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The influence of N_2-fixation on the carbon balance of leguminous plants

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Abstract. Biological dinitrogen fixation by legume-rhizobia symbiosis is very important both from the economic and from the ecological point of view. Theoretically, the reduction of the N_2-molecule to ammonia requires at least 16 ATP and 1.5 mg C per mg N fixed (N_{fix}). These values are difficult to determine in situ as this necessitates the determination of that part of root respiration which drives N_2-fixation. New approaches to such determinations and the results obtained are described. The values vary, depending on the plant species studied, the developmental stage of the plants and the genetic variability of macro- (and micro-?) symbionts. The values range between 1.5 and 4 mg C/mg N_{fix}. In some species (e.g. *Vicia faba* L. cv. Fribo), the apparent CO_2 assimilation is enhanced in order to meet this high energy need. In others (e.g. *Pisum sativum* L. cv. Grapis), root growth is restricted. Physiological criteria are discussed which allow an early diagnosis of the energetic efficiency of various combinations of macro- and microsymbionts as a basis for a selection in plant breeding.

Key words. N_2-fixation, nitrogenase; carbon and energy demand of nitrogen fixation; leguminous plants.

Biological dinitrogen fixation is a process of great economic importance world-wide, since many legumes are grown as crop plants. These plants can be cultivated almost without mineral nitrogen fertilizers. In the last decades, remarkable progress has been made in nitrogen fixation research, especially in the genetic analysis of the symbiosis involved[15]. The reduction of nitrogen in root nodules means a great energy loss for the plants, since N_2-reduction is coupled with a considerable hydrolysis of ATP and a consumption of reducing equivalents. There has been much debate as to whether assimilate supply to the nodules limits nitrogen fixation and plant growth, or whether the plants can compensate from this demand by enhanced photosynthesis[32]. Situations were observed experimentally where nitrogen fixation was limited by the assimilate supply to the nodules[29]. The present paper analyses the carbon demand resulting from nitrogen fixation under various conditions, and investigates the effects on the carbon balance of the host plants. Furthermore, possible methods will be shown to identify such assimilate deficiencies by means of simple indicators. The findings could give valuable information to plant breeders for overcoming deficiencies in the plant-*Rhizobium* symbiosis.

Theoretical considerations on the carbon demand of N_2-fixation

In dinitrogen fixation, the reduction of molecular nitrogen to ammonia is catalyzed by the enzyme nitrogenase. In leguminous plants this process takes place in the bacteroids in root nodules. A simplified outline of the process is given in figure 1. The enzyme complex nitrogenase consists of two parts[9]. Molecular nitrogen is bound to a Mo-cofactor. Reducing equivalents come (in most cases) from ferredoxin, and are transported via the enzyme complex, finally reducing nitrogen to ammonia. The electron flux is coupled with ATP hydrolysis by the enzyme[36]. According to measurements by several authors[18, 35], the minimum amount of ATP hydrolysis required for the process is 4 ATP per electron pair transferred. Thus, the reduction on the enzyme can be expressed by the following equation:

$$N_2 + 6e^- + 8H^+ + (12 + x)ATP \rightarrow 2\,NH_4^+ + (12 + x)ADP + (12 + x)P_i \qquad (1)$$

In order to calculate the minimum carbon need for N_2-reduction from this equation, it has to be taken into account that the generation of reducing equivalents (Fd_{red}) additionally requires the expenditure of a certain amount of energy which must be produced by the oxidation of carbohydrates in roots. Since under optimal conditions one mole of glucose generates 12 reducing equivalents ($NADH/H^+$, $FADH_2$), according to equation 1 at least 0.25 moles of glucose is required to produce the reducing equivalents for N_2-reduction. Furthermore, assuming the maximum P/O ratio of 3 in oxidative phosphorylation, at least 0.33 moles of glucose have to be oxidized via glycolysis and the Krebs cycle to obtain the 12 ATP required (see equation 1). Therefore, the minimum carbon demand for the reduction of one mole of N_2 is 0.58 moles of glucose or 1.49 mg C/mg N_{fix}. However, this minimum value can only apply to optimal (theoretical) conditions. In practice, several factors

