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Symbiotic N$_2$ fixation activity in relation to C economy of *Pisum sativum* L. as a function of plant phenology

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Abstract

The relationships between symbiotic nitrogen fixation (SNF) activity and C fluxes were investigated in pea plants (*Pisum sativum* L. cv. Baccara) using simultaneous ^{13}C and ^{15}N labelling. Analysis of the dynamics of labelled CO$_2$ efflux from the nodulated roots allowed the different components associated with SNF activity to be calculated, together with root and nodule synthetic and maintenance processes. The carbon costs for the synthesis of roots and nodules were similar and decreased with time. Carbon lost by turnover, associated with maintenance processes, decreased with time for nodules while it increased in the roots. Nodule turnover remained higher than root turnover until flowering. The effect of the N source on SNF was investigated using plants supplied with nitrate or plants only fixing N$_2$. SNF per unit nodule biomass (nodule specific activity) was linearly related to the amount of carbon allocated to the nodulated roots regardless of the N source, with regression slopes decreasing across the growth cycle. These regression slopes permitted potential values of SNF specific activity to be defined. SNF activity decreased as the plants aged, presumably because of the combined effects of both increasing C costs of SNF (from 4.0 to 6.7 g C g^{-1} N) and the limitation of C supply to the nodules. SNF activity competed for C against synthesis and maintenance processes within the nodulated roots. Synthesis was the main limiting factor of SNF, but its importance decreased as the plant aged. At seed-filling, SNF was probably more limited by nodule age than by C supply to the nodulated roots.

Key words: C partitioning, ^{15}N and ^{13}C labelling, *Pisum sativum* L., respiration, symbiotic nitrogen fixation.

Introduction

Despite numerous studies, the relationships between symbiotic nitrogen fixation (SNF) and carbon supply to the nodulated roots still remain unclear. It has long been shown that SNF relies on photosynthate supply (Hardy and Havelka, 1976; Sprent *et al.*, 1988) and labelling experiments showed that current photosynthates were rapidly (within 1 h) transferred to the nodules (Warembourg, 1983; Kouchi and Nakaji, 1985; Gordon *et al.*, 1985; Thorpe *et al.*, 1998). Consequently, SNF should vary together with photosynthesis. Experimental conditions that enhance photosynthesis, for example, the increase of CO$_2$ concentration in the atmosphere (Hardy and Havelka, 1976; Finn and Brun, 1982; Murphy, 1986; Soussana and Hartwig, 1996; Schortemeyer *et al.*, 1999) and the increase in light intensity (Lawn and Brun, 1974; Hardy and Havelka, 1976; Bethlenfalvay and Phillips, 1977*a*) are usually associated with an increase in SNF. Similarly, treatments that limit photosynthesis, for example, the partial defoliation (Fujita *et al.*, 1988), shading (Tricot, 1993) and limited light intensity (Feigenbaum and Mengel, 1979) decrease SNF. Although photosynthesis usually affects SNF through the modulation of nodule number or growth, Hardy and Havelka (1976) reported a large increase of SNF specific activity (N fixed per unit of biomass) by either carbon dioxide enrichment of the leaf canopy of soybean plants or an increase in light intensity. Furthermore, it was suggested that SNF could be more precisely related to C availability within the plant. Variations of SNF across the growth cycle were sometimes related to growing conditions (Jensen, 1987; Sparrow *et al.*, 1995) and the steep decrease of SNF at the end of the growth cycle was often considered to be due to C competition with the shoot (Lawrie and Wheeler, 1974; Bethlenfalvay and Phillips, 1977*b*) due to the high demand for seed filling (Jeuffroy and Warembourg, 1991).

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Similarly, regulation of nodule number also occurs, in part, through systemic control involving photosynthate availability (Atkins *et al.*, 1989), even if there is evidence for intrinsic autoregulation of nodule number (Caetano-Anollès and Gresshoff, 1990, 1991) through shoot signal (Francisco and Harper, 1995; Harper *et al.*, 1997). Moreover, nodule development on a root segment is linked to the growth rate of this segment (Tricot *et al.*, 1997).

However, other results showed adverse relationships between SNF and C. The decline of SNF resulting from a water stress was related to increased total carbohydrate (Fujita *et al.*, 1988) or increased soluble sugar concentration (Minchin *et al.*, 1986). Vance and Heichel (1991) concluded that SNF should not be directly limited by C supply, but rather would be O_2 limited (Hunt and Layzell, 1993). Carbon limitation of SNF should arise from impaired C utilization in the nodules (Vance *et al.*, 1998), because key enzymes of nodule C metabolism, such as sucrose synthase, are limited (Gordon *et al.*, 1993, 1997; Gonzales *et al.*, 1995).

SNF not only depends upon C fluxes but also upon environmental factors such as nitrate availability (Sprent *et al.*, 1988). Nitrate inhibition affects SNF by impairing nodule growth rather than through a limitation of SNF activity (Streeter, 1988). Nitrate inhibition of nodule growth depends upon both the amount of nitrate supplied, the period when it is provided to the plant and the legume species (Davidson and Robson, 1986). It has been recently demonstrated that nitrate inhibition of nodule growth results from the decrease of the amount of photoassimilates supplying nodules, which leads to the cessation of cell expansion within nodules (Fujikake *et al.*, 2003).

