ACTUAL AND POTENTIAL NITROGEN FIXATION IN PEA AND FIELD BEAN

AS AFFECTED BY COMBINED NITROGEN

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van bodem en water

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
hoogleraar in de veeteeltwetenschap,
in het openbaar te verdedigen
op vrijdag 6 november 1981
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

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STELLINGEN

1. De feitelijke stikstofbinding van erwt en tuinboon is lager dan de potentiële stikstofbinding, hetgeen berust op onvoldoende toevoer van fotoassimilaten.

   Dit proefschrift

2. Het belang van waterstof-oxydatie voor de aktiviteit van nitrogenase in symbiontische systemen wordt schromelijk overschat.

   Evans,H.J. *et al.* 1981. pp. 84 - 96 *in* Current perspectives in nitrogen fixation (eds. A.H. Gibson and W.E. Newton) Canberra

   Dit proefschrift

3. Toediening van stikstofmeststof gedurende de periode van peulvulling kan de zaadopbrengst van vroege erwterassen verhogen.

   Sinclair,T.R. and De Wit, C.T. 1976. Agronomy J. 68 : 319 - 324

4. De verbouw van gewassen voor de produktie van ethanol als brandstof is in Nederland zinloos en in ontwikkelingslanden misdadig.

   P. van Zijl, Intermediair 26 juni 1981

5. Teelt van veldbonen voor eiwitrijk ruwvoer, als tussengewas bij de herinzaai van grasland, kan in Nederland jaarlijks evenveel energie besparen als een stad van 20.000 inwoners verbruikt.

6. Daar de bouw van rioolwater-zuiveringsinstallaties in Nederland vrijwel voltooid is, moet de heffing voor huishoudens op basis van de wet 'Verontreiniging Oppervlaktewateren' worden afgeschaft teneinde meer menskracht vrij te maken voor andere taken in het milieubeheer.

7. Zij die de invoering van referenda bepleiten om aktievoerders de wind uit de zeilen te nemen, houden er onvoldoende rekening mee dat vaak de rechtmatigheid van het overheidsbeleid wordt aangevochten en niet zozeer de steun van de bevolking voor dat beleid.

Couwenberg, S.W., Intermediair 29 mei 1981

8. Bij de recente diskussie over het al dan niet verbieden van politieke partijen met een racistische inslag zijn de partijen met een theokratische inslag ten onrechte buiten beschouwing gebleven.

Michiel van Mil
Actual and potential nitrogen fixation in pea and field bean as affected by combined nitrogen.
Wageningen, 6 november 1981

CONTENTS

VOORWOORD

Ieder onderzoek is afhankelijk van een samenwerking tussen degene die het uitvoert en vele anderen. In versterkte mate geldt dit voor een promotie-onderzoek. Het onderwerp is immers al in grote lijnen bepaald, de voornaamste methoden zijn al in huis en het geld komt ook ergens vandaan. Het spreekt dus eigenlijk vanzelf dat velen mij in woord en daad geholpen hebben.

Het projekt 'Stikstofbemesting van vlinderbloemige gewassen' waaraan ik vier jaar heb gewerkt, was een lang gekoesterde wensdroom van prof. Mulder. Zijn steeds weer nieuwe ideeën en kritisch kommentaar zorgden ervoor dat de vaart er niet uit ging.

Het welslagen van dit onderzoek is ook in belangrijke mate te danken aan Lie Tek-An die mij veel suggesties gegeven heeft, waaronder de goede greep in de stammenkollektie. Frank Houwaard, ex-lotgenoot in knollenland, heeft mij van begin tot einde op vele manieren bijgestaan. Voor adviezen en hulp met methoden kon ik altijd terecht bij Wim Roelofsen en Antoon Akkermans. Het ijverige en nauwgezette werken van Piet Dijksman heeft alle stikstof-analyses van dit proefschrift opgeleverd. De planten werden met toewijding verzorgd door de heren O. van Geffen, E. van Velsen en A. Houwers. Annie Mol-Rozenboom wiste alle ongewenste sporen van mijn arbeid uit, waarbij geen overstroming haar te veel was. De mooie plaatjes werden vervaardigd door de heren J. van Velzen en A.Wessels. De laatste schakel werd gevormd door mw. C. Möller-Mol die het meestal deerlijk toegetakelde manuscript op uitmuntende wijze wist om te toveren tot het boekje dat nu voor u ligt.

Graag wil ik iedereen die in de afgelopen jaren meegewerkt heeft van harte bedanken.

Tenslotte mag niet onvermeld blijven dat het onderzoek voor een aanzienlijk deel gefinancierd werd door het Landbouwkundig Bureau der Nederlandse Stikstof-meststoffen Industrie.

GENERAL INTRODUCTION

Grain legumes and protein production

The yield of grain legumes in the past decades has not taken advantage of modern agricultural growing practices, breeding and research like other important crops, for example cereals. Both in developed and in developing countries, the yield of grain legumes amounts to less than one-half of that of cereals (Fig. 1) (24). The low yields of grain legumes as compared with cereals can be explained by the differences in seed composition. Legume seeds contain more lipids and protein than cereal seeds, and consequently require a higher amount of photosynthesized carbohydrates for their synthesis (*e.g.* with groundnuts and barley 0.43 and 0.75 g of seed produced per g of carbohydrates, respectively) (72). The rates of yield increase are also different for the two groups of crops: in the past decade, yields of maize increased by 15%, whereas those of beans (*Phaseolus* spp) rose only by 3% (92). However, with the economically more attractive soybeans and groundnuts, yield increases amounted to 20 and 37%, respectively, indicating that grain legumes may respond to research, breeding and improved management.

The area used for growing grain legumes has decreased by 21% in the past decade in developed countries, whereas in developing countries this area (excluding that with soybeans and groundnuts) has risen at an equal rate as the total arable land (approx. 10%). The soybean area in developing countries increased by 55% in this period (FAO production yearbooks).

The reduced interest in grain legumes in developed countries is coupled with an increased consumption of animal protein, as can be seen in Table 1 from the decreasing ratio of consumed to consumable protein. This ratio is strongly dependent on income per capita, a high income leading to a high protein consumption as well as a substitution of animal for vegetable protein (29). In developing countries, population is growing but also agricultural exports are increasing by allocating more land to cash crops. This tends to a change of the type

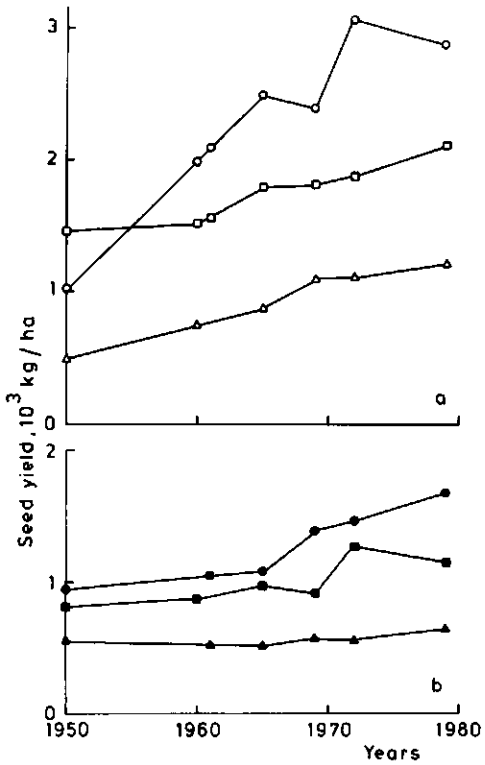

Fig. 1a,b. Seed production of cereals (o, •), pulses (excluding soybeans and peanuts) (Δ, ▲) and soybeans (□ ,■) in developed countries (o, Δ,□) and developing countries (•, ▲,■). Source: FAO Production Yearbooks.

of crops grown for local food, the high-yielding cereals replacing the low-yielding legumes (92). The resulting diet may contain sufficient carbohydrates for the daily energy requirement, but it is too low in protein. In general, the ratio $(\frac{W}{W})$ of cereals to legumes in the diet of developing countries is 9, whereas it should be 7 to 5 to prevent malnutrition (10, 92). Thus, a decreasing interest in grain legumes, occurring in developed countries, is a symptom of an affluent society, whereas in developing countries it is a sign of a low standard of living.

The agricultural pattern in The Netherlands shows the characteristics of an affluent society. Together with the build-up of an extensive system of dairy and meat production, the area of grain legumes shrank considerably, *e.g.* that

Table 1. Production and consumption of protein in 1965. Source: (29)

	Production of consumable protein [1]	Consumption of consumable protein [1]	Consumed protein [2]	Ratio of consumed to consumable protein
	(kg/capita. year)			
North America	152	104	35	0.34
Western Europe	49	81	33	0.41
Developed countries (total)	76	80	34	0.42
Latin America	47	43	24	0.57
South-East Asia	24	26	19	0.73
Africa	29	26	22	0.85
Developing countries (total)	29	29	21	0.72
Income class ($)[3]				
< 100	24	24	19	0.80
100-200	26	27	20	0.73
200-400	32	32	22	0.69
400-800	52	49	29	0.59
800-1600	57	70	33	0.47
> 1600	106	98	35	0.35

[1] Protein in vegetable products, fit for human consumption plus protein in animal products from vegetable fodder not fit for human consumption.

[2] From vegetable and animal origin.

[3] Countries classified according to average income per capita. year in 1965 based upon data of 85 countries.

of green peas grown for dry-seed production from 30000 ha in 1960 to 2400 ha in 1979. Typically, the area of peas grown for the canning industry increased slowly, from 5000 ha in 1960 to 5400 ha in 1980, indicating that a low-yielding crop grown for luxury consumption is less affected when income rises. Field beans have almost disappeared as a food crop, but recently, growers have resumed interest, now as a feed crop for silage (source: Landbouwcijfers 1980).

Agricultural production in the Netherlands has greatly benefitted by the increased use of nitrogen fertilizers (97 kg N per ha in 1960, 216 kg N per ha in 1977, values that are higher than those of other developed countries). However, with increasing energy prices agricultural practices that use lower levels of combined nitrogen than at present are likely to be propagated. This might well lead to a renewed attention to legume crops in developed countries. In developing countries, the use of grain legumes in food production will probably also depend on improved yields of these crops as compared with cereals.

Nitrogen fertilizer use has been of crucial importance to the enhanced productivity of cereals. One reason for the fact that yields of legume crops lag behind those of cereals is that in general they do not respond to added fertilizer nitrogen. Combined nitrogen interferes with the biological nitrogen fixation process of the legume-*Rhizobium* symbiosis. Legume crops seem to be close to a 'yield barrier' resulting from the limits of photosynthetic capacity and of the capacity of nitrogen supply of the symbiotic system. Both aspects will have to be investigated when attempting to surmount this barrier. Therefore, in this introduction the data available from the present literature will be briefly summarized with regard to the nitrogen and carbon limitations to seed production of grain legumes.

Nitrogen limitation and combined-nitrogen dressings

If the growth of legumes, capable of fixing atmospheric nitrogen by means of root nodules, would always be nitrogen-limited, consistent yield increases would be obtained by the supply of combined nitrogen. However, the data available from literature on this topic are often conflicting.

Suboptimum symbioses Dressings of combined nitrogen nearly always enhance yield of legumes growing under circumstances unfavourable for the establishment of a fully effective, nitrogen-fixing symbiosis.

The formation of root nodules by *Rhizobium* strains does not occur at a low soil pH, the value of which is different for each host species (53), while also the rhizobial strains may vary in acid tolerance (39, 40). In these cases, yield of legumes is increased by nitrogen fertilizer dressings (9, 18). Also, a deficiency of trace elements like B (50) or Mo (52) prevents the development of a fully effective symbiosis. The addition of combined nitrogen can partly overcome the resulting nitrogen limitation to growth.

The nitrogen-fixing capacity of the symbiosis might also be impaired by infestation with plagues and diseases. Combined nitrogen was found to increase seed yield of pea plants when the root nodules were invaded by *Sitona* larvae (50), when the plants were attacked by *Fusarium* (50) or by insects. Lodging of the plants cannot be alleviated by combined nitrogen but is even aggravated at high dressings (51).

Legume seed yields can also be decreased if *Rhizobium* strains are present in the soil which do not establish a fully effective symbiosis with the host plant. *Rhizobium* strains that produce ineffective nodules (without nitrogen fixation) or moderately effective nodules (with low levels of nitrogen fixation)

considerably reduce the yield of plant nitrogen and seed as compared with strains which produce highly effective nodules with a specific host plant. Nitrogen fertilizer dressings may improve yield of plants infected by *Rhizobium* strains of poor nitrogen-fixing capacity (27).

Apart from fertilizer dressings, inoculation of the sowing-seed with a highly effective strain can produce good results if few indigenous rhizobia are present in the soil (11, 37). However, typical problems may be encountered with inoculant strains regarding:(a) competition with the indigenous rhizobia for nodulation sites on the legume roots (42); (b) survival in the soil as free-living bacteria (11, 55); (c) a narrow host-plant range of the inoculant strain leading to an ineffective symbiosis when a different host species of the same cross-inoculation group is grown at the inoculated plot (4).

The introduction of new plant varieties to areas outside the region where they originated may also result in symbioses possessing a poor nitrogen-fixing capacity with the locally occurring rhizobia (17). Host plants may require a specific *Rhizobium* strain for an effective symbiosis, whereas other *Rhizobium* strains produce ineffective nodules (41, 42) or inhibit nodule formation by other strains without producing nodules themselves (43). Nitrogen dressings are likely to increase yield in all these cases, although no reports are available from field experiments.

Optimum symbioses It is hardly surprising that yield increases are obtained by the supply of combined nitrogen to suboptimum symbioses. To a certain extent, it is a consequence of the definition of 'suboptimum', although always specific factors can be earmarked as being responsible for the low nitrogen gain of the symbiosis. Therefore, a more interesting problem, at least from a theoretical point of view, concerns the question whether also the yield of a fully effective symbiosis with a high rate of nitrogen fixation would respond to combined nitrogen. With regard to field experiments many conflicting reports can be found in the literature, which is not exhaustively treated here.

No enhancement of seed yield was found in trials with combined nitrogen dressings of pea (21, 50); soybean (1, 36, 81, 85, 87) and field bean (*Vicia* sp.; 15, 28, 63). But yield increases were met in other experiments, *e.g.* with soybean (15, 16, 36); cowpea (14); pea (15, 75); field beans (*Vicia* sp.; 65) and beans (*Phaseolus* sp.; 79).

In growth chamber and greenhouse experiments, uninoculated legume plants supplied with combined nitrogen from sowing consistently show a higher dry matter

production than plants inoculated with an effective *Rhizobium* strain but without added fertilizer. This phenomenon has been attributed to higher energy costs for the fixation of atmospheric nitrogen than for assimilation of combined nitrogen. It may be true of plants growing with NH_4^+-N, but it has not convincingly been established that growth with NO_3^--N would require much less energy than symbiotic growth with atmospheric N_2 (equal costs: 22, 49; lower costs of NO_3^--N assimilation than of N_2 fixation: 45, 57, 67, 71). The problem is complicated by the fact that a considerable part of the nitrate taken up is reduced to ammonia in the leaves, using reducing equivalents derived from photosynthetic activity, which thus decrease the respiratory costs of nitrate reduction (3, 84). On the other hand, a high carbohydrate requirement of nitrogen-fixing roots might provide a stronger sink for photoassimilates leading to an increased photosynthesis as more carbohydrates can be exported from the leaves (44, 66).

The growth rates of nitrogen-fixing plants are usually lower than those of plants growing with combined nitrogen from sowing. Seedlings grown without added nitrogenous fertilizer face a period of 'nitrogen hunger' before the infection process and nodule formation start, whereas seedlings growing with combined nitrogen maintain a constantly high growth rate (19, 69). Inoculated seedlings that are supplied with a low level of combined nitrogen (not interfering with nodule formation) produce a higher shoot mass and leaf area which are capable of supporting a higher nitrogen-fixing activity than inoculated seedlings without added nitrogen (6). This 'starter dose' effect is often aimed at in agricultural practice (13, 64), but results can only be detected if both the naturally available nitrogen level of the soil and the residual nitrogen from the preceding crop are low.

Physiological effect of combined nitrogen on nitrogen fixation

Combined nitrogen exerts a detrimental effect on symbiotic nitrogen fixation, as was already found in the beginning of nitrogen fixation research (48, 62, 64, 86). However, the exact way in which combined nitrogen suppresses both the formation of new nodules and the activity of existing nodules, has evoked a good deal of controversy.

Nodule formation The process of nodule formation by invasion of root hairs by free-living rhizobia has been described in detail (38). During this complicated process, *Rhizobium* bacteria enter the root *via* an infection thread, induce cell divisions in the root cortex, and are normally transformed into large, non-motile,

and often branched bacteroids, capable of fixing nitrogen. The inhibition of the initiation and growth of nodules by combined nitrogen was shown to be a local effect in a series of experiments with split-root systems of which one half was kept nitrogen-free and the other half was supplied with nitrate (90).

The hypothesis that nitrate inhibition of nodule formation would be exerted *via* the production of nitrite has to be discarded as this does not explain the similar effect of ammonium ions (23, 47). Also the theory that the C/N ratio in the plant determined the extent of nodule formation has to be amplified as it offers no sufficient physiological interpretation. A relationship between carbohydrate supply and the effect of combined nitrogen on nodule formation is apparent (61). The restraint of nodule formation by combined nitrogen was counteracted by the addition of carbohydrate compounds (91).

Plant growth regulators may play an important part in nodule formation. The addition of nitrate to legume seedlings decreases root hair curling and nodule initiation by lowering the level of the auxin indolyl-acetic acid (IAA)(80). This adverse effect of nitrate could be reversed by external supply of IAA (54, 82). Furthermore, cytokinins might be involved in the interaction between host plant and rhizobial microsymbiont, as free-living rhizobia were shown to excrete sufficiently large quantities of cytokinins to induce cell divisions in a soybean-callus test (59). Phytohormone activity has been demonstrated both in nodule formation with actinomycetal symbioses (88) and in rhizobial symbioses (26, 77, 78) although no clear-cut solution was offered to the problem of external influences (*e.g.* combined nitrogen) on the process of infection and nodule growth. However, nodules contain cytokinins and auxins in concentrations well above those found in the adjacent root tissue (26), similar to root tips (70). This indicates that further research into the role of phytohormones in nodule formation is necessary to elucidate the effect of combined nitrogen on nodule formation.

Nodule activity The decline of nitrogenase activity upon the supply of combined nitrogen to already nodulated roots is not due to an accumulation of amino acids in the nodules (32) or to a direct inhibition of nitrogenase by added ammonium ions (30) as bacteroids do not take up ammonium ions (33). In medium-term experiments with nodulated pea plants the nitrogenase content of the bacteroids was not affected by combined nitrogen; nitrogenase could be re-activated by supplying the bacteroids with ATP and reducing equivalents (30). However, leghemoglobin synthesis was strongly repressed resulting in the

colour of the nodules turning from pink to green (8). The adverse effect of combined nitrogen on nitrogen fixation of nodulated pea plants could be counteracted by a high light intensity or by added sucrose (31).

In experiments with shoots of leguminous plants exposed to $^{14}CO_2$, addition of combined nitrogen evoked a change in the translocation pattern of photosynthates from the shoot to the roots, resulting in a lower proportion of carbohydrates supplied to the nodules and a higher to other parts of the root system (34, 74). This is consistent with the involvement of plant growth regulators as discussed above. The addition of combined nitrogen enhanced the cytokinin levels of root tips (68, 83) and probably increased photosynthate supply to these tips which would in turn lead to a lower supply of carbohydrates to the nodules.

Carbohydrate limitation to nitrogen fixation

As the photosynthate supply of the nodules seems to be of crucial importance for the explanation of the effect of combined nitrogen on nitrogen fixation, it is worthwhile to subject the relation between photosynthesis and nitrogen fixation to a closer examination. The fact that optimum legume-*Rhizobium* symbioses do not respond to nitrogen fertilizer dressings in an unequivocal way, points to the possibility of a photosynthate-limited growth during the entire growth cycle or part of it. Actually, carbon dioxide enrichment of the atmosphere resulted in a strongly increased nitrogen fixation and yield (25, 58). Furthermore, source-sink manipulations including defoliation and shading reduced nitrogen fixation (5, 12, 35, 60). The same effect occurred when light intensity was low, whereas an increased light intensity raised nitrogen fixation (2, 7, 46) as did grafting of two shoots on a single root (76).

However, the 'nitrogen-hunger' period during the early phases of seedling growth was prolonged and growth was retarded by a high light intensity because the nitrogen deficiency was aggravated (20, 56, 89). Later on, the plants grown at high light intensity showed a higher dry matter production as well as a higher nitrogen fixation than plants grown at a lower light intensity (89). Also in the generative growth phase of legumes, an enhancement of photosynthesis might eventually result in a lower seed yield. A high rate of photosynthesis would accelerate seed production but also increase the demand for nitrogen, which would lead to a mobilization of nitrogenous compounds from vegetative tissues for translocation to the seeds. Thus, photosynthesis would be reduced soon, in turn lowering nitrogen fixation and ending up in 'self-destruction' of the plant (72).

Outline of the investigations

The investigations reported in this paper are focused on the question whether growth and yield of legume-*Rhizobium* symbioses are determined by nitrogen or carbohydrate limitation, with emphasis on the former. The study was carried out with *Rhizobium* strains selected for differences in nitrogen-fixing capacity, and with host species and cultivars with different seed-production rates and photosynthetic capacities.

In Chapters 1 and 2, the *in vitro* nitrogenase activity of isolated bacteroids was compared with the *in vivo* nitrogenase activity of nodulated pea and field bean plants in order to find out whether nitrogen fixation in these plants was limited by an intrinsic factor of the bacteria or by external factors, such as photosynthate supply. Chapter 3 reports on the efficiency of energy-yielding respiratory processes in the root system in relation to the nitrogen source (atmospheric or combined nitrogen). Chapter 4 deals with a study on nitrogen fixation in a symbiosis containing a bacterial uptake hydrogenase, capable of recirculating the hydrogen produced by nitrogenase concomitantly with nitrogen fixation. In Chapter 5, nodule formation and nitrogen-fixing capacity of a number of *R. leguminosarum* strains in association with pea plants were described in detail. Finally, Chapter 6 contains a study on the influence of combined nitrogen on nitrogen fixation of symbioses of pea plants with the *R. leguminosarum* strains studied in Chapter 5. A tentative model was presented for the interactions between nitrogen fixation and photosynthesis.

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1 ACTUAL AND POTENTIAL NITROGEN FIXATION IN *PISUM SATIVUM* L. AND *VICIA FABA* L.

ABSTRACT

In this study, actual nitrogen fixation of pea (*Pisum sativum* L.) and field bean (*Vicia faba* L,), measured as the *in vivo* acetylene reduction of nodulated roots, was compared with potential nitrogen fixation, measured as the *in vitro* acetylene reduction of isolated bacteroids of *Rhizobium leguminosarum*. Actual nitrogen fixation of pea and field bean growing in jars or in the field equalled potential nitrogen fixation during vegetative growth. In the generative phase, however, the actual nitrogen fixation was much lower than the potential nitrogen fixation.

In field beans supplied with nitrate at sowing, values of actual and potential nitrogen fixation were much lower than in those of control plants until pod formation. Actual nitrogen fixation of nitrate-grown plants increased slowly due to a gradual formation of nodules on lateral roots. Specific nitrogenase activities of these nodules were twice as high as compared to nodules on primary roots. From pod formation, potential nitrogen fixation of nitrate-grown plants became equal to that of plants grown without added nitrate. A second nitrate dressing at mid-pod fill causes a ready drop of the actual nitrogen fixation. However, potential nitrogen fixation remained constant for ten days before collapsing.

During growth of pea and field bean, nodule mass increased whereas the available nitrogenase was only partly utilized. Therefore, in the long run, the amount of nitrogen fixed seems to be regulated by nodule mass rather than by nodule activity.

From the data presented in this paper it is concluded that in pea and field bean, vegetative growth is nitrogen-limited, whereas during the generative stage nitrogen fixation is limited by an inadequate carbohydrate supply.

INTRODUCTION

The question if growth of leguminous crops is restrained by carbon or nitrogen limitation has since long attracted the attention of many scientists (*e.g.* Wilson, 17). In the present paper, attention is focused on the nitrogen-fixing capacity of the legume-*Rhizobium* symbiosis, in particular on the question whether this capacity is restricted by the amount of nitrogenase in the plant or by other factors, such as the energy supply of the enzyme.

Quantitative data on nitrogen fixation by legume-*Rhizobium* symbioses are usually derived from momentary acetylene-reducing values of the root system or from long-term changes in nitrogen content of the plant material. In this study, the *in vivo* acetylene reduction by nodulated roots (actual nitrogen fixation) is compared with the *in vitro* acetylene reduction by isolated bacteroids (potential nitrogen fixation). In the latter case ATP and reducing power are supplied to bacteroids treated with EDTA-toluene (15) to ensure maximum nitrogenase activity. This *in vitro* acetylene-reducing technique was used earlier (Houwaard,8, 9) to assess the influence of ammonia on nitrogen fixation by bacteroids of *Rhizobium leguminosarum* in symbiosis with pea plants. Upon the addition of ammonia to pea plants, the *in vitro* nitrogenase activity of the bacteroids remained unchanged whereas the *in vivo* activity declined, presumably due to a shortage of photoassimilates in the nodules.

In the present investigation the *in vitro* method was used to determine whether during the growth cycle of leguminous plants the potential nitrogen fixation differs from the actual nitrogen fixation. Plants used for these tests were grown under optimum conditions comparable to those of plants cultivated in agricultural practice, *i.e.* in soil and in open air. In such plants the influence of combined nitrogen on actual and potential nitrogen fixation has been studied.

MATERIALS AND METHODS

Plant material and growth

Enamelled Mitscherlich jars of 6 liter capacity, sterilized with alcohol, were filled with a mixture ($\frac{w}{w}$) of 1 part of heat-sterilized sand and 2 parts of non-sterile clay. In preliminary experiments the clay soil, obtained from newly reclaimed polder land, had been shown to have a very low population

of indigenous *R. leguminosarum*. The effective strains PRE and RB1 of *R. leguminosarum* were used for peas and field beans, respectively, and the ineffective strain P8 for both peas and field beans. All strains were obtained from the culture collection of our laboratory. For inoculation, 50 ml of a 7-days old culture was mixed with 6 liter of soil. A basal dressing of 540 mg of P_2O_5 and 900 mg of K_2O was applied per pot.

Seeds of pea (*Pisum sativum* L., cultivar Rondo) and field bean (*Vicia faba* L., cultivar Minica) were surface-sterilized by immersion in a solution containing 4% of H_2O_2 and a few drops of Teepol (detergent) for 25 min before sowing. The soil was then covered with sterilized gravel to prevent clogging after watering. Plants were kept in the open air under wire for protection against birds. In each jar ultimately 8 pea plants or 3 field bean plants were retained.

In a separate experiment, field bean seeds were inoculated with *R. leguminosarum*, strain RB1, and sown in a field plot in rows of 45 cm. A basal dressing of 18 g of P_2O_5 and 29 g of K_2O was applied per m^2.