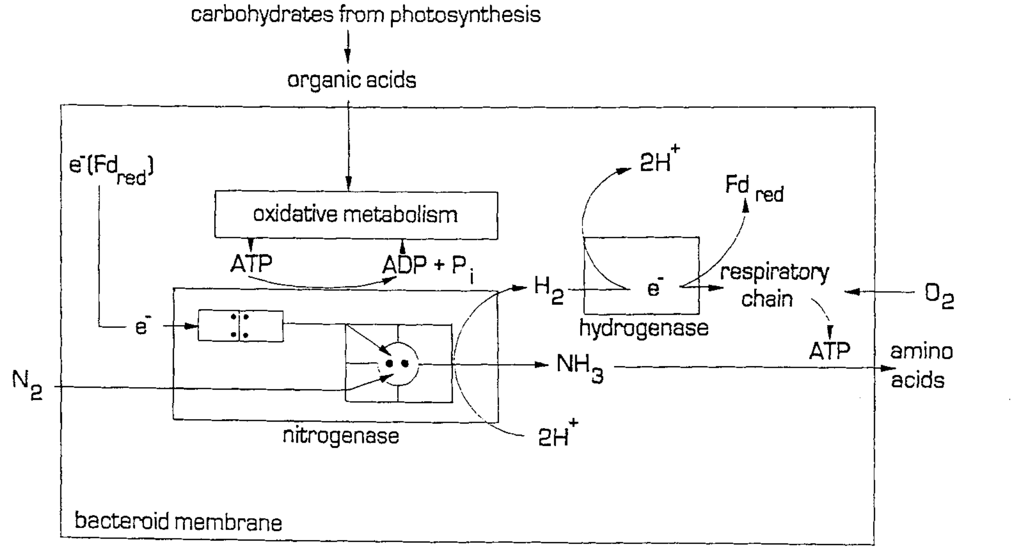

Figure 1. Simplified scheme of N_2-reduction by nitrogenase (according to Müntz[25], modified).

might lead to a higher C consumption. First of all, one has to take into account that the reduction of N_2 is coupled with a simultaneous reduction of H^+ by the enzyme[2] (compare figure 1). The ratio between N_2 and H^+ reduction can vary, and a so-called 'electron allocation coefficient' (EAC) has been proposed[10] and defined as follows:

$$EAC = \frac{e^- \text{ to } N_2}{e^- \text{ to } H^+ + e^- \text{ to } N_2}$$

The EAC is about 0.75 when dinitrogen is available under physiological conditions, but the value may decline to 0.5 (cf. ref. 10).

Assuming an EAC of 0.75 along with the reduction of N_2 described by equation 1, the following process would take place:

$$2e^- + 2H^+ + (4+x)\,ATP \rightarrow H_2 + (4+x)\,ADP + (4+x)\,P_i \qquad (2)$$

The above calculation indicates a further minimum requirement of 0.19 moles of glucose (0.11 moles for ATP generation, 0.08 moles for reduction equivalents) or 0.49 mg C/mg N_{fix}. This brings the total carbon demand up to 1.98 mg C/mg N_{fix}.

Some *Rhizobium* strains possess an uptake hydrogenase which is able to oxidize the H_2 evolved, and thereby a certain fraction of the energy 'lost' according to equation 2 is recycled. In some *Rhizobium* strains the oxidation of H_2 is coupled with ATP production, whereas the reaction is considered to be marginal in other strains[26,31]. Probably in these latter cases, reducing equivalents are generated and used up in processes other than oxidative phosphorylation. Dixon[14] estimated that oxidation of the H_2 evolved in parallel with the reduction of one molecule of N_2 might generate an amount of energy equivalent to 2 ATP. This would decrease the carbon demand in the above calculations by 0.14 mg C/mg N_{fix} or 7.1%.

As indicated in equations 1 and 2, the amount of ATP hydrolyzed by nitrogenase might be higher than 12 + 4 = 16 ATP per molecule of N_2. This strongly depends on environmental conditions such as temperature and pH-value[37]. At low temperatures and pH-values, the amount of hydrolyzed ATP would increase considerably[18,35]. In *C. pasteurianum*, Daesch and Mortenson[12] measured a consumption of about 20 ATP per reduced N_2 whereas Hill[17] obtained values close to 30 for *K. pneumoniae*.