It is now well established that nitrate inhibition of SNF occurs through O_2 limitation due to variations in the O_2 diffusion barrier (Minchin, 1997), but the mechanisms involved remain controversial: control by the N demand through phloem composition (Hartwig *et al.*, 1994; Neo and Layzell, 1997; Parsons *et al.*, 1993; Serraj *et al.*, 1999) or regulation through carbohydrate availability (Vance *et al.*, 1998; Faurie and Soussana, 1993) have been suggested. Besides, SNF has higher C costs than nitrate absorption (Minchin and Pate, 1973; Ryle *et al.*, 1979; Silsbury, 1977). However, experimental measurements of the relative costs are highly variable because of methodological difficulties, and they are difficult to distinguish from those associated with maintenance and synthetic processes within nodulated roots (Phillips, 1980). Cytosolic PEP carboxylase (PEPC) in nodules catalyses the conversion of PEP and HCO_3^- (in equilibrium with CO_2) into oxaloacetate, which is used for the bacteroid respiration during nitrogen fixation (King *et al.*, 1986; Rosendahl *et al.*, 1990). Carbon dixoide fixation by PEPC produces significant savings of respired CO_2, but this complicates the study of carbon cost associated with N_2 fixation (Warembourg and Roumet, 1989). For soybean, Coker and Schubert (1981) estimated CO_2 fixation by PEPC to be in the range of 1–3.4 mol mol^{-1} N_2 fixed and this was confirmed by the estimate of 1.5 mg C mg^{-1} N_2 fixed found by Warembourg and Roumet (1989).

The priorities for assimilates between root, nodules and shoots, and C allocated to nodulated roots according to phenology were previously examined in relation with nitrate availability (Voisin *et al.*, 2003). Whatever the nitrate availability, rhizosphere respiration was demonstrated to account for more than 60% of the amount of C allocated to nodulated roots across the growth cycle (Voisin *et al.*, 2003). Nodulated root respiration includes not only C loss for growth and maintenance processes of roots and nodules, but also C devoted to SNF activity and nitrate uptake activity, as nitrate reduction is known to occur mainly in the roots in temperate legumes (Wallace, 1986).

The first objective of this study was to analyse the dynamics of labelled respiratory CO_2 efflux after simultaneous ^{15}N and ^{13}C exposure, in order to separate the different components of rhizosphere respiration (Warembourg, 1983; Warembourg and Roumet, 1989). In this study of the respiration costs, no nitrate was provided to the plants during the labelling experiments. Thus, labelled C respired by nodulated roots only originated from SNF activity and associated growth and maintenance processes of roots and nodules. Since microbial degradation of root exudates might also have contributed to measured respiration, the term 'rhizosphere respiration' will be employed instead of 'nodulated root respiration'.

The second objective was to establish how variations of SNF activity of *Pisum sativum* L. (cv. Baccara) during growth and according to nitrate supply, are related to C flux towards or within nodulated roots. For this, both the amount of C allocated to the nodulated roots and the C used within the nodulated root were considered in relation to the N source and to the extent of SNF activity. This paper was meant to provide tools for modelling *Pisum sativum* growth under various N nutrition regimes.

Materials and methods

Plant material and growing conditions

Four sets of plants were grown in a naturally lit greenhouse (Dijon, France, 47°20′ lat., temperate climate) with delayed sowing dates as a means of varying growing conditions because of natural variations in temperature, radiation and photoperiod. Eight seeds of pea (*Pisum sativum* L. cv. Baccara) were sown in 5.0 l PVC pots and inoculated with *Rhizobium leguminosarum* bv. vicieae on four occasions: 15 September 1999, 2 March, 16 March, 26 May 2000. The mean temperature was 20 °C for the sowing date of 26 May 2000, while it was close to 15 °C for the other sowing dates. Photoperiod length was 12.5 h for the 1999 sowing date and 11.0, 11.9 and 12.3 h for the sowing dates of 2 March, 16 March, 26 May 2000, respectively. The growing medium was a 1/1 (v/v) mixture of sterilized attapulgite and

clay balls (diameter 2–6 mm). This medium has no buffering effect, allows gases to diffuse easily, and is inert to gas so that it avoids interference with CO_2 and O_2 measurements. At emergence, plants were thinned to four per pot. The genotype used has facultative vernalization requirements. The temperature in the greenhouse was maintained over 5 °C during winter and the roof opened automatically when the temperature exceeded 20 °C in order to avoid extreme heat. Temperature never exceeded 30 °C during the day. Supplemental artificial light was provided in autumn 1999 (up to total day length of 16 h d^{-1}) to induce flowering. An automatic watering system ensured that plants were regularly supplied with the nutrient solutions according to plant transpiration needs determined by the automatic weighing of one pot per treatment which triggered watering of all pots.

Experimental treatments

Two treatments were applied in the greenhouse: half of the plants (90 pots) were provided with N-free nutrient solution (N_0 treatment) and the other half (90 pots) with 5 mol m^{-3} KNO_3 in the nutrient solution (N_5 treatment). Nutrient solutions were adjusted to give a neutral pH, containing 0.8 mol m^{-3} K_2HPO_4, 1.0 mol m^{-3} $MgSO_4$, 0.2 mol m^{-3} NaCl, 0.05 mol m^{-3} iron versenate, and oligo-elements. In addition, the nutrient solutions for the N_0 and N_5 treatments, respectively, contained 0 and 1.25 mol m^{-3} KNO_3, 0 and 1.875 mol m^{-3} $Ca(NO_3)_2$, 0.7 and 0.075 mol m^{-3} K_2SO_4, 2.5 and 0.625 mol m^{-3} $CaCl_2$. The level of nitrate supply (i.e. 5 mol m^{-3} KNO_3) was chosen to limit, but not suppress, nodulation and symbiotic nitrogen fixation. It was determined in a preliminary experiment that this concentration of nitrate supply limits symbiotic N_2 fixation by approximately 50% and drastically reduces nodule biomass without impairing it totally: plants supplied with nitrate produced 60–70% less nodule biomass compared with those only fixing N_2.