Hydrogen production and acetylene reduction by nodulated roots

The shoots of the plants were detached and the roots freed from soil particles by gentle shaking. The whole root system, or in some cases the primary and the lateral roots separately, were incubated in stoppered 1-liter Erlenmeyer flasks. After 25 min, a 100-µl gas sample was assayed for hydrogen, using a thermal conductivity detector. Subsequently, 100 ml of acetylene was added and a 100-µl gas sample was taken after 15 min. Ethylene production was determined with a gas chromatograph equipped with a hydrogen flame detector. Rates of hydrogen production and acetylene reduction were linear with time up to 80 min.

Nitrogenase determinations in bacteroids

In vitro nitrogenase activity of bacteroids was determined with the EDTA-toluene method (EDTA = ethylenediaminetetra-acetic acid) according to van Straten and Roelofsen (15). After completion of the *in vivo* assay, roots were washed in tap water and the nodules carefully collected. These nodules were squeezed in a Bergersen press (2) under argon at 0°C in a buffer solution containing TRIS (50 mM) (TRIS = tris(hydroxymethyl)aminomethane), $MgCl_2$ (2.5 mM), 4% ($\frac{w}{v}$) PVP (PVP = polyvinylpyrrolidone) and $Na_2S_2O_4$ (20 mM). The pH was adjusted to 7.2 with HCl. Bacteroids were centrifuged for 10 min at 5500 x g under argon, washed with a buffer solution (same buffer without PVP),

centrifuged again, and resuspended in the buffer solution without PVP (1 ml of buffer per 80 mg of nodule fresh weight).

Nitrogenase activity of the bacteroids was restored by regeneration at 25°C for 30 min. Subsequently, 1 ml of buffer containing 1 µmole of EDTA and 3 drops of toluene was added to 1 ml of bacteroid suspension, and the mixture vigorously shaken for 1 min. After 1 min of settlement, a 0.5-ml aliquot of the aqueous layer was transferred to a 16.5-ml Hungate tube finally containing 50 µmoles of Tris-HCl, 15 µmoles of $MgCl_2$, 18.4 µmoles of creatine phosphate, 5.6 µmoles of adenosine-5-triphosphate (disodium salt), 30 µg of creatine kinase and 20 µmoles of $Na_2S_2O_4$, in a volume of 1.0 ml, pH 7.2. The gas phase consisted of 10% acetylene in argon. Ethylene production was measured by flame detection after 10 min incubation on a shaker bath of 25°C, 200 strokes per min.

RESULTS

Nitrogenase activity during ontogenesis

Profiles of actual and potential nitrogen fixation by field beans and peas growing in jars during ontogenesis are shown in Fig. 1. In field beans, there was a peak in nitrogenase activity coinciding with the start of the pod-filling stage. No such a pattern was observed in peas. Both in field beans and in peas, potential nitrogen fixation was equal to the actual fixation during the vegetative stage. Starting from flowering in peas and from pod formation in field beans, the potential nitrogen fixation became significantly higher than the actual fixation in a one-sided t-test at the 0.001 level (14).

Actual nitrogenase activity per plant in field beans was about five times higher than in peas. The difference was mainly due to the higher nodule weight of the field bean-*Rhizobium* symbiosis (Fig. 2a), resulting from the higher plant weight of field beans. The amount of bacteroid protein in field bean nodules decreased during the growth season from 90 to 40 mg per g of nodule fresh weight (Fig. 2b). Pea nodules retained a constant level of about 40 mg of bacteroid protein per g of nodule fresh weight (Fig. 2b).

Based upon the data of Figs. 1, 2a and 2b, the specific activity of nitrogenase was calculated (Fig. 2c). In pea plants, the decreasing specific

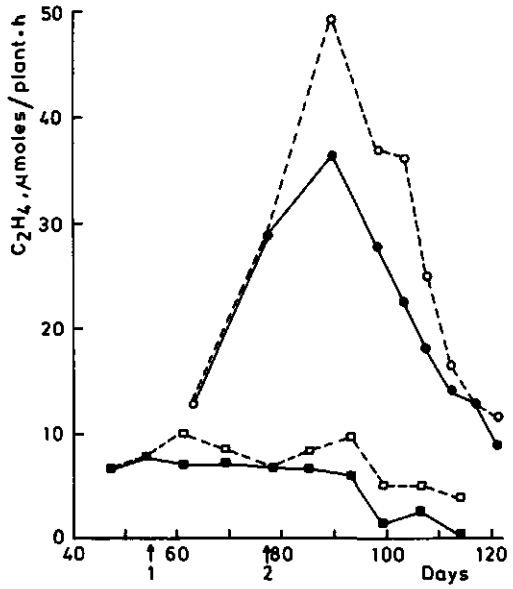

Fig. 1. Actual (■, ●) and potential (□, ○) nitrogenase activity of pea (■,□) and field bean (●, ○) during ontogenesis. Plotted values of actual nitrogen fixation are means of 2 sets of 8 plants each (pea) and of 2 sets of 3 plants each (field bean). Values of potential nitrogenase activity per plant were calculated from nodule fresh weight of 3 plants and triplicate nitrogenase determinations in bacteroids obtained from a known quantity of nodules. Arrow 1 indicates the start of flowering in pea and arrow 2 the start of pod formation in field bean.

activity was compensated by an increasing nodule mass, resulting in a constant level of *in vivo* nitrogenase activity during the growth season (Fig. 1). In contrast, the peak of nitrogenase activity per plant in field beans was due to a rise of specific activity as well as to an increased nodular mass.

Effect of combined nitrogen

To study the effect of combined nitrogen on actual and potential nitrogen fixation, field beans growing in jars were given 1130 mg of N as $NaNO_3$ both at sowing and at mid-pod fill (Fig. 3). Actual and potential nitrogen fixation of nitrate-grown field bean plants were much lower than those of plants grown without added nitrate, *e.g.*, at day 62 1.2 and 12.4 µmoles of C_2H_2 reduced/plant.h (Figs. 3 and 1, respectively). From the beginning of pod formation at

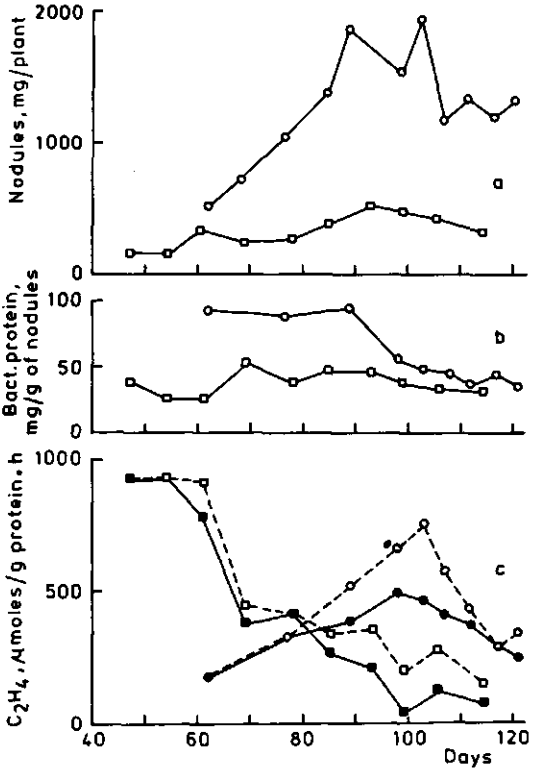

Fig. 2 (a). Nodules, fresh weight, mg/plant. Pea (□), field bean (o); means of duplicate determinations.
(b). Bacteroidal protein per unit of fresh weight nodules. Means of duplicate determinations of pea (□) and field bean (o).
(c). Nitrogenase, specific activities, µmoles C_2H_4/g of bacteroidal protein.h; mean values of triplicate determinations, of pea (*in vivo* ■, *in vitro* □) and field bean (*in vivo* ●, *in vitro* o). For start of flowering (pea) and of pod fill (field bean) see Fig. 1.

day 77, potential nitrogen fixation of nitrate-grown plants approached that of control plants. Actual nitrogen fixation of nitrate-grown plants rose at a lower rate, so that the deviation from the potential nitrogen fixation became more pronounced than in the control plants. For example, at day 89 actual nitrogen fixation amounted to 29.2 µmoles of C_2H_2 reduced/plant.h as compared to 36.3 µmoles of C_2H_2 reduced/plant.h in control plants, whereas potential nitrogen fixation values were 48.8 µmoles of C_2H_2 reduced/plant.h in both cases (Figs. 1 and 3).

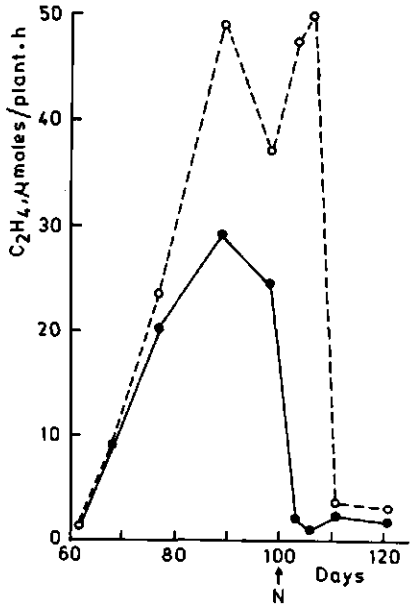

Fig. 3. Actual (•) and potential (o) nitrogenase activity of field beans supplied with 1130 mg N as $NaNO_3$ at sowing and at mid-pod fill (arrow). For sample sizes see legend of Fig. 1.

The second nitrate dressing at mid-pod fill drastically lowered the actual nitrogen fixation (Fig. 3) but left the potential fixation unaffected for 10 days. After that, an almost complete loss of activity took place within 4 days.

Location of nitrogenase activity

Addition of nitrate at sowing delayed nodule formation by field beans, as demonstrated in Table 1. The morphology of the root systems of field bean plants, which possess a well-developed tap root, enabled us to differentiate between nodules on primary and lateral roots. Owing to the delay in nodule formation, significantly more nodules were formed on lateral roots of nitrate-grown plants as compared to plants grown without nitrate ($p < 0.05$). This resulted in a considerably larger contribution of the activity of nodules on lateral roots to total nitrogenase activity in nitrate-grown plants than found in control plants without added nitrate ($p < 0.01$). Acetylene-reducing activity of lateral-root nodules, when calculated per unit of nodule fresh weight, was higher than

Table 1. Actual nitrogenase activities of nodules from primary and lateral roots of field beans, as affected by the addition of nitrate at sowing [a]

Time (days after sowing)	Nodules on primary roots		Nodules on lateral roots		Contribution of nodules on lateral roots to total nitrogenase activity (%)
	Fresh wt. (mg/plant)	Nitrogenase activity [b]	Fresh wt. (mg/plant)	Nitrogenase activity [b]	
Without nitrate					
62	515	14.9	0	-	0
75	881	18.4	36	36.9	8
77	973	26.5	85	35.5	10
89	1570	19.9	306	16.5	14
98	1380	17.3	139	28.0	14
With nitrate					
62	0	-	78	16.3	100
75	235	12.4	222	12.4	49
77	407	22.2	353	32.1	56
89	295	20.3	776	29.9	79
98	125	17.5	995	22.4	91

[a] Data represent means of duplicate measurements. For statistical inference see text.

[b] µmoles of C_2H_4/g of nodules fresh weight.h.

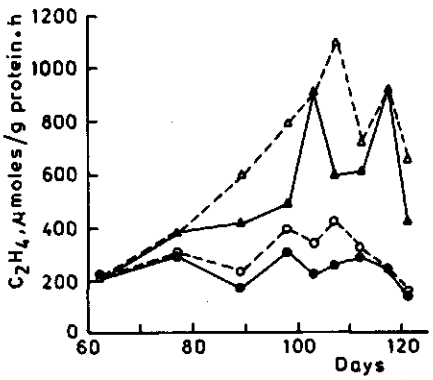

Fig. 4. *In vivo* (●, ▲) and *in vitro* (o, Δ) specific activities of nitrogenase of bacteroids from primary (●, o) and lateral (▲, Δ) root nodules of field bean plants grown without added nitrate. Values represent means of duplicate determinations.

Table 2. Nitrogenase activity in pea and field bean during the entire growth period

	Nitrogenase activity							
	Actual (*in vivo*)				Potential (*in vitro*)			
	Production of:		Fixation of:		Production of:	Fixation of:	Actual as % of potential N_2 fixation	
	C_2H_4 (mmoles/ plant)	H_2	N_2 (mg/ plant)	N_2	C_2H_4 (mmoles/ plant)	N_2 (mg/ plant)		
	1	2	3	4	5	6	7	8
Experiment I								
Pea	11	4	106	65	14	132	81	50
Field bean	40	15	374	235	48	447	84	53
Field bean grown with NO_3^-	18	4	167	134	35	324	51	41
Experiment II								
Field bean (jar)	24	11	227	127	37	346	66	37
Field bean (field)	31	13	291	174	45	416	70	42

1) In air + 10% C_2H_2
2) In air
3) Calculated from 1
4) Calculated from 1 minus 2
6) Calculated from 5
7) Values of 3 as % of those of 6
8) Values of 4 as % of those of 6

that of primary-root nodules in an F test at the 0.05 level (Table 1). When the nitrogenase activities were expressed per unit of bacteroid protein, differences between nodules of primary and lateral roots were more significant ($p < 0.01$) (Fig. 4). Specific nitrogenase activity of nodules from lateral roots was often 50-100% higher than that of primary-root nodules.

Actual and potential nitrogen fixation

Based upon the momentary data of nitrogenase activity, as presented above, the amounts of nitrogen fixed during the entire growth period were calculated (Table 2). Under the conditions of the *in vivo* nitrogenase assay with 10% of acetylene in air, acetylene reduction represents the entire nitrogenase activity. However, in air, in the absence of acetylene, a fraction of the

total electron flow through nitrogenase is not used in nitrogen fixation but gives rise to the production of hydrogen. Therefore, in the calculations of nitrogen fixation, ethylene production was corrected for hydrogen evolved in air. A stoichiometrical factor of 3.0 was used to convert acetylene reduction into nitrogen fixation. For simplicity, nitrogen fixation was assumed to continue at the same level for 24 h a day.

Actual nitrogen fixation amounted to 51-84% of the potential nitrogen fixation in the cases studied. When the distribution of energy among hydrogen production and nitrogen reduction occurring *in vivo* was taken into account, the actual nitrogen fixation even declined to 37-53% of the potential nitrogen fixation (Table 2).

The data on nitrogen fixation discussed above, were obtained from plants growing in jars. In a separate experiment field-grown field bean plants were tested for actual and potential nitrogen fixation (Table 2). In general, field-grown plants produced more dry matter (not shown) and possessed a higher level of nitrogen fixation than plants growing in jars. The ratio of potential to actual nitrogen fixation, however, was equal in both cases.

DISCUSSION

Actual and potential nitrogen fixation

The central theme of this paper is the distinction between actual and potential nitrogen fixation, as calculated from *in vivo* and *in vitro* nitrogenase activities, respectively. The limitations of the *in vivo* acetylene reduction assay of nodulated roots have been extensively discussed by various authors (5, 10). Therefore, this discussion is restricted to the *in vitro* method.

A major disadvantage of the EDTA-toluene technique of assaying *in vitro* nitrogenase activity of isolated bacteroids is its sensitivity to oxygen. Even traces of oxygen irreversibly lower nitrogenase activity. Removing the plants from the soil might have damaged the nodules.

Explaining the difference between *in vivo* and *in vitro* nitrogenase activity as a result of scratching to the nodules is unlikely because oxygen probably decreases both values of the same degree.

The conditions of the EDTA-toluene method have been carefully optimized (15).

Nevertheless, the question may be raised whether the *in vitro* activity measured in this way really is the maximum activity, or that maximum activity is even higher, as it was recently shown that electron donors to nitrogenase may differ in efficiency (16). However, our *in vitro* method was adequate to demonstrate that starting from flowering, an excess amount of nitrogenase is present, presumably not utilized in nitrogen fixation (Figs. 1 and 2c).

To date, indirect evidence for an excess of nitrogenase in root nodules has been derived from Arrhenius plots of nitrogenase activity versus temperature (5, 6). Our direct nitrogenase determinations corroborate this conclusion for plants in the generative growth phase only. The assumption that below 20°C nitrogenase content was limiting nitrogen fixation (6) was not substantiated in the present study in which field beans growing in jars were compared with those growing in the field. In jars, soil temperatures were almost equal to ambient air temperature (15-30°C), while in the field at 10 cm depth, soil temperatures ranged from 13-18°C. However, in jar-grown as well as in field-grown plants, potential nitrogen fixation was higher than actual nitrogen fixation (Table 2).

Addition of nitrate to field beans with full-grown nodules at mid-pod fill induced an immediate drop of *in vivo* nitrogenase activity, but the *in vitro* activity remained unchanged, conformable to the results of Houwaard (8) with added ammonium chloride to peas. Finally, the *in vitro* nitrogenase activity collapsed within 4 days (Fig. 3), in accordance with Bisseling *et al.* (3). To their estimates, nitrogenase turn-over in *R. leguminosarum* bacteroids amounts to 50% within 2 days.

Nodule mass and activity

Dry matter production in pea plants was about five times lower than that in field beans (data not shown). This difference was also observed in nodule fresh weight of these plants and in nitrogenase activity per plant (Figs.1, 2a).

Addition of nitrate at sowing mainly reduces nodule mass and not nitrogenase activity of the nodules (Table 1). Some authors have found that an enhancement of photosynthesis by increasing light intensity or atmospheric carbon dioxide concentration initially increased the nitrogenase activity of the nodules. However, after several days of treatment, a rise of the nodular mass accounted for the major part of the increased nitrogen fixation (1, 11, 12). Thus, in the long run, nitrogen fixation seems to be regulated by nodule mass rather than by nodule activity. Nodule formation and growth even continue when energy supply to the nodules is insufficient for a full utilization of nitrogenase (Figs. 2a,c).

The specific (*in vitro*) nitrogenase activities of nodules on primary and lateral roots show pronounced differences (Fig. 4). Van den Bos *et al.* (4) demonstrated that the *in vitro* nitrogenase activity of mature bacteroids decreased with age. In the present experiment, lateral-root nodules may have contained young bacteroids of a higher degree of nitrogen-fixing activity as compared to the bacteroids of primary-root nodules, which would account for the higher *in vivo* and *in vitro* activity of lateral-root nodules as compared with primary-root nodules.

Nitrogen or carbohydrate limitation

The *in vitro* method of determining nitrogenase activity in bacteroids is based upon an optimized supply of ATP and reducing equivalents (15). Therefore, if the *in vitro* activity is higher than the *in vivo* activity, the difference may be interpreted in terms of an energy shortage of the nodules. This view is corroborated by comparing the present results with literature data. During the generative stage of growth, flower primordia and developing pods act as carbohydrate sinks, reducing the translocation of photoassimilates to the root nodules (7). In the present experiments, the potential nitrogen fixation deviated from the actual fixation from the onset of flowering (pea) or pod fill (field bean) (arrows 1 and 2, respectively, of Fig. 1). Addition of nitrate also causes a decline of photosynthate translocation to the nodules (13). Concomitantly, the ratio of the actual to the potential nitrogen fixation is lower in nitrate-grown plants than in plants growing without combined nitrogen (Fig. 3).

If our interpretation of the physiological significance of potential nitrogen fixation is correct, then leguminous plants are growing under nitrogen-limitation during the vegetative stage. During the generative stage nitrogen fixation is limited by carbohydrate supply leading to excess of nitrogenase in the nodules. During the growth cycle of the legumes the average nitrogenase utilization amounted to 66 - 81% in the absence of combined nitrogen (Table 2). Therefore, in order to increase yields of legume-*Rhizobium* symbioses, efforts should probably be directed to the utilization of the entire potential nitrogen fixation by the enhancement of photosynthesis, rather than by creating *Rhizobium* strains of a higher nitrogen-fixing capacity.

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2 ACTUAL AND POTENTIAL NITROGEN FIXATION BY PEA ROOT NODULES AS AFFECTED BY CYTOKININ AND ETHYLENE

ABSTRACT

In this paper, the *in vivo* nitrogenase activity (actual nitrogen fixation) of nodulated pea roots (*Pisum sativum* L.) has been compared with the *in vitro* nitrogenase activity (potential nitrogen fixation) of bacteroids of two strains of *Rhizobium leguminosarum*, one with and one without hydrogenase. The actual nitrogen fixation of nodules of 28 days old plants, kept in a growth chamber, amounted to 77-82% of the potential nitrogen fixation. Treatment of the nodules or the lower leaves with benzyladenine, a synthetic cytokinin, raised the nitrogenase activity of pea plants to 100% of the potential value. Ethephon, an ethylene-releasing compound, raised the nitrogen fixation of pea plants only when it was supplied to the nodules. The beneficial effect of both ethephon and benzyladenine on nitrogen fixation was maximal after 2 days and decreased afterwards.

Addition of nitrate to the nutrient solution (final concentration 10 mM) decreased the actual nitrogen fixation of the nodules to 55% of the potential nitrogen fixation of the bacteroids within 48 hours. Simultaneous addition of benzyladenine to the nodules counteracted the nitrate effect, raising the actual nitrogen fixation to 81% of the potential value.

Treatment with benzyladenine raised the energy supply to the nodules, as it was shown in translocation experiments with assimilated $^{14}CO_2$. No quantitative relationship between energy supply and nitrogenase activity was found.

INTRODUCTION

Until now, little attention has been paid to the question whether all or only part of the nitrogenase present in *Rhizobium* bacteroids of leguminous root nodules is active during growth of the legume under various environmental conditions.

In this paper, the *in vivo* nitrogenase activity of root nodules (actual nitrogen fixation) was compared with the *in vitro* nitrogenase activity (potential nitrogen fixation) of isolated bacteroids, supplied with ATP and reducing equivalents. In a previous Chapter (Ch.1, pages 21-33),the course of the nitrogenase activity during ontogenesis of pea and field bean was reported. The results obtained indicated that in the vegetative stage of legumes, growing in the open air, nitrogenase was fully utilized, but in the generative stage actual nitrogen fixation amounted to 51-84% of the potential nitrogen fixation.

Energy supply of the enzyme is considered to be a major factor regulating actual nitrogenase activity (19). Therefore, in this paper an attempt was made to alter the ratio of actual to potential nitrogen fixation by treatments that either would lower photosynthate supply to the nodules (addition of nitrate) or would enhance photosynthate availability to the nodules, such as local application of plant growth regulators. The ratio of actual to potential nitrogen fixation might also be influenced by a more efficient use of the energy available, such as functioning of a hydrogen uptake system in the bacteroids. This system enables the bacteroids to recover part of the energy lost by hydrogen production concomitantly to nitrogen fixation. Therefore, also a comparison was made of nitrogenase utilization of pea plants inoculated with strains of *Rhizobium leguminosarum* with or without an uptake hydrogenase.

MATERIALS AND METHODS

Plant material

Pea seeds (*Pisum sativum* L. cultivar Rondo) were sterilized by immersion in a 4% solution of H_2O_2 and a few drops of Teepol (a detergent). After 20 min, seeds were sown in heat-sterilized gravel which was inoculated with a strain of *Rhizobium leguminosarum*. Strain S310a, containing an uptake hydrogenase, and strain PRE, without a hydrogenase, were obtained from the culture collection of our laboratory. The gravel was soaked before sowing with a nitrogen-free nutrient solution containing (mg/l of tap water) $K_2HPO_4.3H_2O$, 360; KH_2PO_4, 120; $MgSO_4.7H_2O$, 250; $CaSO_4.2H_2O$, 250; Fe-citrate, 30; $MnSO_4.4H_2O$, 1; $ZnSO_4.7H_2O$, 0.25; $CuSO_4.5H_2O$, 0.25; H_3BO_3, 0.25; $Na_2MoO_4.2H_2O$, 0.05. Plants were grown in a growth chamber at 20°C, with a 16 h light - 8 h dark period and a light intensity (W/m^2) of 8.8 (blue), 7.2 (red) and 12.0 (far-red).

At 21 days after sowing, plants were transferred to 300-ml Erlenmeyer flasks containing 100 ml of the nutrient solution plus 75 mg of KCl. Each flask con-

tained 4 plants, supported by a plug of cotton wool, keeping the root nodules above the nutrient solution.

Treatments

Five days after transferring the plants to Erlenmeyer flasks, the root nodules or the first and second leaves (from the bottom) were treated with 1 ml/4 plants of an aqueous solution of benzyladenine (10, 100 and 1000 µg/ml, pH 9 with KOH) or ethephon (2-chloroethylphosphoric acid, 250 and 1000 µg/ml); both solutions contained a trace of Teepol. Control plants of the benzyladenine experiment received 1 ml of a 670 µg/ml solution of adenine under similar conditions. In some cases, the nitrogen-free nutrient solution was replaced by a solution containing KNO_3 (10 mM) instead of KCl. Nutrient solutions were renewed daily.

Hydrogen production and acetylene reduction

Sets of 4 intact plants were placed in a stoppered 300-ml Erlenmeyer flask and assayed for hydrogen production after 20 min,using a gaschromatograph equipped with a thermal conductivity detector. Subsequently, 30 ml of acetylene was added and ethylene production determined after 15 min by a gaschromatograph equipped with a hydrogen flame detector. The rate of acetylene reduction was linear for 60 min.

In vitro *nitrogenase assay of bacteroids*

Bacteroids were isolated from nodules after the *in vivo* assay as described by van Straten and Roelofsen (16). Isolated bacteroids were treated with ethylenediaminetetraacetic acid and toluene, supplied with reducing equivalents ($Na_2S_2O_4$) and an ATP-generating system, and incubated at 24°C under an atmosphere of 90% argon and 10% acetylene. Ethylene production was determined after 10 min.

RESULTS

Actual nitrogenase activity as affected by ethephon and benzyladenine

Local application of ethephon to root nodules of pea plants markedly affected nitrogenase activity after 2 days, as shown in Fig. 1. When 65 µg of ethephon was applied per plant, nitrogenase activity was enhanced significantly (F test; $p < 0.05$). However, when nodules were treated with 250 µg of ethephon per plant, nitrogenase activity was significantly lowered ($p < 0.05$) and the nodules turned green. Treatment of the first and second leaves (from the bottom) did not produce significant changes in nitrogenase activity.

Application of benzyladenine significantly raised nitrogenase activity (F test; $p < 0.05$) both when applied to nodules or to the first and second leaves (Fig. 2).

