentage of total plant-N. However, in the

field the Ndfr represented around 90% of the total BGN and the labelling frequency may substantially change the proportion of total plant N allocated to BG.

4.5. *Improvement of the protocol of root collection and sampling*

In the protocol of root collection and sampling, all visible roots and root fragments are generally collected from the soil by hand with either the held of a sieve or tweezers. Then roots are rinsed with deionised water before being dry and the slurry is added to the soil (Jensen, 1996; Mayer et al, 2003; Schmidtke, 2005). In contrast, Janzen and Bruinsma (1989) did not wash the roots to avoid N leaching and Yasmin *et al.*, (2005) froze dried at - 40°C for 2 days the collected roots to detached adhering soil and then carefully brushed the roots to obtain a clean root fraction. In our experiments, we tested to rinse the collected roots with a solution of $CaCl_2$ at 4°C which is generally used to inhibit nitrate transporters for the measure of nitrate influx (Gojon *et al.*, 1998; Faure-Rabasse *et al.*, 2002). In comparison with the use of deionised water (data not shown), we measured a Ndfr of 21.7% of the BGN though it was around 16.7% of the BGN when roots were rinse with $CaCl_2$ indicating that N leaching may occur when roots are rinsed with water.

II.4. **Conclusion**

The cotton-wick method did not alter neither plant biomass and total N content nor N and biomass partitioning. Although some variations in the ^{15}N distribution were observed between N treatments for each one of Frisson and P2, the method seems provide consistent results whatever the fertilisation N and the growth conditions. However, the assessment of the Ndfr was influenced by the labelling frequency and the concentration of the labelling solution. The fortnightly labelling probably leads to a disproportional ^{15}N distribution at the origin of an underestimation of the Ndfr. Moreover, the use of a concentrate labelling solution when plants are treated by intermittent pulse of ^{15}N may generate an over enrichment of N compound released into the soil. The maximum concentration of the labelling solution varies with the plant N content. The effect of the concentration of the labelling solution is not visible on non fixing plants since plants re-absorb N from the soil solution and then homogenise roots and soil ^{15}N enrichment.

Finally, the results obtained with a continuous labelling are more reliable than with intermittant labelling. However, the Ndfr assessment could be improved by i) beginning the labelling very early in the vegetative development ii) maintaining a similar root enrichment from the beginning to the end of the labelling period.

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As in the previous comparison, the ratio of Ndfr : plant-N was higher when plants were labelled continuously with a diluted solution of urea than when plants were labelled fortnightly. It appears that the results need to be interpreted with care when the ^{15}N roots enrichment of plants labelled fortnightly was higher than 1.5% though the results were reliable when plants were labelled continuously with a diluted ^{15}N urea solution.

Chapter II. Effects of genetic characteristics and abiotic factors on the BGN

It is assumed that the share of N compound allocated to the below ground parts varies according to the mobilization of N for seed filling. In order to highlight relationships between BGN, N sources, and the mobilization of root N for pod filling, we compared BGN of different legume varieties and isolines similarly in the field and in the greenhouse. Two varieties of pea, with contrasted NHI, Frisson and Baccara, and P2 (non nodulating isoline of Frisson) were cultivated on soil fertilized with different levels of mineral N.

Effect of water deficit was also explored as this factor is one of the most important affecting crop production. It is known to generate a decrease in nodular biomass and N_2 fixation but above all to influence the growth of the root system. In a growth chamber, pea cv. Solara (So) were either not stressed (NS) or exposed to a severe drought at flowering (16 days, SF) or during pod filling (15 days, SPF). Ndfr was measured using the split root method (Jensen; 1996) and plants were harvested at three dates: end of flowering (59 day after sowing, DAS-59), end of drought period for SPF (DAS-74) and at maturity (DAS-101).

I. Effects of N source, N content and plant N partitioning on the below ground contribution of pea to the soil N pool

A short communication in preparation to be submitted to Soil Biology & Biochemistry

Stéphanie Mahieu , Yves Crozat, Joëlle Fustec

Laboratory of plant Ecophysiology and Agroecology, ESA, 55 rue Rabelais, BP 30748, 49007 Angers cedex 01, France. s.mahieu@groupe-esa.com

Abstract

Our aim was to investigate the influence of N sources, N content and biomass and N partitioning on below ground N (BGN : root N + N rhizodeposition) contribution of legumes to the soil N pool. For this, Peas cv. Frisson (Fr), P2 (isoline nod- of Fr) and cv. Baccara (Ba) were grown on soil fertilized with different levels of mineral N in the greenhouse and in the field. In the both conditions, the amount of N rhizodeposition (Ndfr) increased with the total plant-N content and a positive relationship was obtained between the both variables. For a given plant-N content, Ndfr of Fr and P2 were similar and it is concluded that BGN does not depend on the N source. For a given plant-N content, Ndfr measured in the field was substantially higher than in the greenhouse. However we could not find any relationship between BGN and N partitioning or biomass partitioning explaining the difference between field and greenhouse.

Keywords: N rhizodeposition, NHI, N_2 fixation, shoot-labelling method, legumes

I.1. **Introduction**

Legumes may reduce the need for applications of N fertiliser and hence may play a major role in the development of sustainable agriculture (Crozat and Fustec, 2006). In the literature, values of below ground legume N (BGN : N rhizodeposition + N roots) obtained under field conditions are scarce and vary between 24% and 68% of total plant N depending on the growing conditions and the species (Rochester *et al*., 1998; Khan *et al*., 2003; Mahieu *et al*., 2007). For a given species, effects of growth conditions (such as N availability) or sources of N nutrition (nitrogen fixation or inorganic N uptake) have not been investigated. In the

presence of mineral N in the soil, it is known that symbiotic nitrogen fixation is inhibited and that biomass partitioning within the nodulated roots is altered. Moreover, root growth and root biomass density are enhanced leading to more numerous but finer roots (Voisin *et al*., 2002). However, to a large extent, factors regulating the allocation of N to the below ground parts are not well known. The proportion of N allocated to different plant parts may vary according to the N source, mineral N or symbiotically fixed N and to the pattern of plant N accumulation during seed fill. Indeed, N for seeds can be obtained either by further N accumulation or by N transfer from the vegetative component of the plant (Lecoeur and Sinclair, 2001). Our aim was to investigate the influence of N sources, N content and of biomass and N partitioning on below ground contribution of legumes to the soil N pool.

I.2. **Material and method**

Experiments were carried out on peas cv. Frisson (Fr), P2 (isoline nod- of Fr) and the less leafy and high yielding pea cv. Baccara (Ba) in the field and in the greenhouse. The greenhouse experiment was conducted from March to May 2006. Pea plants were grown in 2 l pots filled with a sandy loam, and provided daily either with a N-free (N0), 5 m*M* N (N5) or 15 m*M* N (N15) nutrient solution. From the seven leaf stage, plants were treated using cotton-wick (Mahieu *et al.*, 2007) and labelled continuously of 0.2% ^{15}N urea solution (99 atom% ^{15}N). The field experiment was conducted from March to June 2006. It was designed in 4 blocks, each consisting of one plot per pea cultivar. Fr and P2 were grown either on unfertilized soil (FrN0, BaN0 & P2N0) or on soil fertilized with 100 kg N ha^{-1} at sowing and 150 kg N ha^{-1} at the stage eight leaves (FrN250 & P2N250). Plants were labelled every ten days with either 0.5 ml of 1.3% ^{15}N urea solution or 1% in P2N0. For each N treatment, microplots of six labelled pea plants were marked out by pushing four plastic sheets into the soil at a depth of 30 cm to prevent lateral losses of applied ^{15}N. Control microplots were designed similarly to those of plants treated using cotton-wick and were 0.35 m in width and 0.225 m in length. Plants were harvested at maturity. The proportion of the total N derived from rhizodeposition (NdfR) was calculated using the equation : % NdfR=((at.%^{15}N soil - at.% ^{15}N background A) / (at.% ^{15}N roots - at.% ^{15}N background B))×100. The soil and the roots of the non ^{15}N-fed legumes were chosen as backgrounds A and B respectively. The Nitrogen Harvest Index (NHI) was calculating as follows: NHI = seed-N / (plant-N + Ndfr).

I.3. **Results and discussion**

I.3.1. Effect of N fertilisation on plant N content and N distribution

In the both experiments, plant-N partitioning and the proportion of N derived from fixation (Ndff) varied with level of N fertilisation. In the field, total plant-N and DW of BaN0, FrN0, FrN250 and P2N250 were similar (Table II.1). These results are consistent with those generally observed in field conditions, optimal yield and biomass production are observed regardless of N fertilisation of legumes at sowing (Sagan *et al.*, 1993; Crozat *et al.*, 1994; Voisin *et al.*, 2002a). Conversely, in the greenhouse, dry weight and total plant N of P2 and Fr increased with the N fertilization level (table II.1). Similar difference between field and greenhouse were reported by Voisin *et al.* (2002) and may be due to a transitory nitrogen deficiency at the beginning of the growth cycle before symbiotic fixation is able to sustain growth of unfertilized peas in the greenhouse. In the field, soil mineral N availability is generally sufficient to satisfy N demand through root absorption at the beginning of the growth cycle (Jensen, 1997a). In the both conditions N concentration and plant N partitioning in each plant part remained generally unchanged whatever the N source; only biomass is likely to increase in all organs except the seeds (Voisin *et al.* 2002). However, in our greenhouse conditions, the root N concentration increased in fertilised treatments though root DW tended to decrease. Therefore the proportion of total DW and N in the roots of P2 and Fr significantly dropped with increasing fertilisation (table II.1).This difference between our results and those of Voisin *et al.* (2003a) seems not to come from a problem of fixation since the proportion of N derived from fixation (Ndff) in the plants of FrN0 was around 80% of the total plant N. Differences in N fertiliser application may explain these differences between our results and those of Voisin et *al.* (2002) (Jensen, 1997a).

Total N and DW content in the different plant parts were lower in P2N0 than in the other treatments in the field and greenhouse (Table II.1), and proportion of total plant N allocated to the root were significantly higher than in other treatments.

For each N5 and N15 fertilisation treatments in the greenhouse or N250 in the field, root and seed N concentration and DW of Fr and P2 were similar.

Table II.1 : Total N content (mg plant^{-1}), roots N (% of plant N), Nitrogen derived from fixation (Ndff) (%), Nitrogen derived from rhizodeposition (Ndfr) expressed as a % of the total below ground N (BGN) and BGN expressed as a % of the total plant-N in Frisson, isoline P2 or Baccara measured at maturity in the greenhouse and in the field experiment. In the greenhouse, plants were provided daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution. In the field, peas were grown either on unfertilized soil (N0) or on soil fertilized with 250 kg N.ha^{-1} (N250). Values are means ± SE, n = 6 (greenhouse), n=4 (field). Veg are 'pods, leaves and stems'.

treatments		Total N (mg plant^{-1})	roots N/ of plant N(%)	Ndff/ plant N (%)	Ndfr/ BGN (%)	BGN / plant N (%)
Greenhouse						
N0	P2	18.5d	35.7a		21.7b	39.9a
	Fr	76.0c	20.2b	79.6 (3.0)	15.3b	17.5b
N5	P2	90.6bc	18.2b		23.2b	15.1b
	Fr	105.6b	19.7b	32.6 (5.9)	18.7b	16.0b
N15	P2	140.6a	12.6c		35.0a	16.3b
	Fr	132.1a	13.2c	22.2 (5.2)	32.9a	16.8b
P		*	*		***	***
Field						
N0	P2	37.9b	11.2a		90.2	56.2a
	Fr	204.8a	5.9b	43.4 (3.6)	91.2	37.0b
	Ba	252.3a	5.2b	62.5 (3.6)	88.1	28.6b
N250	P2	248.3a	4.7b		87.4	34.9b
	Fr	251.7a	5.0b	12.8 (3.4)	90.2	30.8b
P		***	*		n.s.	***

P indicates a statistical significance across treatments within a harvest stage. n.s. non-significant. * $P < 0.01$, ** $P < 0.005$. *** $P < 0.0005$. Different letters indicate significant differences between treatments.

