Die Bedeutung der CO_2-Fixierung von Leguminosenknöllchen für ihre Aktivität und Effizienz

Dissertation

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Einleitung

Die N_2-Fixierung ist unter vielfältigen Aspekten Gegenstand gegenwärtiger Forschung. Im Fokus der hier versammelten Arbeiten liegt die Bedeutung der CO_2-Fixierung von Leguminosenknöllchen für ihre Aktivität und Effizienz. Um die Funktionsweise und die Relevanz der CO_2-Fixierung im Gesamtkontext der N_2-Fixierung einordnen zu können, sollen neben einem kurzen Überblick zum Thema „symbiontische N_2-Fixierung" vor allem die für die Thematik der Arbeit relevanten Aspekte näher beleuchtet werden.

Symbiontische N_2-Fixierung

Unter symbiontischer N_2-Fixierung wird eine Form der biologischen N_2-Fixierung verstanden, bei der ein Bakterium in Symbiose mit einer Pflanze lebt, und im Gegenzug gegen von der Pflanze gelieferte Assimilate diese mit gebundenem Stickstoff versorgt.

Zur N_2-Fixierung sind nur prokaryotische Lebewesen befähigt, da die stabile Dreifachbindung des N_2-Moleküls für Eukaryoten inert ist. Aus diesem Grund ist das Eingehen einer Symbiose mit einem Bakterium eine Möglichkeit für die Pflanzen, sich indirekt den für sie ansonsten unverwertbaren Luftstickstoff anzueignen. In der vorliegenden Arbeit wurde die symbiontische N_2-Fixierung am Beispiel von Pflanzen aus der Familie der *Fabaceen* (*Pisum sativum* L. und *Medicago sativa* L.) und von Bakterien aus der Familie der *Rhizobiaceen* (*Rhizobium leguminosarum* und *Rhizobium meliloti*) untersucht.

Die symbiontische N_2-Fixierung findet in sogenannten Wurzelknöllchen statt. Das Knöllchen ist das Resultat eines komplexen Infektionsvorgangs (Parniske & Downie, 2003), bei dem folgende Teilschritte zur Entstehung eines Wurzelknöllchens führen. Leguminosenwurzeln scheiden Flavanoide aus, die Bakterien anlocken und in diesen die Expression der *nod*-Gene auslösen. Die *nod*-Gene wiederum führen zur Synthese der Nod-Faktoren, die von den Bakterien ausgeschieden werden und an der Pflanzenwurzel die Knöllchenbildung initiieren. Über einen von der Pflanze gebildeten Infektionsschlauch dringen die Bakterien in Zellen des inneren Wurzelkortex ein. Hier werden sie ins Zytoplasma entlassen und von einer pflanzenbürtigen Membran umschlossen; das gebildete Kompartiment wird als Sybiosom bezeichnet. Intensive Zellteilung führt nun zur Ausformung eines Wurzelknöllchens.

In den Knöllchen durchlaufen die Bakterien gewisse morphologische Veränderungen, beispielsweise den Verlust der Zellwand, außerdem werden für die Stickstofffixierung relevante Gene zur Nitrogenasesynthese exprimiert. Die adaptierte symbiontische Form der Bakterien wird als Bakteroid bezeichnet.

Das Ergebnis erfolgreicher Infektion ist das Wurzelknöllchen, ein für die N_2-Fixierung hochspezialisiertes Organ. Bei den Leguminosenknöllchen werden zwei grundsätzliche Knöllchentypen differenziert (Brewin, 1991): der determinierte Knöllchentyp (z.B. bei Sojabohne und Phaseolusbohne) und die indeterminierte Form (z.B. bei Erbse und Luzerne). Diese beiden unterscheiden sich grundlegend hinsichtlich ihrer Morphologie (Abb.1).

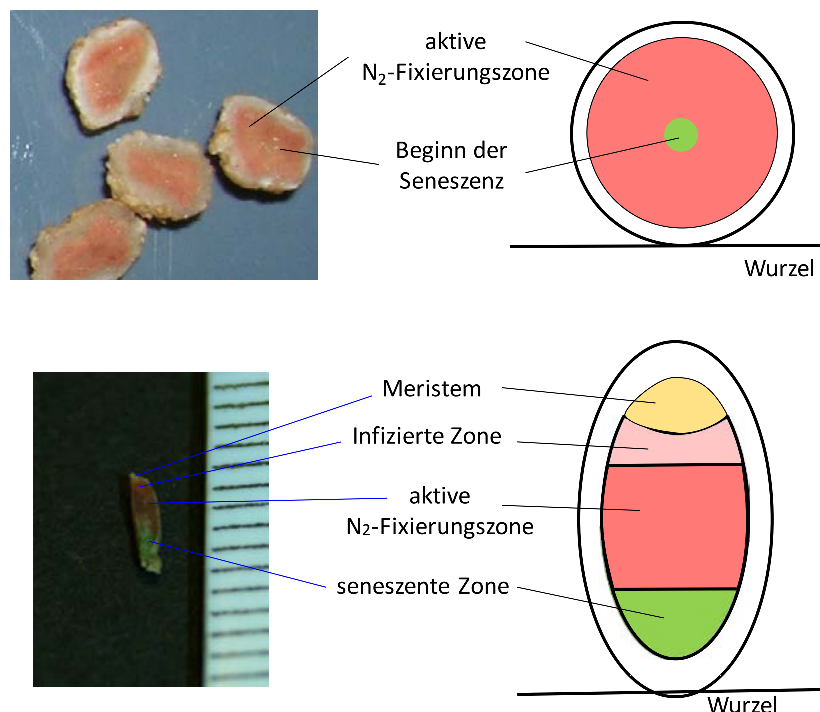


Abb.1: **Einteilung der Knöllchentypen hinsichtlich ihrer Morphologie.**

Neben der schematischen Abbildung rechts, sind im oberen Bild Phaseolusbohnenknöllchen und im unteren Bild ein Erbsenknöllchen im Querschnitt zu sehen.