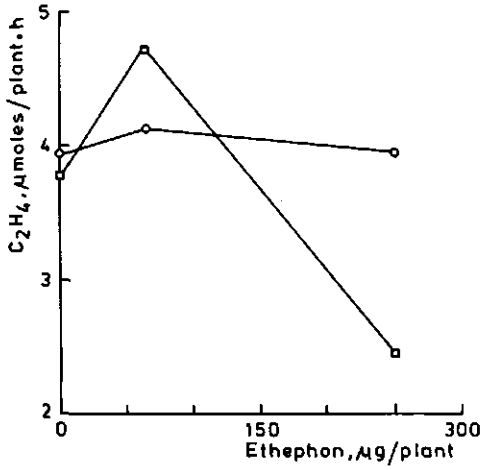

Fig. 1

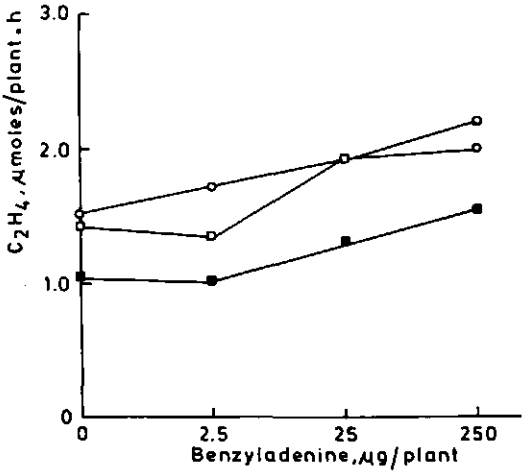

Fig. 2

Fig. 1. Nitrogenase activity of 28 days old pea plants inoculated with *R. leguminosarum* PRE. Data represent means of triplicate measurements of sets of 4 plants, 48 h after application of ethephon to the first and second leaf from the bottom (○) or to nodules (◘).

Fig. 2. Nitrogenase activity of 28 days old pea plants inoculated with *R. leguminosarum* PRE. Data represent means of triplicate measurements of sets of 4 plants, 48 h after application of benzyladenine to the first and second leaf from the bottom (○), to nodules of plants growing on a nitrogen-free nutrient solution (□) and to nodules of plants transferred to a nutrient solution containing 10 mmoles of KNO_3 per l (■). Control sets were given 150 µg of adenine per plant. Control plants without adenine (not shown) possessed an equal nitrogenase activity as compared to adenine-treated control plants.

The site of application did not produce significant differences. The effect of benzyladenine on nitrogenase activity was dependent on concentration. The highest values were reached when 250 µg of benzyladenine was used per plant. Increasing concentrations to 500 µg per plant lowered nitrogenase activity (data not shown).

Both in the experiments of Figs. 1 and 2, nitrogenase activity was measured 2 days after the application of ethephon and benzyladenine, respectively. In preliminary experiments, a maximum response was obtained 2 days after treatment with growth regulators. After 4 days, no effects on nitrogen fixation were observed in comparison with the control plants. A repeated application of

benzyladenine, 4 days after the initial treatment, again raised nitrogenase activity of plants growing without combined nitrogen after an incubation period of 24 hours.

In plants, transferred to a nutrient solution containing 10 mmoles of KNO_3 per l, nitrogenase activity was significantly reduced ($p < 0.05$) (1.06 and 1.42 µmoles of C_2H_4 produced/plant.h by plants with and without nitrate, respectively). Treatment of the nodules with benzyladenine counteracted the nitrate effect by increasing nitrogenase activity (1.55 µmoles of C_2H_4 produced/plant.h with 250 µg/plant of benzyladenine).

Actual and potential nitrogen fixation

The way in which benzyladenine and nitrate treatments affect nitrogenase activity was studied with two *R. leguminosarum* strains, S310a and PRE, by comparison of the actual nitrogenase activity of nodulated roots and the potential nitrogenase activity of isolated bacteroids, the latter being supplied with reducing equivalents ($Na_2S_2O_4$) and an ATP-generating system. The ratio of the actual to the potential nitrogen fixation was distinctly influenced by the two treatments, as shown in Table 1. Untreated pea plants utilized the nitrogenase

Table 1. Effect of strain of *R. leguminosarum*, benzyladenine and nitrate treatments on actual and potential nitrogen fixation, photosynthate supply and relative efficiency of nitrogen fixation by pea plants[1)]

Strain of micro-symbiont	Treatment	Nitrogenase activity (µmoles of C_2H_4 produced/g nodules fresh wt.h)		Ratio of actual to potential nitrogenase activity (%)	Nodule label[2)] (kcpm/plant)	Relative efficiency (%)[3)]
		Actual	Potential			
S310a	Control	16.3 *d*	21.1 *c*	77	94 *l*	95 *p*
	BA	21.9 *c*	21.9 *c*	100	125 *m*	95 *p*
PRE	Control	27.9 *b*	33.9 *a*	82	111 *lm*	71 *q*
	BA	34.1 *a*	35.1 *a*	97	155 *n*	60 *r*
	NO_3^-	19.0 *cd*	34.6 *a*	55	61 *k*	68 *q*
	NO_3^- + BA	27.7 *b*	34.2 *a*	81	72 *k*	62 *r*

1) Values represent means of triplicate measurements, 2 days after treatment of nodules with 250 µg/plant of benzyladenine (BA) or transfer of plants to a nutrient solution containing 10 mmoles of KNO_3/l. Values followed by the same letter are not significantly different in Tukey's test at the 5% level.

2) For explanation see text.

3) Calculated as $\left(1 - \frac{H_2 \text{ evolved in air}}{C_2H_4 \text{ produced air} + 10\% \ C_2H_2}\right).100\%$ (14)

present in the bacteroids for only 80%. Benzyladenine (250 µg per plant) raised the actual nitrogen fixation to 100% or nearly 100% of the potential values.

In plants supplied with nitrate, the ratio of actual to potential nitrogen fixation dropped to 55%. Simultaneous application of benzyladenine to nodules of such plants raised the actual nitrogen fixation as well as the ratio actual-potential nitrogen fixation to values similar to those of control plants without combined nitrogen (Table 1).

In order to check whether the favourable effect of benzyladenine on nitrogenase activity was coupled with an increased photosynthate supply of the nodules, a pulse-label experiment with $^{14}CO_2$ was carried out. Shoots of pea plants were exposed in light to $^{14}CO_2$ for 15 min, 24 h after the start of the nitrate and benzyladenine treatments. Roots were carefully excluded from labelling by sealing them with plastic foil and beeswax. Plants were killed in liquid nitrogen 24 h after labelling, *i.e.* 48 h after the start of the treatments, to ascertain a possible correlation with the nitrogenase data obtained from other sets of plants. Root nodules were crushed in liquid nitrogen and aliquots counted in a liquid scintillation counter, using Instagel (Packard Inc.) as a scintillation liquid.

The results of this experiment show (Table 1) that with strain PRE, more radioactivity was recovered in the nodules than with strain S310a. Benzyladenine treatment of nodules gave a pronounced rise of recovered radioactivity with both S310a and PRE. Benzyladenine (250 µg per plant) increased nodule counts per min significantly in 2 out of 3 treatments. Specific radioactivity of nodules was equal to that of shoots, whereas that of roots was 3-5 times lower (data not shown). Nitrate in the nutrient solution significantly lowered nodule counts per min as compared to the control.

The nitrogenase activity of nodules formed with strain S310a, which contains an uptake hydrogenase, responded to benzyladenine similarly to that of nodules formed with strain PRE (Table 1). The presence of the hydrogenase in S310a resulted in a higher relative efficiency than with strain PRE, *i.e.* fewer moles of hydrogen evolved in air per mole of ethylene produced under acetylene. With strain PRE, a decrease of the relative efficiency was observed when the nitrogenase activity was increased by benzyladenine treatment, both in the presence and absence of nitrate in the nutrient solution.

The ratio of actual to potential nitrogen fixation with S310a was slightly below that with PRE. The lower actual nitrogen fixation with S310a was due to a lower potential nitrogenase activity of the bacteroids. Expressed on a protein

basis, *in vitro* nitrogenase activity amounted to 365 µmoles of C_2H_4 produced/g of protein.h in S310a bacteroids, as compared with 732 µmoles of C_2H_4 produced/g of protein.h in PRE bacteroids.

DISCUSSION

Mode of action of benzyladenine and ethephon

Plant growth regulators belonging to the cytokinin group, as benzyladenine, are known to attract assimilates to the site where the hormone is applied. They reduce protein degradation (10), increase ion uptake and transport, and induce cell division (8, 17).

Ethephon, an artificial compound, is degraded within the plant to ethylene at pH values above 3.5. Ethylene mobilizes storage products, directs them to the site of fruit growth and promotes fruit ripening and senescence (15).

In this study, benzyladenine and ethephon were used in an attempt to alter photosynthate availability to the nodules which might influence the ratio of actual to potential nitrogen fixation. Fairly large quantities of benzyladenine were used as compared to literature data on leaf application (11). No data were available on local application of growth regulators to nodules. As nodules are considered to be impermeable to water, the uptake of benzyladenine and ethephon probably has to occur through the root cortex. With our method of brush application of an aqueous solution to the nodules, inevitably also adjacent root parts were treated. However, both with leaf and nodule treatments, the quantities of growth regulator actually taken up are unknown.

In line with literature data (1), the observed effect of ethephon on nodules (Fig. 1) might be explained in terms of mobilization of assimilates. Consequently, a low concentration of ethephon would stimulate nitrogenase activity by mobilizing storage products (*e.g.* starch). A high concentration of this growth regulator initially might have enhanced nitrogen fixation, but would later on cause an exhaustion of substrates and consequently a decrease of nitrogen fixation. Treatment of nodules with benzyladenine may enhance the sink function of the nodules and stimulate nitrogenase activity due to a higher supply of photosynthates (Fig. 2, Table 1).

Lower leaves have been shown to act as source of substrates to nodules (11, 13). Application of ethephon or benzyladenine to these source leaves was aimed at increasing photosynthate supply of the nodules in an indirect way. Ethephon was thought to mobilize storage carbohydrates in the leaves, making them available

for translocation to the nodules. Even if this would have happened, no effect of it on nitrogenase activity was observed (Fig. 1). Benzyladenine treatment of the source leaves was intended to reduce protein degradation and thus to enhance photosynthesis in the leaf. Alternatively, the translocation pattern in the plant could be changed, diverting photosynthate transportation from the shoot apex to the nodules. In either way, the observed rise in nitrogen fixation after leaf application of benzyladenine is likely to be due to an increased supply of photosynthate to the nodules.

Actual and potential nitrogen fixation

Benzyladenine application to nodules increased the actual nitrogenase activity (Table 1). This phenomenon was correlated with an increase in energy supply. However, no quantitative relationship could be established between the additional photosynthates translocated to the nodules and the rise in nitrogenase activity. The same difficulty was encountered by other authors (7, 14); it might be attributed to the synthesis of storage products, to the accumulation of assimilates in the plant tissue of the nodules (7) or to differences in respiratory activity of the nodules, leading to a smaller pool of labelled compounds.

A considerable gap exists between the occurrence of maximum nitrogenase activity, 48 h after treatment with benzyladenine, and the translocation time of ^{14}C labelled photosynthate (3-9 h)(6). One explanation of this deviation might be the occurrence of uptake problems of externally applied growth regulators as discussed above.

On a cellular level, benzyladenine is known to increase respiration, proton extrusion and transmembrane potential in pea plants (8, 9, 10). On the other hand, Laane *et al.* (5) showed that in bacteroids of *R. leguminosarum*, transmembrane potential was acting as a major force regulating nitrogenase activity by directing the electron flow to nitrogenase. Combining these results, the effect of benzyladenine on nitrogenase activity might be explained by a higher supply of photosynthates, an enhanced respiration, leading to a higher transmembrane potential and, consequently, a higher nitrogenase activity.

Combined nitrogen decreased the ratio of actual to potential nitrogen fixation (Table 1), as was found earlier in this laboratory (3; Chapter 1, pp. 21 - 33) Addition of sucrose to the nutrient solution alleviated the effect of combined nitrogen on nitrogenase activity (4) partly by overcoming a shortage of energy in the nodules but also by decreasing nitrogen uptake. In our experiment, a decrease of nitrate uptake was unlikely because in general, benzyladenine stimulates ion uptake and transport. However, the benzyladenine effect on photosyn-

thate supply (Table 1) was lower in nitrate-grown plants as compared to plants growing in a nitrogen-free nutrient solution.

The possibility of increasing nitrogen fixation by the use of cytokinins and other growth regulators (especially those synthetized in roots), as indicated by the results of the present investigation,might open interesting perspectives to agriculture if the short duration of the higher activity and the impractical application site might be overcome. Another approach would be to increase the rate of natural cytokinin synthesis in root nodules. The main cytokinin activity of nodules seems to be located in the nodule meristem, *Rhizobium*-infected cells possessing a lower activity (17). But also free-living rhizobia have been shown to be capable of synthetizing cytokinin-like substances in sufficient quantity to induce cell division in a soybean-callus test (12). Whether also bacteroids produce such substances is unknown. However, bacteroids might influence photosynthate supply to the nodules either directly by excretion of cytokinins, or indirectly by triggering off synthesis of cytokinins in the plant (18).

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3 ALTERNATIVE, CYANIDE-RESISTANT, RESPIRATION IN ROOT SYSTEMS OF *PISUM SATIVUM* L. AS AFFECTED BY STRAIN OF *RHIZOBIUM LEGUMINOSARUM* AND NITRATE SUPPLY

ABSTRACT

A study has been made of the alternative, cyanide-resistant, respiratory pathway in non-nodulated pea plants (*Pisum sativum* L., cultivar Rondo) as well as in pea-*Rhizobium* symbioses of different nitrogen-fixing capacity. Alternative respiratory activity was low in root systems of 26-days old non-nodulated or nodulated plants in the absence of nitrate, regardless of the nitrogen-fixing capacity of the nodules.

Supply of the plants with nitrate (10 mM) raised the respiration of the root system due to alternative-respiration activity. In non-nodulated root systems respiration rate was doubled, 48 h after the supply of nitrate, and alternative respiration increased to 60% of the electron flow to oxygen. In nodulated root systems, the nitrate-induced enhancement of the alternative respiration was inversely related to the nitrogen-fixing capacity.

Carbohydrate levels in ineffectively nodulated plants were higher than those in effectively nodulated plants in the absence of nitrate. When nitrate was supplied, carbohydrate levels fell concomitantly with the rise in alternative respiration. In nitrogen-fixing plants, they remained unchanged upon the addition of nitrate.

The response of growth rate and the induction of nitrate reductase to nitrate supply were lower with plants lacking the capacity to fix nitrogen than in nitrogen-fixing plants. Nitrate supply reduced nitrogenase activity both in highly and moderately effective *Rhizobium* strains. Upon nitrate supply, hydrogen production by nitrogenase decreased more rapidly than total nitrogenase activity, leading to an increased relative efficiency. Inhibition of the alternative respiration of isolated bacteroids led to a sharp decline of nitrogenase activity due to oxygen inactivation.

Pea plants with and without nitrogen fixation, responded to added nitrate by increased alternative respiration consuming 6-10%and 24-43%, respectively, of the carbohydrates supplied to the roots by the shoots within 48 h. A main function of alternative respiration in pea roots seems to be the degradation of excess carbohydrates supplied by the shoot following a growth stimulus by the supply of nitrate. As alternative respiration was also found in pea plants with a high nitrogen-fixing capacity upon the supply with nitrate, it is concluded that these plants were growing under nitrogen limitation.

From respiration measurements of detached nodules it is concluded that utilization of the alternative pathway in these nodules decreases the amount of ATP generated with 8-21% of the maximum ATP production from glucose. Therefore, the alternative respiration should be taken into account when calculating energy requirements of nitrogen fixation, based on the differences in respiration between nitrogen-fixing and non-nodulated nitrate-utilizing plants.

INTRODUCTION

In plant mitochondria, an alternative respiratory pathway is known to function in addition to the cytochrome pathway (Bahr and Bonner, 1973a,b). This alternative pathway is insensitive to cyanide but can be inhibited by hydroxamic acids. The alternative respiratory pathway branches from the cytochrome pathway at ubiquinone (Storey, 1976), which easily transfers electrons to the cytochrome pathway, but which reduces the first carrier of the alternative pathway only when it is in its largely reduced state (Bahr and Bonner, 1973b). The alternative pathway is generally assumed to yield no ATP during electron transfer to oxygen (Solomos, 1977). In this aspect, alternative respiratory activity may be regarded as a 'wastage' of energy.

In a recent review, Lambers (1980) suggested that the alternative route might function as an 'overflow mechanism' to get rid of excessive amounts of carbohydrates, not utilized for growth, maintenance or formation of storage products in plants. According to De Visser and Lambers (1979), roots of nodulated, nitrogen-fixing pea plants possess a very low alternative-pathway activity. In contrast, alternative respiration in roots of non-nodulated pea plants grown on ammonia or nitrate, amounted to 25-50% of total respiration (Lambers, 1980). These results show that nitrogen source and nitrogen supply are important factors in determining respiratory efficiency in legumes.

In experiments with nodulated legumes, the quantity of carbohydrates supplied to the roots was estimated by Pate and co-workers to amount to 55-74% of the carbohydrates generated in net photosynthesis.Nodules acquired 32% of the net photosynthate in *Pisum sativum* (Minchin and Pate, 1973). Twelve per cent of the net photosynthate was used in nodule respiration for maintenance and nitrogen fixation; the remainder would be used for synthesis and transport of amino acids. In spite of the large quantities of carbohydrates supplied to nodulated roots, a low alternative respiratory activity was found (De Visser and Lambers, 1979). Due to the high costs of energy for nitrogen fixation and synthesis of amino acids no excess of carbohydrates is generated which would be respired via the alternative pathway.

In order to find out whether a relation exists between alternative respiration and nitrogen fixation, in the present paper this type of respiration was determined in non-nodulated and nodulated root systems of pea plants with different nitrogen-fixing capacity and with different relations between growth, storage and respiration. Also separated nodules and bacteroids were investigated. The influence of the nitrogen source on respiration, growth and nitrogen fixation was analyzed in a series of experiments in which nitrate was added to nodulated and non-nodulated plants. The data on 'energy loss' by the alternative respiration were compared with the data on energy costs of nitrogen fixation and hydrogen production by nitrogenase.

ABBREVIATION: SHAM (salicylhydroxamic acid).

MATERIALS AND METHODS

Plant growth

Seeds of pea plants (*Pisum sativum* L. cultivar Rondo) were sterilized by immersion in an aqueous solution of 4% H_2O_2 and a few drops of Teepol (detergent). After 20 min, seeds were sown in sterilized gravel or perlite, which was inoculated with a strain of *Rhizobium leguminosarum*. All strains used in these experiments were obtained from the culture collection of the Laboratory of Microbiology, Wageningen.

The plants were watered with a sterile nitrogen-free nutrient solution containing(mg/l of tap water) K_2HPO_4, 360; KH_2PO_4, 120; $MgSO_4.7H_2O$, 250; $CaSO_4$, 250; Fe(III) citrate, 30; $MnSO_4.4H_2O$, 1; $ZnSO_4.7H_2O$, 0.25; $CuSO_4.5H_2O$, 0.25; H_3BO_3, 0.25; $Na_2MoO_4.2H_2O$, 0.05. Plants were kept in a growth chamber at a light/dark period of 16/8 h at 20°C and a relative humidity of 70%. At 21 days after sowing,

they were transferred to 300-ml Erlenmeyer flasks, containing 100 ml of nutrient solution plus KCl (10 mM). Experimental sets consisted of 4 plants supported by a cotton wool plug. In some experiments, the nutrient solution was replaced by a solution containing KNO_3 (10 mM) instead of KCl, 6 days after transfer of the plants.

Plant respiration

Measurements started 5 days after transfer of the plants to the water culture. Respiration was determined according to Lambers and Steingröver(1978).Root systems were detached from the shoots and fitted into a 300-ml vessel equipped with a Clark-type electrode and a magnetic stirrer. The vessel was filled with an air-saturated nutrient solution plus 10 mmoles of KCl or KNO_3 per 1, but without Fe(III) citrate, and sealed with a mixture of equal parts of vaseline and beeswax. Respiration measurements were performed in a water bath at 20°C. Linear rates of oxygen consumption were monitored for at least 10 min. Then the solution was replaced by an equal one containing in addition 25 mmoles of SHAM per 1, and oxygen consumption measured again for 10 min.

Respiration of root nodules, 30-50 mg of fresh weight, was determined polarographically in a YSI magnetic-stirrer bath at 20°C. Nutrient solutions of the composition as described above were saturated with O_2 before use in order to improve linear response.

Bacteroid respiration

Nodules were detached from the roots and squeezed in a Bergersen press (Bergersen, 1966) in a buffer containing 50 mmoles of Tris(hydroxymethyl)-aminomethane, 2.5 mmoles of $MgCl_2.7H_2O$, 300 mmoles of sucrose and 4% by weight of polyvinylpyrrolidone(PVP), in 1 l of demineralized water. The pH was adjusted to 7.2 with HCl before use. The nodule brei was centrifuged at 5500 x g for 10 min and the supernatant (nodule cytosol) used in respiration measurements. The bacteroids (pellet) were washed in the same buffer without PVP and resuspended to a concentration of 80 mg of nodule fresh wt per ml of buffer. Respiration measurements were carried out in a YSI magnetic-stirrer bath at 20°C, using succinate (10 mM) as the substrate. Inhibitor constants (apparent Ki) were calculated from the reciprocal of respiratory rate against concentration of SHAM (0.1-10 mM) (Bahr and Bonner, 1973a).

Hydrogen production and acetylene reduction

Detached roots of 4 plants were incubated in a stoppered 300-ml Erlenmeyer flask. After 20 min, a 100 µl gas sample was taken and hydrogen production determined with a gas chromatograph equipped with a thermal conductivity detector.

Subsequently, 30 ml of acetylene was added to the flask and after 15 min a 100 µl gas sample was analyzed for ethylene production by hydrogen-flame detection.

Nitrate reductase

Nitrate reductase was assayed in the first unfolded leaf from the top using NADH as an electron donor, as described by Stulen (1974). Leaf extracts were incubated at 27°C in the presence of NADH and KNO_3. Nitrite production was determined colorimetrically after 30 min, using the sulphanilamide method (Snell and Snell, 1949).

Chemical analyses

Ethanol-soluble carbohydrates ('sugars') were determined by the anthrone method (Trevelyan and Harrison, 1952) in ethanol-extracted plant dry matter. The residue was heated at 100°C for 2 h with 0.1 N HCl. The content of ethanol-insoluble carbohydrates ('starch') was measured in the supernatant using the anthrone method.

Plant nitrogen was determined by the Kjeldahl method, using $CuSO_4$ and Se as catalysts.

RESULTS

Alternative respiration and nitrogen fixation

Pea-*Rhizobium* symbioses with different nitrogen-fixing capacity were obtained by inoculation of *Pisum sativum* L. (cultivar Rondo) with different strains of *Rhizobium leguminosarum*. As shown in Table 1, these symbioses displayed different properties with regard to nodule formation, hydrogen production and acetylene reduction, and growth.

Table 1. Characteristics of pea-*Rhizobium* symbioses, 28 days after sowing. Values represent means of 7 determinations of sets of 4 plants. Values in one column followed by the same letter are not significantly different in Tukey's test at the 0.05 level

Strain of micro-symbiont	C_2H_4 production (µmoles/g nod fr wt.h)	H_2 production (µmoles/g nod fr wt. h)	Relative efficiency (%)	Nodule fresh wt (mg/ plant)	Plant nitrogen (mg/ plant)	Shoot to root ratio
Non-nodulated	0 *c*	0 *e*	–	0 *d*	4.9 *c*	1.5
P8	0 *c*	0 *e*	–	54 *c*	5.8 *c*	1.6
S313	11.1 *b*	5.5 *c*	50	197 *a*	10.6 *b*	1.7
S310a	14.1 *b*	1.6 *d*	89	56 *c*	9.8 *b*	1.9
PRE	28.1 *a*	10.3 *b*	63	107 *b*	12.2 *b*	1.8
PF2	25.1 *a*	14.0 *a*	44	102 *b*	16.1 *a*	2.0

With strain S310a, the ratio of hydrogen evolved in air to ethylene produced in air with 10% acetylene was lower than with other strains owing to the presence in this strain of a hydrogenase that enabled the utilization of H_2 produced by the nitrogenase system (Table 1). Assuming full utilization of hydrogen taken up by hydrogenase-containing rhizobial strains, Schubert and Evans (1976) calculated relative efficiencies according to the formula $(1-\frac{H_2 \text{ evolved in air}}{C_2H_2 \text{ reduced}}).100\%$. Thus a high relative efficiency indicates that little energy is lost in hydrogen production by nitrogenase activity.

Respiration measurements of root systems of these symbioses were performed at 26 days after sowing (Table 2). Root systems without a nitrogen-fixing symbiosis generally showed a lower respiration rate than those with such a system. A significant difference between total respiration and cytochrome (SHAM-resistant) respiration was found only in the symbiosis Rondo x PF_2, which also possessed the highest shoot to root ratio (Table 1). With the other *R. leguminosarum* strains no significant differences were found, in spite of the distinctly different nitrogen-fixation parameters.

Table 2. Total and SHAM-resistant respiration of 26-days old pea root systems grown without nitrate. Values represent means of 3 sets of 4 plants. Values followed by the same letter are not significantly different in Tukey's test at the 0.05 level

Strain of micro-symbiont	Respiration (mg O_2/g dry wt.h)		Ratio (1:2)
	Total (1)	SHAM-resistant (2)	
Non-nodulated	3.8 *d*	3.5 *de*	1.1
P8	3.0 *ef*	2.9 *f*	1.0
S313	4.7 *ab*	4.5 *ab*	1.0
S310a	4.8 *ab*	4.4 *bc*	1.1
PRE	3.9 *cd*	3.9 *cd*	1.0
PF2	5.1 *a*	4.4 *bc*	1.2

Effect of nitrate supply

Alternative respiration. As pea plants with a low nitrogen-fixing capacity were likely to grow under nitrogen limitation, a series of experiments was carried out in which nitrogen limitation was overcome by the addition of nitrate. Immediately after the initial respiration measurements at day 26, the plants were transferred to a nutrient solution containing 10 mmoles of KNO_3 instead of 10 mmoles of KCl per l.

As shown in Fig. 1a., alternative respiration was generated within 24 h after the supply of nitrate. An analysis of variance showed that the ratio of total to SHAM-resistant respiration increased more sharply in non-nodulated plants than in nodulated plants with a low nitrogen-fixing capacity, which in their turn produced a high ratio as compared with nodulated plants with a high nitrogen-fixing capacity (F test, $p < 0.01$). The alternative respiratory activity was generated in addition to the cytochrome (SHAM-resistant) respiration (Fig. 1b)

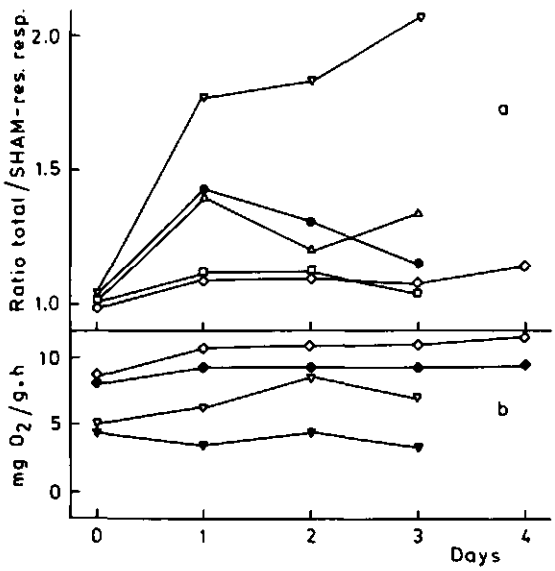

Fig. 1a. Ratio of total to SHAM-resistant respiration of root systems of pea plants (non-nodulated, ▽; inoculated with *R. leguminosarum* strains P8, ●; S313, △; PRE, □ ; and PF_2, ◇) as affected by nitrate supply (10 mM) (day 0, 26 days after sowing). Values are means of 9 determinations of sets of 4 plants from 3 experiments. For statistical interference see text.