I.3.2. Effect of N source and N fertilisation on BGN

In Fr and P2, Ndfr was positively correlated with total plant dry matter (data not shown) as well as with the total plant N content (Fig. II.1, R^2= 0.70, P<0.0001 in the field and R^2= 0.80, P<0.0001 in greenhouse). In the greenhouse, conversely to the allocation of total N in the roots, the proportion of Ndfr to total BGN released into the soil increased with increasing plant N content (table II.1). In the field, the proportion Ndfr: BGN was similar for all treatments, it was around 90% of total BGN whatever the treatment. Except of P2N0 treatment, the proportion of the total plant-N allocated below ground was around 16% for all treatments in the greenhouse and around 30% in the field. Therefore the N fertilisation is likely to influence the ratio Ndfr : BGN but do not affect the N partitioning between above and below ground parts.

For a given plant N content, no difference was found between Fr and P2 or between the different Fr treatments. Such results suggest that BGN does not depend on the N source.

The % Ndfr/ BGN of P2N0 was not affected by the N deficiency but the % BGN: plant-N of P2N0 was more than twice higher than in FrN0 in the both growth conditions.

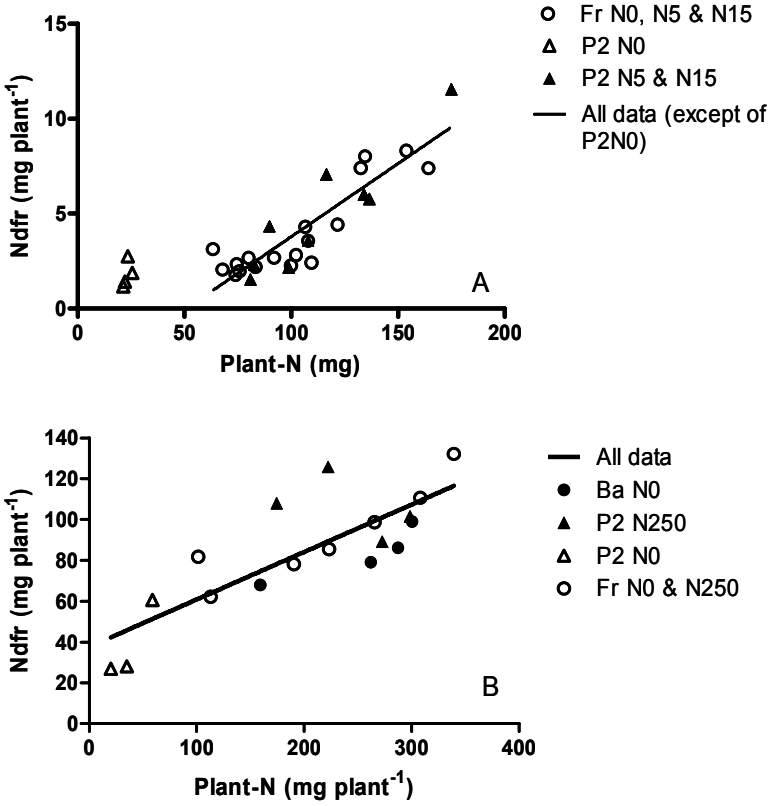

Fig. II.1: Relationships between the amount of N derived from rhizodeposition (Ndfr) (mg plant-1) and plant-N (mg plant-1) of Frisson, isoline P2 or Baccara measured at maturity (A) in the greenhouse and (B) in the field. In the greenhouse, plants were provided daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution. In the field, peas were grown either on unfertilized soil (N0) or on soil fertilized with 250 kg $N.ha^{-1}$ (N250).

I.3.3. Effect of plant N partitioning on BGN

Total plant N and DW of BaN0 and FrN0 were similar as well as the proportion of Ndfr: BGN. Hence any relationship was found between BGN and N partitioning or biomass partitioning of BaN0 and that of FrN0.

Although similar total plant-N was obtained for some plants in the both growth conditions (Fig. II.2), the Ndfr was substantially higher in the field than in the greenhouse. Since the proportion of BGN: plant N of P2 and Fr grown on the same N fertilisation level were similar despite of a quite different root system, this difference between field and greenhouse is not due to the architecture of below ground parts. In the field and greenhouse, the % Ndfr : plant-N was correlated with the NHI (Fig. II.2). In each one of the growth conditions, there were no difference between the slope and intercept of Fr, P2 and Ba (Field) data and a single linear relationship was obtained for the different treatment (Fig.II.2). In the both conditions the NHI

ranged from 0.25 to around 0.6 and the higher was the NHI the lower was the % Ndfr : plant-N. However for a given NHI, the % Ndfr: plant-N was higher in the field than in the greenhouse.

The proportion of N remaining in the Veg (pods, leaves and stems) was lower in the field than in greenhouse (data not shown) though the proportion of Ndfr: plant-N was quite higher. Therefore, the difference between the both growing conditions may likely results from a difference in N remaining to the Veg.

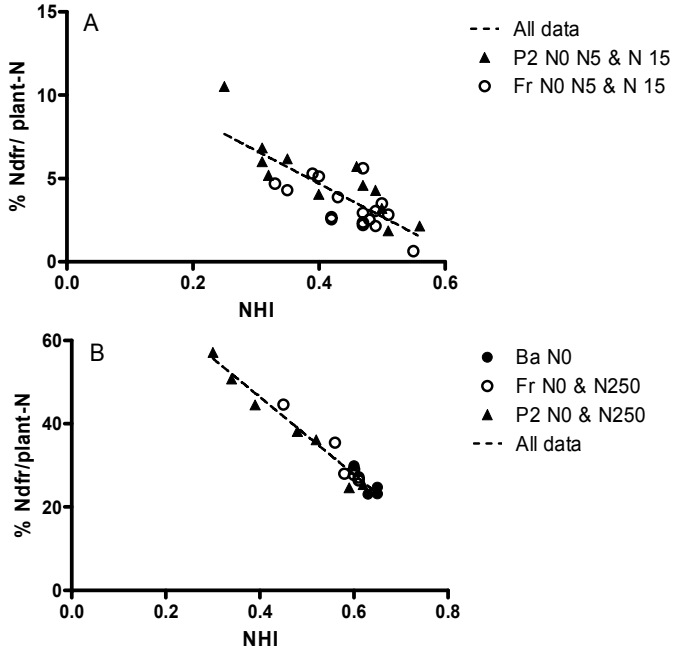

Fig. II.2: Relationships between the proportion of total plant N derived from rhizodeposition (% Ndfr: plant-N) with the Nitrogen Harvest Index (NHI) of Frisson, isoline P2 or Baccara measured at maturity (A) in the greenhouse and (B) in the field. In the greenhouse, plants were provided daily either with a N-free (N0), 5 mM N (N5) or 15 mM N (N15) nutrient solution. In the field, peas were grown either on unfertilized soil (N0) or on soil fertilized with 250 kg $N.ha^{-1}$ (N250).

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II. The influence of water stress on the below-ground N partitioning during reproductive growth of pea (*Pisum sativum* L.)

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S. Mahieu[1*], F. Germon[2], A. Aveline[1], H. Hauggaard-Nielsen[2], P. Ambus[2] and E. S. Jensen[2]

[1]Laboratory of Plant Ecophysiology and Agroecology, ESA, 55 rue Rabelais, BP 30748, 49007 Angers cedex 01, France. [2]Biosystems Department, Risø DTU, The Technical University of Denmark, DK-4000 Roskilde, Denmark.

Corresponding author:

*Stéphanie Mahieu

Lab. of Plant Ecophysiology and Agroecology,

ESA, 55 rue Rabelais, BP 30748,

49007 Angers cedex 01, France

Tel 33 2 41 23 55 55

Fax 33 2 41 23 55 50

s.mahieu@groupe-esa.com.

Abstract

Rhizodesposition of N from legumes roots constitutes a significant source of N, which should be quantified to improve estimates of biological N_2 fixation, N balances and the N benefit for following crops. This work aimed to quantify the rhizodeposition of nitrogen from pea (*Pisum sativum* L.) when influenced by water stress, which is considered one of the most frequent abiotic stresses influencing pea. In a controlled environment, pea plants were exposed to a severe drought or not stressed, either at flowering or during pod filling. N rhizodeposition was measured using the split root method and plants were harvested at the end of flowering (59 days after sowing, DAS-59), at the end of the drought period at SPF (DAS-74) and at maturity (DAS-101). Water stress strongly affected pea dry weight and N accumulation. In both stress treatments, nodule biomass and N content were reduced by about 65% in the absence of stress. Regardless of the treatment, total below-ground N (Root N + N rhizodeposition; BGN) and N rhizodeposition were correlated with total plant-N content and the proportion of BGN to total plant-N was similar among treatments at each sampling date. At DAS 59 and 74, the N contained in rhizodeposits represented around 30% of the total BGN and increased to around 60% at maturity though BGN decreased from around 20% to 13% of the total plant N between DAS 74 and maturity. The results suggest that water stress has no specific effect on the proportion of total plant N allocated to below-ground N.

Key words :N rhizodeposition, water stress, roots, nodules, N partitioning, legumes, *Pisum sativum* L., Split root

II.1. **Introduction**

One way for farming systems to be sustainable and to remain productive in long term is to exploit the ability of legumes to fix atmospheric N_2 (Jensen and Hauggaard-Nielsen, 2003; Peoples, 1995). However, enhancing the use of legumes as a means of N acquisition in agroecosystems depends on improving the accuracy with which the amount of N fixed can be assessed. Recent studies conducted in the field have revealed that below-ground nitrogen (BGN), including root N and N rhizodeposition, may represent a substantial part of total plant N and therefore is vital for accurately calculating N balances (Dubach and Russelle, 1994; Khan et al, 2003; Peoples, 1995; Rochester *et al.*,1998; Russell and Fillery, 1996b). Measurements of BGN using different labelling methods vary around 24% to 40% of total plant-N for Faba bean (Khan *et al.*, 2003; Rochester *et al.*,1998), 68% for chickpea (Khan et al, 2003), between 38 and 49% for lupin (McNeill and Fillery, 2008), 30% to 40% for pea (Mahieu *et al.*, 2007; Wichern *et al.*, 2007). As a consequence, factors regulating the allocation of N below ground are important if estimates of N benefit from biological N_2 fixation by a grain legume crop are to be improved.

Effects of abiotic factors on root growth and carbon rhizodeposition has been intensively studied (Keith *et al*., 1986; Jones et Darrah, 1993; Jensen, 1993; Devries *et al.* 1989a; Benjamin and Nielsen, 2006). Until now, experiments on N rhizodeposits have mainly focused on methodological problems and to a large extent, factors regulating the allocation of N to the below-ground parts are not well understood. Previous experiments in pea crops from Sawatsky and Soper (1991), Jensen (1996), Mayer *et al.* (2003) and Mahieu *et al.* (2007) have shown that the proportion of total below-ground plant N in rhizodeposits can vary between 40 and 80% at maturity. Those assessments were carried out in non-limiting growth conditions either in controlled or in field experiments. In the field, grain legumes crops may experience abiotic stresses such as water deficit, and it is not known how such stress may influence N rhizodeposition.