Seed development is characterized by three developmental stages: fertilization, the final stage in seed abortion which corresponds to the beginning of reserve compounds storage, and physiological maturity (Ney *et al*., 1993). The three developmental stages progress linearly along the stem versus cumulative degree-days (Ney and Turc, 1993). The temperature sum since emergence for each phenological stage is indicated in Table 1. For each plant set, three pots of each treatment were selected for ¹³C-¹⁵N labelling at these three characteristic stages (Table 1): 'vegetative' (ten nodes on main stem), 'flowering' (flowers on the 6th node of the main stem, before the beginning of seed filling) and 'seed filling' (between the final stage of seed abortion and the start of physiological maturity).

In 2000, in order to obtain a range of photosynthetic rates for a given phenological stage, three different concentrations of CO_2 in air (150, 360 and 750 µl l^{-1}) were chosen during ¹³C labelling for the different plant sets (three sowing dates). The changes in the photosynthetic rate with increasing concentrations of CO_2 in air

were verified in a preliminary experiment with similar plants (data not shown).

¹³CO₂ and ¹⁵N₂ labelling

Pots of each treatment selected in the greenhouse for ¹³C-¹⁵N labelling were watered with a large volume of nitrate-free nutrient solution and allowed to drain normally. The amount of nitrate in the last volumes of nutrient solution leaving the pots was then measured and found to be negligible. This ensured that all nitrate has been removed from the pots prior to their installation in the labelling chamber. In this study, no nitrate was provided to the plants during the labelling experiments, thus precluding any root assimilation of nitrate.

Equipment used for the labelling experiments was similar to that used by Warembourg *et al*. (1982) and described by Voisin *et al*. (2000) and so is only briefly described here. Roots and shoots were maintained in separate compartments: the pots of plants grown in the greenhouse were inserted in individual air-tight PVC containers to separate root and shoot atmospheres. Air tightness was ensured using physiological moulding material (Qubitac, Qubit systems inc., Kingston, Canada) and silicone rubber (RTV, Zundel Kohler, France). The soil atmosphere was circulated with CO_2-free air entering the pots at a rate of 6.0 l h^{-1}. The CO_2 produced by rhizosphere respiration was trapped by bubbling air exhausted from the pots through NaOH solution. The traps were calibrated for daily measurements (50 ml NaOH 2 N, two pots per treatment). Two additional root circuits were used in 1999 (one for each treatment) to obtain a dynamic measurement of CO_2 respiration: the efflux of each pot was directed to an automatic electro-valve sampler composed of 16 CO_2 traps (30 ml NaOH 0.5 N) sequentially used for 1 h. Oxygen concentration of the root atmosphere was maintained at 20±1% during labelling to compensate for O_2 consumption due to respiration. Expansion bags regulated pressure variations due to gas variations resulting from samplings or injections.

The pots (three pots per treatment) with separate root atmospheres were placed in a transparent air-tight labelling chamber (0.95×0.95×1.5 m) made of Plexiglas. At each chosen air CO_2 concentration (150, 360 and 750 µl l^{-1}), the shoots were exposed to a ¹³C-enriched atmosphere for 10 h: the air CO_2 concentration was continuously measured with an infrared gas analyser (IRGA Ciras, PP system, Montigny le Bretonneux, France) and maintained by automatic CO_2 injection as needed. To obtain a constant and uniformly labelled atmosphere, the whole CO_2 content of the atmosphere within the chamber was rapidly trapped at the beginning of the experiment. The required CO_2 concentration (150, 360 or 750 µl l^{-1}) was then achieved and maintained using injection of a mixture of CO_2 with a constant ¹³C/¹²C (10 atom% ¹³C).

Plants were regularly irrigated with a nitrate-free nutrient solution during the labelling experiment. Nodulated roots were simultaneously exposed to a ¹⁵N₂-enriched atmosphere for 24 h (day of

Table 1. *Temperature sum for the different stages at the different sowing dates and temperature sum when plants were inserted in the labelling chamber*

Sowing date	Phenological stage			Labelling		
	Flowering (Cumulative degree-days since emergence)	Final stage of seed abortion	Physiological maturity	Vegetative	Flowering	Seed-filling
15 September 1999	937–1430	1481–1683	1792–1889	558	1332	1629
2 March 2000	761–1005	1023–1242	1413–1563	557	960	1371
16 March 2000	762–1029	1045–1321	1427–1706	641	923	1333
26 May 2000	715–1172	1036–1304	1357–1595	613	915	1356

exposure to ^{13}C and the following night). The ^{15}N$_2$-enriched atmosphere around the roots was obtained by injecting ^{15}N$_2$, the amount depending on the phenological stage: 2 atom%^{15}N at the vegetative and flowering stages and 3–4 atom%^{15}N at seed-filling. These ^{15}N enrichments of the root atmosphere were calculated prior to the experiment using the equation for isotopic dilution and previously acquired data, concerning the evolution of plant biomass and of its N content as a function of phenology (Warembourg, 1993). To be on the safe side, the detection limit of the mass spectrometer was multiplied by ten (Warembourg, 1993). As the quantity of N$_2$ fixed by the plant was small compared with the total amount of N in the atmosphere, the amount of N$_2$ in the enclosed atmosphere was assumed constant during the experiment.