Fig. 1b. Total (▽,◇) and SHAM-resistant (▼,◆) respiration (mg O_2/g dry wt.h) of root systems of pea plants (non-nodulated, ▽, ▼ and inoculated with strain PF_2 ◇ , ◆) as affected by nitrate supply (day 0, 26 days after sowing). Values are means of 3 determinations of sets of 4 plants, from one experiment out of the 3 experiments of Fig. 1a.

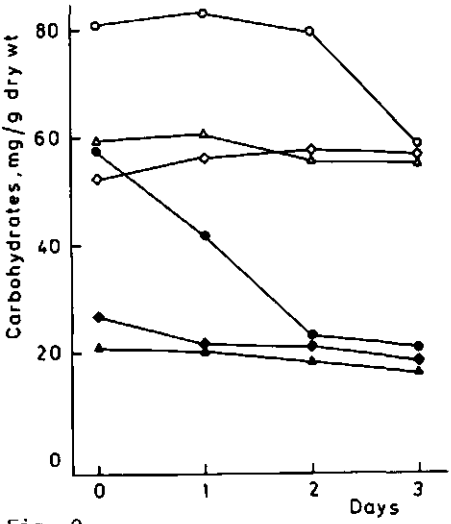

Fig. 2

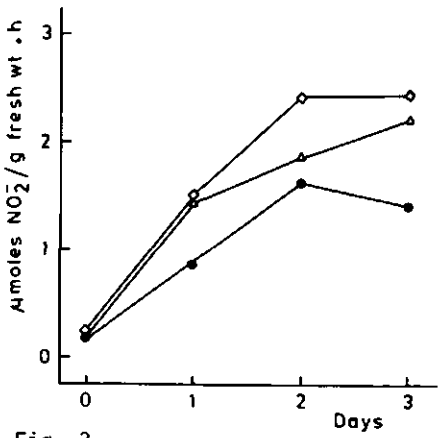

Fig. 3

Fig. 2. Ethanol-soluble (●, ▲, ◆) and ethanol-insoluble (o, Δ, ◇) carbohydrates (mg glucose eq./g dry wt) in roots of pea plants inoculated with *R. leguminosarum* strains P8 (●, o); S313 (▲, Δ) and PF_2 (◆ , ◇) as affected by nitrate supply (10 mM) (day 0, 26 days after sowing). Values are means of triplicate determinations.

Fig. 3. Nitrate-reductase activity (µmoles NO_2^-/g fr wt.h) in the first unfolded leaf from the top of pea plants, inoculated with *R. leguminosarum* strains P8 (●), S313 (Δ) and PF_2 (◇), as affected by nitrate supply (10 mM) (day 0, 26 days after sowing). Values are means of duplicate determinations of 4 leaves.

Carbohydrate content. In the ineffective symbiosis (P8), carbohydrate levels in roots as well as in shoots were much higher than in effective symbioses (Fig. 2, roots only). On the addition of nitrate, however, both ethanol-soluble and ethanol-insoluble carbohydrates dropped to the levels of effectively nodulated plants within 48 and 72 h, respectively. Carbohydrate levels in nitrogen-fixing plants remained unaffected by nitrate supply.

Nitrate reductase. Synthesis of nitrate reductase in leaves was induced by nitrate (Fig. 3). A more rapid synthesis was observed in leaves of plants with a high nitrogen-fixing capacity. Growth rates of plants without nitrogen fixation responded to nitrate 2-3 days after transfer to the nitrate-containing nutrient solution, and thus lagged behind the synthesis of nitrate reductase.

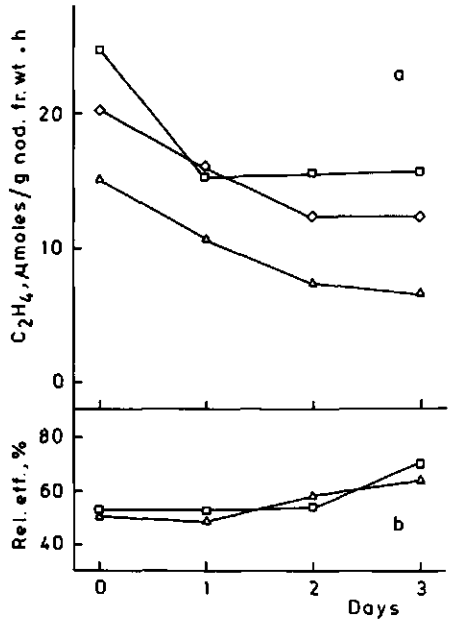

Fig. 4a. Ethylene production of pea plants inoculated with *R. leguminosarum* strains S313 (Δ), PRE (□), and PF_2 (◇), as affected by nitrate supply (10 mM) (day 0, 26 days after sowing).

Fig. 4b. Relative efficiency of pea plants inoculated with *R. leguminosarum* strains S313 (Δ) and PRE (□) as affected by nitrate supply (10 mM) (day 0, 26 days after sowing).

Nitrogenase activity. Transfer of nodulated pea plants to the nutrient solution with nitrate (10 mM) caused a decrease of nitrogenase activity in symbiotic associations with a high as well as a low nitrogen-fixing capacity (Fig. 4a). Hydrogen production decreased more rapidly than acetylene reduction, resulting in a rise of the relative efficiency (Fig. 4b).

Alternative respiration in bacteroids

Although SHAM-sensitive respiration has been described in mitochondria of higher plants (Solomos, 1977), it became interesting to check whether not only the plant part of the nodules but also the bacteroid fraction contributes to this type of respiration. Respiration of nodule cytosol (nodule brei minus bacteroids) was sensitive to SHAM, as shown in Table 3, but SHAM-sensitive respiration was also present in bacteroids supplied with succinate (10 mM). Respiration rates of

Table 3. Inhibitor constants (apparent Ki) of KCN and SHAM in *R. leguminosarum* bacteroids and nodule cytosol. Values represent means of 6 determinations. Values followed by the same letter are not significantly different in Tukey's test at the 0.05 level

Strain of microsymbiont	Nodule cytosol (mM SHAM)	Bacteroids (mM SHAM)	Bacteroids (mM SHAM in the presence of 0.8 mM KCN)	Bacteroids (mM KCN)
P8	N.D.	27.1 *a*	4.4 *c*	2.1 *q*
S313	N.D.	25.7 *a*	N.D.	N.D.
PRE	9.4 *bc*	15.8 *b*	4.9 *c*	0.2 *p*

N.D. = Not Determined

bacteroids of strain PRE, capable of nitrogen fixation, were higher but K_i values were lower than those of bacteroids of strain P8 lacking the capacity to fix nitrogen.

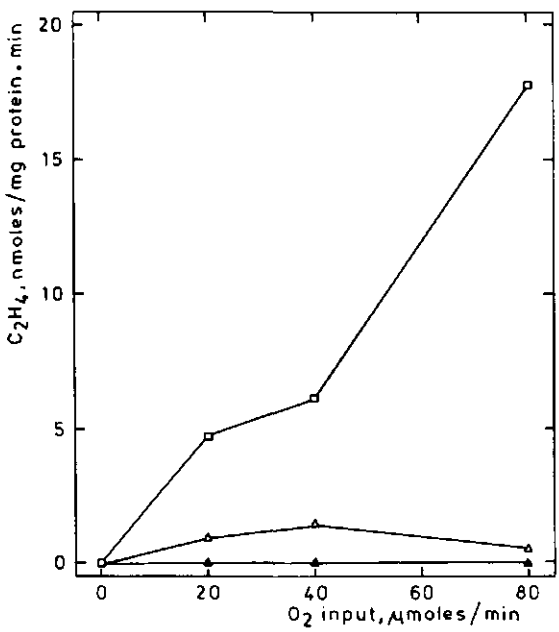

Fig. 5. Ethylene production (nmoles/mg protein.min) by bacteroids of *R. leguminosarum* PRE as affected by O_2 input and concentration of SHAM (o, □, 12.5 mM, Δ and 25 mM, ▲) For experimental conditions see text.

Nitrogen fixation was absent under the experimental conditions of Table 3, due to oxygen inactivation. Therefore, the function of the alternative respiration in nitrogen fixation was investigated in a separate experiment, in which respiration of PRE bacteroids was measured under nitrogen-fixing conditions (Fig. 5). Bacteroids were incubated under varying oxygen tension in the presence of reduced myoglobin and bovine serum albumine, using succinate (20 mM) as the substrate, as described by Laane *et al.* (1978). With SHAM (12.5 mM), respiration was much lower as compared to the control, resulting in a lower maximum of nitrogenase activity at a lower oxygen input (cf. Fig. 5, Table 3).

DISCUSSION

Alternative respiration and nitrate supply

In roots of pea plants growing without nitrate, alternative respiration was only found in plants inoculated with *R. leguminosarum* strain PF_2, which had a high growth rate and a high nitrogenase activity (Tables 1 and 2). Although the symbiosis with PRE was as active as that with PF_2 regarding N_2 fixation, its activity started a number of days later, as can be seen from its lower yield of plant nitrogen. When nitrate was supplied, the growth rates of all plants were enhanced and alternative respiration was generated (Fig. 1). This happened in addition to the cytochrome-linked respiration (Fig. 1b; see also Bahr and Bonner, 1973b). Alternative respiration of nitrogen-fixing plants was stimulated by nitrate supply to a much lower extent than that of plants without nitrogen fixation (Fig. 1). The latter plants, which had been growing under nitrogen limitation, contained high levels of carbohydrates that declined (Fig. 2) before a growth response became manifest. With the ineffective strain P8, the ratio of total to SHAM-resistant respiration decreased to the values of the effective strain after the excess of carbohydrates had been decreased (Figs 1a and 2). Therefore, the degradation of excess carbohydrates is probably a function of the alternative respiratory pathway, as suggested by Lambers (1980). According to his 'overflow'-hypothesis, alternative respiration is generated if there is an imbalance between the carbohydrates supplied by the shoot and the carbohydrate requirements of the roots for growth, maintenance, storage or osmoregulation. As alternative respiration was also generated upon the supply of nitrate to pea plants with a high nitrogenase activity, this would mean that under the experimental conditions employed, these plants were not growing under C limitation but probably under N limitation.

Alternative respiration and carbohydrate metabolism

Based upon the data on respiration, carbohydrate levels and plant growth, as presented in the Results section, the contribution of the alternative respiration to the carbohydrate metabolism of the pea plant was calculated (Table 4). To calculate the amounts of substrate used as carbon skeletons for the synthesis of cell material, a coefficient of 1.4 g was used to convert g of dry weight produced into g of glucose utilized (Penning de Vries, 1974).

Carbohydrate supply of the root by the shoot was calculated from the substrate used in the respiration of the root and in the synthesis of root material. This value therefore does not include the amount of carbohydrates recirculated to the shoot in amino acids, which was estimated at 14% of total carbohydrate supply of the root in cowpea (Herridge and Pate, 1977) and 20% in pea (Minchin and Pate, 1973). To convert the respiration data from oxygen consumed per h into glucose consumed per day, a respiratory quotient of 1.0 was assumed and diurnal variation was neglected. In a recent paper, Lambers *et al.* (1980) observed diurnal variations in roots of *Lupinus albus* L. both in carbon dioxide production and oxygen consumption. Therefore, the conversion employed in the present study must be considered as a rough estimate. Net photosynthesis was determined from the substrate used in the synthesis of shoot material and in translocation to the root.

In nitrogen-fixing plants, both net photosynthesis and carbohydrate supply of the roots were higher than in plants without nitrogen fixation owing to the improved nitrogen supply (Table 4). Although carbohydrate supply of the roots is increased by added nitrate, the distribution pattern in the root system is altered (Small and Leonard, 1969), resulting in less carbohydrates being supplied to the nodules. This explains the moderate decrease of nitrogenase activity due to the supply of nitrate (Fig. 4). Similarly, Bethlenfalvay *et al.* (1978b) found that photosynthesis was enhanced by the addition of ammonia to non-nodulated pea plants. With different *Rhizobium* strains, photosynthesis was positively correlated with nitrogen fixation (Bethlenfalvay *et al.* 1978a).

The alternative respiration of the root system was active in catabolizing 1-7% of the carbohydrate supply of the root when the plants were growing without nitrate (Table 4). With nitrate, however, the proportion of the alternative respiration in carbohydrate catabolism was much higher (5-11% in nitrogen-fixing plants and 15-31% in non-nodulated or ineffectively nodulated plants). Therefore, the alternative respiration should be taken into account in calculations of energy requirement of nitrogen fixation and nitrate reduction, based upon respiration measurements of nitrogen-fixing and nitrate-reducing plants.

Table 4. Proportion of the alternative respiration in carbohydrate metabolism of pea plants (non-nodulated or infected by a strain of *R. leguminosarum*), growing without nitrate (day 0, 26 days after sowing) and 48 h after the supply of nitrate (10 mM; day 2). For explanation see text.

Item	Non-nodulated		P8		S313		PRE	
	0	2	0	2	0	2	0	2
1a. Total root respiration (mg O_2/g dry wt.h) (cf Fig. 1b)	5.0	8.5	5.3	7.0	9.0	9.8	7.8	8.7
1b. Alternative respiration as % of total respiration (cf Fig. 1a)	8	52	4	31	4	20	2	10
2. Shoot dry wt (mg/plant)	164	179	174	190	260	354	241	298
3. Root dry wt (mg/plant)	109	112	108	112	157	176	133	148
4. Carbohydrate supply of root by shoot (mg glucose/ plant.day) (1a + 3)	15	36	17	37	42	69	43	57
5. Net photo-synthesis (mg glucose/ plant.day) (2 + 4)	19	60	23	70	60	130	79	113
6. Alternative respiration of the root system								
a. as % of carbohydrate supply (1b)	7	31	3	15	3	11	1	5
b. as % of net photo-synthesis (1b)	5	19	2	8	2	6	1	3

Alternative respiration and nitrogen fixation

Nitrogen fixation was calculated from ethylene production under air with 10% of acetylene (representing total nitrogenase activity) minus hydrogen production in air, representing that part of nitrogenase activity not utilized in nitrogen fixation (Table 5). The carbohydrate requirement of nitrogen fixation was calculated from respiration measurements of detached nodules. The resulting values of 3.1 - 4.2 mg of carbon utilized per mg of nitrogen fixed with strain PRE correspond to the values of 4.1 mg C/mg N in pea nodules (Minchin and Pate, 1973) and 2 - 5 mg C/mg N in cowpea nodules (Herridge and Pate, 1977). The large amount of nodular tissue formed with strain S313 enhanced the overall cost of nitrogen fixation to 6.3 - 11.1 mg C/mg N owing to a higher nodule respiration at equal amounts of nitrogen fixed.

Energy costs of total nitrogenase activity were expressed in terms of moles of ATP derived from nodule respiration per mole of nitrogen fixed (Table 5). One mole of glucose was assumed to yield 36 moles of ATP when respired *via* the cytochrome pathway as compared with 12 moles when the alternative pathway was utilized. The possible influence of wound respiration was neglected, so that the reported values must be considered as rough estimates. The values of moles of ATP utilized per mole of nitrogen fixed are considerably higher than the value of 28 mol ATP/mol N_2 as reported by Evans *et al.* (1981), as in our case also nodule maintenance respiration is accounted for.

A calculation of the amount of ATP 'lost' by hydrogen production simultaneously with nitrogen fixation was based upon the assumption that production of 1 mole of hydrogen requires 7 moles of ATP (Pate *et al.*, 1981). The amount of ATP 'lost' in the production of hydrogen is of the same order of magnitude as compared with the amount of ATP lost by the alternative respiration with strain S313, producing a large amount of nodular tissue. However, with strain PRE considerably more ATP was lost in hydrogen production than by use of the alternative pathway (Table 5).

Alternative respiration was found not only in the plant part of the nodule (cytosol), but also in the bacteroids (Table 3). The inhibitor constants for SHAM in bacteroids were almost 50-100 times higher as compared to those reported from plant mitochondria (Tomlinson and Moreland, 1975; Henry and Nyns, 1975). Althought the alternative oxidase is less sensitive to SHAM when succinate is used as substrate, permeability difficulties in addition might explain this difference. Similar to the data obtained with mitochondria, an increased electron flux via the alternative pathway yielded a lower K_i for SHAM (*e.g.* presence or absence of KCN; PRE in comparison to P8) (Wedding *et al.* 1973).

Table 5. Costs of nitrogen fixation in pea-*Rhizobium* symbioses growing without nitrate (day 0, 26 days after sowing) and 48 h after the supply of nitrate (10 mM; day 2). For explanation see text.

Item	P8		S313		PRE	
	0	2	0	2	0	2
1. Production (µmoles/plant.day) of (cf Table 1)						
a. ethylene	0	0	72	50	67	49
b. hydrogen	0	0	36	21	39	23
c. nitrogen fixed (1a,b)	0	0	12	10	9	9
2. a. Total respiration of nodule (mg glucose/plant.day)	1.0	1.0	5.3	7.5	2.6	2.5
b. Alternative nodule respiration as % of total	52	38	19	23	26	11
c. Total nodule respiration as % of carbohydrate supply of roots (Table 4; 2a)	6	3	12	11	6	4
3. Overall costs of nitrogen fixation (mg C respired/mg N) (1c, 2a)	-	-	6.3	11.1	3.1	4.2
4. ATP (µmoles/plant.day)						
a. production in nodules (2a,b)	136	145	927	1270	424	467
b. 'lost' in alternative respiration (2b)	72	49	135	230	89	37
c. 'lost' in hydrogen production (1b)	0	0	252	147	273	161
5. Overall costs of nitrogen fixation (moles of ATP/moles N_2 fixed)						
a. actual ATP production (4a,1c)	-	-	77	131	46	54
b. assuming full phosphorylation efficiency (no alternative respiration)(4a,b;1c)	-	-	89	155	55	58

Treatment of nitrogen-fixing bacteroids with SHAM, final concentration 12.5 mM, significantly reduced nitrogenase activity (Fig. 5) by decreasing respiration. As an inhibition of alternative respiration does not involve a decrease in ATP production during electron transfer from succinate to oxygen (Storey and Bahr, 1969), this decrease might point to a respiratory protection as a possible function of the alternative respiration in bacteroids. The alternative oxidase is known to be less sensitive to oxygen than cytochrome oxidase (Solomos, 1977). In analogy, Appleby *et al.* (1976) reported respiratory protection by an oxygen-insensitive oxidase in *R. japonicum* bacteroids. Also, a non-phosphorylating, oxygen-insensitive oxidase was found in *Azotobacter vinelandii* (Ackrell and Jones, 1971; Eilermann, 1973) and assumed to function in protecting nitrogenase against high pO_2.

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4 NITROGEN FIXATION BY A PEA-*RHIZOBIUM* SYMBIOSIS CONTAINING A BACTERIAL UPTAKE HYDROGENASE

ABSTRACT

Pea plants (*Pisum sativum* cv. Rondo) were sown in soil with low numbers of indigenous rhizobia and inoculated with *Rhizobium leguminosarum* strain S310a containing an uptake hydrogenase (Hup^+), or strain PRE, without hydrogenase (Hup^-).Roots of pea plants grown in the open air and inoculated with S310a did evolve traces of hydrogen in air, whereas with strain PRE (Hup^-) 38% of the electron flow through nitrogenase was allocated to hydrogen production during the entire growth cycle of the plants. However, total plant nitrogen of the symbiosis with strain S310a was considerably lower than that with PRE (720 and 1380 mg N per pot, respectively). This was due to a lower nitrogenase activity of the nodules of S310a, to a lower bacteroid content of these nodules and to an early senescence of the nodules in comparison with PRE. Also potential nitrogen fixation (as calculated from *in vitro* nitrogenase activity of isolated bacteroids, supplied with ATP and reducing equivalents) was lower with S310a than with PRE. From the later part of the vegetative phase of the pea plants, an excess amount of nitrogenase not utilized in nitrogen fixation was present both in the nodules of S310a and in those of PRE which amounted to 27% in S310a (Hup^+) and 19% in PRE (Hup^-). The *in vitro* hydrogenase activity of S310a bacteroids decreased during the pod-filling period of the plant.

Pea plants inoculated with S310a, cultivated in a growth chamber, showed no positive effects on nitrogen fixation by enrichment of the root atmosphere with 1% of hydrogen, even so when the energy supply of nitrogenase was minimized by combination of low light intensity and nitrate addition. Nitrogenase activity of isolated S310a bacteroids kept under aerobic conditions was not enhanced when 10% of hydrogen was present as a substrate. However, hydrogen oxidation provided a respiratory protection of nitrogenase against high oxygen levels.

As the energy gain by the oxidation of hydrogen was estimated at 0.6 - 4.7% of the ATP produced in the nodules by the respiration of carbohydrates, it is concluded that the occurrence of hydrogenase plays only a minor role in the energy supply of nitrogenase.

INTRODUCTION

Most *Rhizobium* species possess a number of strains which contain a hydrogenase capable of oxidizing all or part of the hydrogen produced by nitrogenase simultaneously with nitrogen fixation (*e.g.* Schubert and Evans, 1976; Evans *et al.* 1980). The energy loss by this ATP-dependent H_2 production might be minimized by hydrogenase activity, although it has been assumed that the production of 1 mole of H_2 requires 7 moles of ATP (Pate, Atkins and Rainbird, 1981) but that the oxidation of 1 mole of H_2 would yield only 2 moles of ATP (Dixon, 1972)

The greater part of the papers published on the subject deal with the hydrogenase of *R. japonicum*. This hydrogenase can virtually recycle all of the H_2 produced by nitrogenase and the presence of the enzyme has been shown to increase nitrogenase activity of isolated bacteroids supplied with H_2 (Ruiz-Argüeso, Emerich and Evans, 1979). Dry-matter production as well as nitrogenase activity were higher in greenhouse-grown soybean plants inoculated with hydrogenase-positive (Hup^+) strains than in similar plants inoculated with hydrogenase-negative (Hup^-) strains (Schubert, Jennings and Evans, 1978). However, when nitrogenase activity and dry matter-production of a Hup^--mutant strain were compared with the values of the Hup^+ wild-type strain, only minor differences of weak significance were found (Zablotowicz, Russell and Evans, 1981; Lepo *et al.*, 1981). In a field experiment with soybean, the use of a Hup^+ strain of *R. japonicum* as inoculant resulted only in a low increase in seed protein but not in a higher seed yield than was obtained with soybean inoculated with a Hup^- strain (Hanus *et al.*, 1981).

With the less-explored Hup^+ strains of *R. leguminosarum*, the hydrogen uptake rates usually are not sufficient to recycle all of the hydrogen generated by nitrogenase activity (Ruiz-Argüeso, Hanus and Evans, 1978; Bethlenfalvay and Phillips, 1979). The alleged beneficial effects of hydrogen oxidation to energy supply of nitrogenase start from the assumption that energy supply is the rate-limiting step of nitrogen fixation. In a previous Chapter (Ch. 1, pp.21-33) it

was shown that nitrogenase content was limiting nitrogen fixation during the main part of vegetative growth of pea plants with a Hup^- strain of *R. leguminosarum*, whereas energy supply was likely to be rate-limiting during generative growth. In order to ascertain whether the presence of hydrogenase could increase nitrogen fixation in *R. leguminosarum*, in the present paper both actual and potential nitrogen fixation values are reported in pea plants inoculated with Hup^+ strain S310a during the growth cycle in open air in soil and under energy-limiting conditions in a growth chamber.

MATERIALS AND METHODS

Plant material and growth

Seeds of pea plants (*Pisum sativum* L. cv. Rondo) were sown in gravel as described earlier (Chapter 2, p. 36) , and inoculated with *R. leguminosarum* S310a, which contains hydrogenase. The plants were kept in a growth chamber at 20°C and 70% relative humidity, with a 16 h light/8 h dark cycle. At 14 days after sowing, when nodule formation had started, plants were transferred to a 4.2-l container with 2.5 l of nitrogen-free nutrient solution and an additional supply of KCl, 10 mmoles per l. The nodules were kept in the gas phase. Ten plants, supported by rubber stoppers, were placed in a plastic lid. Subsequently, the lid was closely fit to the container and the nutrient solution aerated with CO_2-free air containing 1% of H_2 (treatment) or with CO_2-free air (control). After the hydrogenase and nitrogenase measurements at 21 days after sowing, the nutrient solution was replaced by a solution containing KNO_3 (10 mmoles per l) instead of KCl. The nitrogen-containing nutrient solution was refreshed every two days.

In a separate experiment, plants were grown in the open air in pots in soil containing low numbers of indigenous rhizobia, as described before (Chapter 1, pp. 21-33).

Hydrogenase and nitrogenase measurements

In vivo hydrogen production and nitrogenase activity in nodulated roots were determined as described before (Chapter 1).

For the *in vitro* anaerobic assay of hydrogenase and nitrogenase, nodules were picked from the roots after completion of the *in vivo* assay. The nodules were squeezed in a press under argon, at 0°C, in a buffer containing (mmoles/l): Tris(hydroxymethyl)aminomethane, 50; $MgCl_2$, 2.5; sucrose, 300; 1,4 dithiotreitol, 5; and polyvinylpyrrolidone (PVP), 4% ($\frac{w}{v}$). The pH was adjusted to 8.0 with HCl.

Bacteroids were centrifuged for 10 min at 5500g under argon, washed with a buffer solution (same buffer without PVP) and resuspended in this buffer solution. Bacteroids from 1 g of nodules were transferred to a 16.5 ml Hungate tube with 2 ml of buffer containing 10 mM of methylene blue, under an atmosphere of 0.6% H_2 in argon. H_2 consumption at 25°C was followed gas-chromatographically at 30 min intervals after pre-incubation for 10 min (Roelofsen and Akkermans, 1979). *In vitro* nitrogenase was determined in bacteroids treated with EDTA and toluene, and supplied with ATP and sodium dithionite as described before (Van Straten and Roelofsen, 1976; Chapter 1).

RESULTS

In a preliminary experiment, only 2 Hup^+ strains were found among 47 strains of *R. leguminosarum* of the culture collection of this laboratory, showing that the occurrence of a hydrogenase is a rare feature in *R. leguminosarum*. The hydrogenase found in free-living *R. leguminosarum* S310a showed derepression characteristics similar to those of *R. japonicum* 110 when grown in a medium as described by Lim and Shanmugam (1978). For example, uptake hydrogenase activity in cells of S310a with succinate and cyclic AMP amounted to 1.8 mmoles H_2/g.h protein.h, as compared with 2.3 mmoles H_2/g.h with cells of strain 110 with malate and cyclic AMP, reported by Lim and Shanmugam.

Nitrogenase and hydrogenase activities of plants growing in open air

To assess the influence of an uptake hydrogenase on nitrogen fixation and growth, a pot experiment was carried out in which pea plants were cultivated in soil inoculated with *R. leguminosarum* S310a (Hup^+) and PRE (Hup^-). During the entire growth period, considerably less nitrogen was fixed by pea plants grown in symbiosis with S310a than by plants grown with PRE, as evidenced by total plant nitrogen values (720 and 1380 mg N per pot at maturity, respectively).