During plant growth, N is released from roots into the soil by exudation of specific nitrogeneous compounds (Paynel *et al.*, 2001; Whipps, 1990) and due to roots and nodules senescence. In the absence of stress, the proportion of total plant N in rhizodeposits increases with plant age (Jensen, 1996b). Dubach and Russelle (1994) quantified the amount of N

released into the soil due to root and nodule senescence by measuring root length and nodule number that decomposed during a season in the absence of stress. They concluded that the highest amounts of N were released into the soil either as a result of senescence of the finest roots or due to nodule senescence, depending on the plants species. They observed that the N content of decaying nodules in Birdsfoot trefoil was significantly lower than in functioning nodules suggesting that N was remobilised during senescence. However, specific N content in dead undecomposed fine roots was not reduced indicating either that no significant N remobilisation had occurred or that N import was equal to N export in these organs.

Water deficit is one of the major factors limiting plant productivity and symbiotic nitrogen fixation in many arid regions of the Mediterranean basin. In dry conditions, several mechanisms contribute to limiting nitrogen fixation by affecting either nodulation initiation, nodule development or function (Busse and Bottomley, 1989; Mnasri *et al.*, 2007; Serraj *et al.*, 1999; Zahran and Sprent, 1986). Accumulation of biomass and N are reduced and roots N partitioning is modified, due to a decrease in nodule biomass. Field peas exposed to water stress have finer root systems which grow deeper into the soil and lower root dry weight compared with unstressed plants (Benjamin and Nielsen, 2006; Devries *et al.*, 1989a).

In the above ground parts, biomass accumulation is generally reduced due to reduced leaf area, decreased net photosynthesis and stomatal closure (Guilioni *et al.*, 2003; Lecoeur *et al.*; 1995; Lecoeur et Sinclair, 1996; Monteith, 1977). The effect on seed yield depends on when the water stress occurs and on the intensity and duration of the shortage. If stress occurs during flowering, the duration of flowering is shorter and some flowers are aborted (Acosta Gallegos and Kohashi Shibata, 1989; Ney et al, 1994). As a consequence pod and seed numbers are reduced though only seed number is affected when the stress occurs during seed-filling period. In both cases less N is remobilised from the different plant parts for seed filling (Devries *et al.*, 1989b). Those phenomena could be a result of the effect of stress on biomass or nitrogen accumulation rather than of any specific effect of water stress on developing organs during the period of constraint (Jeuffroy et Warembourg, 1991, Guilioni *et al.*, 2003).

The aim of this study was to determine the effect of water stress occurring either during flowering or the pod filling period on below-ground N partitioning within roots, nodules and

rhizodeposits. We also provide an overview of the dynamics of plant N partitioning during the reproductive period as influenced by water stress. A continuous split root ^{15}N labelling technique was used to quantify N rhizodeposition.

II.2. **Material and method**

II.2.1. Experimental unit

The results reported here were obtained using a direct split-root ^{15}N labelling method allowing simple and continuous labelling of pea plant (*Pisum sativum* L.) as shown successfully by Jensen (1996b), Sawatsaky and Soper (1991) or Schmidtke (2005a et b). The peas were grown in experimental units consisting of a labelling compartment (LC) and a transfer compartment (TC) (Fig. 1). The LC consisted of a 200-ml glass cylinder with light expanded clay aggregates (LECA) as the inert substrate and labelling nutrient solution, while the TC was a 1,5-l. pot holding 1.7 kg soil (dry matter: 97.7%). The soil surface was covered with nylon mesh and a mixture of granite and flint so as to regulate evaporation.

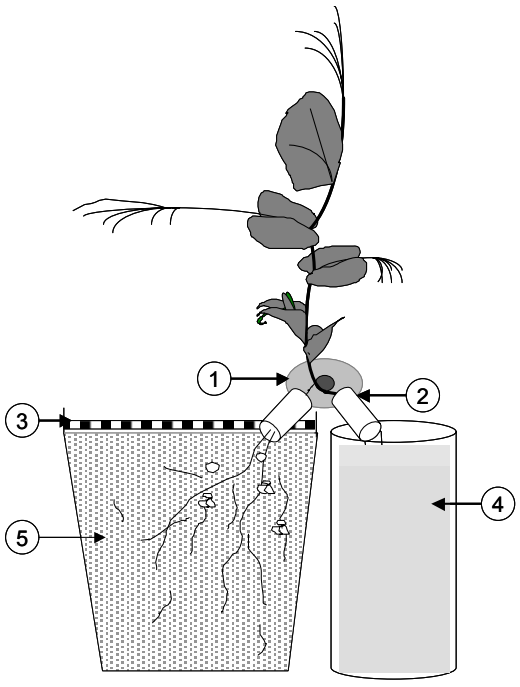

Fig. 1: Split root method used to label pea plants. (1) Terostat and (2) Teflon tube protecting roots from dessication (3) Stones and flint laid on a mesh of nylon (4) Labelling compartment containing light-expended clay aggregates and filled with ^{15}N labelling solution (5) Transfer compartment filled with sandy soil in which rhizodeposition was measured.

II.2.2. Soil and nutrients

The soil used was a 3:1 (w:w) mixture of a sandy loam soil with washed dutch river quartz sand. The sandy loam soil consisted of 11% clay, 14% silt, 49 % fine sand and 25% coarse sand was obtained from a depth of 0 to 20 cm in the Risø experimental field.

The soil mixture contained 0.11% total N and 1.12% total C. The pH(H_2O) after correction with $CaCO_3$ was 6.7. Nutrients, except N, were added before potting in the following amounts (mg kg^{-1} soil): KH_2PO_4 (266.7), KCl (66.7), $MgSO_4.7H_2O$ (133.4), $CuSO_4.5H_2O$ (6.7), $MnSO_4.H_2O$ (4.7), $Na_2MoO_4.2H_2O$ (1.1) and $CoCl_2.6H_2O$ (1.3).

The labelling nutrient solution containing basic nutrient and 10 atom%^{15}N excess was prepared using the following concentrations (mM) : $MgSO_4.7H_2O$ (0.75), $Na_2HPO_4.2H_2O$ (0.67), K_2SO_4 (1), $CaCl_2.2H_2O$ (2), Fe(III)NaEDTA (0.027) and $K^{15}NO_3$ (5)(Rosendahl and Jakobsen, 1987).

II.2.3. Plants

Pea (*Pisum sativum* L. cv. Solara) seeds of 300 ± 10 mg were germinated for 3 days in moist vermiculite. The tap root was cut, leaving the upper 1 cm intact. The seedlings were replanted and grown for 10 more days in vermiculite, before transplantation. One pea plant was planted per experimental unit. The root system was divided equally and rooted in both compartments through a bent Teflon tube, the stem base being fixed to the pot with a sealing compound (Terostat X).

II.2.4. Growth conditions

Experimental units were placed in a growth chamber and the peas were grown with a 16-8 h day-night regime at 17 and 13°C respectively. Metal halide lamps provided an average photosynthetic photon flux density of 500 μE m^{-2} s^{-1} above the plants. In non-limiting conditions, plants were watered with deionised water as required to maintain a level of 75% water holding capacity (WHC) in the transfer compartment whereas the LC was fully replenished with ^{15}N labelled nutrient solution. During drought periods, watering was reduced to stabilize soil moisture at 24% WHC and the supply of labelling nutrient solution was

limited to 20% of normal volume. Special arrangement of experimental units was randomized in the growth chamber three times a week.

Pea plants were grown for 101 days and labelling was terminated at day 81 by filling up the glass cylinder with deionised water instead of labelled nutrient solution.

II.2.5. Experimental design

To quantify rhizodeposition during growth and determine post-harvest N dynamics under the influence of a variable water supply, three treatments with 16 replicates each were set up. An unstressed reference treatment, benefiting from unlimited water (Control, C), was maintained constantly at 75 % WHC. The second treatment was exposed to a 16 day severe water stress at flowering (SF), whereas a 15 day severe water stress during pod filling (SPF) was applied to the third treatment at the beginning of pod filling. Drought stress was initiated by limiting water supply and maintained in such a way that plants remained closed to wilting point.

Plant materials (aerial parts and roots) and soil of four replicates of each treatment were sampled at the end of the two drought periods and at maturity corresponding to 59, 74 and 101 days after sowing (DAS-59, DAS-74 and DAS-101).

Aerial plant parts of four replicates from each treatment were removed from the remaining experimental units at DAS-101 and the transfer compartments were then incubated for 35 days in the dark at 15°C. WHC was adjusted to 55% and soil was sampled at DAS-136.

II.2.6. Sampling and analytical methods

At DAS-59, 74 and 101, plants aerial parts were cut away from the root system at the stem base. For each replicate, the transfer compartment and the labelling compartment were detached from each other to be analysed separately.

The soil from the TC was then spread on a paper sheet and all visible roots and root fragments were removed carefully with the aid of tweezers. All roots + nodules from the transfer compartment ($TC_{root+nod}$) were washed with 200ml deionised water. At DAS-59 and DAS-74, roots (TC_{roots}) and nodules (TC_{nods}) were separated for further determination of ^{15}N enrichment and N content. The slurry was dried for 24 hours at 80°C before being

incorporated to the soil for further measurements. The root system from the labelling compartment ($LC_{root+nod}$) was extracted, washed in 100ml deionised water and weighed.

A $TC_{root+nod}$ sub-sample of ca. 1g was weighed out at DAS-59, 74 and 101, and stored in acetic acid so as to determine root length (Newman,1966). Root length of the whole sample was established by extrapolation.

At DAS-101, grains were harvested to determine grain N yield. All plant materials harvested during growth: $TC_{root+nod}$, $LC_{root+nod}$, stems and leaves, grains, were dried for 20 hours at 80°C, weighed and then milled to obtain <1 mm particles.

At DAS-136, soil from incubated pots was blended to break all remaining roots into small pieces and then homogenized before analysis.

Nitrate and ammonium concentrations in the soil samples from DAS-59, 74, 101 and 136 were determined with a Technicon Autoanalyser II, using the sodium salicylate-sodium nitroprusside hypochlorite method for NH_4^+ and the sulfanilamide-naphtyl-ethylenediamine method for NO_3^-, (Technicon, 1974; Jensen, 1994). Analyses were carried out on 20g of fresh soil sample extracted with 200ml of 2M KCl shaker for 1 hour and filtered through a Whatman n°40 filter.

Ground air dried soil (99.2% dry matter) and all plant materials from DAS-59, 74, 101 and 136 were analysed using an automated elemental analyser (Carlo Erba 1110 CONS) coupled on-line with a ratio mass spectrometer (Finnigan MAT Delta+), to simultaneously determine total N content and ^{15}N enrichment (Jensen, 1991). The ^{15}N enrichment of the inorganic pool, at DAS-101 and 136, was measured by elemental analysis and mass-spectrometry after diffusion to a polytetrafluoroethylene trap (Sørensen and Jensen, 1991).