To study the fate of photosynthates, labelling was followed by a chase period of 4 d in 1999 and 2 d in 2000 (Montange *et al.*, 1981; Kouchi *et al.*, 1986), with the plants exposed to a concentration of CO$_2$ in air of 360 μl l^{-1}. A chase period of 4 d was used in 1999 in order to study the components of rhizosphere respiration (see below). Moreover, such a duration of the chase period allowed the verification that, for peas, isotope partitioning among plant parts was similar after 4 d to that observed after 2 d of chase (data not shown). Within the chamber, temperature and humidity of the air around the shoot were maintained using an air conditioning unit (Voilot, Dijon, France). Day and night temperature and humidity in the chamber during each labelling experiment were chosen to match the mean temperature and humidity observed the previous day in the greenhouse. Light came from four 400 W sodium lamps placed on each side of the enclosure and two 1000 W mercury lamps situated above the enclosure. The resultant photosynthetic active radiation varied from 600 to 900 μmol m^{-2} s^{-1} from the bottom to the top of the canopy. Photosynthetic active radiation in the chamber was similar to that encountered during the same period by plants kept in the greenhouse. Soil water was adjusted daily gravimetrically. The whole system was monitored by a computer using Dasylab software (Newport Omega, USA).

Harvesting and measurements

Shoot and root atmospheres were sampled every 2 h during the labelling period for mass spectrometry measurements (Fison Isochron, Micromass, Villeurbanne, France) of their ^{13}C and ^{15}N enrichment, respectively. Rhizosphere respiration was measured by titration of the NaOH solution used to trap the evolved CO$_2$. The ^{13}C enrichment of the respired CO$_2$ was measured using a mass spectrometer on a gaseous sample produced by acidification of the trap solutions.

All the plants from each pot were harvested together. Samples were taken at the beginning of the labelling experiment (control plants) and at the end of the chase period (labelled plants). They were separated into shoots, roots and nodules. Dry matter was determined after oven-drying at 80 °C for 48 h and the C and N concentrations determined by the Dumas procedure on ground samples. Their ^{13}C and ^{15}N enrichments (atom%) were measured using a dual inlet mass spectrometer. An example of the isotopic data is given in Table 2.

Calculations for ^{13}C and ^{15}N

Isotopic determinations were done according to the isotope dilution principle. The percentage of carbon (%^{13}C) that was derived from the labelled source can be calculated:

$$\%^{13}C = 100 \times \frac{(\text{atom\% }^{13}\text{C sample} - \text{atom\% }^{13}\text{C control})}{(\text{atom\%}^{13}\text{C labelled atmosphere} - \text{atom\%}^{13}\text{C control})}$$

where atom%^{13}C (of either labelled plants or non-labelled control plants) is the ^{13}C abundance of plant material (shoot, roots, nodules) and respired C (Deléens *et al.*, 1994). The %^{15}N was calculated similarly. The amount of C and N newly acquired could be calculated using dry matter and C and N concentration measurements.

Carbon costs associated with nodulated roots of strictly N$_2$-fixing plants

The total amount of C initially incorporated in root and nodule biomass during the labelling period (*QCi*), before any loss by exudation and turnover, corresponded to the amount of C remaining in the biomass at harvest, to which was added a calculated maintenance respiratory component *Rm*. The difference between total rhizosphere respiration and *Rm* was calculated and associated with SNF and root plus nodule synthesis activities (*Rfix+Rs*). When expressed as a percentage of *QCi*, the difference between activity components of respiration (*Rfix+Rs*) of both N treatments was supposed to arise only from a difference in SNF activity (*Rfix*). The corresponding amount of respired C was related to the differential amount of fixed N between N treatments and the ratio between these values represented the C costs associated with SNF activity (mg C respired mg^{-1} N fixed) (Fernandez and Warembourg, 1987). *Rfix* could then be calculated using the amount of N$_2$ fixed (*Rfix*=C costs of SNF × mg N fixed). *Rs* was the remaining component of the total 'activity' component (*Rs+Rfix*) of the nodulated root respiration.

The maintenance and synthesis components of roots (*Mr* and *Sr*) and nodules (*Mn* and *Sn*) were then calculated using the following equations:

Maintenance components: $Mr \times Wr + Mn \times Wn = Rm$

where *Wr* and *Wn* are the amounts of labelled C remaining in root and nodule biomass at harvest, respectively.

Synthesis components: $Sr \times QCi,r + Sn \times QCi,n = Rs$

where *QCi,r* and *QCi,n* are the amounts of labelled C initially incorporated in root and nodule biomass, respectively (*QCi,x=Wx* $(1+Mx)$).

With two unknowns each, these equations were solved using the two sets of data arising from the two N treatments and allowed the carbon costs of SNF, together with those associated with synthesis and maintenance of nodulated roots, to be calculated.

Statistics

Analysis of variance was performed with the GLM procedure of SAS and means were compared using the least significant difference test at 0.05 probability (SAS Institute, 1987).

Results

Components of rhizosphere respiration for plants only fixing N$_2$

Rhizosphere respiration that originated from carbon acquired during the labelled photoperiod (12 h) was calculated using labelled CO$_2$ efflux. Respiration was plotted against time, from the beginning of the labelling period to the end of the chase period (120 h) (Fig. 1). In order to compare N treatments which differed in biomass partitioning (Voisin, 2002), values were expressed as% of shoot carbon assimilation (Fig. 1) that was calculated from isotopic data, as C increment in biomass plus nodulated root respiration. Two distinct phases could be distin-

Table 2. *Data of isotopic composition of ^{13}C and ^{15}N ($\delta^{13}C$ and $\delta^{15}N$) given as an example for plants only fixing N$_2$ (N$_0$ treatment) of the group of plants sown on the 26 May 2000*

These data are average values for three pots before (control plants) and after (labelled plants) exposure to simultaneous $^{13}CO_2$ and $^{15}N_2$ labelling.