Actual nitrogen fixation (*in vivo* nitrogenase activity of nodulated roots) and potential nitrogen fixation (*in vitro* nitrogenase activity of isolated bacteroids) are shown in Fig. 1a for the entire growth cycle of the plants. With strain S310a, both actual and potential nitrogen fixation were significantly lower than with strain PRE ($p < 0.001$). During the main part of vegetative growth, actual was equal to potential nitrogen fixation with both strains, but from 61 days after sowing the potential was higher than the actual nitrogen fixation ($p < 0.01$). Actual and potential nitrogen fixation per unit of nodule weight decreased with time (Fig. 1a).

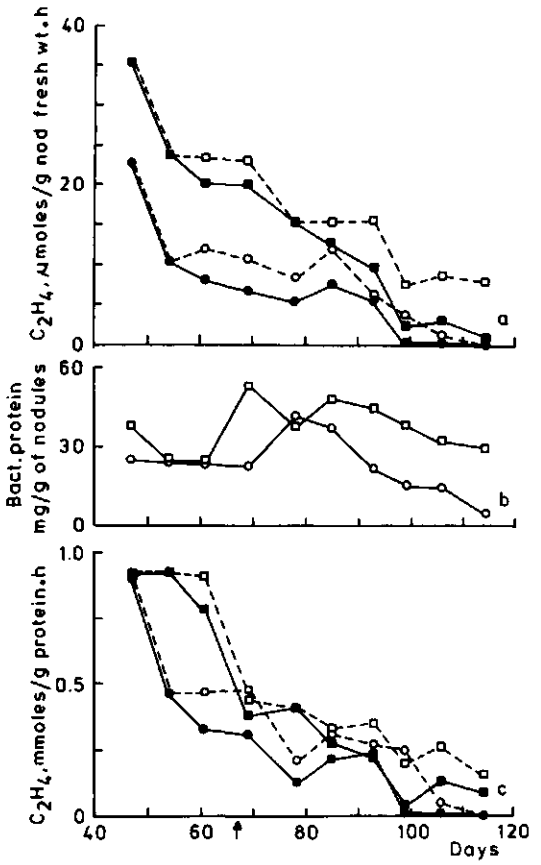

Fig. 1a. Actual (●, ■) and potential (o, □) nitrogenase activity during ontogenesis of pea plants inoculated with *R. leguminosarum* strains S310a (●, o) and PRE (■, □). Values are means of triplicate determinations (potential nitrogenase activity) or duplicate determinations of 8 plants (actual nitrogenase activity) growing in pots in the open air.

Fig. 1b. Bacterial-protein content of nodules from pea plants inoculated with *R. leguminosarum* strains S310a (o) or PRE (□). Values are means of duplicate determinations.

Fig. 1c. Specific nitrogenase activity (*in vivo*, ●, ■; *in vitro*, o, □) of pea plants inoculated with *R. leguminosarum* strains S310a (●, o) and PRE (■, □). Values are means of duplicate determinations.

When nitrogen fixation was calculated for the entire growth season, assuming a nitrogenase activity of 24 h per day without diurnal variation, and a factor of 3.0 to convert moles of ethylene produced into moles of nitrogen fixed, actual and potential nitrogen fixation in pea plants inoculated with S310a would amount to 2.3 and 3.1 mmoles N_2 per plant, respectively. Thus, with the Hup^+ strain S310a 27% of the nitrogen-fixing capacity would not be utilized. In plants inoculated with the Hup^- strain PRE actual and potential nitrogen fixation during the growth period would amount to 3.8 and 4.7 mmoles of N_2 per plant, respectively, which means that 19% of the nitrogen-fixing capacity would not be utilized.

The differences in nitrogenase activity between S310a and PRE can partly be explained by differences in bacterial-protein content of the nodules (Figs. 1b,c), especially during the pod-filling period. However, during the vegetative growth phase the bacterial protein present in S310a was clearly less active in nitrogen fixation than the protein of PRE (Fig. 1c). This might be caused by a less complete transformation of bacteria (rods, no nitrogenase activity) into bacteroids (branched shape, high nitrogenase activity) during nodule development with strain S310a as compared with strain PRE. An almost equal nitrogenase activity per individual bacteroid at a greatly different activity per unit of nodule weight was found earlier in this laboratory with field beans growing with *R. leguminosarum* strains PRE and PF_2 (Van den Berg, 1977).

Hydrogen production in air by nodulated roots was significantly lower in symbiosis with S310a than with PRE (Fig. 2a) resulting in a pronounced difference in relative efficiency (Schubert and Evans, 1976) during the growth period (95% in S310a and 62% in PRE). Bacteroid hydrogenase activity, as determined with the methylene-blue assay, was found in S310a only (Fig. 2b). Similar to nitrogenase activity, specific hydrogenase activity declined with time. If the nitrogenase of strain S310a is assumed to produce hydrogen in the same proportion to ethylene as was found with strain PRE, the hydrogenase values of Fig. 2b are too low to explain the almost absent net hydrogen production in air by nodulated roots with S310a up to 70 days after sowing.

Nitrogenase and hydrogenase activities under reduced light conditions

In order to be able to notice even a minor positive effect of the hydrogenase activity on the energy supply of nodules with strain S310a, an experiment was carried out with pea plants inoculated with this strain, kept in a growth chamber under suboptimal conditions. As a low nitrogenase activity under poor energy conditions might lead to a hydrogen production which could be too low for satu-

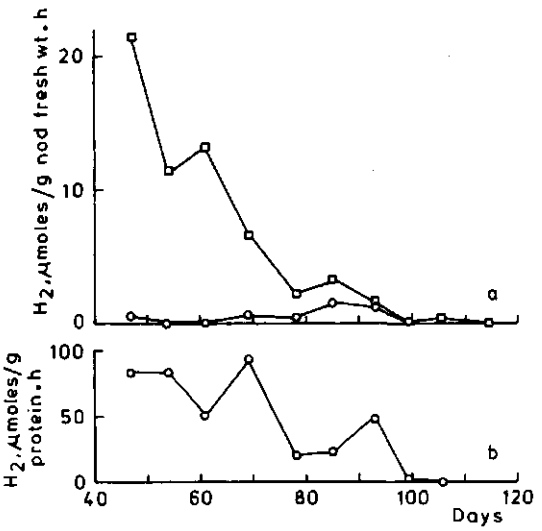

Fig. 2a. Hydrogen production in air by pea roots nodulated with *R. leguminosarum* strains S310a(o) and PRE (□). Values are means of duplicate determinations in sets of 8 plants from pots in the open air.

Fig. 2b. Specific *in vitro* hydrogenase activity of pea root nodules formed with *R. leguminosarum* strain S310a (o). Values are means of duplicate determinations.

ration of the hydrogenase, treatment groups were given 1% of H_2 in the root atmosphere. At a light intensity of 90 W/m^2 no differences were observed in nodule weight per plant and *in vivo* and *in vitro* nitrogenase activity due to growth with 1% of H_2 during 7 days, as compared to control plants grown without added hydrogen. When nitrate (10 mM) was supplied, nodule weight and both *in vivo* and *in vitro* nitrogenase activity decreased equally in hydrogen-treated plants and in plants without added hydrogen. Therefore, experiments were carried out in which light intensity was reduced to 50 W/m^2 and after 7 days nitrate (10 mM) was added in order to minimize energy supply to nitrogenase (Table 1). Under this reduced light intensity, the *in vivo* nitrogenase activity and nodule fresh weight amounted to only one-third of the values found at high light intensity, but no differences in these parameters were found that were associated with hydrogen enrichment. Also the *in vitro* hydrogenase activity was equal, but the *in vitro* nitrogenase activity was clearly higher when the plants were treated with H_2; nitrate partly eliminated this effect.

Table 1. Hydrogenase and nitrogenase activities of pea plants as affected by the supply of 1% of H_2 to the root atmosphere from 14 days after sowing. The plants were inoculated at sowing with *R. leguminosarum* S310a and were kept on an N-free nutrient solution in a growth chamber at 50 W/m^2. At 21 days after sowing the plants were supplied with a similar nutrient solution containing KNO_3, 10 mmoles/l

Item	Days after sowing			
	21 (- NO_3^-)		28 (+ NO_3^-)	
	no H_2	+ H_2	no H_2	+ H_2
In vivo C_2H_4 production (µmoles/plant.h)	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.4 ± 0.3
Nodule fresh weight (mg/plant)[1]	75 ± 8	72 ± 6	143 ± 6	166 ± 10
In vitro hydrogenase activity (µmoles H_2 consumed/g nodules, fresh wt.h)[2]	1.2 ± 0.3	0.9 ± 0.1	0.4 ± 0.2	0.3 ± 0.0
In vitro nitrogenase activity (µmoles C_2H_4 produced/g nodules, fresh wt.h)[2]	5.7 ± 1.3	9.8 ± 1.4	5.2 ± 1.0	6.5 ± 0.2

1) Means and standard deviation of duplicate samples of 10 plants.

2) Means of triplicate samples.

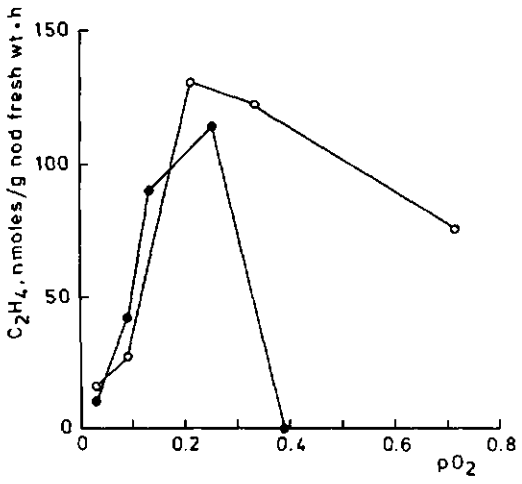

Fig. 3. Effect of pO_2 on the nitrogenase activity of isolated bacteroids of *R. leguminosarum* strain S310a, supplied with 10% of H_2 (o) or without added H_2 (•).

Nitrogenase activity of bacteroids supplied with H_2

The *in vitro* nitrogenase assay determines the activity of the enzyme under anaerobic conditions, using ATP and sodium dithionite as source of energy and of reducing equivalents, respectively. Therefore the contribution of hydrogen uptake to energy supply for nitrogen fixation had to be measured under aerobic conditions with isolated bacteroids capable of using the hydrogen added as substrate. Washed bacteroids of strain S310a from 630 mg of nodular tissue were incubated under 10% of acetylene and varying pO_2, in the presence of reduced myoglobin and of bovine serum albumine (final volume of the incubation mixture 2 ml), as described by Laane, Haaker and Veeger (1978). The effect of 10% of H_2 on nitrogenase activity of bacteroids without added carbon substrate is shown in Fig. 3. The hydrogen uptake system in S310a bacteroids did not raise nitrogenase activity above the rate of bacteroids dependent on endogenous respiration. However, the activity of nitrogenase in bacteroids in the presence of 10% of H_2 is maintained at a higher pO_2, showing that in S310a bacteroids the hydrogenase system only offers some respiratory protection.

DISCUSSION

Hydrogen uptake and utilization by bacteroids of the Hup^+ strain S310a of *R. leguminosarum* did not increase nitrogenase activity but only provided respiratory protection (Fig. 3), in contrast to the results obtained with *R. japonicum* bacteroids (Ruiz-Argüeso *et al.*, 1979). It is doubtful, however, to what extent a respiratory protection is functional in the legume nodule, where bacteroids are present in compartments, buffered as to high oxygen level by leghemoglobin.

No stimulation of nitrogen-fixing characteristics was found in the experiment with nodulated pea plants in hydrogen-enriched environment (Table 1), except the increased *in vitro* nitrogenase activity of plants growing at reduced light intensity. Even in that case, however, the benefit of hydrogen is doubtful as the *in vitro* nitrogenase activity was lower than the *in vivo* activity. This has been found earlier with plants growing in water culture (Houwaard, 1978). As the nodules produced in water culture had a whitish surface due to the formation of lenticel tissue, the oxygen content of this tissue might have caused a partial inactivation of nitrogenase during preparation of the bacteroids.

The *in vitro* nitrogenase activity was higher than the *in vivo* activity during the generative phase of pea plants growing in pots in the open air (Fig. 1a), supporting the concept of potential nitrogen fixation (Chapter 1, pp. 21-34) The hydrogenase present in strain S310a prevented the generation of large amounts of hydrogen in air (Fig. 2a) but did not contribute to improved utilization of the potential nitrogen fixation which amounted to 73% with S310a (Hup^+) and to 81% with PRE (Hup^-). In the generative phase with PRE as well as with S310a, nitrogen fixation seems limited by photosynthate supply, regardless of nitrogen-fixing capacity and regardless of the presence of hydrogenase (Fig. 1a). Both with PRE and S310a, the full capacity of the nitrogen-fixing system was only realized by an enhanced photosynthate supply (Chapter 2, page 39). Even under energy-limiting conditions the presence of hydrogenase had no detectable effect on nitrogen fixation.Thus, hydrogen oxidation will certainly not enhance nitrogenase activity when energy supply is not the limiting factor, *e.g.* during early vegetative growth under optimum conditions when the content of the enzyme is limiting nitrogen fixation.

In a previous paper (Chapter 3) the energy consumption of the nodules of the highly effective strain PRE in acetylene reduction was estimated

at 6.3 moles of ATP per mole of C_2H_2 reduced, whereas in nodules of the moderately effective strain S313 a value was calculated of 12.9 moles ATP consumed per mole of C_2H_2 reduced. When one of these values is assumed to be valid for the nodules of the moderately effective strain S310a, and when the oxidation of 1 mole of H_2 is assumed to yield 2 moles of ATP (Dixon, 1972), the contribution of hydrogenase to energy generation in the nodule can be calculated. Assuming that the costs of acetylene reduction with the moderately effective strain S310a are equal to those with the highly effective strain PRE, hydrogenase activity would produce 1.1 - 2.9% of the ATP generated in respiration in the experiment with added hydrogen (Table 1). In the pot experiments with soil these values amounted to 3.3 - 9.7% during the period when hydrogenase activity was detected (Fig. 2b). However, a comparison with the intermediate strain S313 is more realistic, as nodule weight per plant as well as nodule activity are similar to those with S310a. In that case, the hydrogenase contributes 0.6 - 1.4% of the ATP generated in respiration during the experiment in hydrogen-enriched air, whereas in the pot experiment 1.6 - 4.7% of the ATP production would be provided by hydrogenase activity. These benefits of hydrogenase activity are lower than those calculated by Evans *et al.* (1981) for *R. japonicum* (9.1% of ATP supply to nitrogenase) based on a different set of assumptions.

Whatever the benefits of hydrogenase activity may be, they do not compensate for other,less favourable, characteristics. The shorter span of nodule activity with S310a as compared with strain PRE is one of such characteristics. With hydrogenase-containing strains of *R. leguminosarum*, nitrogen fixation and dry matter production are lower than it is true of most of the hydrogenase-less strains (*e.g.* Fig. 1, S310a *vs* PRE; strain 128C53 *vs* 175G11, Ruiz-Argüeso *et al.*, 1978). In addition, hydrogenase-positive strains might be weak competitors (*e.g.* S310a *vs* PRE, Van Mil, in prep.). Summarizing, it may be concluded that the presence of hydrogenase should only play a minor role in selecting a *Rhizobium* strain for use as an inoculant.

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5 NODULE FORMATION AND NITROGEN FIXATION IN SYMBIOSES OF PEA (*PISUM SATIVUM* L.) AND DIFFERENT STRAINS OF *RHIZOBIUM LEGUMINOSARUM*

ABSTRACT

Symbioses of one pea variety (*Pisum sativum* L., cultivar Rondo) inoculated with different strains of *Rhizobium leguminosarum* showed marked differences in nitrogen-fixing characteristics. Nitrogen fixation, as calculated from plant-nitrogen data, was highest with strain PRE which produced a low nodular mass with a high nitrogenase activity. With this strain red nodules were located mainly on the primary roots, but lateral-root nodules were steadily produced. Pea plants inoculated with strains S313 and S310a fixed less nitrogen than those inoculated with strain PRE. Their nodule formation pattern was different from that of PRE, strain S313 producing large amounts of pink, whitish nodular mass on the primary root but virtually no nodules on the lateral roots, whereas with strain S310a the primary (red) root nodules rapidly were replaced by large amounts of red lateral root nodules. Pea plants inoculated with the ineffective strain P8 produced small, white nodules predominantly on the lateral roots.

Nodule numbers on the primary roots were equal with all strains, whereas numbers of nodules on lateral roots were inversely correlated to nodule weight on the primary root. From the data presented it is concluded that nodule formation is connected with nitrogenase activity, a low activity being compensated for by the production of large nodules (S313) or by the formation of many new nodules (S310a).

Nitrogenase activity of the nodules declined with time. Less electrons were spent in hydrogen production as compared with acetylene reduction, resulting in a rise of relative efficiency with proceeding growth period.

The values of nitrogen fixation, as calculated from acetylene-reduction data, using a conversion factor of 3.0, were lower than those derived from the differences between yield of plant nitrogen of the effective symbioses (with strains

PRE, S313 and S310a) and that of the ineffective symbiosis with strain P8. When the hydrogen produced in air by effectively nodulated roots was subtracted from the acetylene-reduction values, nitrogen fixation was even more seriously underestimated, but in addition severely biased in favour of the hydrogenase-containing strain S310a, which produced a low plant-nitrogen yield.

The real values of the conversion factor, as obtained by division of the acetylene-reduction figures by the yield of plant nitrogen derived from nitrogen fixation, decreased gradually during the growth period. With all strains, nitrogen fixation was highest during the pod-filling stage.

In a separate experiment 3 pea cultivars, differing in seed-production rates, were inoculated with a single *R. leguminosarum* strain. Nitrogen fixation with an early variety (Florix) was lower than that with an intermediate variety (Rondo) or a late variety (Mercato). Mercato fixed more nitrogen than Rondo but its seed yield was less because of a poorer translocation of nitrogen to the seed from vegetative matter.

INTRODUCTION

In agriculture, yields of leguminous crops are determined to a considerable extent by the quality of the symbioses, *i.e.* by the matching of the host cultivar with the rhizobial strain (5,11). As plant breeding programmes are usually aimed at increased yield, and at improved resistance against pests and diseases, a high nitrogen fixation capacity is selected in an indirect way.

A direct approach to enhance nitrogen fixation is to find new *Rhizobium* strains with better nitrogen-fixing characteristics. In the present paper, the relation between nitrogen fixation, yield and symbiotic characteristics has been investigated in associations of peas with a number of selected strains of *Rhizobium leguminosarum*. As low levels of nitrogen fixation have to be compensated by the supply of combined nitrogen for obtaining satisfactory yields, the influence of combined nitrogen on nodulation, nitrogen fixation and yield is described in a second paper.

MATERIALS AND METHODS

Plant material and growth

Enamelled Mitscherlich jars of 6-l capacity, sterilized with alcohol, were filled with a mixture ($\frac{w}{w}$) of 1 part of heat-sterilized sand and 2 parts of non-sterile clay, obtained from newly reclaimed polder land. For inoculation, 50 ml

of a 7-days old culture of *Rhizobium leguminosarum* was mixed with 6 l of soil. A basal fertilizer gift of 3.8 mmoles of phosphorus and 9.6 mmoles of potassium was applied per pot.

Seeds of pea plants (*Pisum sativum* L.), cultivars Florix, Mercato and Rondo, were surface-sterilized by immersion in a solution containing 4% of H_2O_2 and a few drops of Teepol (detergent) for 25 min before sowing. The soil was then covered with sterilized gravel to prevent clogging after watering. Plants were kept in the open air under wire for protection against birds. In each pot finally 8 plants were retained.

Hydrogen production and acetylene reduction

The shoots of the plants were detached, the roots freed from soil particles by gently shaking and incubated in stoppered 1-l Erlenmeyer flasks. After 25 min a 100-µl gas sample was assayed for hydrogen production using a gas chromatograph equipped with a thermal conductivity detector. Subsequently, 100 ml of acetylene was added and a 100-µl gas sample was taken after 15 min. Ethylene production was determined with a gas chromatograph equipped with a hydrogen-flame detector.

Rates of hydrogen production and acetylene reduction were linear with time up to 80 min.

Nitrogen analyses of the plant material

Nitrogen in oven-dried plant material was determined by the Kjeldahl method, using $CuSO_4$ and selenium as catalysts.

RESULTS

In a preliminary experiment, the success of inoculation of the natural soil used in the experiments, which was assumed to contain low levels of indigenous rhizobia, was tested with mutant strains of *R. leguminosarum* resistant against streptomycin as well as acriflavine (Table 1). Seeds were sown in small 0.5-l test pots as described in the Methods section, using 5 ml of a 4-days old bacterial culture grown on yeast-mannitol broth as an inoculum (approximately 5.10^5 bacteria per g of soil). The nodules of the pea plants were checked 40 days after sowing by pricking a needle first into a surface-sterilized nodule and then into yeast-mannitol agar plates without and with streptomycin and acriflavine. Inoculation with *R. leguminosarum* strains PRE^{SA} and $S313a^{SA}$ proved to be successful,

Table 1. Nodule formation on pea (*Pisum sativum* cv. Rondo) by indigenous rhizobia and by inoculated strains (antibiotic-resistant), 40 days after sowing in clay soil. Values represent means and standard deviations of duplicate determinations

Strain	Pink nodules per plant	Antibiotic-resistant colonies (%)	C_2H_2 reduction (nmoles/pl.h)
P8	2 ± 1	0	80 ± 50
S310a^{SA}	15 ± 5	34 ± 9	350 ± 80
S313^{SA}	15 ± 2	88 ± 12	370 ± 170
PRE^{SA}	20 ± 4	78 ± 15	240 ± 120
Uninoculated	7 ± 2	0	100 ± 50

whereas strain S310a^{SA} was less able to compete with indigenous *Rhizobium* strains for nodulation sites. Inoculation with the wild-type strain P8 gave many small white ineffective nodules on pea plants but a few larger pink nodules which were apparently derived from indigenous strains. From these results it was concluded that in a large-scale experiment inoculation should be heavier (5.10^6 bacteria per g of soil) in order to prevent competition difficulties. In the large-scale experiment only wild-type strains were used.

Nodule formation and nitrogen fixation

Nodulation brought about by three *R. leguminosarum* strains showed clearly different patterns (Plate 1). The ineffective strain P8 produced small white nodules in a great number on the lateral roots. In contrast, strain S313 formed big, branched nodules with a faintly pink colour in a great mass, virtually only on the primary roots. Strain PRE occupied an intermediate position with medium-sized, red nodules spread over primary and lateral roots in a more even way. Strain S310a (not shown) behaved similarly to PRE, except for the nodule colour which changed from red to green more rapidly, indicating early senescence.

There was no difference in the number of nodules on primary roots produced by each rhizobial strain (Fig. 1a). The numbers of lateral-root nodules formed during growth were significantly different with the four strains (F test, $p < 0.01$, Fig. 1b). The highest numbers of lateral-root nodules were found with S310a and P8.

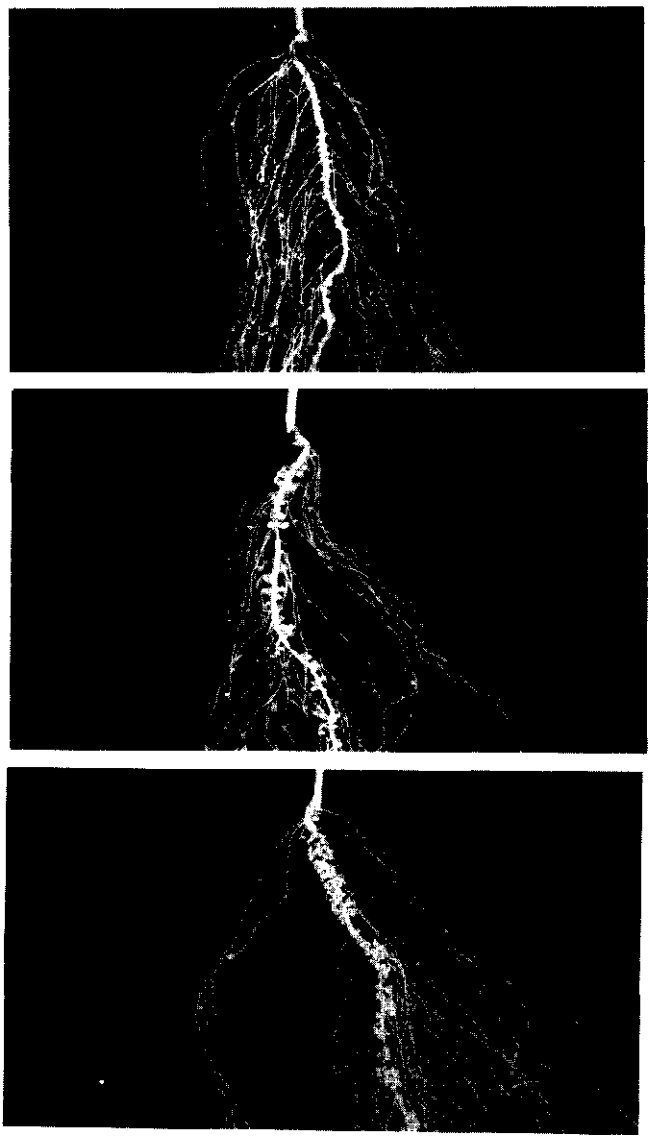

Plate 1. Root systems of 64-days old pea plants inoculated with *Rhizobium* strains P8 (a), PRE (b), and S313 (c).

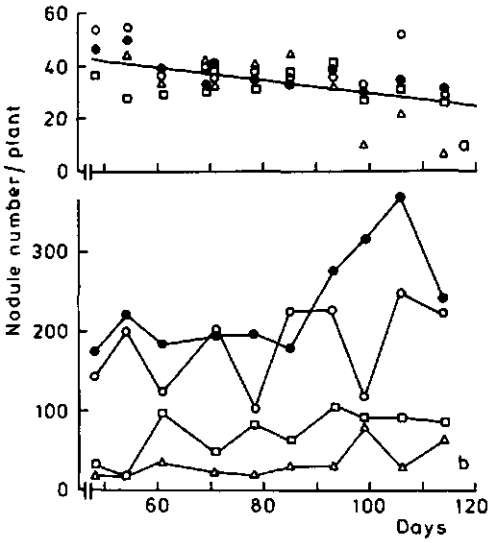

Fig. 1. Root nodule numbers during ontogenesis of pea plants, cv. Rondo inoculated with *R. leguminosarum* strains P8 (●), S310a (o), S313 (Δ), and PRE (□). Values represent means of duplicate samples of 4 plants from different pots. (a) Nodule numbers on primary roots. (b) Nodule numbers on lateral roots.

Nodule fresh weight per plant increased gradually until mid-pod fill, as shown in Fig. 2a. With S313 and S310a nodule weight per plant was significantly higher ($p < 0.01$) than that with PRE. From mid-pod fill, nodule weights decreased slowly with PRE and rapidly with S313. With strain S310a, a considerable proportion of the nodules turned green early. The contribution of primary-root nodules to total nodule weight decreased during plant growth, the greatest decrease being found with S313 (Fig. 2b). Primary-root nodules contributed significantly ($p < 0.01$) more to total nodule weight per plant in the sequence S313 > PRE > S310a, P8.