II.2.7. Calculation

The rhizodeposition of N quantified in this experiment is the net amount of N derived from rhizodeposition (NdfR), i.e. the residual N of three concomitant phenomena: gross rhizodeposition, reassimilation by the plants of N previously deposited and losses by denitrification. Net rhizodeposition was defined as root-derived N present in the soil after removal of visible roots and root fragments (Jensen, 1996).

For the calculations of rhizodeposition, the following assumptions were made: 1) roots growing in the labelling compartment provided a constant proportion of the total N absorbed by the plant; 2) uptake and use of N by the plant was identical for ^{14}N and ^{15}N; 3)

rhizodeposition rate was constant through time; 4) The N deposited had the same enrichment as the root nitrogen.

All ^{15}N enrichments of samples were corrected for the background value of ^{15}N: 0.37098 atom% ^{15}N for the soil samples and 0.3663 atom% ^{15}N for pea parts; the background values being derived from unlabelled replicates.

The proportion of total and inorganic soil nitrogen derived from rhizodeposition of N (*p*NdfR) was calculated using the following equation (Janzen and Bruinsma, 1989):
*p*NdfR = atom % ^{15}N excess soil (at.% ^{15}N soil) / atom % ^{15}N excess roots (at.% ^{15}N roots)

In order to calculate the percentage of inorganic N derived from rhizodeposition (%$NdfR_{In\text{-}N}$) at DAS-136, the at.% ^{15}N root value of samples harvested at maturity (DAS-101) was used. The amounts (mg) of N rhizodeposition in the total and inorganic N pools were obtained by multiplying the amounts of N in these pools by respective %NdfR values.

Statistical analyses were carried out using Statbox Pro software. When possible analysis of variance was used for multiple comparison of means using Newman and Keuls test with a confidence level of 0.05. When data normality or equality of variances was not respected, comparisons of means were carried out using non parametric Kruskal-Wallis tests with a confidence level of 0.05. GraphPad Prism 4.03 Software was used for linear regression analysis and comparisons.

II.3. **Results**

II.3.1. Dry weight, nitrogen content, biomass and nitrogen partitioning

Water stress reduced the nitrogen (N) and dry weight (DW) accumulation while stress during flowering reduced yields more than stress during pod-filling (Table II.2). Total DW and N content of plants stressed during flowering were around half of C or SPF treatments at DAS 59 and DW and N accumulation remained very low until maturity though plants were re-watered. Whatever the treatment, there was no significant change in total DW and N content between DAS 74 and 101 (Table II.2).

At each sampling date, the proportion of total plant N present in the roots was similar among treatments. The proportion of biomass in the roots of the C and SPF treatments was also similar at each sampling date and was slightly lower than in the roots of the SF treatment

at DAS 59 and 101. Between DAS 59 and 101, the proportion of DW and N allocated to the roots decreased in all treatments (Table II.2; $P < 0.0001$).

Table II.2: Pea total dry weight (g plant^{-1}), total N content (g plant^{-1}) and N and dry weight partitioning (%) measured 59, 74 and 101 days after sowing (DAS). (C) Control, (SF) water stressed during flowering, (SPF) water stressed during pod-filling.

Treatment		Total dry weight (g plant^{-1})	Total N (g plant^{-1})	Dry weight partitioning (%)			N partitioning (%)		
				Roots	Veg*	Seeds	Roots	Veg*	Seeds
DAS 59	C	14.3a	0.37a	14.3b	85.7a		15.8	84.2a	
	SF	8.3b	0.20b	18.6a	81.4b		15.9	84.1a	
	SPF	14.3a	0.37a	14.3b	85.7a		15.8	84.2a	
P		**	***	*	*		n.s.	n.s.	
DAS 74	C	23.7a	0.59a	11.3	88.7		12.8	87.2a	
	SF	11.6c	0.24c	13.1	86.9		13.9	86.1a	
	SPF	17.4b	0.40b	13.3	86.7		12.2	87.8a	
P		****	****	n.s.	n.s.		n.s.	n.s.	
DAS 101	C	20.7a	0.53a	5.1b	35.8b	59.1a	4.7	15.4b	79.9a
	SF	11.1c	0.25c	8.5a	47.3a	44.2b	7.4	23.9a	68.7b
	SPF	17.4b	0.43b	6.4b	46.1a	47.6b	5.6	24.4a	70.0b
P		****	***	**	***	****	n.s.	**	**

Presented values are means (n = 4).
P indicates a statistical significance across treatments within a harvest stage. n.s. non-significant, * $P < 0.01$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$. Different letters indicate significant differences between treatments.
Veg are 'pods, leaves, stems'.

At DAS 101, the proportions of DW and N in 'pods, leaves and stems' and in the seeds were similar in the SF and SPF treatments. In these treatments, the proportion of biomass or nitrogen in the seeds and the N re-mobilised from 'pods, leaves and stem' were lower than in the C treatment.

II.3.2. Dry weight and nitrogen contents of roots and nodules

Roots + nodules from the labelling compartment ($LC_{root+nod}$) represented around 22.2 ± 9.4 % (n=4 ± SE) of the total root DW and there were no significant difference among treatments and date of sampling ($P > 0.05$; data not shown). At each harvest stage, when the DW (data not shown) or N content (Fig. 1A) of the total roots from the transfer compartment ($TC_{root+nod}$) were plotted against total plant-N content, significant linear relationships were found. At each sampling date, DW and N content of the $TC_{root+nod}$ increased with total plant-N content independently of treatment. Analysis of the linear regressions did not show any statistical difference between the relationships obtained at DAS 59 and DAS 74, so the two datasets were pooled (DAS 59 + 74; intercept 5.19; pooled slope 0.14; $r^2 = 0.70$; $P < 0.0001$; Fig. 1A). Intercepts and slopes of the linear regression obtained from DAS 59 + 74 and DAS 101 datasets (intercept 7.86; slope 0.04; $r^2 = 0.46$; $P < 0.05$) were significantly different ($P <$

0.005). As a consequence, for a given plant-N content, DW and N content of $TC_{root+nod}$ decreased between DAS 74 and DAS 101 for all treatments (C, SF and SPF) and the proportion of $TC_{root+nod}$ N of stressed plants either remobilised for seed filling or released into the soil, due to root senescence, was lower than from $TC_{root+nod}$ of C treatments.

When plants were stressed the total root DW production and N content as well as total plant-N content were reduced (Fig.II.3; Table II.3). At DAS 59, in the SF treatment, DW and N content of the $TC_{root+nod}$ were approximately half that in C peas ($P < 0.005$), and remained lower until maturity. In the SPF treatment, DW and N accumulation in $TC_{root+nod}$ were also reduced between DAS 59 and 74 compared to the unstressed treatment (Table II.3; $p < 0.005$). At DAS 74, there was no significant difference between root and nodule DW or N content of peas SF and SPF. For the two treatments, the decreases in $TC_{root+nod}$ biomass and N content were mainly due to a decrease in DW and N content of nodules. Indeed, nodule DW and N content of the SF treatment were reduced by about 65% at DAS 59 and did not increase between DAS 59 and DAS 74. In the SPF treatment, nodule DW and N content were reduced by about 65% between DAS 59 and DAS 74 ($p < 0.05$). After stress application, in the two treatments, root DW was 25% lower than in C treatments, and N content was reduced by about 30% and 35% in the SF and SPF treatments, respectively. In the SF treatment, root density was also reduced compared with the C treatment though not in the SPF treatment. At DAS 101, root DW and N content of the SF treatment were lower than in C and SPF treatments, and there was no difference between these two treatments.

Table II.3: Roots and nodules dry weight (g $plant^{-1}$) and total N content (mg $plant^{-1}$) and root density measured 59, 74 and 101 days after sowing (DAS) in the transfer compartment (TC). (C) Control, (SF) water stressed during flowering, (SPF) water stressed during pod-filling.

		Dry weight (g $plant^{-1}$)		N content (mg $plant^{-1}$)		Root density
Treatment		TC roots	TC nodules	TC roots	TC nodules	($cm.cm^{-3}$)
DAS 59	C	1.02a	0.86a	26.8a	41.0a	11.4a
	SF	0.75b	0.30b	16.7b	13.2b	5.6b
	SPF	1.02a	0.86a	26.8a	41.0a	11.4a
P		*	***	*	*	***
DAS 74	C	1.45a	1.01a	36.8a	37.7a	12.8a
	SF	0.90b	0.33b	22.2b	13.4b	6.8b
	SPF	1.13ab	0.36b	26.6b	13.5b	10.6a
P		*	***	**	*	***
DAS 101	C	0.91ab	ND	25.5	ND	9.1
	SF	0.69b	ND	16.2	ND	6.5
	SPF	0.92a	ND	23.6	ND	9.5
P		**		n.s.		n.s.

Presented values are means (n = 4).

P indicates a statistical significance across treatments within a harvest stage. n.s. non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. Different letters indicate significant differences between treatments.

II.3.3. Root and nodule ^{15}N enrichment and N concentrations

Atom % ^{15}N excess of the soil was similar between C, SF and SPF treatments at each sampling date ($P > 0.05$; data not shown) and mean values (*100) increased from 0.61 ± 0.16% to 1.57 ± 0.41% (n=4 ± SE) between DAS 59 and 101 ($P < 0.0001$). In the transfer compartment, atom % ^{15}N excess of roots was higher than atom % ^{15}N excess of nodules whatever the treatment (Table II.4). Water stress induced an increase in atom % ^{15}N excess in both roots and nodules of the SF and SPF treatments compared to C plants. At DAS 101, the entire root system was collected without sorting roots and nodules and mean atom % ^{15}N excess of $TC_{root+nod}$ of the C treatment was similar to atom % ^{15}N excess of TC roots at DAS 74 (Table II.4). This indicates that between DAS 74 and 101, N from C treatment nodules was probably released into the soil, due to nodule senescence. Nodule N concentration at DAS 74 was lower than at DAS 59 indicating that N remobilisation occurred between the two sampling dates.

Table II.4: ^{15}N excess (%) and N concentration (%) of roots and nodules measured 59, 74 and 101 days after sowing (DAS) in the transfer compartment. (C) Control, (SF) water stressed during flowering, (SPF) water stressed during pod-filling.

		^{15}N excess (%)			N concentration (%)	
Treatment		roots	nodules	total roots	TC roots	TC nodules
C	DAS 59	0.70	0.32	0.49b	2.68b	4.78
	DAS 74	0.71	0.29	0.49b	2.57b	3.72
	DAS 101	ND	ND	0.70a	2.85a	ND
P		n.s.	n.s.	*	*	ns
SF	DAS 59	1.13	0.58	0.90	2.45ab	4.36
	DAS 74	1.02	0.45	0.80	2.28b	3.97
	DAS 101	ND	ND	0.89	2.58a	ND
P		n.s.	n.s.	n.s.	*	ns
SPF	DAS 59	0.70	0.32	0.49b	2.68a	4.78
	DAS 74	0.95	0.42	0.76a	2.36b	4.85
	DAS 101	ND	ND	0.68a	2.58ab	ND
P		n.s.	n.s.	*.	*	ns

Presented values are means (n = 4).
P indicates a statistical significance across harvests stages for a treatment. n.s. non-significant, * $P < 0.05$. Different letters indicate significant differences between treatments.

In the SF and SPF treatments, mean atom % ^{15}N excess of $TC_{root+nod}$ increased after water depletion and remained high until maturity (Table II.4). However at maturity, it was lower than atom % ^{15}N excess of TC roots at DAS 74 indicating that either N from nodules was re-absorbed by the plant or nodule structure was maintained later in the growth cycle.