On the other hand, the assumption of a P/O ratio of 3 for oxidative phosphorylation probably does not reflect the real conditions. Phillips[28] used a value of 2. If cyanide-resistant respiratory chains play a role[13], even lower values or a nonphosphorylating electron transport[20] are possible. Nevertheless, direct measurements of this ratio under various conditions are still missing. A P/O ratio of only 2 will considerably increase the carbon requirement for N_2 reduction as calculated in this paper. This would mean a carbon need of 0.66 moles of glucose, plus 0.33 moles for the reducing equivalents, resulting in a need for 2.55 mg C/mg N_{fix}. If plants need 30 moles ATP/mole N_2, then the C requirement under such conditions will be 4.06 mg C/mg N_{fix}.

To sum up, the carbon need for N_2 reduction may vary considerably, depending on the environmental conditions and also on the characteristics of micro- and macrosymbiont (e.g. presence of hup-genes). The lowest limit is probably reached with 1.5 mg C/mg N_{fix}, whereas an upper limit can hardly be fixed theoretically. One has to bear in mind that these values only reflect the carbon need for the direct reduction process and do not include the energy expenses for further nitrogen assimilation (see the review by Atkins[3]).

Methods to estimate the carbon demand of N_2-fixation

The carbon demand of N_2-fixation can be defined as the CO_2-evolution caused by that part of respiration which drives N_2-fixation in nodulated roots. The ratio of this amount to the amount of molecular nitrogen incorporated in a certain period of time has to be calculated. Measuring this fraction of root respiration causes several problems, since it is difficult to separate it from the respiration rates for other growth and maintenance processes. Furthermore, N_2-fixation must always be measured simultaneously. Despite these obstacles, the whole-plant carbon demand resulting from nitrogen fixation has been estimated by many authors, using various techniques, which emphasises the importance of such determinations.

A first approach was to calculate the carbon requirement for nitrogen fixation by comparing the rates of dry matter formation and N-accumulation of N_2-fixing leguminous plants with those in plants supplied with mineral-N. The experiments were carried out in liquid cultures. Plants supplied with mineral nitrogen were kept free of infection, while the others were grown in a nitrogen-free solution and developed nodules. Dry matter formation and N-accumulation were determined as the difference between two consecutive harvests. Assuming that the difference in dry matter accumulation between plants with and without mineral nitrogen represented the amount of carbon respired for nitrogen fixation, it is possible to calculate the carbon demand of nitrogen fixation. However, this approach will only furnish correct data if the photosynthetic activity in both groups of plants is the same. It is known that N_2-fixing plants often develop increased photosynthesis (compare the next section of this paper), so the values will not represent true estimates of the carbon demand of nitrogen fixation in all cases. Values ranging from 2.9–19 mg C/mg N_{fix} were calculated[1,8,11].

Other approaches took into account CO_2 evolution rates of roots and nodules. One principle of measurement was to assess the CO_2 evolution and $^{15}N_2$ incorporation of detached nodules[4,5]. Relatively low values were reported such as 1.5 mg C/mg N_{fix} in *Pisum sativum* L. and 1.7 mg C/mg N_{fix} in *Lupinus albus* L.[4]. Bergersen[5] obtained an extremely high value of 19.4 mg C/mg N_{fix} in *Glycine max.* (*Merr.*) L. with a similar technique. This value may be explained by a low nitrogen fixation activity caused by the detachment of the nodules. Such calculations with detached nodules will hardly permit an exact determination of the carbon demand of N_2-fixation in vivo. The isolation of nodules will probably affect their activities to a certain extent.

Another principle was used by Mahon[21], who determined the carbon costs of nitrogen fixation by measuring the root and nodule respiration rates in relation to acetylene reduction in intact nodulated pea plants. Values ranging from 6 to 6.8 mg C/mg N_{fix} for *Pisum sativum* L. were found. Applying a similar method, Minchin and Pate[24] and Pate and Herridge[27] obtained values ranging from 4 to 6.5 mg C/mg N_{fix} for *Pisum sativum* L. and *Lupinus albus* L. There are several factors which limit the validity of this method. For example, the calculation of nitrogen fixation rates using the acetylene reduction technique is problematic. Furthermore, respiration driving N_2-fixation and the respiration driving other processes were not separately determined.