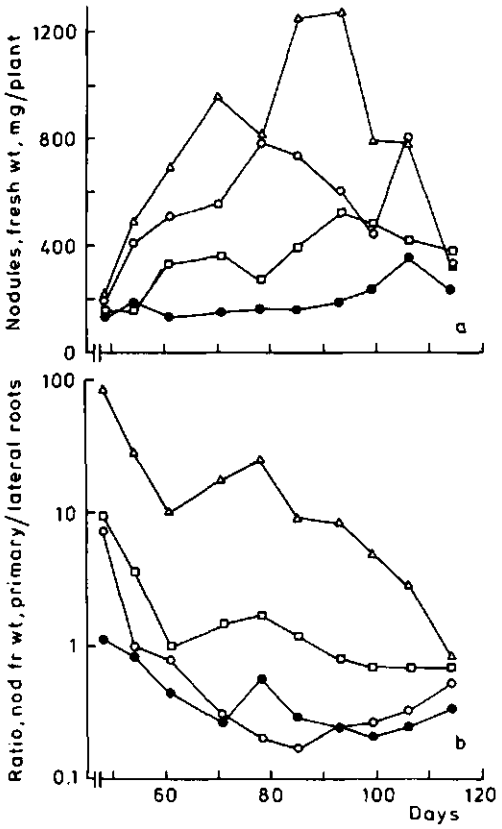

Fig. 2. Root nodule fresh weight during ontogenesis of pea plants, cv. Rondo inoculated with *R. leguminosarum* strains P8 (●), S310a (o), S313 (Δ), and PRE (□). Values represent means of duplicate samples of 4 plants from different pots. (a) Total nodule fresh weight per plant. (b) Weight ratio of primary to lateral root nodules.

Nitrogenase activity of the nodules formed by PRE, S313 and S310a with cv. Rondo declined during growth, the greater fall being found with PRE (Fig. 3a). Roots nodulated with P8 displayed no nitrogenase activity. The quantity of hydrogen produced in air by nitrogenase decreased more rapidly as compared with the decrease in ethylene produced under acetylene, resulting in a steady rise of the relative efficiency $(1-\frac{H_2 \text{ produced in air}}{C_2H_4 \text{ produced in air} + 10\% \ C_2H_2})$(see 13; Fig 3b).

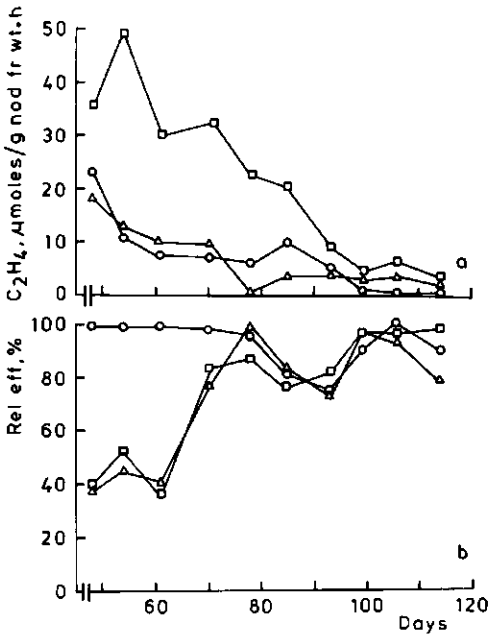

Fig. 3. Nitrogenase activity of root nodules during ontogenesis of pea plants, cv. Rondo, inoculated with *R. leguminosarum* strains S310a (o), S313 (Δ), and PRE (□). Values are means of duplicate pots with 8 plants each. (a) Ethylene produced under air + 10% acetylene, expressed per g of nodule fresh weight. (b) Relative efficiency (see text).

Strain S310a on the other hand displayed a high relative efficiency owing to the presence of a hydrogenase capable of utilizing part of the hydrogen evolved by nitrogenase (Chapter 4, pp. 63-75). Relative efficiency with this strain initially amounted to 96-99% but decreased to the values of the strains without a hydrogenase at pod fill (day 78). This occurred together with the formation of a few large nodules on the lateral roots, which were morphologically different from the nodules initially produced with S310a. When these deviating nodules were assayed separately (day 93) a relative efficiency of 48% was found indicating that they were formed by one of the few rhizobia originally present in the soil.

The *R. leguminosarum* strains studied in this experiment showed distinct differences in the supply of nitrogen to the plant (Fig. 4c). With the ineffective stra P8, the plant was fully dependent on seed and available soil nitrogen, resulting in a very low yield of nitrogen at maturity (15 mg N/plant). The amounts of plant

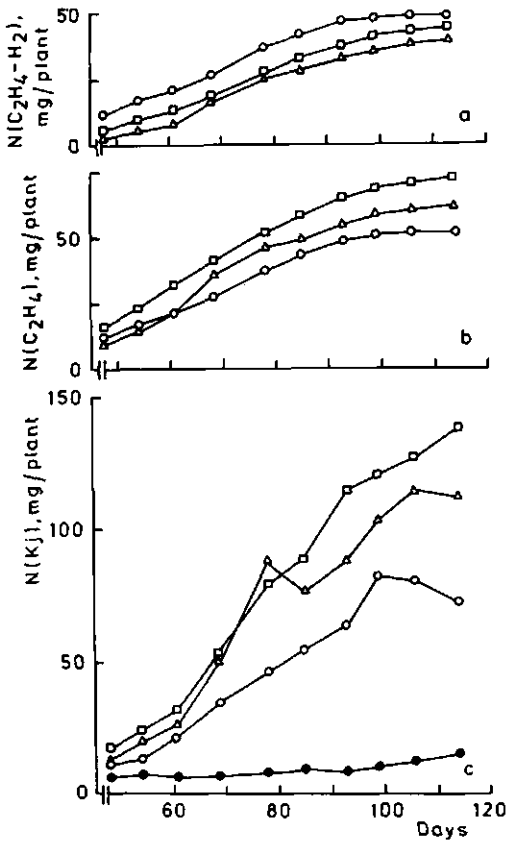

Fig. 4. Nitrogen fixation during ontogenesis of pea plants, cv. Rondo, inoculated with *R. leguminosarum* strains P8 (●), S310a (o), S313 (Δ), and PRE (□). Values represent means of duplicate samples. (a) Calculated from ethylene production (under 10% of acetylene in air) minus hydrogen production in air. (b) Calculated from ethylene production under 10% of acetylene in air. (c) Total plant nitrogen as determined by the Kjeldahl method.

nitrogen contained in cv. Rondo differed significantly ($p < 0.01$) with the rhizobial strains in the sequence PRE > S313 > S310a > P8.

The fact that pea plants inoculated with strain P8 displayed no nitrogenase activity, enabled us to calculate the amount of nitrogen fixed by nitrogen-fixing symbioses by subtracting the nitrogen yield of the P8-associations from the values of the effective symbioses. During the entire growth period, nitrogen fixation of the 3 associations tested amounted to 122, 96, and 56 mg N per plant with PRE, S313 and S310a, respectively (fig. 4c).

Nitrogen fixation during the growth period was also estimated from acetylene-reduction data, assuming an activity of the nitrogen-fixing system during 24 h per day without diurnal variation, and using a factor of 3.0 to convert moles of ethylene produced under acetylene into moles of nitrogen fixed (Fig. 4b). These values underestimated nitrogen fixation as compared with the plant nitrogen data: 71, 61, and 52 mg N per plant with PRE, S313 and S310a, respectively. This underestimation was more serious with strains PRE and S313 (37-42%) than with strain S310a (8%).

Hydrogen production in air by nitrogenase is usually considered to occur in competition with nitrogen fixation. Therefore, nitrogen fixation was also estimated from ethylene production under air + 10% of acetylene (representing total nitrogenase activity) minus hydrogen production in air (Fig. 4a). According to this method, nitrogen fixation during the growth period would amount to 43, 38 and 48 mg N per plant with strains PRE, S313 and S310a, respectively. Thus, the inclusion of the hydrogen production not only aggravated the underestimation of nitrogen fixation but even caused a misrepresentation of the nitrogen-fixing capacity in favour of the hydrogenase-positive strain S310a.

The distribution of the nitrogen-fixing activity over the growth period was influenced by the rhizobial strain (Table 2). With S310a, nitrogen fixation declined rapidly during pod formation, whereas with S313 and PRE a considerable part of the total nitrogen fixation took place during the pod-filling period. When the conversion factors of ethylene production under acetylene into nitrogen fixed were calculated on a growth season basis, assuming an activity of 24 h per day without diurnal variation, the values deviated considerably from the theoretical value of 3.0 (Table 2). The ratio was high during vegetative growth but decreased afterwards. When the allocation of electrons between nitrogen and proton reduction was taken into account, the ratio dropped below 3.0 except for strain S310a. This strain, which contains an uptake hydrogenase, gave rise to a consistently higher ratio between ethylene produced and nitrogen fixed than the other strains. However, also with S310a this ratio declined during the growth period.

Table 2. Nitrogen fixation during ontogenesis of pea (cv. Rondo)-*Rhizobium* symbioses[1)]

Growth stage	Strain of microsymbiont		
	S310a	S313	PRE
Vegetative (1-68 days)			
N_2 fixed (mmoles/plant)[2)]	1.00	1.61	1.60
C_2H_4/N_2[3)]	4.33	3.54	4.14
$(C_2H_4-H_2)/N_2$[4)]	4.25	1.67	1.82
Flowering and pod formation (69-86 days)			
N_2 fixed (mmoles/plant)[2)]	0.64	0.71	1.12
C_2H_4/N_2[3)]	4.07	2.87	2.27
$(C_2H_4-H_2)/N_2$[4)]	3.83	2.37	1.89
Pod-fill and ripening (87-114 days)			
N_2 fixed (mmoles/plant)[2)]	0.49	1.24	1.62
C_2H_4/N_2[3)]	2.86	1.67	1.37
$(C_2H_4-H_2)/N_2$[4)]	2.31	1.41	1.13
Entire growth period (1-114 days)			
N_2 fixed (mmoles/plant)[2)]	2.11	3.56	4.38
C_2H_4/N_2[3)]	3.90	2.76	2.62
$(C_2H_4-H_2)/N_2$[4)]	3.68	1.72	1.58

1) Values are averages of duplicate samples of 8 plants

2) Calculated from plant nitrogen of the effective symbiosis minus plant nitrogen of the ineffective symbiosis (Rondo x P8)

3) Conversion factor for converting C_2H_4 produced in air + 10% of C_2H_2 into N_2 fixed

4) Conversion factor for converting C_2H_4 produced in air + 10% of C_2H_2, minus H_2 produced in air, into N_2 fixed.

Host influence

In a separate experiment, the influence of the host plant was briefly checked in 3 pea cultivars with different rates of seed production: Florix (early), Rondo (intermediate), and Mercato (late). The plants were inoculated with a single strain of *R. leguminosarum*, PF_2. The distribution of nitrogen fixation during the growth period is shown in Table 3. The considerably shorter growth period of Florix resulted in a lower yield of fixed nitrogen and seed yield (Fig. 5a) as compared with Rondo and Mercato. The late-flowering cultivar Mercato apparently had a higher nitrogen-fixing capacity than Rondo, when calculated from values of ethylene produced under acetylene, using a conversion factor of 3.0 to convert moles of ethylene produced into nitrogen fixed (Fig. 5b). However, the actual difference in plant nitrogen between Mercato and Rondo was not as large as suggested by the ethylene production data (Fig. 5c). With Mercato, seed yield was lower than with Rondo (Fig. 5a) but final yield of plant nitrogen was higher owing to a higher nitrogen content of the leaves (at maturity 35.4 and 18.6 mg N per plant with Mercato and Rondo, respectively).

Table 3. Nitrogen fixation during ontogenesis of pea cultivars inoculated with *R. leguminosarum* strain PF_2

Growth stage	Cultivar		
	Florix	Rondo	Mercato
Vegetative (days)	1 - 47	1 - 57	1 - 64
N_2 fixed (mmoles/plant)[1)]	1.13	1.79	2.00
Flowering and pod formation (days)	48 - 58	58 - 73	65 - 86
N_2 fixed (mmoles/plant)[1)]	0.37	0.70	1.40
Pod-fill and ripening (days)	59 - 86	73 - 100	87 - 105
N_2 fixed (mmoles/plant)[1)]	0.54	1.45	0.67
Entire growth period (days)	1 - 86	1 - 100	1 - 105
N_2 fixed (mmoles/plant)[1)]	2.04	3.94	4.07

[1)] Calculated from plant nitrogen of the effective symbiosis minus plant nitrogen of the ineffective symbiosis Rondo x P8. Values are averages of duplicate determinations of 8 plants

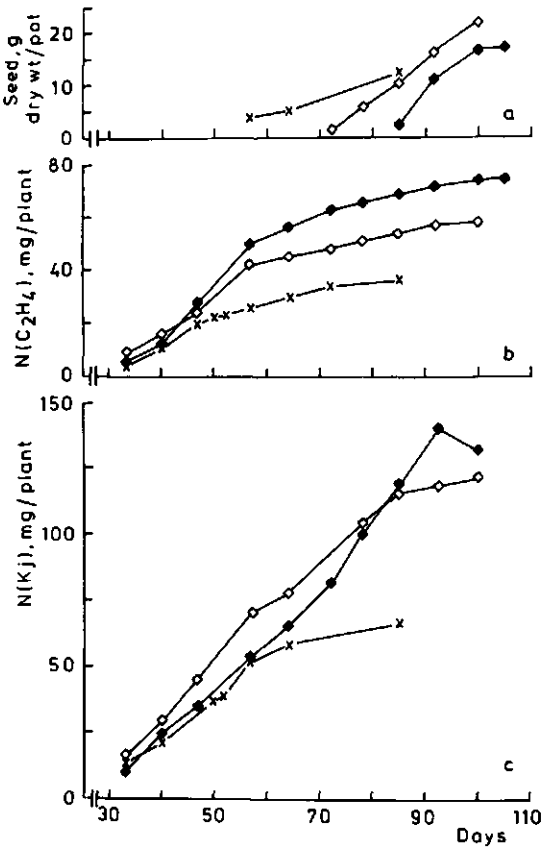

Fig. 5. Seed production and nitrogen fixation during ontogenesis of pea cultivars (Florix, x; Rondo, ◇; and Mercato, ◆) inoculated with *R. leguminosarum* strain PF_2. Values represent means of duplicate samples. (a) Seed production. (b) Nitrogen fixation as calculated from acetylene reduction values. For explanation see text. (c) Plant nitrogen as determined by the Kjeldahl method.

DISCUSSION

Nodule formation

The difference in nodule formation between legumes infected with an effective strain of *Rhizobium* and legumes infected with an ineffective strain was reported early in the study of nitrogen fixation (6). However, differences in nodule formation between effective strains have received less attention (18). With strain PRE, a high nitrogenase activity of the nodules (Fig. 3a) was associated

with a relatively low nodular mass, mainly located on the primary root (Fig. 2). With strain S313, the lower nitrogenase activity of the nodules was almost compensated by the high nodular mass produced (compare Figs 2, 3 and 4) whereas with strain S310a the low nitrogenase activity led to a constant formation of nodular tissue on lateral roots (Fig. 2). However, with S310a this did not result in a nitrogen-fixing capacity as high as that of PRE, as the older nodules aged at an early stage.

The nodulation pattern of strain S310a was similar to that of strain P8 (Figs 1, 2). As with S310a and P8 nodule numbers were equal but nodule weights were different (Figs 1, 2), the hypothesis that nodule formation is regulated by the synthesis of an unknown inhibiting compound is unlikely, as this hypothesis cannot explain the occurrence of different nodule weights at equal nodule numbers (12). An explanation of the observed differences in nodulation patterns might be offered by assuming differences in synthesis of growth-stimulating phytohormones, such as cytokinins. A function of phytohormones in nodule formation as has been proposed by Libbenga (10), has so far not been verified adequately. Therefore, we restrict to the general view that nitrogenase activity and nodule formation are related by strategies that are different with each strain. A high nitrogenase activity is assumed to be associated with a relatively low amount of nodular tissue (PRE) and *vice versa* (S313), whereas a rapid senescence is compensated by the formation of new nodules. These patterns are of course dependent on the interaction between host and microsymbiont. A large supply of photo-assimilates increases nodule mass, as was discussed earlier in a comparison of pea with field bean (Chapter 1, page 31).

Hydrogen production

Hydrogen production in air decreased relatively to ethylene production (Fig. 3b) with strains PRE and S313 during the first 60 days of growth. Subsequently, it declined much more sharply than the ethylene production, so that the relative efficiency of the symbioses with these strains rose to approximately 90%. The relative efficiency with strain S310a was nearly 100% owing to the presence of hydrogenase which recycled the hydrogen produced by nitrogenase, but obviously this process did not compensate for the low nitrogenase activity and unfavourable nodule formation pattern with this strain (Fig. 4).

The observed rise in relative efficiency of nitrogen-fixing nodules (Fig. 3b) during the generative growth phase of the pea plants coincides with the presumably decreasing supply of photosynthates to the nodules due to the formation of pods (9). This observation is in agreement with results reported in a

previous Chapter 1,(pp.21 - 34) which showed that energy supply of nitrogenase during the generative phase is insufficient for a maximum nitrogenase activity, in contrast with the vegetative phase, during which nitrogenase is entirely utilized. The decrease in hydrogen production during presumably reduced photosynthate supply of the nodules seems to contradict evidence from experiments with isolated nitrogenase (16, 7, 17). These experiments generally indicate that under low energy conditions, *e.g.* when ATP levels are low (16), or when electron flow through nitrogenase Mo-Fe component is low (7), electrons are allocated to proton reduction rather than to the fixation of nitrogen. Also more hydrogen is produced when the ratio of Fe component (II) to Mo-Fe component (I) of nitrogenase is low, *i.e.* when few electrons are transferred by the Fe protein to the Mo-Fe protein (17).

Van den Bos *et al.*(3) have shown that the ratio of Fe protein to Mo-Fe protein increases with the age of bacteroids of *R. leguminosarum* strain PRE. Therefore, the increase in relative efficiency during the growth period of pea plants (Fig. 3; 1, 2) might be associated with the altered ratio of nitrogenase components rather than to the inadequate photosynthate supply of the nodules.

The observation that nitrogenase is not fully utilized during generative growth of pea plants and field bean plants is likely to be explained by the lower photosynthate supply, as in short-term experiments an excess of nitrogenase, present in the nodules, can be activated by increasing photosynthate supply (Chapter 2)

The calculation of nitrogen fixation from acetylene-reduction figures is hazardous, as can be seen from Fig. 4 and Table 2. Even when nitrogenase activity was assumed to continue for 24 h per day at its daytime level , the plant nitrogen yields were underestimated by the use of a conversion factor of 3.0 (Fig. 4; 8). In any case, hydrogen production should not be taken into account when comparing strains with and without hydrogenase, as not only an underestimation occurs but even a misrepresentation is found in favour of a strain with hydrogenase but of low nitrogen-fixing capacity. The data of Table 2 also indicate that a conversion factor of 3.0 underestimates nitrogen fixation more seriously when applied to plants in the generative growth phase than to plants in the vegetative phase (15). In the latter case nitrogen-fixation data are overestimated when derived from acetylene-reduction values.

Host plants

Some desirable features of pea varieties with regard to nitrogen fixation have been described in the literature (4). From Fig. 5 it can be derived that the early variety Florix showed 'self-destructive' traits (14) due to a rapid seed production, resulting in a low overall level of nitrogen fixation. The late flowering variety Mercato fixed more nitrogen than the intermediate variety Rondo, especially during the prolonged stages of vegetative growth and flowering. However, seed production of Mercato was lower than that of Rondo, stressing once more that nitrogen-fixing capacity is just one of the main features to be reckoned with in the selection of host plants.

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6 EFFECT OF N-FERTILIZERS ON NITROGEN FIXATION AND SEED YIELD OF PEA-*RHIZOBIUM* SYMBIOSES OF DIFFERENT NITROGEN-FIXING CAPACITY

ABSTRACT

The supply of nitrate to pea (cultivar Rondo)-*Rhizobium* symbioses with different nitrogen-fixing capacity growing in natural soil, lowered nitrogen fixation more seriously in symbioses with the highly effective strain PRE than with the moderately effective strain S313. Nitrogen fixation with strain S310a was lower than that with S313, but was enhanced by low levels of nitrate supply. In pea plants inoculated with strain PRE, nitrogen fixation was decreased by the addition of combined nitrogen irrespective of the time of dressing during the growth period.

Leaf dry weight and seed yields were significantly increased by the supply of nitrate, even in the case of symbioses with the highly effective strain PRE. Seed yield increases owing to the application of nitrate with strains S310a and S313 were higher than those with PRE. Pea plants inoculated with S313 produced more seed at high nitrate levels than plants infected with PRE, associated with a higher leaf mass and nitrogen fixation. The differential effects of combined nitrogen on nitrogen fixation and seed yield were described in a model. According to a path analysis, the fact that nitrogen fixation of a less effective strain is relatively unaffected by combined nitrogen could be attributed to increased photosynthesis. The harvest index was dependent on the rate of seed production by the pea cultivar and was not affected by the rhizobial strain or by nitrate supply. Only in the case of the ineffective symbiosis Rondo x P8 the harvest index increased owing to nitrate supply.

Nodule numbers and nodule weight per plant decreased with all strains after the supply of nitrate, whereas the weight per nodule was raised with strains PRE and S310a. With those strains, the fewer nodules also showed a higher nitrogenase activity per nodule than those of the plants without added nitrate. In the xylem exudate of plants inoculated with effective strains, asparagine

and aspartic acid accounted for 50 and 20% of the total amino-acid nitrogen, respectively. With the ineffective symbiosis Rondo x P8 less asparagine and more aspartic acid was produced, *viz.* 10 and 50%, respectively, of the total amino-acid nitrogen. The amino-acid pattern did not vary with nitrate supply.

Neither ethanol-soluble nor insoluble carbohydrate content of the roots of an effective symbiosis was influenced by nitrate supply. With an ineffective strain, however, the ethanol-soluble carbohydrates increased with nitrate supply up to 800 mg N per pot but decreased thereafter. This phenomenon was attributed to an increased production of photosynthate which could not be used in growth, due to lack of nitrogen.

From the data presented in this paper it is concluded that strains whose nitrogen fixation has a low sensitivity in symbioses to combined nitrogen offer no favourable prospects for use as an inoculant.

INTRODUCTION

In a previous paper, a number of pea-*Rhizobium* symbioses were described with distinctly different nitrogen-fixing capacities. The results obtained with those symbioses gave rise to the hypothesis that associations with a poor nitrogen-fixing capacity,giving plants with a low nitrogen content and a low seed yield, might respond to combined nitrogen differently from a symbiosis with a high nitrogen-fixing capacity.

Combined nitrogen is known to exert a detrimental effect on nodule formation and on nitrogen fixation, as was already observed in the beginning of nitrogen fixation research (17, 24). It has no direct effect on the nitrogen-fixing system (11) but reduces the photosynthate supply to the root nodules, leading to a lower nitrogenase activity (18; Chapter 2, page 39). The addition of combined nitrogen therefore often leads to a simultaneous decrease in nitrogen fixation without increasing yield (2, 21). However, also beneficial effects of combined nitrogen on nitrogen fixation have been reported, for example the addition of a 'starter dose' which increases nitrogen fixation by enhancing photosynthesis (4, 12, 13, 15). Since these effects so far have not been examine in a coherent study, the investigationsreported in the present paper deal with the effect of combined nitrogen on pea-*Rhizobium* symbioses with high and low nitrogen-fixing capacities in relation to nitrogen fixation, photosynthetic capacity and seed yield.

MATERIALS AND METHODS

Plant growth and nitrogen fertilizer

The various pea-*Rhizobium* symbioses were grown in non-sterile soil as described in the previous paper. Rye grass, Westerwolds, cv. Tewera (*Lolium perenne*) was sown in soil without gravel cover.

A solution of NH_4NO_3 or $NaNO_3$ was given at different growth stages of the plant. In the ^{15}N experiment a solution of $K^{15}NO_3$ was supplied at sowing and at pod fill (enrichment percentages varying from 1.28-2.79 atom % ^{15}N).

Amino acids in xylem exudate

Pots were watered and the plants were decapitated after 2 h. Xylem sap was collected in glass capillary tubes for 1 h and stored at -20°C until analysis. Protein was removed from the samples by addition of sulphosalicylic acid, 2% by weight, followed by centrifugation. The supernatant was analyzed for amino acids with a Biotronik Amino Acid Analyzer.

Carbohydrates

Ethanol-soluble carbohydrates ('sugars') were determined in ethanol extracts of roots by the anthrone method (20). The residue was heated at 100°C for 2 h with 0.1 *N* HCl to hydrolyze the ethanol-insoluble carbohydrates ('starch') which were subsequently measured in the supernatant with the anthrone method.

Nitrogen determinations

Nitrogen content of shoot, root, pod wall and seed was determined by the Kjeldahl method. A 100 mg sample of dry plant material was digested for 3 h at 360°C with 3 ml of concentrated H_2SO_4, using a mixed catalyst of $CuSO_4$, Se and K_2SO_4. Subsequently, the ammonia generated in digestion was steam-distilled into 10 ml of H_3BO_3 (40 g/l) and titrated with 0.02 *N* H_2SO_4, using a mixed indicator of methylred and bromocresolgreen.

When samples were analyzed for ^{15}N, the ammonia generated in the digestion was steam-distilled into 10 ml 0.1 *N* HCl. Ammonia was determined in a 1-ml aliquot with Nessler's reagent. The remaining distillate was analyzed for ^{15}N by emission spectrometry, using the Rittenberg method of freeing N_2 from NH_4Cl (1). Enrichment of ^{15}N was calculated by comparison with a standard set supplied by VEB Chemie, Berlin, D.D.R. (7).

RESULTS

Nitrogen fixation and combined nitrogen supply

The nitrogenase activity of effectively nodulated pea plants (cv. Rondo x PRE) was reduced by the supply of combined nitrogen at sowing (Fig. 1). Even a low dose of ammonium nitrate decreased *in vivo* nitrogenase activity and delayed the occurrence of the maximum enzyme activity. In plants without added nitrogen, maximum nitrogenase activity was found at the beginning of the pod-filling stage.

The effect on nitrogen fixation of split dressings of combined nitrogen at various moments of the growth period was studied in a separate experiment with pea plants inoculated with *R. leguminosarum* strain PRE (effective) and strain P8 (ineffective). Nitrogen fixation was calculated as the difference in total plant nitrogen between the effective and ineffective symbiosis (Fig. 2).