Isotopic data	Vegetative stage		Flowering		Seed filling	
(δ‰)	Control	Labelled	Control	Labelled	Control	Labelled
$\delta^{13}C$ data						
Leaves and stems	–28.7	+597	–29.5	+528	–22.0	+157
Pod walls	–	–	–28.3	+1022	–24.0	+71
Seeds	–	–	–27.3	+1289	–27.9	+452
Roots	–27.5	+127	–28.4	+122	–28.6	+94
Nodules	–28.3	+396	–28.5	+96	–29.0	+206
$\delta^{15}N$ data						
Leaves and stems	–1.2	+129.6	+0.1	+118.9	–2.8	+1.8
Pod walls	–	–	+0.4	+240.6	–1.7	+9.1
Seeds	–	–	+2.4	+267.0	+0.6	+9.7
Roots	–3.0	+46.6	+0.4	+67.4	+1.1	+10.3
Nodules	–0.7	+89.0	+1.6	+82.0	+3.8	+17.8

guished (Warembourg, 1983; Fernandez and Warembourg, 1987): at first, there was a phase with high rates of labelled CO$_2$ production that started soon after the beginning of ^{13}C feeding (Fig. 1). Rates reached a maximum during the night that followed the day of ^{13}C exposure with a second peak at midday the next day, and sharply decreased by the end of the second day (around 35 h); There was then a regular decrease with a succession of day and night fluctuations that can be attributed to temperature changes (Warembourg, 1983). Root temperature was, however, not recorded in this experiment. Despite that, on average, over several days, this phase was characterized by a slow exponential decrease of rates with time that can be significantly described by an expression of the type (Fig. 2A):

$$y=Ae^{-kt} \ (P < 0.05) \tag{1}$$

where A and k were the initial and average hourly respiration rates, respectively. This second phase was attributed to the turnover of labelled structures in the nodulated roots and associated with maintenance activities (Ryle *et al.*, 1976), while the first phase included biosynthesis of root and nodule tissues (McCree, 1970; Penning de Vries, 1975; Ryle *et al.*, 1976) and SNF activity (Warembourg, 1983). It is noteworthy that part of the second respiration phase will have been due to microbial degradation of labelled root exudates. Since root exudation and rhizomicrobial respiration of root exudates has been found to be a constant proportion of root growth for a given phenological stage (Warembourg and Estelrich, 2001), this component has been integrated into maintenance activities. It was also hypothesized that rhizomicrobial activity was independent from SNF by nodules.

Integration of equation (1) makes it possible to account for the total amount of labelled C lost by the nodulated roots in maintenance (*Rm* component). The decrease of labelled C loss due to *Rm* (Fig. 2B) was of the form:
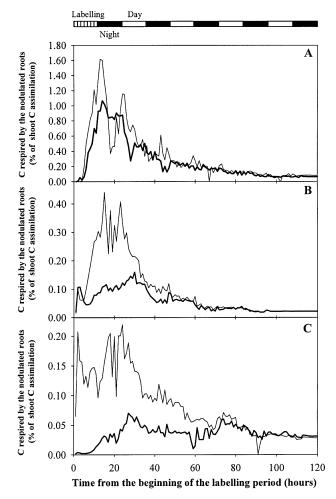
$$Rm(t)=A/k \ e^{-kt} \tag{2}$$

where *Rm(t)* is the remaining C to be respired in maintenance processes at a given time, and *kt* the hourly decrease rate.

In order to account for the delay for labelled C transfer, integration into nodulated root biomass, and turnover, *Rm* was only calculated from 30 h after the beginning of the ^{13}C labelling period (Fig. 2B). This was done by extrapolation of equation (2) back to time zero. The difference between the total labelled C lost by nodulated roots in respiration and the initial calculated value of *Rm* was attributed to activity processes that included SNF activity of the nodules (*Rfix* component) and synthetic processes of both roots and nodules (*Rs* component) (Fig. 2B) (Ryle *et al.*, 1976).

These results were significantly expressed by equation (1) ($R^2 >0.85$), except for the N$_5$ treatment at seed-filling ($R^2=0.67$). Integration following equation (2) smoothed the daily variations and significantly described the data ($R^2 >0.97$), thus allowing intermediate respiration components *Rm*, and (*Rs+Rfix*) to be calculated for each labelling period and each treatment (data not shown).