II.3.4. N rhizodeposition

At each sampling date, mean values of %Ndfr of total BGN and of %BGN of total plant N were similar among treatments (Table II.5). The proportion of BGN allocated to rhizodeposition was around 30% for all treatments at DAS 59 and 74 and increased to 60% at DAS 101 ($P < 0.0001$; Table II.5). The proportion of total plant N allocated to BGN significantly decreased from around 20% at DAS 59 and 74 to 13% at DAS 101 ($P < 0.0001$; Table II.5).

Table II.5: Nitrogen derived from rhizodeposition (Ndfr) (g plant^{-1}), Ndfr expressed as a % of BGN and BGN expressed as a % of total plant N measured 59, 74 and 101 days after sowing (DAS). (C) Control, (SF) water stressed during flowering, (SPF) water stressed during pod-filling.

Treatment		Ndfr (mg plant^{-1})	Ndfr / BGN (%)	BGN / plant-N (%)
DAS 59	C	28	31	21
	SF	12	28	21
	SPF	28	31	21
P		n.s.	n.s.	n.s.
DAS 74	C	29	29	19
	SF	17	31	20
	SPF	22	31	17
P		ns	ns	ns
DAS 101	C	43	64	12
	SF	28	60	14
	SPF	45	65	15
P		n.s.	n.s.	n.s.

Presented values are means (n = 4).
P indicates a statistical significance across treatments within a harvest stage. n.s. non-significant.

As for the total TC roots N content, significant linear relationships were obtained between amount of Ndfr and the total BGN per plant as a function of total plant-N content (Fig. II.3 B & C). At each sampling date, the amount of Ndfr per plant increased with total plant-N content independently of treatment. The slopes of the lines obtained at DAS 59 + 74 and at DAS 101 ($r^2 = 0.71$; $P < 0.005$) were similar ($P = 0.08$; pooled slope 0.049) but intercepts differed very significantly (7.10 and 8.06 respectively; $P < 0.0001$). As a consequence, for a given plant-N content, Ndfr is higher at DAS 101 than at DAS 59 or 74. When total BGN per plant was plotted against total plant-N content (Fig. II.3 C), the linear regressions obtained from DAS 59 + 74 ($r^2 = 0.78$; $P < 0.0001$) and DAS 101 ($r^2 = 0.61$; $P < 0.005$) datasets had similar slopes ($P = 0{,}261$; pooled slope 0.155) while intercepts were different (10.21 and 7.35; $P < 0.0005$). However, contrary to the relationship between Ndfr per plant and a given

plant-N content, the amount of BGN per plant decreased by around 26% between DAS 74 and 101.

Because of variability in the data, we could not find any significant differences between mean Ndfr per plant across treatments (C, SF, SFP; Table II.5). Nevertheless, the amounts of N released into the soil and the total plant-N content of stressed plants were lower than in C plants (Fig. II.3B). Mean Ndfr values of SF plants were more than two times lower than those of C plants at DAS 59 and remained lower than those of C and SPF plants until maturity (Table II.5). Mean Ndfr values of SPF plants were also slightly lower than those of C plants at DAS 74 but similar at DAS 101.

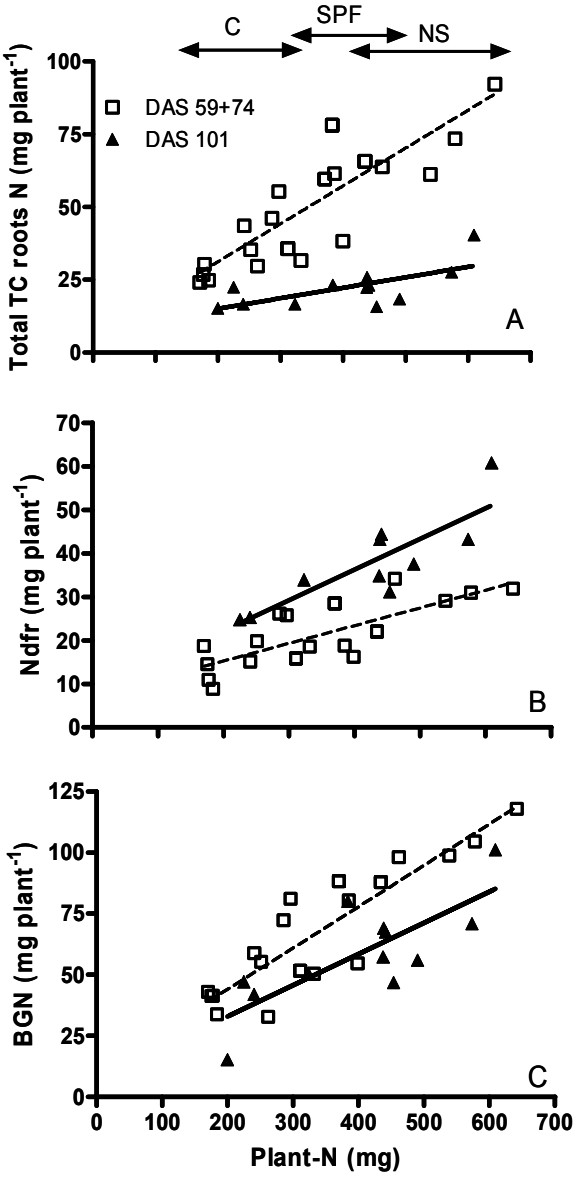

Fig. II.3: Relationships between below ground nitrogen (BGN) and plant-N. (A) Increase in the total N content in TC roots (mg plant-1) as a function of total plant-N (mg plant-1). (B) Increase in the N derived from rhizodeposition (Ndfr) (mg plant-1) as a function of total plant-N (mg plant-1). (C) Increase in BGN (mg plant-1) as a function of total plant-N (mg plant-1) - (DAS 59 + 74) plants collected 59 and 74 days after sowing, (DAS 101) plants collected 101 days after sowing. (C) Control, (SF) water stressed during flowering, (SPF) water stressed during pod-filling.

Finally, Ndfr per plant showed a significant relationship with root density (Fig. II.4A). For a given density, the amount Ndfr per plant was higher ($P = 0.01$) at DAS 101 (intercept 7.86; slope 3.85; $r^2 = 0.61$; $P < 0.005$) than at DAS 59 or 74 (intercept 8.64, slope 1.38, $r^2 = 0.49$; $P < 0.0005$). Root density was correlated with the total plant-N content and there were no significant differences between sampling dates (intercept 3.15; slope 0.02; $r^2 = 0.57$; $P < 0.0001$; Fig. II.4B).

II.3.5. *Amount of inorganic N in the soil and mineralization of N rhizodeposits*

The amount of inorganic N in the soil was measured after plant harvests (DAS 59, 74, 101) and 35 days after maturity (DAS 136). Soil inorganic N had almost been taken up by the growing plants by DAS 59 and 74 and subsequently increased up until DAS 136 (Data not shown). However, in the SF treatment, the amount of inorganic N measured in the soil at DAS 101 and DAS 135 was lower than that of the C treatment ($P = 0.02$). The amounts of mineralised N in the soil of C and the SF treatments were not significantly different ($P > 0.05$). Between DAS 101 and 135, 30-35% of the total amount of mineralised N came from Ndfr in the C and SPF treatments whereas around 50% came from this source in the SF treatment. At DAS 136, in SF and SPF, the proportion of mineralised Ndfr was lower than that of the C treatment (Data not shown).

II.4. **Discussion**

II.4.1. Total pea dry weight, nitrogen content and yield

Water stress applied either during flowering or during the seed filling period caused a significant reduction in pea DW and N accumulation (Table 1). Specific effects on yield induced by water stress occurring during these periods were observed. The duration of flowering was reduced and several flowers were aborted in the SF treatment as observed by Acosta Gallegos and Kohashi Shibata (1989) and Ney *et al.* (1994). Pod and seed numbers were reduced though only seed number was reduced when the stress occurred during the seed filling period. Water consumption by control pea plants was highest during flowering and therefore pea DW and N accumulation were more strongly inhibited by water stress during

this growth phase. Around 85% of this inhibition reduction was caused by a decrease in above ground plant production (Table 1).

In water limited conditions, biomass accumulation is generally lower due to reduced leaf area (Guilioni, 2003; Lecoeur *et al.* 1995) and a decrease in net photosynthesis caused by stomatal closure (Guilioni *et al.*, 2003; Jones, 1992; Lecoeur and Sinclair, 1996). However, Guilioni *et al.* (2003) have shown that photosynthetic ability can be recovered a short time after water stress, the results presented here showed that accumulation of biomass between DAS 59 and 74 remained very low after re-watering in plants stressed during flowering (3.3 g $plant^{-1}$) compared with C (9.4 g $plant^{-1}$). This lack of ability to reactivate photosynthetic activity has also been observed by Ney *et al.*, 1994. However, the causes are not yet fully understood.

In our experiment nodule dry weight was reduced by about 65% during the water limited period and there was no accumulation of N until maturity. Lecoeur and Sinclair (2001) observed that water deficit treatments, both in the field and greenhouse, accumulated less N compared with well-watered treatments, and that accumulation ceased earlier during seed filling. The N_2 fixation process is a plant process particularly sensitive to water stress and several mechanisms limiting nodulation initiation, nodule development or function have been observed under water limiting conditions (Busse and Bottomley, 1989; Mnasri *et al.*, 2007; Serraj *et al.*, 1999; Zahran and Sprent, 1986). Morever, according to Patterson *et al.* (1979), recovery of nitrogenase activity after rewatering requires 4-5 days. Consequently, reduced biomass accumulation after re-watering may be caused both by internal reduction in the pea plants energy investment in N_2 fixation symbiosis, partly caused by water stress, and by the stage of the pea growth cycle.

II.4.2. Effect of water stress on above and below-ground N partitioning between the end of flowering and maturity

Althoug the N content of pea plants was influenced by the drought treatments, N partitioning within the different plant parts appeared to be unaffected by water stress until DAS 74 (Table 1) with no difference between the slopes and intercepts of the DAS 59 and 74 (Fig. 1A, B, C). The proportion of total plant N was around 80% in the above ground parts and 20 % in the below ground parts (Table 1 and 3).

The main effects of water stress occurred between DAS 74 and 101. During this late growth stage and independently of treatment, total plant N did not increase. More than 90% of the N for seed filling was remobilised from the above ground plant parts (Table 1). In stressed plants, the proportion of N in the seeds re-mobilised from 'pods, leaves and stem' was significantly lower than in C plants (Table 1). The slopes of the correlations between root N and total plant N content at DAS 74 and at DAS 101 were significantly different (Fig. 1A) showing that the amount of N remobilised from roots for seed filling or released into the soil was also lower in stressed plants compared to unstressed plants.

During this period, the proportion of BGN released into the soil increased from around 30% to 60% though simultaneously the proportion of total plant N allocated to BGN decreased from 20% to around 10%. This indicates that while the part of root N is released into the soil, another part is remobilised for seed filling.

II.4.3. Dry weight and nitrogen content of roots and nodules

After stress induction, total root DW and N content in SF and SPF treatments were reduced, mainly due to water stress affecting nodule development. Although nodule biomass and N content were greatly reduced by the water limitation, the amount of Ndfr was lower in stressed plants (Fig. 1A, Table 2) and the proportion of total plant N allocated to the roots and released into the soil remained similar to control plants (Table 3). Between DAS 59 and 74, nodule N content of plants stressed during pod-filling decreased from 41 to 13.5 mg N plant^{-1} but the amount of Ndfr did not increase in parallel (Table 2 and 4).