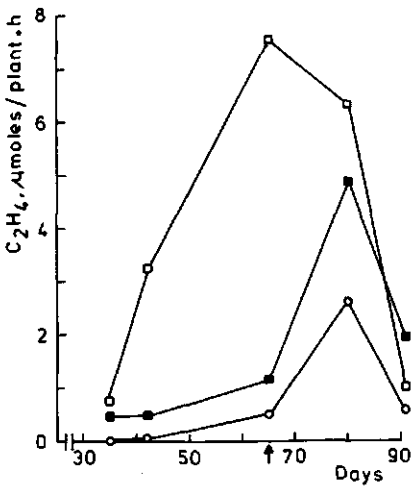

Fig. 1. Profiles of nitrogenase activity in the symbiosis pea (cv. Rondo) x *R. leguminosarum* PRE during ontogenesis (1978 experiment), as affected by the supply of NH_4NO_3 at sowing: no added N, □ ; 314, ■ ; and 942 mg N/pot, ○. Arrow indicates the beginning of the pod-filling stage, 65 days after sowing. Values are means of duplicate samples of 8 plants.

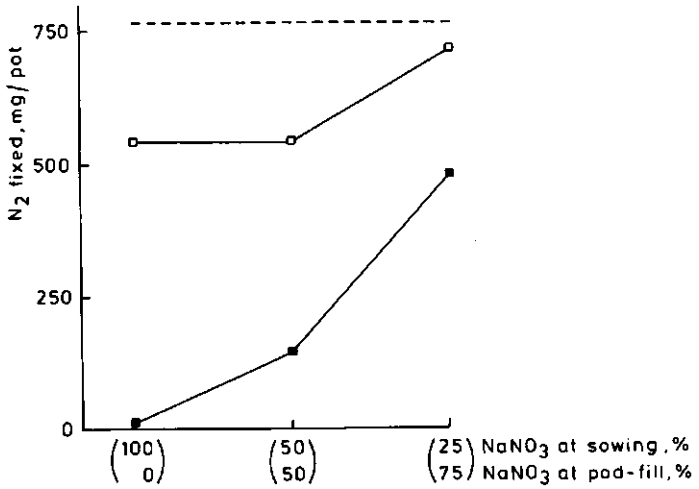

Fig. 2. Yields of nitrogen fixed in the symbiosis pea (cv. Rondo) x *R. leguminosarum* PRE (1979 experiment) as affected by the addition at various growth stages of $NaNO_3$: 515, □ ; 1130 mg N/pot, ■ . N (Kj) analyses were performed at maturity of the plants. The broken line indicates the amount of nitrogen fixed by plants growing without added $NaNO_3$. Values represent means of duplicate determinations.

Obviously, nitrogen fixation is reduced less seriously by the supply of combined nitrogen late in the growing season than by supply at an earlier date.

Seed yield of effectively nodulated pea plants was hardly affected by varying the moment of (split) dressings of combined nitrogen during the growth period (Table 1). Supply of the nitrogen during flowering and pod filling tended to give higher seed yields.

Experiments with $^{15}NO_3^-$. The response to combined nitrogen of nitrogen fixation in symbioses with different nitrogen-fixing capacities was studied in an experiment in which ^{15}N-labeled nitrate was used in order to differentiate between fixed nitrogen and plant nitrogen derived from combined nitrogen. Nitrate was given in split dressings of equal quantities at sowing and at pod fill to prevent damage by high doses of the fertilizer.

Yield of plant nitrogen of all symbioses had increased substantially by the supply of nitrate.The highest amount of nitrate had been taken up by the moderately effective symbiosis of cv. Rondo with *R. leguminosarum*, strain S310a (column 6 of Table 2).

Table 1. Effect of the moment of split dressings with NH_4NO_3 on the seed yield of pea plants (cv. Rondo) inoculated with *R. leguminosarum* strain PRE [1)]

Total amount of NH_4NO_3 added (mg N/pot)	Split dressings of NH_4NO_3 (mg N/pot) during the stage of				Seed dry weight (g/pot)
	Sowing	Vegetative growth	Flowering	Pod-filling	
0	0	0	0	0	13.0 *c*
314	314	0	0	0	15.0 *c*
	0	314	0	0	18.1 *b*
	0	0	314	0	18.2 *b*
	0	0	0	314	17.8 *b*
628	314	314	0	0	17.7 *b*
	314	0	314	0	19.8 *ab*
	314	0	0	314	18.1 *b*
	0	314	314	0	22.0 *a*
	0	314	0	314	19.2 *b*
	0	0	314	314	19.3 *b*

1) Values are means of determinations of 5 pots. Values followed by the same letter are not significantly different in Tukey's test at the 0.05 level.

To estimate the amount of nitrogen fixed by the various symbiotic systems, total plant nitrogen of the effective symbiosis was diminished with the amount of plant nitrogen of the ineffective symbiosis dressed with the same amount of combined nitrogen as the effective association (column 4 of Table 2). As alternative for the ineffective symbiosis, the nitrogen yield of rye grass plants was employed for calculating the yields of fixed N (column 5 of Table 2).

A somewhat more accurate way to estimate the amount of fixed nitrogen in symbioses grown in natural soil and dressed with combined nitrogen consists of the calculation of total plant nitrogen minus (a), the nitrate-derived nitrogen of the plant material and (b) the amount of nitrogen taken up from soil and seed. The nitrate-derived nitrogen in plant material can be directly measured by supplying a labeled nitrogen compound (column 6 of Table 2). Available soil + seed N was derived from the ineffective symbiosis cv. Rondo x P8, dressed with an equal amount of nitrate (column 7 of Table 2). The results of these calculations are given in columns 4, 5 and 8 of Table 2. Comparison of both ways of estimation shows that at low levels of combined nitrogen the first-mentioned method gave somewhat lower values than the last-mentioned,whereas at high levels the results tended to be the other way round.

Table 2. Estimations of nitrogen fixed during the growth period of pea (cv. Rondo)-*Rhizobium* symbioses grown in natural soil and dressed with $K^{15}NO_3$, half of which was applied at sowing and the other half at the beginning of the pod-filling period. All values are means of duplicate determinations, and are given as mg N per pot

Plant and strain of microsymbiont (1)	$K^{15}NO_3$ supplied (2)	Total plant N (3)	Yield of fixed N_2= total plant N minus plant N of		$K^{15}NO_3$ utilized by plants (6)	Available soil + seed N (7)	Yield of fixed N_2 = (3) minus (6) and (7, Rondo x P8) (8)	'A' value (9)
			Rondo x P8 (4)	Rye grass (5)				
Rye grass	0	131	-	-	0	131	-	
	800	548	-	-	386	162	-	
	1600	1258	-	-	1098	169	-	
Rondo x P8	0	129	-	-	0	130	-	
	400	482	-	-	348	134	-	
	800	598	-	-	524	74	-	
	1200	994	-	-	899	95	-	
	1600	1170	-	-	1091	79	-	
Rondo x S313	0	1125	997	995	0	-	997	
	400	1357	876	1018	288	-	936	
	800	1448	850	900	464	-	910	
	1200	1851	856	948	983	-	773	
	1600	1755	585	497	1141	-	535	531
Rondo x S310a	0	720	592	589	0	-	592	
	400	1065	583	726	287	-	645	
	800	1389	791	841	496	-	819	
	1200	1539	545	636	831	-	613	
	1600	1677	507	419	1259	-	339	347
Rondo x PRE	0	1356	1227	1225	0	-	1228	
	400	1501	1020	1162	249	-	1118	
	800	1450	853	902	652	-	724	
	1200	1492	498	589	870	-	527	
	1600	1549	379	291	1093	-	377	376

A simplified method of calculating nitrogen fixation from ^{15}N-fertilizer trials has been proposed by Fried and Middelboe (8). According to their method, the amount of fixed nitrogen (A value) is calculated as

$$\left(1 - \frac{\text{excess atom \% } ^{15}N \text{ in legume}}{\text{excess atom \% } ^{15}N \text{ in reference crop}}\right) \times \text{total N legume}$$

The calculated A values of our experiment correspond closely to the nitrogen fixation values found by subtraction of nitrate-derived nitrogen and available soil nitrogen from total nitrogen of the effective symbioses (Table 2, column 9).

Of the three effective *R. leguminosarum* strains tested the highly effective strain PRE in symbiosis with cv. Rondo fixed the highest amounts of nitrogen in the absence of added nitrate or with a low amount of this N compound. With high amounts of added nitrate (800, 1200 and 1600 mg N per pot) nitrogen fixation with strain PRE was considerably reduced. With the moderately effective strain S313 lower amounts of nitrogen were fixed than with PRE in the absence of combined nitrogen, but the reverse was true at high levels of combined nitrogen. The low nitrogen-fixing capacity with strain S310a was enhanced by 38% by nitrate supply up to 800 mg N per pot, whereas with higher levels of combined nitrogen, nitrogen fixation decreased, similar to that of the other strains.

Nitrogen fixation during the growth period. The greater part of nitrogen fixation occurred during the pod-filling period (Table 3) as has been found earlier in pea (19), soybean (9) and cowpea (6). The higher nitrogen fixation during the pod-filling stage coincided with a somewhat higher efficiency of nitrate uptake than during the period of vegetative growth and flowering. Nitrogen fixation of pea-*R. leguminosarum* strains with a low or intermediate nitrogen-fixing capacity (S310a and S313, respectively) responded favourably to added nitrate during the generative phase of the growth cycle. The yields of fixed N until maturity were increased by the supply of nitrate up to a dose of 800 mg N per pot at the beginning of the pod-filling period, whereas nitrate supply at sowing decreased nitrogen fixation with all strains.

Table 3. Uptake of $K^{15}NO_3$ and N_2 fixation by pea plants (cv. Rondo) from sowing tothe beginning of pod-fill (1-72 days) and from pod-fill until maturity (73-114 days). Values represent means of duplicate samples of 8 plants and are given as mg N/pot

Strain of microsymbiont	$K^{15}NO_3$ supplied 1)	Uptake of $^{15}NO_3^-$		N_2 fixation	
		(1-72)	(73-114)	(1-72)	(73-114)
P8	0	-	-	-	-
	400	108	240	-	-
	800	157	367	-	-
	1200	378	521	-	-
	1600	524	567	-	-
S313	0	-	-	429	568
	400	121	167	262	674
	800	218	246	164	746
	1200	452	531	85	688
	1600	491	650	171	364
S310a	0	-	-	278	314
	400	106	181	174	471
	800	218	278	139	680
	1200	374	457	196	417
	1600	504	755	125	214
PRE	0	-	-	442	786
	400	137	112	436	682
	800	241	411	246	478
	1200	392	479	174	353
	1600	525	568	102	275

1) Split dressing, 50% at sowing and 50% at day 73.

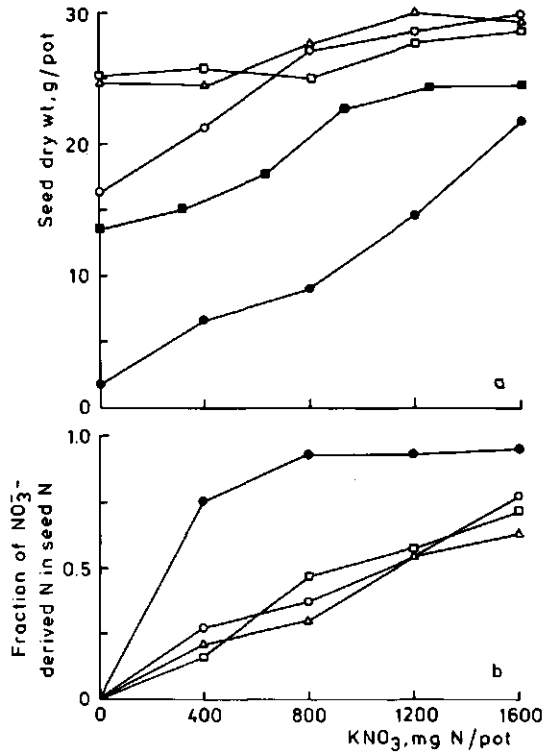

Fig. 3. Seed yields at maturity of pea-*Rhizobium* symbioses as affected by split dressings of KNO_3 *viz.* 50% at sowing and 50% at pod fill. Values represent means of yields of 4 pots.
(a) Pea cv. Rondo inoculated with *R. leguminosarum* strains P8 (●), S310a (○), S313 (△), and PRE (□)(1980 experiment). PRE (1978) supplied with NH_4NO_3 (■).
(b) Fraction of NO_3^--derived N in seed N, at maturity of pea cv. Rondo, inoculated with *R. leguminosarum* strains P8 (●), S310a (○), S313 (△), and PRE (□).

Seed yield

N dressings with KNO_3 of pea plants (cultivar Rondo), inoculated with different *Rhizobium* strains, raised seed dry weight (Fig. 3a). With strains P8, S310a and S313, seed yields were significantly enhanced ($p < 0.001$) by nitrate supply (1980). Strain PRE produced a high seed yield in the 1980 experiment without added nitrate; supply of the nitrogen compound gave only a low, weakly significant ($p < 0.05$) additional seed yield. However, in the 1978 experiment, the seed dry weight with PRE was much lower than in 1980, but it was considerably enhanced by added NH_4NO_3 ($p < 0.001$). This points out that seasonal factors exert an important influence on the yield response to combined-nitrogen application.

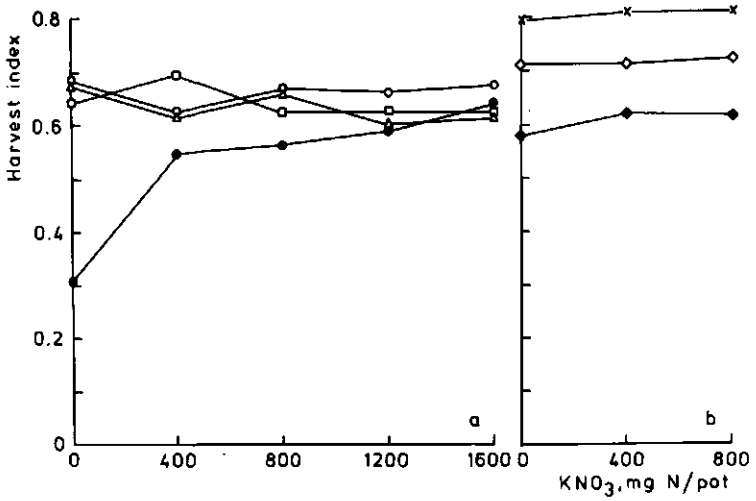

Fig. 4. Harvest indices (ratios of seed nitrogen to total plant nitrogen) of pea-*Rhizobium* symbioses as affected by split dressings of nitrate, 50% at sowing and 50% at pod fill.
(a) Pea cv. Rondo, inoculated with *R. leguminosarum* strains P8 (●), S310a (o), S313 (Δ), and PRE (□).
(b) Pea cvs Florix, early (x), Rondo, intermediate (◇), and Mercato, late (◆), inoculated with *R. leguminosarum* PF_2.

The contribution of nitrate-derived nitrogen to the seed yield was higher in pea plants inoculated with the ineffective strain P8 than in nitrogen-fixing plants (Fig. 3b).

The harvest index of the various symbioses, which normally is a measure of the efficiency of redistribution of nitrogen from vegetative parts and pod walls to the seed was independent of nitrate supply and of rhizobial strain (Figs 4a,b). However, with the ineffective strain P8 the harvest index of the unfertilized plants deviated from those dressed with nitrate owing to nitrogen deficiency so that little nitrogen was available for redistribution (Fig. 4a, Table 1). The harvest index was only dependent on the pea variety as can be seen by comparing Figs 4a and b.

Table 4. Effect of nitrate on nodule formation and nitrogenase activity in pea (cv. Rondo)-*Rhizobium* symbioses, 70 and 113 days from sowing [1]

Strain of microsymbiont	Added nitrate[2] (mg N/pot)	Total nodule fresh wt (mg/plant)	Nodules on primary roots		Nodules on lateral roots		Nitrogenase activity[3] (μmoles C_2H_4/ h.g of nodule fresh wt)
			Numbers per plant	Fresh wt(μg) per nodule	Numbers per plant	Fresh wt(μg) per nodule	
70 days from sowing (pod fill)							
P8	0	158	35	134	147	755	0
	800	39	22	59	69	377	0
S313	0	950	33	26545	21	3524	9.5
	800	177	16	7313	28	2143	7.3
S310a	0	554	41	312	201	2119	16.1
	800	212	11	4090	41	4073	26.2
PRE	0	367	37	4351	87	1218	13.4
	800	175	16	7812	38	1316	25.1
113 days from sowing (maturity)							
P8	0	223	27	2074	213	784	0
	1600	72	29	552	126	444	0
S313	0	311	7	17571	59	3186	2.1
	1600	248	17	8294	35	3057	7.3
S310a	0	330	30	3766	206	1053	0.7
	1600	180	14	1786	65	2385	3.6
PRE	0	367	30	5033	87	2483	3.1
	1600	128	9	4000	47	1957	1.7

[1] Data on nodule number and wt represent means of triplicate samples of 3 plants; the nitrogenase activity was determined in duplicate samples of 8 plants each.

[2] Split dressings at sowing (800 mg N per pot) and at pod fill, 73 days from sowing (an additional 800 mg N per pot).

[3] Nitrogenase activity based on total nodule fresh wt.

Nodule formation

Addition of nitrate to pea plants cv. Rondo at sowing had reduced nodulation as it was observed at pod fill. Numbers of nodules on primary as well as on lateral roots of almost all of the symbioses tested had considerably decreased (Table 4). The same was true of total nodule weight per plant. In the case of weight per nodule the effect of combined nitrogen was much more variable as concerns different symbioses. With *R. leguminosarum* strain S313, which forms large amounts of nodular tissue (up to 40% of total root wt), weight per nodule on primary roots had dropped to approximately 1/3 on the nitrate-dressed plants as compared to the plants without added nitrate. Weight per nodule on lateral roots was also adversely affected by nitrate supply at sowing but the reduction was much less pronounced than it was true of the nodules on primary roots. In the case of strain S310a and to a lesser extent PRE, weight per nodule on primary roots was much higher when nitrate had been supplied. The beneficial effect of nitrate supply on weight of individual nodules on lateral roots with those two strains was much less pronounced than that on primary roots.

The second supply of nitrate at the start of pod filling, has adversely affected numbers and total weight of nodules on both primary and lateral roots as observed at maturity of the plants. Weight per nodule on lateral roots had only slightly decreased as compared with those on primary roots. In the case of S310a the nodules had clearly increased in weight by the dressing with nitrate.

Nitrogenase activity of the S313 nodules, measured at pod filling had declined slightly due to nitrate supply at sowing, as contrasted to the S310a and PRE nodules whose nitrogenase activity was considerably higher than that of nodules of unfertilized symbioses. Nitrogen-fixing activity of the various associations dropped to low values during maturity of the plants. Nodules from plants dresses with nitrate tended to be more active than those without supply of nitrate. This was particularly true of the nodules formed with S313.

Carbohydrate content and amino acid composition. A comparison of the carbohydrate contents of the roots of ineffectively nodulated (P8) and effectively nodulated (PRE) plants showed that the contents of ethanol-insoluble carbohydrates (mainly starch) in both associations were equal, and declined slightly with increased nitrate supply (Fig. 5). Ethanol-soluble carbohydrates (sugars) of symbioses with the effective strain PRE were lower than the ethanol-insoluble

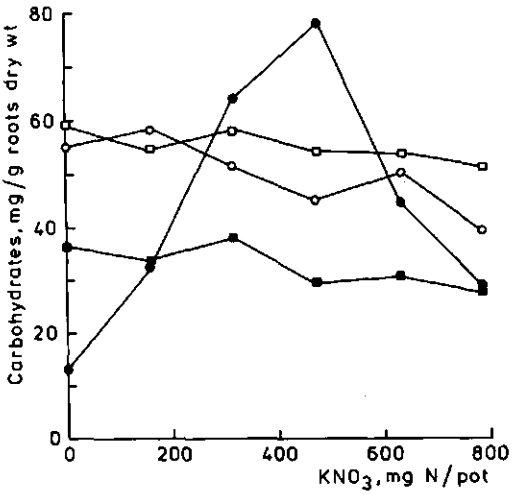

Fig. 5. Ethanol-soluble (●,■) and ethanol-insoluble (o,□) carbohydrates in roots of pea plants (cv. Rondo), 68 days after sowing, inoculated with *R. leguminosarum* strains P8 (●, o) and PRE (■,□), as affected by nitrate supply at sowing. Values represent means of triplicate determinations.

carbohydrates. In symbioses with the ineffective P8 strain, nitrate supply caused a distinct maximum in the content of ethanol-soluble carbohydrates, which decreased to the value found with PRE at the highest level of nitrate added. A similar phenomenon has been observed in effectively nodulated soybean plants (14). It was attributed to an increase in photosynthetic activity of the plant, without sufficient nitrogen being present for the utilization of the additonal sugars for growth, resulting in an increase in sugar concentration. No explanation can be given why no increase in ethanol-insoluble carbohydrates ('starch') was found along with the rise in ethanol-soluble carbohydrates since the contents of these carbohydrates are usually related (see Chapter 3, Fig. 2)

The amino-acid content of the xylem exudate was hardly affected by the supply of nitrate to the plants (Figs. 6a,b), as was found earlier with peas (6). The amount of nitrogen present as amino acid in the xylem sap of the ineffective symbiosis Rondo x P8 was lower than that of the effective symbioses Rondo x PRE, x S310a and x S313, but increased with nitrate supply (Fig. 6a).

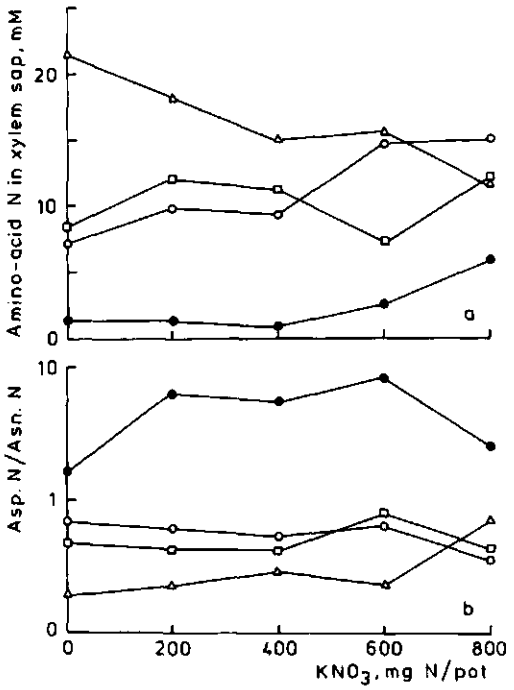

Fig. 6. Amino-acid nitrogen in the xylem exudate of pea (cv. Rondo) inoculated with *R. leguminosarum* strains P8 (●), S310a (o), S313 (Δ), and PRE (□), as affected by nitrate supply at sowing. Analyses made 68 days after sowing.
(a) Total amino-acid N concentration.
(b) Ratio of Asp.-N to Asn.-N.

A similar slight response to nitrate occurred in the exudate of pea plants associated with the poorly N_2-assimilating *R. leguminosarum* strain S310a. The only difference in the amino acid pattern between the strains (not shown) was formed by the aspartic acid and asparagine concentrations as was found earlier in this laboratory (22). With the ineffective strain P8, nitrogen was transported in the plant as aspartic acid rather than as asparagine, whereas the reverse was true with the effective strains (Fig. 6b). These two amino acids together accounted for 58-89% of the total amino-acid nitrogen of the xylem exudate of all symbioses.

DISCUSSION

Differential response to combined nitrogen of pea plants inoculated with different rhizobial strains

Seed yield of pea plants was increased by the supply of combined nitrogen, even with strain PRE which possesses a high nitrogen-fixing capacity without added nitrate (Table 2, Fig.7b). Nitrate uptake and utilization by plants inoculated with strains with a low nitrogen-fixing capacity (S313, S310a) were higher than it was true in plants inoculated with strain PRE (Table 2). At high

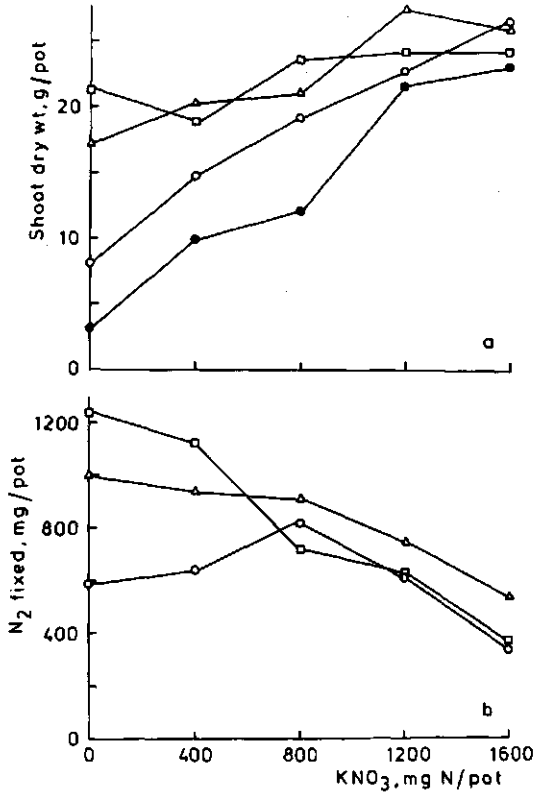

Fig. 7(a). Dry weight of shoots (= leaves + stem) at maturity of pea plants, inoculated with *R. leguminosarum* strains P8 (●), S310a (o), S313 (Δ), and PRE (□), as affected by nitrate supply. Values are means of 4 pots of 8 plants each.
(b). Nitrogen fixation during growth of pea plants, inoculated with *R. leguminosarum* strains S310a (o), S313 (Δ), and PRE (□). Values derived from Table 2.

nitrate supply, seed yields with S313 and S310a were higher than those with PRE (Fig. 3a). The pea cv. Rondo association with strain PRE fixed the highest amounts of nitrogen at low nitrate supply (Table 2, Fig. 7b). At high levels of added nitrate, the yield of fixed nitrogen decreased sharply to 31% of the value without added nitrate. The shoot dry weight of this symbiosis increased slightly, due to nitrate supply (Fig. 7a). In contrast, the shoot dry weight of plants inoculated with strain S310a rose steeply when nitrate was added. The poor nitrogen-fixing capacity of the pea symbiosis with S310a was enhanced from 100 to 138% at a nitrate supply of 800 mg N/pot, but fell to 57% of the initial value when higher amounts of nitrate were supplied. Nitrogen fixation with the moderately effective strain S313 was only lowered to 54% at high nitrate supply. The shoot dry weight of this symbiosis responded to added nitrate to a lower degree than with S310a but more than with PRE.

The dissimilar response to the supply of combined nitrogen with highly effective and moderately effective rhizobial strains is explained by assuming the occurrence of different levels of C- and N-limited growth simultaneously. A symbiosis growing under N limitation might react to combined nitrogen supply by increased photosynthesis, with an enhanced nitrogen fixation as an indirect result. However, also a symbiosis growing under C limitation might react to combined N supply, as simultaneously with a C limitation nitrogen fixation is limited owing to a shortage of carbohydrates. A similar concept has recently

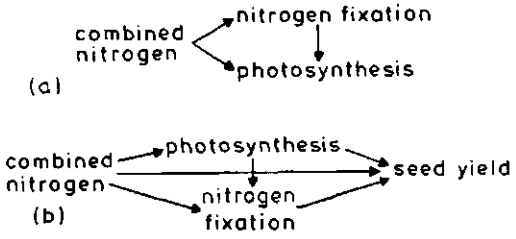

Fig. 8. Model of relations between combined nitrogen supply, photosynthesis and nitrogen fixation with regard to seed yield of plants.
(a) Vegetative phase.
(b) Generative phase.

been proposed by Phillips and co-workers (16, 23). These relations are visualized in the summary models presented in Fig. 8 for the vegetative and the generative growth phases.