The method proposed by Warembourg et al.[33] is based on the simultaneous short-term exposure of shoots to $^{14}CO_2$ and roots to $^{15}N_2$ in order to study nitrogen fixation in relation to carbon assimilation and use. In parallel with the short-term exposure of shoots to $^{14}CO_2$, the $^{14}CO_2$ release from the root space was measured continuously. The assumption was made that the $^{14}CO_2$ release shortly after labelling represents the respiratory demand for nitrogen fixation, since Warembourg and Roumet[34] assume that N_2-fixation takes place at the expense of recently assimilated carbon. The maintenance respiration of roots was measured as $^{14}CO_2$ release 24 h after labelling and substracted from the $^{14}CO_2$ release shortly after labelling. The $^{15}N_2$ incorporation was determined simultaneously with the shoot exposure to $^{14}CO_2$. This method seems to allow a precise assessment of the carbon demand for N_2-fixation, because the equipment permits continuous measurements and control of environmental conditions such as air temperature, humidity and CO_2 concentration. The values obtained with these techniques ranged from 2.5 to 7.6 mg C/mg N_{fix} in several leguminous species[34]. However, all these experiments were only short-term tests.

To determine the carbon need for nitrogen fixation over longer periods, nodulated plants were grown in quartz sand[23,30]. The purpose of the experiments was to determine that part of respiration which drives N_2-fixation, and to relate its C-consumption to the simultaneous N_2-fixation. The latter was determined as the increase of the amount of N in the nodulated leguminous plants between two consecutive harvests (intervals of 7 or 14 days). In order to assess the respiration demand of N_2-fixation ($A_{N\text{-fix}}$) the CO_2 release from the root space of nodulated plants (CF_{wr}) had to be measured during this period. The sum of root maintenance respiration of the N_2 fixing plants (A_{we}), microbe respiration (A_{mr}) and that fraction of root growth respiration which drives the incorporation of reduced N into proteins and other endergonic processes ($A_{N\ \text{incorporation}}$) had to be substracted from this value, to give the carbon demand of N_2-fixation ($A_{N\text{-fix}}$):

$$A_{N\text{-fix}} = CF_{wr} - (A_{we} + A_{mr} + A_{N\ \text{incorporation}})$$

$A_{N\ \text{incorporation}}$ was assessed by measurements on non-nodulated plants grown simultaneously. These control

Table 1. Carbon demand for N_2-fixation in various stages of plant development. According to Schulze[30]

Measuring period	*Pisum sativum* L. 'Grapis'	*Vicia faba* L. 'Fribo'
1) 2 to 4 weeks after emergence	1.5	4.2
2) 4 weeks after emergence until flowering	3.5	4.0
3) between flowering and ripening	no N_2-fixation	2.1

Values are given as mg C/mg N_{fix} as mean value of 6 replicates.

plants were nourished by mineral nitrogen (e.g. urea; nitrate was possible only if it could be shown that it was reduced in the shoots, coupled with the light reaction of photosynthesis under the conditions employed[30]). Such plants should have the same C requirement per mg N for N utilization as nodulated plants (the C needed for the uptake of mineral N is very low[3]).

By replacing $A_{N\ \text{incorporation}}$ by $A'_{N\ \text{incorporation}} \cdot x/y$, we get the equation:

$$A_{N\text{-fix}} = CF_{wr} - (A_{we} + A_{mr} + A'_{N\ \text{incorporation}} \cdot x/y)$$

where $A'_{N\ \text{incorporation}}$ = root growth respiration of the mineral-fed plants, x = N-assimilation in the N_2-fixing plants and y = N-assimilation in the mineral N-fed controls. The calculated amount of carbon must then be related to x. Maintenance respiration (A_{we}) of roots plus microbe respiration (A_{mr}) were determined as CO_2 release from the root space of plants grown in quartz sand 48 h after shoot removal (for details and validity tests-compare[22,30]). Using this experimental approach, several values were determined for *Vicia faba* L. and *Pisum sativum* L. varieties of temperate regions (table 1).