Carbon costs associated with nodulated roots of strictly N$_2$-fixing plants

Carbon costs of SNF increased during the growth cycle (Table 3). Carbon costs of roots and nodules synthesis were close and decreased similarly with time, with root synthesis being slightly more C expensive than nodules synthesis (Table 3). Root maintenance increased with time while nodule maintenance decreased (Table 3). Root maintenance was lower than nodule maintenance until flowering, but higher at seed filling.
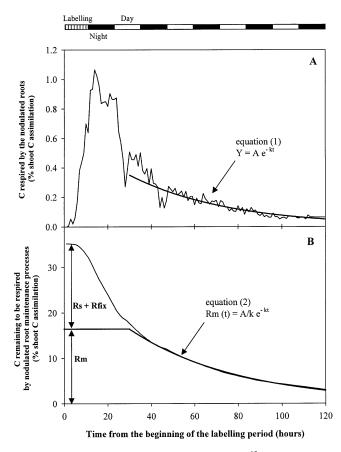
Fig. 1. Hourly C respiratory efflux (A, B, C) calculated from ^{13}C respired by the nodulated roots of *Pisum sativum* L. cv. Bacarra as a function of time from the beginning of the labelling period to the end of the chase period (120 h), as influenced by the N source (broad line: N_5 treatment; narrow line: N_0 treatment) at the vegetative (A), flowering (B) and seed filling (C) growth stages of the 1999 plant set. Hourly rates are expressed as a percentage of shoot carbon assimilation calculated from ^{13}C labelling as the sum of C increments in biomass plus rhizosphere respiration. The labelling, night and day periods are indicated by a dotted, closed and open boxes, respectively, at the top of the figure.

Symbiotic nitrogen fixation activity as related to nitrate, nodule biomass and carbon

The interdependence of SNF activity and C availability within the plant was investigated in the 2000 experiment: simultaneous ^{13}C and ^{15}N labelling with several atmospheric CO_2 concentrations were performed on three different plant sets with delayed sowing dates, which permitted a range of photosynthetic rates for each growth stage to be obtained.

Variations of SNF with phenology were first examined using average values obtained at the three sowing dates (Fig. 3): SNF activity was measured as the amount of nitrogen fixed over one day using ^{15}N data. At each phenological stage, SNF was lower in the presence of

Fig. 2. Calculation of respiration components from ^{13}C labelling using the N_5 treatment of the vegetative stage of the 1999 plant set as an example. Hourly respiration of the nodulated roots (A) and amount of C remaining to be respired due to maintenance (B) as a function of time from the beginning of the labelling period to the end of the chase period (120 h). *Rs*: synthesis component; *Rfix*: SNF component; *Rm*: maintenance component. The labelling, night and day periods are indicated by a dotted, closed and open boxes, respectively, at the top of the figure.
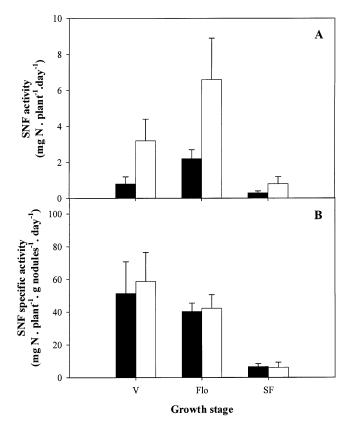
nitrate (N_5 treatment, Fig. 3A). For both N treatments, SNF was maximal at flowering and declined to low values at seed filling (Fig. 3A). SNF activity per unit nodule biomass (SNF specific activity) decreased throughout the growth cycle, but was not affected by nitrate supply (Fig. 3B).

SNF was then related to nodule biomass. SNF activity increased with nodule biomass for both N treatments at the vegetative and at flowering stages (Fig. 4) whereas it remained low and similar regardless of nodule biomass at seed filling. A linear relationship could be established between SNF activity and nodule biomass at each phenological stage regardless of the N treatment (Fig. 4).

As it is known that SNF directly depends upon current photosynthate supply (Kouchi *et al.*, 1986), SNF specific activity was related to the amount of newly photosynthetic C that was allocated to the nodulated roots (Fig. 5). It was calculated from labelled C as the sum of C increment in nodulated root biomass plus that evolved in their respiration. At each growth stage, SNF specific activity linearly increased with the amount of C allocated to the nodulated

Table 3. *Components of respiratory activity of nodulated roots calculated using curves of ^{13}C-labelled nodulated root respiration after ^{13}C labelling of the shoot atmosphere*

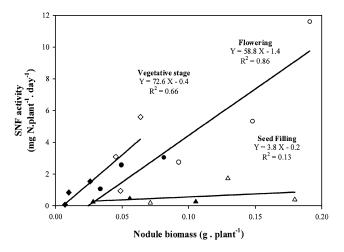| Respiratory costs		Vegetative stage	Flowering stage	Seed-filling stage
Synthesis costs				
C respired/C initially	Roots	0.68	0.40	0.070
incorporated in biomass	Nodules	0.53	0.37	0.001
Maintenance costs				
Proportion of C in biomass	Roots	0.23	0.48	0.76
initially formed that is	Nodules	0.91	0.79	0.59
respired=turnover				
Symbiotic N$_2$ fixation costs				
g C respired g^{-1} N fixed		4.0	6.4	6.7

Fig. 3. Daily SNF activity (A) and SNF activity per unit nodule biomass (SNF specific activity) (B) during growth of *Pisum sativum* L. cv. Bacarra (V: vegetative stage; Flo: flowering stage; SF: seed-filling stage) as influenced by the N source (black: 5 mol m^{-3} KNO$_3$ supplied plants (N$_5$ treatment); white: plants only fixing N$_2$ (N$_0$ treatment). Vertical bars represent standard error deviation (*n*=9).