The model of Fig.8a for vegetative growth is based upon the assumption that growth of legumes in soil in this phase is principially N-limited. There is support from literature to justify this assumption from fertilizer trials (*e.g.* 12, 15, 24), but it has also been shown that under such conditions the available amount of nitrogenase is limiting nitrogen fixation (Chapter 1, pp. 21 - 34) The model of Fig. 8b for generative growth depends on the assumption that principally a C limitation exists during this growth phase. This assumption is also supported by experimental evidence, *e.g.* the response to carbon dioxide enrichment of the atmosphere (9) and the response to a higher light intensity (3). Furthermore by the observation that during the generative growth phase, an excess of nitrogenase is present in the nodules, not used in nitrogen fixation (Chapter 1)

In order to provide a quantitative description of the effect of carbon and nitrogen limitation on growth of the various symbioses studied, a pathway analysis was made according to the models of Fig. 8. The results of this analysis using the data of Table 2 for nitrogen fixation and combined nitrogen uptake (columns 8 and 7, respectively)and Figs 3 and 7 for seed yield and shoot weight, respectively, are listed in Table 5. Photosynthesis has been roughly estimated by using dry weights of plant mass with photosynthetic activity, *i.e.* shoot, leaves and pod walls. This is inaccurate because photosynthetic activity per unit of leaf mass differs with rhizobial strain (5; Chapter 3, Table 4) and changes with age, but it is suitable for an approximate evaluation of the relationship between the variables.

The path regressions of Table 5 are derived from a series of linear multiple regression equations, assuming a causal order as shown in Fig. 8. In path regressions, the influence of variables which intervene according to the causal order assumed is accounted for. For example, the path regression of photosynthesis (dependent variable) on combined nitrogen (independent variable) during the vegetative period (Table 5, item 3) includes the direct effect of combined nitrogen on photosynthesis as well as the indirect effect *via* nitrogen fixation.

The decrease of nitrogen fixation due to combined nitrogen supply was significantly different between the *Rhizobium* strains (Table 5, items 1 and 6) in the sequence PRE > S313 > S310a. During the vegetative growth phase, photosynthesis with strain PRE appeared to be mainly dependent on nitrogen fixation,

Table 5. Path regressions of nitrogen fixation, photosynthesis and seed yield on combined nitrogen [1)]

Path regression	Strain of microsymbiont			
	P8	S313	S310a	PRE
Vegetative growth (Fig. 8a)				
1 N_2 fixation on combined N	0	-0.48 b^{**}	-0.23 c^{**}	-0.73 a^{**}
2 Photosynthesis on N_2 fixation	0	16.67 b^{**}	15.93 b^{**}	25.51 a^{**}
3 Photosynthesis on combined N	30.28 a^{**}	14.00 b^{**}	18.09 b^{**}	6.28 c^{**}
4 Fit of multiple regressions (R^2)	0.85	0.69	0.77	0.75
Generative growth (Fig. 8b)				
5 Photosynthesis on combined N	21.00 a^{**}	6.58 c^{**}	10.95 b^{**}	4.12 d^{**}
6 N_2 fixation on combined N	0	-0.51 b^{**}	-0.27 c^{**}	-0.78 a^{*}
7 Seed yield on combined N	19.01 a^{**}	4.71 c^{**}	11.75 b^{**}	3.08 c^{**}
8 N_2 fixation on photosynthesis	0	0.06 a^{**}	0.05 a^{**}	-0.00 b
9 Seed yield on photosynthesis	-0.28 b	0.78 a^{*}	0.93 a^{*}	-0-33 b
10 Seed yield on N_2 fixation	0	22.18 a^{**}	12.33 b^{**}	22.40 a^{**}
11 Fit of multiple regressions (R^2)	0.91	0.86	0.95	0.77

[1)] Path regressions from multiple linear regression equations (units mg) based upon 25 cases per strain in each part of the growth period. Values in one row followed by the same letter are not significantly different at the 0.05 level in a two-sided t-test. Asterisks indicate that the path regression is non-zero (*, 0.05 level; **, 0.01 level) according toan F test.

whereas with strains S313 and S310a combined nitrogen and nitrogen fixation influenced photosynthesis to an equal extent (Table 5, items 2 and 3).

Photosynthesis during the generative growth period was increased by combined nitrogen in the sequence P8 > S310a > S313 > PRE (item 5). With strains S310a and S313, an enhanced photosynthesis due to the addition of nitrate significantly increased nitrogen fixation (item 8) and seed yield (item 9). Seed yield was not significantly correlated with photosynthesis with strains PRE and P8 owing to the dominating effect of nitrogen fixation in PRE and combined nitrogen in P8.

The fit of the multiple regressions was reasinably good (items 4 and 11), notwithstanding the fact that the assumption of linear relationship was not always justified. For example, the relation between nitrogen fixation and combined nitrogen contained a significant square power term with strain S310a (cf. Fig. 7b),, which was left out of consideration.

The results presented in this paper show that pea plants inoculated with strains of *R. leguminosarum* with a different nitrogen-fixing capacity respond to combined nitrogen in a different way as was found before in a field trial with these strains (10). At high dressings more nitrate is taken up by plants living in symbiosis with a strain of moderate nitrogen-fixing capacity than by plants with a highly effective strain (Table 2). Photosynthesis is enhanced by added nitrate in plants inoculated with a less effective strain. Therefore, at high nitrate dressings of these strains a higher level of nitrogen fixation is found than with a highly effective strain at high nitrate dressings. An increased nitrate uptake, photosynthesis and nitrogen fixation account for the high seed production of less effective strains as compared with highly effective strains at high nitrate dressings. Consequently, the less effective strains possess a nitrogen-fixing capacity that is less sensitive to combined nitrogen. Selection of such strains for use as inoculant, is not likely to produce yield increases with economically feasible fertilizer dressings.

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GENERAL DISCUSSION

Potential nitrogen fixation

The *in vitro* nitrogenase assay (Ch. 1) of isolated bacteroids, treated with EDTA and toluene, and supplied with ATP and sodium dithionite, appeared to be a valuable method for obtaining information on nitrogenase utilization in legume-rhizobia symbioses. The method was used in experiments with various host plants inoculated with *R. leguminosarum* strains of different origin *viz.* PRE, The Netherlands (Chs 1, 3); S310a, Sweden (Ch.3); RB1, Turkey (Ch. 1); TOM, Turkey and HIM, Pakistan (van Mil, unpublished results). However, with some strains, *e.g.* PF_2 and S313, this assay did not provide satisfactory results, rendering these strains unsuitable for the purpose of determining nitrogenase utilization.

The EDTA-toluene method usually yields values of *in vitro* nitrogenase activity that are higher than those of *in vivo* activity, which enabled us to develop the concept of potential nitrogen fixation. The electron donor used, sodium dithionite, was recently shown to be not as efficient in generating nitrogenase activity as a mixture of flavodoxin + ferredoxin I (25). Therefore, the EDTA-toluene method might underestimate potential nitrogen fixation.

According to the results obtained with our assay, pea plants inoculated with *R. leguminosarum* strains PRE and S310a, and field bean plants inoculated with RB1 utilized their full potential nitrogen fixation only during the vegetative phase, when grown in soil in the open air (Chs 1, 3). Actual nitrogen fixation was found to be consistently lower than potential nitrogen fixation in the generative phase, irrespective of shoot mass or of shoot to root ratio (Ch. 1). This discrepancy between actual and potential nitrogen fixation was widened by the addition of nitrate, which lowered actual nitrogen fixation, whereas potential nitrogen fixation remained unaffected for 10 days (Ch. 1). Both the development of generative organs (13) and the addition of combined nitrogen (10, 19) cause a change in photosynthate translocation pattern from the shoot

to the root, resulting in a lower carbohydrate supply of the nodules. The application of benzyladenine to the nodules eliminates the adverse effect of added nitrate on actual nitrogen fixation (Ch. 2), probably by increasing carbohydrate supply to the nodules. From the data presented in Chs 1 and 2 the conclusion can be drawn that in the vegetative phase, nitrogen fixation is limited by the nitrogenase content of the bacteroids, whereas in the generative phase, an inadequate supply of carbohydrates from the host plant to the micro-symbiont causes a suboptimal rate of nitrogen fixation. As these results were also obtained in field-grown bean plants (Ch. 1) it is likely that this conclusion also holds under circumstances relevant to agriculture.

Regulation of nodule formation

Despite the differences in nodule-formation characteristics between the pea-*Rhizobium* symbioses investigated, the maximum amount of nodules per plant were obtained at the beginning of the pod-filling period (Ch. 5; cf. field beans, Ch. 1). The process of nodule formation can be regarded as a continuous adaptation of nodular mass to the increasing shoot mass (Ch. 1), but it is surprising that increase of nodular weight occurs preferentially to the complete utilization of the nitrogenase present in the bacteroids. Nodule formation probably occurs at lower levels of carbohydrate supply than the functioning of nitrogenase at optimum rate (28). The same was found in experiments with enhanced photosynthesis, *e.g.* by carbon dioxide enrichment of the atmosphere (8, 16), by increased light intensity (1) or by grafting of two shoots on one root system (22). Nodule activity was raised immediately after these treatments, but later on nodular mass grew higher and accounted for the major part of the increased nitrogen fixation per plant.

An explanation of these phenomena might be offered by the observation that uninfected cells of the nodules rather than infected cells are supplied with photosynthates (12). This might point to a lower synthesis of photoassimilate-attracting phytohormones in cells infected with rhizobia than in uninfected cells, *e.g.* the nodular meristematic tissue.

Hydrogen production and hydrogenase activity

Gradually less hydrogen was found to be produced by nodules in air relative to total nitrogenase activity during ontogenesis of both bean and pea plants, giving rise to an increased relative efficiency upon starting of pod filling (Chs 4, 5; 3). An enhanced light intensity was shown to stimulate nitrogenase activity, but hydrogen production was raised to a higher degree, resulting in

a decrease of the relative efficiency (4). The addition of combined nitrogen to pea plants caused a lower actual nitrogenase activity coupled with a higher relative efficiency than that of control plants without added nitrate, whereas treatment of nodules with benzyladenine produced opposite results (Ch. 2). Depodding of field beans reduced nitrogenase activity but enhanced relative efficiency. Application of benzyladenine to the lower leaves decreased relative efficiency, but increased nitrogenase activity (van Mil, unpublished results). From these results it might be concluded that a high production of hydrogen by the nitrogenase system is a sign of an excess of energy, whereas under circumstances of a low energy supply of the nodules, hydrogen production would decline more sharply than total nitrogenase activity. However, there are also reports according to which short-term changes in energy supply would have no impact on relative efficiency when influencing total nitrogenase activity (18) or where a rise in nodule activity was associated with a rise in relative efficiency (2, 9). Furthermore, experiments with isolated nitrogenase have convincingly shown that electrons are allocated to hydrogen production rather than to nitrogen fixation under low energy supply (21). No explanation can be given of the widely differing results of the experiments with isolated nitrogenase and those obtained in the present investigation with nodulated pea plants growing in soils, as to the influence of energy supply of the nitrogenase system on hydrogen production and nitrogen fixation. Therefore more research needs to be done to elucidate this effect.

Hydrogenase activity of *R. leguminosarum* strain S310a (Hup^+) was not associated with a high level of nitrogenase utilization, nor did it prevent or delay nodule senescence as compared with strain PRE (Hup^-). As hydrogen oxidation in plants with S310a would only provide a maximum of 0.6-4.7% of ATP supply to nitrogenase (Ch. 4) (*i.e.* less than 0.6% of the energy gained in net photosynthesis), no amazing results can be expected. Evans and co-workers (7) reported an energy gain by hydrogen oxidation in soybeans of 9.1% of ATP supply to nitrogenase, which would account for a rise in dry matter production of 21% as compared with soybeans inoculated with a Hup^- strain after an exponential growth period for 25 days with a 9.1% enhanced relative growth rate. As a high dry matter production in general is coupled with a high nodular mass (Chs 1 and 5), plants inoculated with Hup^+ bacteria would release more Hup^+ bacteria upon senescence than plants inoculated with Hup^- bacteria. These Hup^+ bacteria might also be benefitted by hydrogenase activity when occurring in the soil in a free-living state (6), which would offer them another considerable advantage

over Hup$^-$ bacteria. However, the fact that the vast majority of naturally occurring strains is Hup$^-$ (Ch. 5; 14) indicates that at least one of these statements is substantially incorrect.

Interactions between C and N limitations of growth

A 'starter dose' of combined nitrogen may increase nitrogen fixation *via* increased photosynthesis (see General Introduction). In Ch. 6 the low nitrogen-fixing capacity of pea plants inoculated with *R. leguminosarum* strain S310a was shown to be enhanced by the supply of nitrate up to 800 mg N per pot, correlated with a rise in leaf dry weight. In contrast, the high nitrogen-fixing capacity of pea plants inoculated with strain PRE was reduced by added nitrate, the leaf dry weight hardly being altered. Net photosynthesis, as calculated from respiration measurements of root systems and of plant-growth data (Ch. 3), was increased to a greater extent by nitrate supply in pea plants inoculated with the moderately effective strain S313 than in plants with the highly effective strain PRE. The generation of a low rate of alternative respiratory activity (Ch. 3) due to added nitrate even in plants inoculated with highly effective *Rhizobium* strains might point to an N limitation of growth. The potential nitrogen fixation, however, in pea plants of the same age, kept under identical conditions was not fully utilized (Ch. 2), which indicates a C-limited growth. To explain this apparent contradiction, we might assume that C and N limitations occur simultaneously in nodules and in whole plants. For example, the nodules might be C-limited, with part of the potential nitrogen fixation not utilized, which would result in an N-limited growth of the whole plant. A similar approach has been advocated by Phillips and co-workers (15, 26).

During generative growth leguminous plants are C-limited, as can be concluded from experiments with CO_2 enrichment, which leads to a higher nitrogen fixation and a higher yield (8). However, also combined-nitrogen dressings during the pod-filling period may result in an enhanced yield. Combined-nitrogen supply increased seed yield of a determinate soybean cultivar, whereas seed yield of an indeterminate cultivar was not affected (11). This points to an important influence of the rate of seed production on photosynthesis, nitrogen fixation and yield.

In the present investigation seed yield of pea plants cv. Rondo (pod-filling period 25 days) responded to nitrate added during the pod-filling period (Ch. 6), whereas seed yield of field beans (pod-filling period 35 days) was not raised (van Mil, unpublished results; 17). Due to the rapid seed formation in pea,

nitrogen fixation declined more sharply than in field beans during ontogenesis of plants grown without added nitrate (Chs 1, 4).

Thus, if the rate of seed production is high, the nitrogen demand of the seed will also be high. At the same time nitrogen fixation is limited by the reduced carbohydrate supply, and consequently, nitrogenous compounds are mobilized from vegetative tissue and transported to the growing seeds. Photosynthesis is reduced, which in turn leads to a lower nitrogen fixation, eventually resulting in the 'self-destruction' of the plant (19). In such a case, combined-nitrogen dressings during the generative growth phase,preferably as foliar spray of urea because the nitrate-absorption capacity of the roots is reduced (5, 23, 24, 27), may prevent self-destruction by lengthening the period of high photosynthetic activity and thus may increase yield.

If the rate of seed growth is low (as in field bean), the self-destructive traits are less pronounced and combined nitrogen supply at mid season has no effect on yield (17).

Prospects for agriculture and research

In agricultural practice there seems to be no clear-cut approach as to increase in yields of leguminous crops by combined-nitrogen dressings, except with suboptimum symbioses or when an extremely low quantity of soil nitrogen is available (see General Introduction). The yield of peas with a relatively short pod-filling period might be increased by combined-nitrogen dressings during that period,although there is the risk of lodging. With peas grown for the canning industry yield increases, if any, will be low because a large amount of nitrogen is retained in the leaves due to the early harvest.

The recommendation of 'super-strains' with hydrogenase, does not seem to offer unequivocally good prospects, as hydrogenase-containing strains may have disadvantages (Chs 4, 5).

Thus, perspectives for enhancing the yield of legume crops will be on a long term, and in the field of research. The interactions between C and N limitation need elucidation. To remove the 'yield barrier', attention should be focused on increasing the photosynthetic capacity of the plant, as the nodular tissue seems to be formed in response to the needs of the plant. Another fruitful approach might involve a renewed interest in the role of plant growth regulators, as the supply of the nodules with photosynthates is of crucial importance to nitrogen fixation. Studies on C- and N-limited growth in legumes with different rates of seed production (*e.g.* pea and field bean)

and with different nitrogen requirements for seed production (*e.g.* pea and clover) would provide still another valuable extension of the investigations reported in this thesis.

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SUMMARY

Actual nitrogen fixation of pea and field-bean plants, grown in soil in the open air, was determined as the acetylene reduction of nodulated roots. During the major part of the vegetative growth of these plants, actual nitrogen fixation was equal to the potential maximum nitrogenase activity of the bacteroids present in the nodules. This means that increase of the actual nitrogen fixation could be achieved only if the potential nitrogenase activity of the bacteroids would be enhanced or if more nodules would be present. During the generative growth phase, the potential nitrogenase activity of the bacteroids was not entirely utilized irrespective of the shoot mass of the host plant or the nitrogen-fixing capacity of the *Rhizobium* microsymbiont.

The addition of nitrate to nodulated plants sharply reduced actual nitrogen fixation but did not affect potential nitrogen fixation within 10 days. The nitrate effect was temporarily eliminated by treatment of the root nodules with benzyladenine, a synthetic plant-growth regulator with a photosynthate-attracting action. It is concluded that incomplete utilization of the nitrogenase present in the bacteroids is caused by an inadequate carbohydrate supply of the nodules upon supply of the leguminous plant with nitrate.

In root systems of pea plants growing in symbiosis with *R. leguminosarum* strains of poor nitrogen-fixing capacity, the N-limited growth led to an accumulation of carbohydrates. Upon the supply of such plants with nitrate, the carbohydrate level was decreased concomitantly to an increase in alternative (*i.e.* cyanide-resistant) respiration. Low rates of alternative respiration were found when nitrate was added to pea plants inoculated with highly effective strains, as the N-limitation of the plants was less severe. The carbohydrates respired in the nodules amounted to 6.3 mg C/mg N fixed with a moderately effective strain but only 3.1 mg C/mg N fixed with a highly effective strain.

Some rhizobial strains possess a hydrogenase that is capable of recirculating part of the hydrogen evolved in air by nitrogenase simultaneously with nitrogen fixation. However, the energy gain by hydrogen oxidation was very low, *viz.*

0.6-4.3% of the costs of nitrogen fixation. As hydrogen oxidation did not delay nodule senescence or increase nitrogenase utilization, the presence of hydrogenase in rhizobial strains seems to be an unimportant factor in determining the nitrogen-fixing capacity of the symbiosis.

Strains of *R. leguminosarum* with a low nitrogen-fixing capacity showed a distinctly different nodule formation pattern on primary and lateral roots of pea plants as compared with highly effective strains. The estimates of the yield of fixed nitrogen, derived from the acetylene-reduction method, of plants inoculated with rhizobial strains of different nitrogen-fixing capacity, equally deteriorated during the growth period. When rates of hydrogen production in air were subtracted from the acetylene reduction rates, the estimates of nitrogen fixation were severely biased in favour of a hydrogenase-containing strain.

Nitrogen fixation of pea plants infected with a highly effective strain of *R. leguminosarum* was decreased by the supply of combined nitrogen to a greater extent than that of plants with a moderately effective strain. In plants inoculated with a strain of poor nitrogen-fixing capacity, nitrogen fixation per plant was even stimulated by a low dose of combined nitrogen. This increase was probably due to an enhanced photosynthetic capacity which counteracted the adverse effect of combined nitrogen. Seed yields were increased by nitrate dressings, regardless of the rhizobial strain. Seed yields of plants inoculated with moderately effective strains were slightly higher than those of highly effective strains at high levels of combined nitrogen, owing to higher nitrate uptake, higher nitrogen fixation and increased photosynthesis.

SAMENVATTING

Vlinderbloemige gewassen zoals erwt en tuinboon (veldboon) zijn in staat om stikstof uit de lucht te binden door een samenwerking (symbiose) aan te gaan met bacteriën van het geslacht *Rhizobium*. Deze bacteriën dringen de wortel binnen via de wortelharen en veroorzaken daar de vorming van wortelknollen. De stikstofbindende bacteriën in deze knollen hebben een gedaanteverandering ondergaan van staafvorm naar vertakte of knotsvorm (bacteroïden).

Tijdens de symbiose voorziet de waardplant de bacteroïden van suikers, verkregen door de fotosynthese. Bij erwt en tuinboon die in potten met grond in de open lucht werden geteeld was tot de bloei de maximale stikstofbinding van de bacteroïden (de potentiële stikstofbinding) de beperkende factor van de stikstofbinding van de planten (actuele stikstofbinding). In deze periode kan alleen door betere knollen of door meer knollen de stikstofbinding van de planten worden verhoogd. Dit is in tegenstelling tot de periode van bloei en peulvorming waarin het stikstofbindend vermogen van de bacteroïden meestal aanzienlijk hoger is dan de werkelijke stikstofbinding (potentiële groter dan actuele stikstofbinding). In deze periode krijgen de knollen te weinig suikers van de plant om optimaal te functioneren omdat een aanzienlijk deel van de produkten van de fotosynthese voor zaadvorming wordt gebruikt.

De toevoeging van nitraat aan planten met wortelknollen leidt tot een daling van de stikstofbinding doordat de toevoer van suikers naar de wortelknollen vermindert. Dit effect kan tijdelijk worden tegengegaan wanneer de wortelknollen tegelijk met de nitraatgift behandeld worden met benzyladenine, een synthetisch plantehormoon dat suikers kan aantrekken.

Tuinbonen ontwikkelen zich veel forser dan erwten en binden ook veel meer stikstof. De stikstofbinding van erwt en tuinboon bereikt haar maximale hoogte aan het begin van de peulvullingsfase. Dit komt doordat steeds nieuwe actieve wortelknollen worden gevormd. Het is echter vreemd dat in de symbiose van plant en bacterie de stikstofbinding eerder wordt verhoogd door meer knolweefsel te

maken dan door het stikstofbindend vermogen van de bacteroïden volledig te benutten.

Tijdens de peulvullingsfase daalt de stikstofbinding. Het sterkst gebeurt dit bij de erwten die een korte peulvullingsduur hebben, het minst bij tuinbonen en erwten met een lange peulvullingsduur.

Niet alle bacteriestammen hebben een evengroot vermogen om stikstof te binden in symbiose met een bepaalde waardplant. Erwteplanten die met een matig of slecht stikstofbindende bacteriestam worden geënt groeien bij stikstofgebrek. Er is dan te weinig stikstof in de plant aanwezig waardoor de door de fotosynthese gevormde suikers niet volledig worden benut voor de groei, maar worden opgehoopt in de erwteplanten. Wanneer deze planten dan van nitraat worden voorzien, worden de suikers zodanig verademd dat weinig energie wordt verkregen (verspillende ademhaling). In planten die met een goed stikstofbindende *R. leguminosarum* stam zijn geënt worden minder suikers opgehoopt. Daardoor treedt minder verspillende ademhaling op als nitraat wordt toegevoegd.

Tijdens de stikstofbinding vormen de bacteroïden naast ammoniak ook waterstof die in de lucht verdwijnt, waardoor energie verloren gaat. Sommige *Rhizobium*-stammen vormen niet alleen waterstof, maar kunnen deze verbinding zelf weer opnemen. Op deze manier zou een deel van de verloren gegane energie weer kunnen worden teruggewonnen. In ons onderzoek bleek echter, dat dit waterstofopnemend vermogen van weinig belang is om de stikstofbinding en de groei van de planten te bevorderen.

Erwteplanten die in symbiose met een goede *R. leguminosarum* stam groeien, binden meer stikstof en produceren meer bladmateriaal en zaad dan planten die met een slechte of matige stam knollen vormen. Wanneer nitraat wordt gegeven aan planten geënt met een goede stam, wordt de stikstofbinding verlaagd maar de zaadopbrengst vaak iets verhoogd door de ruime stikstofvoorziening als gevolg van de nitraatbemesting. Bij planten die met een matige stam zijn geënt daalt de stikstofbinding minder dan het geval was bij een goede stam maar de reactie op toegediende kunstmeststikstof is groter. Er kan dan meer blad worden gevormd waardoor de fotosynthese wordt verhoogd en het nadelig effect van nitraat op de stikstofbinding kan worden tegengegaan. Bij planten die door een slechte *Rhizobium*-stam zijn geïnfecteerd kan een lage nitraatgift op deze wijze zelfs de stikstofbinding verhogen. Bij een hoge nitraatgift kan de zaadproductie van dergelijke planten iets hoger zijn dan die van planten met een goede stam omdat de laatste in dat geval minder nitraat opnemen, minder stikstof binden

en daardoor een lagere fotosynthese hebben.

Voor de landbouwpraktijk kunnen de in dit onderzoek verkregen resultaten niet rechtstreeks gebruikt worden. Bij erwten zal in een beperkt aantal gevallen een kleine stikstofbemesting bij het zaaien een gunstige uitwerking kunnen hebben op de bladvorming en uiteindelijk ook op de zaadopbrengst. In principe zou een bemesting tijdens de peulvullingsfase, bijvoorbeeld door een bespuiting met ureum, een opbrengstverhoging kunnen geven, maar vanuit de praktijk kunnen bezwaren ingebracht worden tegen een bemesting van een te velde staand gewas.

Het voornaamste resultaat van dit onderzoek is wel dat de opbrengst van vlinderbloemige gewassen verhoogd kan worden als de fotosynthese verhoogd wordt. Er worden dan extra wortelknollen gevormd waardoor indirect een hogere stikstofbinding kan worden verkregen. Er is op dit moment te weinig bekend omtrent het verband tussen fotosynthese en stikstofbinding tijdens de groeicyclus van de peulvruchten. Nader onderzoek hierover is dan ook noodzakelijk om de opbrengsten van deze gewassen te kunnen verhogen. Voor Nederland kan verwacht worden dat de belangstelling voor peulvruchten zal toenemen als de energieprijzen blijven stijgen. De prijs van stikstofmeststoffen zal er dan toe leiden dat de vlinderbloemige gewassen, die weinig of geen stikstofmeststof nodig hebben, zich uitbreiden ten koste van gewassen die een hoge stikstofgift nodig hebben,zeker als de opbrengsten verhoogd kunnen worden. Te denken valt bijvoorbeeld aan het vervangen van gras als eiwitrijk ruwvoeder door veldbonen of aan het vervangen van vlees voor menselijke consumptie door peulvruchten.

Voor ontwikkelingslanden zijn hogere opbrengsten van peulvruchten,die als voedselgewas verbouwd worden,noodzakelijk, maar niet toereikend, om het bestaande eiwittekort in de voeding op te heffen.

CURRICULUM VITAE

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