The data of table 1 and the values cited in the literature indicate that the values varied considerably. This could be due to:

1) The different methods used. All methods which do not measure the fraction of CO_2-evolution from the root space driving N_2-fixation in situ, and which measure N_2-fixation only indirectly, are problematic. This is the case with measurements in detached nodules, and also with the acetylene reduction test, since the conversion factor for calculating N_2-fixation from acetylene reduction is highly dependent on the experimental conditions[19].
2) The plants themselves and their developmental stage at the time of measuring. The carbon requirement for nitrogen fixation can obviously vary between the various stages of ontogenesis. As discussed in the first section of this paper, the reasons for this effect may be variable and are not yet understood.

Influence of carbon use by nitrogen fixation on whole plant growth

Even relatively low amounts of organic C required for N_2-fixation represent a great expense for the whole plant. When *Vicia faba* L. plants, for example, fix 200 kg N/ha per year, and respire 2.5 mg C/mg N_{fix}, then an additional demand of 500 kg C or about 1.25 t dry matter is required in comparison with plants nourished with reduced nitrogen. Therefore, nitrogen fixation must have considerable effects on the carbon turnover in such plants. The question arises as to how plants meet this high C requirement and whether conditions exist where the C-demand limits plant growth. Legumes have been reported to adapt themselves to a high carbon turnover in the roots by increasing their photosynthetic activity. There is evidence that, at least in some cases, nodulated plants have a higher photosynthetic activity than non-nodulated controls of the same species[6,7,23]. Nodulated leguminous plants often reach the same growth rates and N-assimilation as mineral N-fed plants by means of a more intensive photosynthesis.

In order to ascertain whether this enhanced photosynthesis is able to support N_2-fixation under all conditions and in all plant species, determinations of whole plant carbon balances over longer periods are needed. Schulze[30] performed such experiments with *Pisum sativum* L. and *Vicia faba* L. plants. N_2-fixing plants were compared with mineral N-fed controls. The mineral N-fed controls were nourished with urea in the case of *Vicia faba* L.[30]. In the case of *Pisum sativum* L., nitrate was used in most cases, because reduced nitrogen compounds caused plant damage. In *Pisum sativum* L. plants around 90% of the nitrate was reduced in the shoot. The reduction was coupled to the light reaction of photosynthesis under the experimental conditions used (Adgo, unpublished). The shoot and root respiration in darkness as well as the apparent CO_2-fixation of shoots in light were determined in all experiments in parallel with the dry matter increments from two consecutive harvests. The results for a 14-day-long measuring period before the onset of flowering are shown in table 2. The data are given for the nitrogen-fixing plants both as absolute figures and as a percentage of the figures for the mineral N-fed controls, in order to facilitate comparison.

Obviously in the case of the plant varieties used (and for the environmental conditions and microsymbiont involved) only *Vica faba* L. plants were able to compensate for the enhanced root respiration required for nitrogen

Table 2. Carbon balance of 4-week old *Pisum sativum* L. and *Vicia faba* L. plants. Experiments in Mitscherlich pots with fine quartz sand over 2 weeks. *P. sativum*, 15 plants per pot; *V. faba*, 7 plants per pot. According to Schulze[30]

Measurement	*Pisum sativum* L. ('Grapis')		*Vicia faba* L. ('Fribo')	
	N_2-nourished (absolute values)	N_2-nourished as percentage of the control given mineral N (nitrate)	N_2-nourished (absolute values)	N_2-nourished as percentage of control given mineral N (urea)
Apparent CO_2-fixation of the shoots in light [g CO_2]	19.3	(98)	12.2	**(131)***
Shoot respiration in darkness [g CO_2]	3.3	(97)	0.9	(112)
Root respiration (light and darkness) [g CO_2]	2.8	**(162)***	1.9	**(199)***
Dry matter increment (shoots) [g]	7.7	(108)	3.1	(106)
Dry matter increment (roots) [g]	0.6	**(63)***	0.5	(125)

Data are given per Mitscherlich pot as mean value of 6 replicates, * significantly different in comparison with the mineral N-fed control with $\alpha \leq 5\%$ (t-test).

fixation by higher photosynthesis. In contrast to this, *Pisum sativum* L. plants showed no enhanced photosynthesis. The carbon requirement for nitrogen fixation was obviously met at the expense of root growth. Nevertheless, the shoots revealed no difference in growth whether or not mineral N was supplied. However, the pea plants ceased to fix nitrogen after flowering when pod-filling commenced. Thus different mechanisms exist for meeting the high carbon requirements of nitrogen fixation, and they can limit nitrogen fixation and plant growth in some cases.