At DAS 101, the entire root system was collected without sorting roots from nodules and mean ^{15}N enrichment of total TC roots of control plants was similar to ^{15}N enrichment of TC roots at DAS 74. As a consequence, N from nodules of C plants was probably released into the soil due to nodule senescence. Nodule N concentration at DAS 59 was lower than at DAS 74 indicating that N remobilisation occurred between the two sampling dates. A similar phenomenon was also observed by Dubach and Russelle (1994) studying nitrogen transfer from Birdsfoot trefoil : N concentration of dead nodules was lower than in live nodules indicating N remobilisation during senescence.

In stressed plants, mean ^{15}N enrichment of total TC roots was similar at DAS 74 and 101 and remained lower than that of TC roots at DAS 74. Consequently, either N from nodules was re-absorbed by the plant or nodule structure was maintained until later in the growth cycle. These results are consistent with those of Voisin *et al.* (2003b) suggesting that the main

N uptake pathway has priority for carbon within the root system and that nodule senescence is not directly linked to nodule age or carbon availability but rather depends on N source.

II.4.4. Nitrogen derived from rhizodeposition

Ndfr was likely to decrease with water stress depending on the stage when the stress occured. However, the proportion of total plant N allocated to the roots and released into the soil was similar for all treatments at each sampling date. Therefore, the amount of N released into the soil was correlated with total plant N content and water stress did not have any specific effect on Ndfr. The proportion of N released into the soil was maintained at around 30% of total BGN at DAS 59 and 74, but increased to about twice this amount between pod filling and maturity, as reported by Jensen (1996) and Sawatsaky and Soper (1991). At this late stage of the growth cycle, when root growth has stopped, net sink strengths of roots and nodules are lower than during the vegetative stage (Voisin *et al.*, 2003a) due to the high demand for assimilates for seed filling (Jeuffroy and Warembourg, 1991). Hence, the allocation of C assimilates to below-ground parts was probably too low to maintain the entire root system and the rapid Ndfr increase may have resulted from root senescence at this stage (Voisin *et al.*, 2003c).

When comparing different experiments BGN varied slightly from 15% (Mayer, 2003, Jensen, 1996) to 21% (Schmidtke, 2005), and up to 30% of total plant N (Mahieu *et al.*, 2007, Wichern *et al.*, 2007). It seems that the relationship between Ndfr and total plant N content obtained in the present study is valid under given growing conditions, but also that the amount of N released into the soil does not vary as a function of total plant N content alone. In the present study, Ndfr was also correlated with root density. Rhizodeposition appeared to be closely linked to root system establishment and water stress had an indirect effect on N rhizodeposition, mainly by limiting root system development.

II.4.5. Suitability of the ^{15}N labelling methodology and reliability of the results

The use of ^{15}N labelling methods allows increased accuracy for the assessment of the amount of N released into the soil by a legume crop. However, experiments comparing different ^{15}N labelling techniques have shown that some methods may generate physiological and morphological changes that can lead to either an underestimation or an overestimation of

the amount of N released into the soil (Cadisch *et al.*, 2002). When using the split root technique, the Ndfr assessment results are based only on the consideration of the transfer compartment. When comparing both the split-root and the cotton-wick methods, Mahieu *et al.* (2007) concluded that for a given plant N content, Ndfr results were higher when using the split root method, but they couldn't conclude that one method was more accurate than the other. Using the split-root technique Jensen (1996) and Schmidtke (2005) found Ndfr values of around 20 mg N $plant^{-1}$ at maturity which was considerably lower than the values presented here of around 29 mg N $plant^{-1}$ estimated at DAS 59 and 74 increasing to 43 mg N $plant^{-1}$ at maturity (Table 3). However, the 7% of total plant N released into the soil was consistent with the 7 or 10% measured by Jensen (1996) and Schmidtke (2005), respectively, and with the 13% measured by Mayer et al (2003) using a ^{15}N in situ cotton-stem labelling method in controlled conditions.

Regardless the treatment, ^{15}N enrichment of nodules was lower than of roots (Table 3). Morever, root and nodule ^{15}N enrichment varied considerably during the stress period. As a consequence, ^{15}N enrichment of root and nodule derived N released into the soil during this period may also be different. However it is the total ^{15}N enrichment at sampling that is used for Ndfr calculation. Such variation in ^{15}N enrichment within the root system complicates the calculation of root-derived N in the soil (Khan *et al.*, 2002; Russel and Fillery, 1996b). Assessing proportions of N released into the soil deriving from nodules and roots is even more difficult as the rate of senescence of both sources are not known and can vary according to environmental conditions. In our conditions, to assess Ndfr at the end of the stress period, it may be more appropriate to either apply mean ^{15}N enrichment of roots and nodules at the beginning and at the end of the stress period or to apply the ratios of N content in each compartment, nodule or root, to total root N content. Finally, seven methods to calculating the Ndfr were tested (data not shown). For example, using ratios of N content of each compartment to total root N content gave Ndfr values at DAS 59 and 74 around 10 to 17% higher than the Ndfr values in this article, depending on the treatment. This indicates that the order of magnitude of the results need to be interpreted with care. Regardless of the methods used to calculate Ndfr, similar relationships are obtained between total plant N content and rhizodeposited N proving that our interpretation is reliable.

II.5. Conclusion

For all treatment, Ndfr and BGN were correlated with total plant N. Reduction of Ndfr of stressed plants was due to the effect of water stress on total plant N and biomass accumulation. Therefore, water stress did not have any specific effect on N rhizodeposition. Variation in nodule and root ^{15}N enrichment and N concentration during the reproductive period indicated that nodules of stressed pea plants were maintained later in the growth cycle while nodules of unstressed pea plants were more likely to senescence.

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N fertilisation and water stress affected nodules and roots dry weights and N contents. However, in each experiment (effect of N source or water stress), for each variety of pea, BGN was correlated with the total plant-N content as well as with the plant dry weight. In each experiment, there were no difference between the ratio of BGN : plant-N of plants fertilised or unfertilised and between plants exposed to a water stressed and non-stressed plants. The results suggest that N source and water stress have no specific effect on the proportion of total plant N allocated to the below ground parts (Root N + N rhizodeposition). The effect of the N fertilisation was on the BGN partitioning since the proportion of the total BGN released into the soil increased with the N fertilisation.

For a given plant N content, BGN of Ba and Fr were different and we found a good relationship between the proportion of total plant N released into the soil and the nitrogen harvest index.

At each sampling date and whatever the treatment, BGN and Ndfr increased with the total plant-N content. However, between the end of flowering and maturity, for a given plant N content, the Ndfr increased though the BGN decreased. These results suggest that, between the end of flowering and maturity, one part of the root N content was allocated to the Ndfr and another part was mobilized for pod-filling.

SYNTHESIS AND DISCUSSION

I. Suitability of a ^{15}N labelling method for assessing N rhizodeposition

Root exudation (Paynel *et al.*, 2001; Henry *et al.*, 2005; Nguyen, 2003) and root senescence (Crawford *et al.*, 1997; Dubach and Russelle, 1994) are the mechanisms by which most Nitrogen is released from roots into the soil during plant growth. A substantial proportion of the amount of N rhizodeposition measured may also result from root debris not collected by hand (Mayer *et al.*, 2003). Darwent *et al.* (2003) located and quantified root **C** exudation using an original bacterial biosensor method (*Pseudomonas fluorescens* 10586 pUCD607, marked with the lux CDABE genes for bioluminescence) while Henry *et al.* (2005) achieved the same using a ^{14}C labelling method. With both methods, authors observed that exudation was greater from root tip regions than from the whole root and was correlated with the number of root apices. These experiments could only be carried out in axenic and controlled conditions (microcosms, microlysimiter and in axenic soil or sand pot) and values resulted from short term measurements. Quantification of root N exudation has also been possible in a microlysimeter using a ^{15}N labelling method (Paynel *et al.*, 2001). However, in the soil, no method is available for simultaneously quantifying proportions of N released into the soil deriving from root exudation and from root senescence with such accuracy during an entire growth season. In order to assess the N benefits of introducing a grain legume into rotations in field conditions, it is assumed that all the N released from roots over a season can be quantified if the plant is homogeneously labelled with a ^{15}N tracer. Since rhizodeposition results from several mechanisms, the accuracy of the measurement depends on the homogeneity of the plant enrichment with the ^{15}N tracer.

I.1. Labelling conditions and homogeneity of plant enrichment

In the different experiments, estimation of the N rhizodeposition was influenced by a number of labelling parameters: (i) the concentration of the labelling solution (ii) the frequency of labelling (iii) the start of the labelling period.

I.1.1. Concentration of the labelling solution

Due to the heterogeneity of soil ^{15}N enrichment and the difficulty of obtaining homogeneous soil sampling, it has been assumed that the accuracy with which Ndfr can be measured could be improved by increasing root ^{15}N enrichment. Using a continuous split root labelling method, Schmidtke (2005a) have effectively shown that the variation in measured Ndfr linked to the choice of the background value may be decreased by increasing ^{15}N root abundance. However, when testing a range of solution with increasing ^{15}N urea concentration, we observed that, for a given plant-N content, when plants were labelled with a too high concentration, the estimation of the Ndfr was not consistent and was overestimated. To obtain a consistent assessment of Ndfr when plants are labelled by intermittent pulse, the concentration of the labelling solution has to be calculated in order to obtain a root enrichment lower than 1.5%. Similar results were noticed by Khan *et al.* (2002b) when labelling plants by intermittent pulses, the results were all more heterogeneous than when plants were labelled with a concentrated ^{15}N urea solution.

This effect was not visible in non fixing P2 isoline of Frisson pea. Since these plants relied essentially on N absorption from the soil solution for N acquisition, one part of the N released from roots into the soil may have been reabsorbed creating a homogenisation between root and rhizodeposit enrichment. Therefore, the ^{15}N retrieved from the soil was not representative of the ^{15}N abundance of the rhizodeposits at harvest.

I.1.2. Labelling frequency

In the different experiments, the Ndfr and the Ndfr : BGN ratio were higher in plants labelled continuously than in plants labelled fortnightly despite similar root ^{15}N enrichment. A significant linear relationship between root and soil ^{15}N content was obtained for plants treated continuously with a diluted ^{15}N urea solution and we concluded that only results obtained with continuous labelling were more reliable than with intermittent labelling. This was not the conclusion of Russell and Fillery (1996a) who decided that pulse labelling was more suitable than weekly labelling due to higher ^{15}N root enrichment. Conversely, using ^{14}C, Nguyen (2003) observed that a long chase period was likely to increase ^{14}C allocation to below ground biomass in comparison with intermittent labelling. ^{14}C pulse labelling was shown to lead to an heterogeneous labelling of the plant C pool, as only carbon assimilated at the same time as the ^{14}C pulse is labelled (Merharg, 1994; Paterson *et al.*, 1997; Darwent *et*

al., 2003; Nguyen, 2003). This also led to an underestimation of C flow to below ground parts (Nguyen, 2003).

In our experiment, interpretation of the effect of N fertilisation on N rhizodeposition was different according to the labelling frequency applied. Similar observations were reported in Darwent *et al.* (2003) who suggested that contradictory findings regarding the effect of N supply on C rhizodeposition may be partly due to labelling frequency. Nguyen (2003) also obtained a significant relationship between the coefficient of root respiration and root residue when plants were continuously labelled though no such correlation was observed in pulse labelling experiments.