Fig. 4. SNF activity of *Pisum sativum* L. cv. Baccara as a function of nodule biomass, as influenced by the N source: closed symbols, 5 mol m^{-3} N-KNO$_3$-supplied plants (N$_5$ treatment); open symbols, plants only fixing N$_2$ (N$_0$ treatment), and growth stage: diamonds, vegetative stage; circles, flowering stage; triangles, seed-filling stage.

roots (Fig. 5). Both N treatments followed the same trend and regression slopes between SNF specific activity and the amount of C allocated to the nodulated roots decreased across the growth cycle (Fig. 5).
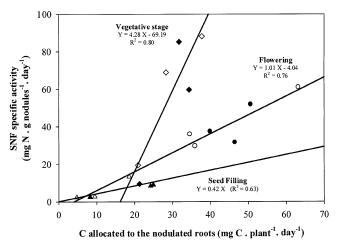
Discussion

Although literature concerning SNF has been documented with relationships between SNF activity and nodule biomass, these studies lack quantitative results, with the exception, however, of the few relationships established at the growth cycle time-scale by Larue and Patterson (1981) or Tricot-Pellerin *et al*. (1994). Quantitative linear relationships between SNF activity and nodule biomass (Fig. 4) for each growth stage were established for the first time in this study. These relationships are valid whatever the N source and, interestingly, they allow for the first time the factors determining SNF activity at the day-time scale and during the whole growth cycle to be investigated further. For this, SNF was considered as the product of SNF per unit nodule biomass (SNF specific activity) by nodule biomass. As SNF specific activity was not significantly different between N treatments (Fig. 3B), with SNF activity being lower in the presence of nitrate (Fig. 3A), for each growth stage the hypothesis was made that there is a potential value for SNF specific activity that does not depend upon the N source (or N treatment). For each growth stage, the potential value of SNF specific activity was indicated by the slope of linear regressions between

Fig. 5. Daily SNF specific activity (SNF per unit nodule biomass) during growth of *Pisum sativum* L. cv. Baccara as a function of the amount of new C (^{13}C) allocated to the nodulated root, as influenced by the N source: closed symbols, 5 mol m^{-3} N-KNO$_3$-supplied plants (N$_5$ treatment); open symbols, plants only fixing N$_2$ (N$_0$ treatment), and growth stage: diamonds, vegetative stage; circles, flowering stage; triangles, seed-filling stage.

SNF activity and nodule biomass that were established regardless of the N treatment (Fig. 4). The potential value of SNF specific activity decreased with phenology (slope of linear regression between SNF and nodule biomass, Fig. 4). This could be related either to variation of nodule efficiency with time (Warembourg, 1983) and/or to C limitation of SNF activity, as in pea C allocation to nodulated root decreases as the plant ages (Voisin *et al.*, 2002*a*). However, to date, it was never clearly shown to what extent C supply actually modulated SNF specific activity and the causal relationships are still required to be clarified.

Carbon cost related to SNF specific activity

Considering SNF efficiency, the carbon costs of SNF increased with time from 4.0 to 6.7 g C respired g^{-1} N fixed from the vegetative to the seed-filling stage (Table 3). Possible explanations for the decrease of SNF efficiency with time are either that electron allocation efficiency to SNF (Bethlenfalvay and Phillips, 1977*a*, *b*; Layzell, 1990) and/or C recycling from PEPC activity (Roumet, 1990) decreased with time.

It remains difficult to compare these values with those of other studies, due to methodological difficulties, especially on temperate species like pea for which nitrate reduction occurs in the roots (Wallace, 1986). Moreover, the C costs of SNF were often calculated including the costs of synthesis of nodules or even roots, together with those associated with SNF (Phillips, 1980). Lastly, the long duration of most measurements may have smoothed variations across time. However, using the same methods, Warembourg and Roumet (1989) measured slightly dif-

ferent values for soybean (2.5–7.6 g C g^{-1} N fixed) and red clover (2.8–4.6 g C g^{-1} N fixed). When compared to their description, the respiratory rates calculated in this study from ^{13}C efflux (Fig. 1) showed some variations that were higher than when ^{14}C was used (Warembourg, 1983; Warembourg and Roumet, 1989). This has been attributed to methodological difficulties associated with the use of the stable isotope ^{13}C: natural ^{13}C enrichment of the air and isotopic discrimination associated with biological processes complicates the calculations and introduces additional sources of experimental errors, due to the need of unlabelled control plants; the enrichment of C-labelled components (such as biomass of the various plant parts and respiratory CO$_2$) is much more precise when radioactive isotope (^{14}C) is used instead of stable isotopes (Morot-Gaudry *et al.*, 1995).

Nevertheless, comparisons can be made with theoretical values. These calculations represented net C cost of SNF including N$_2$ reduction, H$_2$ reduction, N assimilation and transport out of the nodules. Based on ATP requirements, calculated C costs strictly associated with nitrogenase activity were in the range of 1.5–4 g C g^{-1} N fixed, depending on environmental conditions (Schulze *et al.*, 1994). When including N assimilation and transport, 3.96 g C would be respired g^{-1} N fixed (Roumet, 1990). Hence, the values presented are close to the theoretical estimates, even though C fixation by PEP carboxylase (Warembourg and Roumet, 1989) was not accounted for.

Is SNF specific activity limited by C competition within nodulated roots?

Even if nitrogenase activity is O$_2$-limited rather than C-limited (Hunt and Layzell, 1993; Vance and Heichel, 1991), SNF specific activity could still depend upon C supply. The fact that SNF may be related to C supply has been documented by others (Hardy and Havelka, 1976; Finn and Brun, 1982; Zanetti and Hartwig, 1997) but again, there were few attempts if any of quantitative studies, probably because experimental measurements of SNF and C fluxes together require special equipment associated with isotopic measurements. The original linear relationships between SNF specific activity and the amount of C allocated to the nodulated roots (Fig. 5) for the different growth stages are reported here. This indicated that SNF activity was strongly linked to C supply. Thus, differences in growing conditions, affecting C allocation to nodulated roots (Voisin *et al.*, 2002*a*), could explain the variability in SNF values reported by many authors on pea (Jensen, 1986, 1987) or on other species (Herridge and Pate, 1977; Warembourg, 1983; Warembourg and Roumet, 1989; Herdina and Silsbury, 1990; Rennie and Kemp, 1980). The decrease across the growth cycle of the slopes of linear regression between SNF specific activity and the amount of C allocated to the nodulated roots (Fig. 5) may partly be explained by the

decrease of nodule efficiency as the plant aged (Table 3). The decrease across the growth cycle of the regression slopes between SNF and C supply may also indicate a decreased competition for C between SNF and other respiration processes within nodulated roots (i.e. synthesis and maintenance) as a lower slope indicates a lower C limitation of SNF.