Leguminous plants can therefore be grouped according to their ability to fix nitrogen up to the end of the vegetation period[29]. *Pisum sativum* L., *Lupinus albus* L. and *Glycine max.* (*Merr*) L. cease to fix nitrogen after flowering, whereas *Lupinus luteus* L. and *Vicia faba* L. continue to fix nitrogen. The behaviour of the first group is without doubt related to carbon deficiency in the nodules. Evidence for this is provided by the finding that nitrogen fixation did not stop after flowering when growing pods were removed from the first group of plants[23]. These plants could obviously not support growing pods and nodules with enough photosynthates simultaneously. Furthermore, when photosynthesis in such plants was 'artificially' enhanced, e.g. by applying high CO_2-concentrations in the atmosphere around the shoots, nitrogen fixation took place during the pod-filling stage. Daily sugar spraying of the shoots with a solution of 2% sucrose showed the same effect (table 3). The sucrose was partly taken up by the plants. This was demonstrated by experiments with ^{14}C-sucrose. In *P. sativum* L., this treatment enhanced plant growth and allowed nitrogen fixation to continue till the end of the vegetation period in *Pisum sativum* L. On the other

Table 3. Effect of daily sugar spraying of the shoots with 2% sucrose solution on dry matter increment and nitrogen fixation between the beginning of flowering and ripening, in *Pisum sativum* L. ('Erbi') (27 days) and *Vicia faba* L. ('Fribo') (56 days). The experiments were performed by Adgo (unpubl.)

	Pisum sativum L. 'Erbi'		*Vicia faba* L. 'Fribo'	
	without sugar spraying	with sugar spraying	without sugar spraying	with sugar spraying
Shoots and pods dry matter increment [g dry matter]	5.5	22.2*	35.8	38.5
Root dry matter increment [g dry matter]	0.1	0.6*	0.3	1.9*
N increment in both organs [mg N]	120	341*	1102	1100

Data are given per pot as mean value of 4 replicates, * = means a significant difference in comparison with controls without sugar spraying with $\alpha \leq 5\%$ (t-test).

hand, the same treatment had almost no effect in *Vicia faba* L. plants.

Vicia faba L. plants apparently have enough photosynthetic capacity to produce assimilates for both sinks, namely the growing pods and the nitrogen-fixing nodules. It remains to be clarified whether this is true under any environmental conditions and with all plant varieties. Interestingly, some exceptions to the above-mentioned group do seem to exist. Hayas[16] reported that in a Syrian pea variety ('Anward') nitrogen fixation continued up to the end of the vegetation period, in contrast to German varieties. Thus, even within one species different variations may be in different groups. In the case of 'Anward' the continuation of nitrogen fixation parallel with pod filling was probably due to the relatively long time between flowering and ripening. Possibly the 'competition' for assimilates between pods and nodules was, therefore, not as strong as in the 'highbred' German pea varieties where the same plant growth stage was only about half as long.

To sum up, it can be stated that the carbon requirement for N_2-fixation may limit plant growth only under certain conditions. There are, however, differences between plant species and varieties in this behaviour. The optimal partnership between microsymbiont and macrosymbiont seems to be important as well. Plant breeders need criteria which will easily and rapidly allow the efficiency of symbiosis to be assessed. The following criteria, for example, show that there is no limitation in the carbon supply to the nodules.

1) No growth response to mineral nitrogen fertilization at flowering. In such cases the nitrogen fixation would simply be replaced by mineral N[29].

2) A long time between flowering and ripeness. In such cases the 'competition' for assimilates between nodules and growing pods is probably not too strong.

3) The same root growth rate of nitrogen fixing plants in comparison with mineral N-fed ones.

4) Slow dying of the lower leaves after flowering signalizes a good assimilate supply of the roots and the preservation of a strong assimilatory apparatus, which also nourishes the nodules.

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