Therefore, the difference in the assessment of Ndfr due to labelling frequency would seem to result from the fact that fortnightly labelling does not enable an uniform enrichment of the root N content. Indeed, it has been suggested that legume roots can differ in their disproportional ^{15}N distribution in spite of identical ^{15}N abundance in their total plant biomass (Schmidtke, 2005a).

I.1.3. Start of the labelling

At the end of the growth cycle, C requirement for seed filling increases and C investment in root maintain once decreases (Voisin *et al.*, 2003). Therefore, we assumed that rhizodeposition increased at this late stage due to root senescence and plants were labelled from the 6 or 7 leaf stage. In the water stress experiment, the proportion of Ndfr increased from 30% at flowering and pod filling to 60% of the total BGN at maturity. However, this was not the case in the other experiments in 2005 and 2006. The proportion of Ndfr to total BGN was similar at pod filling and at maturity. In each one of our experiments the ratio of recovered ^{15}N in the roots (the proportion of recovered ^{15}N in the roots to the proportion of recovered N) was lower than in the above ground parts. This disproportional distribution of the ^{15}N to the N between above and below ground parts has also been noted in the literature (Russell and Fillery, 1996a; Khan *et al.*, 2002b). This difference was probably due to ^{15}N labelling starting too late since it was started at the six leaf stage when investment of C and N to the roots began to decrease (Voisin *et al.*, 2003a)**.** Moreover, McNeil and Fillery (2008) measured a BGN of 51% and 42% of total plant-N during vegetative growth period of lupin in two field experiments. Therefore ^{15}N labelling should start earlier.

A late start to labelling may also explain the heterogeneous root enrichment along the root axis observed by Khan *et al.* (2002b) since they had also started to label plants around the six leaf stage.

I.2. Homogeneity of root enrichment and suitability of the Janzen and Bruinsma (1989) equation for Ndfr estimation

Ndfr estimation using a labelling method relies on ^{15}N enrichment of rhizodeposits being similar to ^{15}N root enrichment. This assumption is probably partly correct since the majority of measured N rhizodeposition comes from root residue not collected by hand (Mayer *et al.* 2003) and from root senescence during the growth cycle (Dubach and Russelle, 1994; Crawford *et al.*, 1997). However a large part of N rhizodeposition may also come from root exudation whose enrichment may differ from root enrichment if the plant is not homogeneously labelled. Moreover, in the water stress experiment, ^{15}N root enrichment varied between roots and nodules and in the whole root system between flowering and maturity. Variations between roots and nodules were also reported by Russell and Fillery (1996a) and between lateral and tap roots by Khan *et al.* (2002b). In the present study, we showed that the uneven enrichment between root and nodules had no effect on the assessment of N rhizodeposition when comparing Frisson and the non nodulated isoline P2 or the different Frisson N treatment. However, in the water stress experiment, ^{15}N root enrichment varied from 0.5% to 0.7% ^{15}N excess in non-stressed plants from flowering to maturity. As a consequence, ^{15}N enrichment of N released from roots into the soil during this period may also have varied, though only total ^{15}N enrichment at sampling was used for calculating N rhizodeposition. Using mean ^{15}N root enrichment between flowering and maturity, the Ndfr values of the non stressed treatment at maturity were higher than those presented in Mahieu *et al.* (2007). This indicates that the order of magnitude of the results needs to be interpreted with care.

Regardless of the methods used to calculate Ndfr, similar relationships were obtained between total plant N content and rhizodeposited N proving that our interpretations are reliable.

I.3. Suitability of the ^{15}N labelling method for measuring Ndfr

N rhizodeposition results from several mechanisms. In addition to a heterogeneous nutrient influx in time and space, the pattern of root exudation varies along the root axis while root exudate composition and quantity changes with plant age (Wichern *et al.*, 2008). The roots seem to be subjected to a continuous renewal of root tissues during vegetative growth and senescence increases at the end of the reproductive period with simultaneous remobilisation of N for seed filling. Therefore a reliable estimate of Ndfr depends on the homogeneous plant enrichment with the ^{15}N tracer. No significant variations were observed in the ^{15}N root enrichment when plants were either fortnightly or continuously labelled between pod-filling and maturity using the cotton-wick method. However, we did not control ^{15}N enrichment of the different plant parts during the vegetative period though this probably varied since the labelling started at the 6 or 7 leaf stage. In the water stress experiment, large variation in ^{15}N root enrichment was observed between flowering and maturity as described in the former paragraph though only ^{15}N root enrichment at harvest was used for calculating Ndfr. These variations in ^{15}N root enrichment during the growth cycle may lead either to an over or an under estimation of Ndfr (McNeil and Fillery, 2008).

Disproportional ^{15}N distribution along the root axis observed by Khan *et al.* (2003) may also result from variation in the rate of N uptake even when the plant is continuously labelled with a constant labelling solution concentration. A rate of ^{15}N uptake similar to that of N may be achieved using the split-root method. Indeed, we can assume that ^{15}N uptake from the labelling solution contained in the labelling compartment is proportional to the rate of N uptake by the whole roots. However, the two root compartments (soil / nutrient solution) are not exposed to the same N concentration and the ^{15}N root enrichment of each was very different (Mahieu *et al.*, 2007). Moreover, we did not obtain a reliable relationship between root ^{15}N content and soil ^{15}N content and the ^{15}N root enrichment varied throughout the reproductive period. In conclusion, labelling should be carried out in such a way as to keep root enrichment similar from the beginning to the end of the labelling period if the reliability of Ndfr assessment is to be improved. This may be controlled by sampling plants at different stages of the growth cycle.

II. Variation in the allocation of N to below ground parts

II.1. Plant N content and N allocation in the below ground parts

II.1.1. Legumes or plants grown in non N limiting conditions

As far as we know, the effect of N fertilisation on N rhizodeposition has not been investigated yet. Only the effect on C rhizodeposition has been studied and results vary according to the plant species or the method used. The difficulty in assessing the C rhizodeposition comes from the rapid mineralization of C compounds released from roots by soil microorganisms.

In the different field and greenhouse experiments presented in this manuscript, N rhizodeposition increased concomitantly with total plant N content. A positive linear relationship was obtained between the two variables for plants grown in non limiting N conditions. Despite alterations in total plant N content and biomass resulting either from water stress or N fertilisation, the ratio of BGN: plant-N was similar for all treatments compared to control plants. Moreover, in the water stress experiment the ratio of Ndfr: BGN remained unchanged between the different treatments. However this proportion tended to increase for plants grown with increasing N fertilisation. Therefore, for a given set of non N limiting growing conditions, the different treatments would be likely to influence the ratio of Ndfr: BGN rather than that of BGN: plant-N. Our results suggest that whatever the N fertilisation treatments under non N limited conditions, the ratio of BGN: plant-N remains unchanged.

This is not in contradiction with Nguyen's (2003) analysis who pooled nine datasets for continuous ^{14}C labelling experiments and 19 for pulse chalse studies. He observed that C allocation to the roots decreased though the percentage of ^{14}C retrieved in rhizosphere respiration and in the soil residues were both significantly increased by N fertilisation (+25% and 82%, respectively). Therefore, N fertilisation also increases C rhizodeposition relatively to C exported by shoots to belowground. However, no study has so far reported that the proportion of total plant assimilated C allocated below ground (root + rhizodeposition) is similar regardless of N fertilisation.

The first measure of the amount of C and N released simultaneously from roots of field grown peas at maturity was recently conducted by Wichern *et al.* (2007). Plants were labelled

fortnightly using the cotton wick method with both ^{13}C and ^{15}N. The Ndfr and the Cdfr of pea collected at maturity were 36.4% and 29.6% of total plant N and C, respectively. However, Cdfr was likely to have been underestimated since the percentage of ^{13}C recovery was very low. This was probably because the CO_2 issued from mineralization of C rhizodeposits by microorganisms was not taken into account and may have accounted for a large part of the C released into the soil. *It suggests the maintenance of a functional equilibrium between C and N partitionning.*

II.1.2. Plants exposed to a N deficiency

In the P2N0 treatment (i.e plants unable to fix N_2), plants were exposed to N deficiency. In this treatment, the proportion of N allocated below ground was twice as high as in other treatments. In situation of N deficiency, plants may favour root development in order to prospect a greater volume of soil to acquire N from the soil solution. Curiously, the proportion of Ndfr to BGN remain unchanged though it can be expected that plants would have re-absorbed the more N from the soil. Therefore, increasing root exudates at the expense of shoot development in situations of N deficiency may support the hypothesis proposed by Paterson (2003) that plants may favour microbe development and nutrient cycling. The rate of nutrient cycling in the soil is mediated by microbes and this process depends on the input of C from plants growing in the soil.

II.1.3. Effect of N source on N rhizodeposition

For a given plant-N content, similar results were obtained for plants able and unable to fix N_2 (P2). Results are consistent with those of Voisin *et al.* (2003b): they observed that flow of C assimilates towards the roots is not affected by nitrate concentration in the soil solution. Whatever the level of N availability, root growth depends essentially on the availability of C assimilates. Moreover, for a given plant N status, the allocation of C assimilates to roots is similar whatever the N source (Voisin *et al.*, 2003b).

In non limited N conditions, N uptake and allocation to the different plant parts are regulated by the availability of C assimilates and do not depend on the N source (Ourry et al., 1996; Lejay et al., 1999; Malagoli et al., 2004; Voisin et al., 2003). It can therefore be hypothesized that when plant N status is sufficient to guarantee optimal yield, plant N partitioning between

above and below ground is not affected by environmental conditions and N rhizodeposition increases linearly with plant N content. Conversely, in limited N conditions (for plants unable to fix N_2), it can be suggested that allocation of photoassimilates to the different plants parts is regulated by N availability. This hypothesis may also explain that both negative (Merckx et al., 1987; Liljeroth et al., 1990; Paterson and Sim, 2000) and positive (Johansson, 1992) effects of N supply on C rhizodeposition have been reported in the literature.

II.2. Effect of root morphology on BGN partitioning within root and rhizodeposition

In greenhouse conditions, Ndfr amounted less than 35 % of total BGN though under field conditions Ndfr varied around 90% of the total BGN. It has been shown that C exudation is closely linked to root length (Darwent *et al.*, 2003), root surface and to the number of apices (Henry *et al.*, 2005). Such differences in root morphology may explain differences observed in the ratio Ndfr: BGN between field and greenhouse conditions. Indeed, in the field, roots explore a larger volume of soil using a similar root dry weight (0.5 -0.8 g). Therefore in the field, a large part of N rhizodeposition may result from thin roots not collected by hand. A difference in root morphology may also explain the variation in the ratio of Ndfr: BGN observed when increasing N fertilisation in the 2006 greenhouse though the ratio of the BGN: Plant-N was not affected.