Relationships between SNF activity, synthesis and maintenance of roots and nodules

The carbon threshold under which no SNF occurred (X intercept, Fig. 5) may indicate the minimum amount of C necessary for growth and/or maintenance. This threshold was identical for both N treatments at each growth stage, suggesting that total C use for synthesis and maintenance of nodulated roots was similar whatever the N source. However, even if calculated C costs of root and nodule synthesis were similar whatever the growth stages (Table 3), turnover of roots and nodules highly differed (Table 2). At the vegetative stage, C costs for roots and nodules synthesis were similar (Table 3) and synthesis may have been the main component of C consumption due to nodulated root establishment during this period. This is emphasized by the fact that nodule biomass is always low at this early stage compared with root biomass (Pate *et al.*, 1979). Hence, there was probably little difference in actual C used for maintenance between N treatments (with different root/nodule biomass ratio) despite the calculation (Table 3) that demonstrated a high turnover of nodules (Table 3). Synthesis of nodulated roots is usually mostly achieved by flowering (Tricot, 1993; Voisin *et al.*, 2002*b*). For plants only fixing N₂, higher C costs incurred by nodule maintenance at flowering (Table 3) have been shown to occur at the expense of root growth at that stage (Voisin, 2002) so that the total C costs for the whole plant did not increase. Thus, a decrease across the growth cycle of C limitation of SNF as shown by (i) a decrease of the regression slope between SNF and C supply and (ii) a decrease of the C threshold under which no SNF occurred (Fig. 5), may arise from a decrease of the synthesis processes, which are the main limiting factor of SNF, regardless of the N treatment. This limitation mostly occurs at the beginning of the growth cycle, when roots and nodules are constructed.

At seed-filling, SNF specific activity could still be related to the amount of C allocated to the nodulated roots (Fig. 5), indicating that nodule functioning was still effective at this late stage. However, it was less predictable than at earlier stages (comparison of R^2 in Fig. 5), presumably because C allocation to the nodulated roots was no longer sufficient to maintain nodule biomass, resulting in nodule senescence (Fig. 5). Many studies (Lawrie and Wheeler, 1974; Pate and Herridge, 1978) have suggested that the low SNF activity usually observed at the end of the growth cycle was due to C deprivation because

of competition with seed-filling (Jeuffroy and Warembourg, 1991). This study shows that the C costs of SNF vary with nodule age and would determine SNF potential value. This is supported by observations of prolonged SNF ability at the end of the growth cycle in relation to the presence of newly formed nodules (Voisin *et al.*, 2002*c*).

The calculated costs are not actual costs, but only account for new ¹³C incorporated via the photosynthetic process that is respired for the synthesis of structures (synthesis component *Rs*) or resulting from their turnover (maintenance component *Rm*). However, it has been shown that nodule activity mainly relied on current photosynthesis whereas root activity probably involved stored C (Kouchi *et al.*, 1986). This apparent increase of root turnover calculated in this study (Table 3) may therefore result from an increased use across the growth cycle of current photosynthates, instead of stored C that is probably depleted at the end of the growth cycle because of remobilization to the seeds (Munier-Jolain, 1994). However, the values for synthesis C costs were similar to those given by Penning de Vries (1975). This suggests that root growth may be mainly supported by current photosynthates.

The use of isotopes has allowed the relations between SNF activity and C allocated to, and used within, nodulated roots to be investigated. Labelling with ¹⁵N dinitrogen in the root atmosphere is the only way specifically to track the amount and fate of N coming into the plant via SNF. Together with ¹³C labelling of the shoot atmosphere, this study demonstrated the quantitative relationship between SNF specific activity and C entry within the nodulated roots, regardless of nitrate availability. A potential value of SNF specific activity was defined regardless of the N source. Potential SNF decreased with phenology presumably due to nodule age that conditions their efficiency. At each growth stage, potential SNF specific activity was modulated by C supply to the nodulated roots, following different linear relationships depending on C competition with the synthesis processes of the nodulated roots. As these relationships did not depend upon the N source, this study showed that short-term nitrate inhibition of SNF specific activity was negligible. Hence, regulation of SNF activity by nitrate would be mediated through impairment of nodule growth in the long term.

It has been shown previously that the amount of C allocated to nodulated roots did not depend upon nitrate availability but C partitioning between roots and nodules biomass could not be assessed (Voisin *et al.*, 2003). In order to get an operational model of C/N flux within leguminous plants with an emphasis on their nodulated roots, it would now be valuable to investigate how C allocation towards nodule and root biomass is related to nitrate availability: this would allow the amount of N

entering plants as a function of phenology to be predicted dynamically as a function of net photosynthesis. Thus, the relationships between SNF specific activity and C allocated to nodulated roots already provide a predictive tool useful for modelling SNF variations with growing conditions and time through a single variable.

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