II.3. Variations in plant N partitioning between above and below ground parts observed between the different growth conditions

The ratios of BGN: plant-N were quite consistent for a given growing conditions but it differed considerably between the different experiments. Under controlled conditions, BGN approximated i) 26% of total plant N content with both labelling methods (split-root and cotton-wick) in 2005, ii) 16% regardless of N fertilisation level or labelling frequency using the cotton-wick method in 2006, iii) 25% whatever the concentration of ^{15}N urea labelling solution (0.2%, 1.4% and 0.6%) in 2007 and iv) 14% in controlled and stressed plants in the water stress experiment. In both field experiments, BGN was around 30% of total plant-N whatever the method used, the pea variety and the N fertilisation level. Such variations in the proportion of BGN are also observed in the literature varying from 14% of total plant-N in

controlled conditions (Mayer *et al.*, 2003; Jensen, 1996) to 30% in the field (Wichern *et al.*, 2007). Although a good relationship was obtained between Ndfr and total plant N content in each of the 2006 experiments, for a given plant N content, Ndfr was much higher in the field than in the greenhouse. As a consequence, it may be suggested that the amount of N released into the soil does not vary as a function of total plant N content alone. Moreover, this difference seems due neither to water availability nor to N fertilisation level since these factors did not affect the ratio of BGN: plant-N.

In each experiment, a highly correlated relationship between the proportion of Ndfr: plant-N (%) and the proportion of total plant-N allocated to the seeds (NHI) was obtained. The part of N released into the soil decreased with increasing NHI. However, this relationship was specific to each experiment and we cannot explain the difference observed between the different experiments regarding plant N partitioning, even though the same variety was used.

Though experiments conducted under controlled conditions were carried out in a similar volume of soil, BGN varied greatly within the range 14% - 25% of the total plant N. Therefore the differences were not due to variation in the volume of soil explored by the roots or to differences in soil texture since this was very similar in the different experiments.

Since Ndfr is correlated with total plant-N content and N uptake is regulated by the availability of C assimilates in legumes, C seems to be a good candidate for explaining the difference in plant N partitioning between the different experiments. The difference in plant N partitioning between the different experiments might result from the effects of environmental factors on photosynthesis. Nitrate uptake is down- or up- regulated by the nycthemeral and life growth cycle as well as by environmental factors (temperature, radiation) and endogenous variables (day/ night cycle, ontogenetic stage) in rape (Malagoli *et al.*, 2004) and soybean (Dehlon *et al.*, 1996). The authors proposed that regulation may be partly mediated by whole plant C availability and subsequent C allocated to roots.

For example, the 2006 greenhouse experiment started in February during short days (<12h) and low light intensity, while the 2007 greenhouse experiment started in May during long days (<15h) with good light intensity. Therefore, the difference in plant N partitioning between experiments may result from time the plants were exposed to light before maturity

and particularly during vegetative growth. Indeed, allocation of C to below ground parts of pea is higher during vegetative growth (Voisin *et al.*, 2003a) and the ratio of BGN : plant N was higher in 2007 than in 2006.

II.4. Regulation of the N rhizodeposition

Henry *et al.* (2005) showed that root surface area, soluble C concentration in root tissues and number of root apices are important variables mediating effects of N on the release of recently fixed C by roots. Here, a wide range of root morphologies was obtained either by affecting nodule biomass and root dry weight with increasing N fertilisation levels or by generating water stress conditions. In the water stress experiment total plant-N was also correlated with root density and both were correlated with Ndfr. Therefore water stress might have an indirect effect on N rhizodeposition, mainly by limiting root system development. However the proportion of plant N allocated below ground remained unchanged compared to control plants and the ratio of Ndfr: BGN was not altered despite of a reduction of 65% in the nodule biomass of water stressed plants. In annual legumes such as pea or faba bean, nodules are indeterminate and contribution of root and nodule senescence was expected to be higher at the end of the growth cycle. However in stressed plants some nodules were maintained later in the growth cycle probably to meet N requirements during seed filling while all nodules senescenced in control plants. As in the 2006 greenhouse and field experiments, total below-ground N was correlated with total plant-N content and the proportion of BGN to total plant-N was similar among treatments at each sampling date. Therefore, N rhizodeposition appears to be regulated by the availability in photoassimilates.

CONCLUSION AND OUTLOOK

The first objective of the researches developed in this thesis was to compare and evaluate the reliability of ^{15}N labelling methods for measuring N rhizodeposition and to develop an appropriate protocol for BGN assessment of pea in field conditions using an in situ ^{15}N labelling method. The cotton wick method appeared as the most suitable method to measure N rhizodeposition in field conditions. Intermittent labelling probably leads to a disproportional ^{15}N distribution at the origin of an underestimation of the Ndfr though consistent results were obtained when plants were labelled continuously with a diluted labelling solution. However Ndfr assessment may still be improved by i) beginning the labelling earlier in the vegetative development and ii) maintaining a similar root enrichment from the beginning to the end of the labelling period since variation in the ^{15}N roots enrichment along the growth cycle may largely influence the assessment of the Ndfr.

A second objective was to study the effects of genetic characteristics through variation in the Nitrogen Harvest Index (NHI) as well as the effects of N sources and water availability. In the different experiments, the Ndfr and the total BGN were correlated with the total plant N content and was not influenced by the N source. The different environmental factor tested (N fertilisation level and water stress) did not affect the ratio BGN: plant-N when plants were grown in non N limiting conditions regardless of a decrease or an increase in the total plant N content. Otherwise, the ratio BGN: plant-N increased when plants were grown in limiting N conditions. It is assumed that N uptake and allocation to the different plant parts are regulated by the availability of C assimilates in non limited N conditions and conversely, allocation of photoassimilates to the different plants parts is regulated by N availability in limited N conditions. The results are consistent with the effect of N fertilisation on C rhizodeposition reported in the literature; it suggests the maintenance of a functional equilibrium between C and N partitioning.

Although the varieties chosen for our field experiments are known to have contrasted architectures, plant N content and partitioning of the different varieties of peas were similar and a similar Ndfr was measured. Therefore we cannot conclude whether Ndfr varied according to the genetic characteristics.

In field conditions the labelling was realised by intermittent pulse and would have likely be underestimated. Nevertheless, the project has shown that contribution of pea to the soil N pool was substantially higher than it was previously assumed in the litterature. A BGN varying

from 30 to 37 % of the total plant N was measured for the different varieties of pea. This should be taken into account to estimate N benefit from biological N_2 fixation by a grain legume crop and for the prediction of the N economies for the succeeding crops in legume-based cropping systems.

A good relationship was obtained between NHI showing that the Ndfr probably varied according to the N remobilised for seed fill. However, no relationship between BGN and N partitioning or biomass partitioning explaining the difference between field and greenhouse was found. Our hypothesis was that the Ndfr vary according to the pattern of plant N accumulation during seed fill. Nevertheless, the proportion of N remaining in the Veg (pods, leaves and stems) was lower in the field than in greenhouse (data not shown) though the proportion of Ndfr: plant-N was quite higher. Therefore, the difference between the both growing conditions may likely results from a difference in N allocation to the Veg. The both variables were well correlated but the correlation cannot be confirmed after transformation since data from field experiments were obtained by an intermittent labelling. This may be confirmed by measuring Ndfr of field pea grown on different level of N fetilisation after a continuous labelling.

In the field a BGN reaching 30 to 37 % of the total plant N is either not taken into account in the establishment of the N balance or accounted for less than 20% of the total plant N. Calculating the N budget on the basis of pea crop yield measured in France, the mean N balance after a pea crop would vary from + 22 kg N ha^{-1} in rich N soil to + 87 kg N ha^{-1} in low input system when taken a BGN of 30% into account. Khan *et al.* (2003) showed that N balance was increased of 93 kg N ha^{-1} after a Chickpea crop when including the BGN in the calculation compared to the estimation when only above ground N was taken into account (Khan *et al.*, 2003). As a consequence, omit to take into account the BGN in the calculation of the N balance may substantially alter the estimation of N benefit from a legume crop. Moreover, this fraction represents an important pool of N which contributes to soil N fertility and should be taken into account for the prediction of the N economies for the succeeding crops in legume-based cropping systems (Russell and Fillery, 1996b). However, it is now necessary to better assess the amount of N available for plant growth and the soil N turnover (Mayer *et al.*, 2003; Jensen, 1996a)

Mayer *et al.* (2003) and Jensen (1996c) observed that after a pea crop, around 30% of the Ndfr was mineralisable after three months. Similar proportion of mineralised N after a faba bean crop was measured by the end of flowering of the succeeding cereal crop by McNeill and Fillery (2008). During the *winter* following legume crop around 10% was loss as leaching and around 10% was retreieved in the above ground shoot of the following cereal crop. McNeill and Fillery (2008), observed that after a wheat season following a legume crop, a substantial proportion of legume derived BGN (32-55%) was still present as residual insoluble organic N and considered to be an important contribution to structural and nutritional long-term sustainability of these soil. This means that legumes represent a powerfull tool for the remediation and revegetalisation of damage lands; a number of legumes have been used in the remediation of degraded land areas.

Our results also suggest that N source and water stress have no specific effect on the proportion of total plant N allocated to the below ground parts (Root N + N rhizodeposition). The N fertilisation only affected BGN partitioning: the proportion of the total BGN released into the soil increased with the N fertilisation. Moreover the plant is as efficient as non legume to capture N from the soil solution. As a consequence, improving the N benefit from grain legume farming may only be achieve by increasing N_2 fixation either by selecting varieties or choosing better adapapted species to water stress conditions.

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Title: Assessment of the below ground contribution of field grown pea (*Pisum sativum* L.) to the soil N pool

Abstract: Enhancing the use of legumes as a means of N acquisition in agroecosystems depends on improving the accuracy with which the amount of N fixed can be assessed. The assessment of nitrogen contribution of legumes to the soil N pool remains unclear, mainly because of difficulties in assessing below ground N (BGN), especially N rhizodeposition. For given species, effects of growth conditions (such as water or N availability), sources of N nutrition (nitrogen fixation or inorganic N uptake) or genotype on this process have not been investigated.

The aim of my Ph.D. was to adapt and refine a ^{15}N labelling method to measure the amount of N derived from rhizodeposition by a pea with a fine degree of accuracy and to design a protocol for field studies. Two methods (split-root and cotton-wick) and a variety of labelling conditions were tested on different pea varieties and isolines, in the field and in the greenhouse. In addition, plants were grown on either unfertilised or fertilised soil with different levels of mineral N in order to highlight relationships between BGN, N sources, and the mobilization of root N for pod filling. Subsequently, the influence of water stress on below-ground N partitioning was investigated during the reproductive growth of pea.

Results obtained in the field were higher than those measured in the greenhouse. The proportion of N released into the soil was lower than 30% in the greenhouse though it represented around 90% of total BGN in the field. The labelling frequency and concentration of the labelling solution were shown to influence the assessment of N rhizodeposition. However, results obtained with both methods were reliable when plants were labelled continuously with a diluted labelling solution. The ^{15}N stem feeding technique was easier to adapt and monitor in the field than the split-root method and was chosen for further field studies. Several soil collection and sampling conditions tested to attein a fine degree of accuracy in measuring the amount of N derived from rhizodeposition.

N fertilisation and water stress affected nodule and root dry weights and N contents. However, in each experiment (effect of N source or water stress), for each variety of pea, BGN was correlated with the total plant-N content as well as with the plant dry weight. In each experiment, there were no differences between the ratio of BGN: plant-N of fertilised or unfertilised plants and between plants exposed to a water stress and non-stressed plants. The results suggest that N source and water stress have no specific effect on the proportion of total plant N allocated to the below ground parts (Root N + N rhizodeposition). However, N fertilisation influenced BGN partitioning since the proportion of total BGN released into the soil increased with N fertilisation.

In the field, rhizodeposition represented a substantial part of the total plant N. This should be taken into account when estimating N benefits from biological N_2 fixation by a grain legume crop and for the prediction of N economies for succeeding crops in legume-based cropping systems.