Bei den indeterminierten Knöllchen befindet sich ein persistierendes apikales Meristem an der Spitze, welches im Verlauf der Knöllchenontogenese zu einer zylindrischen Wuchsform führt. Daran schließt sich die mit Bakteroiden infizierte Zone, die N_2-Fixierungszone und schließlich die seneszente Zone an. Determinierte Knöllchen sind durch ein nicht per-

sistierendes Meristem gekennzeichnet, welches zu einer runden Form führt. Im Zentrum der determinierten Knöllchen befindet sich eine ontogenetisch homogene Zone von infizierten Zellen, in denen N_2-Fixierung stattfindet. Die Seneszenz beginnt im Kern und breitet sich von der Mitte her radial nach außen hin aus.

Neben den morphologischen Unterschieden weisen diese beiden Knöllchentypen auch Unterschiede im Hinblick auf die N-Exportprodukte aus dem Knöllchen in die Pflanze auf. Die determinierte Form exportiert den Stickstoff in Form von Ureiden (Allantoin und Allantoinsäure). Die indeterminierten Knöllchen, die in dieser Arbeit untersucht wurden, produzieren dagegen hauptsächlich Asparagin als N-Transportform in den Spross. In der N_2-Fixierungszone der Knöllchen befinden sich die durch Bakteroide infizierten Zellen.

Im Zytosol der infizierten Zellen befindet sich der Symbiosom, ein Zellkompartiment, welches im Verlauf der Infektionsvorgänge durch Endocytose der Bakteroide durch die Plasmalemmamembran der Pflanze entstanden ist. Die Symbiosommembran ist somit invers gekehrt, und Transport vom Zytosol der infizierten Zelle in das Zytosol des Symbiosoms ist mit einem Export aus der pflanzlichen Zelle vergleichbar.

Im Zytosol des Symbiosoms befinden sich die Bakteroide, welche wiederum durch die Bakteroidmembran begrenzt sind. Angelieferte Energieträger der Pflanze und Produkte der N_2-Fixierung müssen demnach eine Vielzahl von Membranen passieren. Die diesen Transportvorgängen zugrunde liegenden Mechanismen sind noch nicht vollständig aufgeklärt und sind Gegenstand aktueller Forschung (Lodwig et al., 2003; White et al., 2007).

In den Bakteroiden findet an der Nitrogenase die N_2-Fixierung, also die Reduktion von N_2 zu NH_3, statt. Der Nitrogenasekomplex besteht aus zwei Untereinheiten, der Nitrogenasereduktase (dimeres Fe-Protein) und der eigentlichen Nitrogenase (tetrameres MoFe-Protein) (Thorneley, 1992). Eine für die N_2-Fixierung problematische Eigenschaft dieses Enzyms ist die hohe Sauerstoffsensitivität (Robson & Postgate, 1980). Diese wirkt sich dadurch problematisch aus, dass zur Bereitstellung der für N_2-Fixierung erforderlichen Energiemenge intensive Atmungsprozesse erforderlich sind, was zu einem hohen Sauerstoffbedarf führt. Durch das Vorhandensein von großen Mengen Leghämoglobin in der aktiven Zone der Knöllchen wird beidem Rechnung getragen. Das Leghämoglobin ist ein eisenhaltiges Häm-Protein, das im Knöllchen in mM Konzentrationen vorliegt (Appleby, 1984) und der aktiven Zone die charakteristische rote Farbe verleiht. Es bindet

molekularen Sauerstoff und führt ihn den Mitochondrien und der auf der Bakteroidmembran situierten Atmungkette zur Respiration zu. Das Leghämoglobin wurde bereits früh entdeckt (Kubo, 1939) und ist in seiner Funktion, den Sauerstoffpartialdruck niedrig zu halten und gleichzeitig den hohen Sauerstoffbedarf zu erfüllen, für die N_2-Fixierung unentbehrlich. Im Verlauf der Knöllchenseneszenz wird das Leghämoglobin abgebaut, und das Knöllchen bekommt eine für die Seneszenz charakteristische Grünfärbung, die auf dem Abbauprodukt von Leghämoglobin, Biliverdin, beruht (Abb. 2).

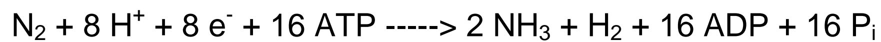
Abb.2: **Erbsen- (rechts) und Luzerneknöllchen (links) im Querschnitt, in unterschiedlich weit fortgeschrittenen Stadien der Seneszenz.**

Aktives Knöllchengewebe weist durch Leghämoglobin eine charakteristische Rotfärbung auf. Grünes Knöllchengewebe dagegen weist auf den Abbau des Leghämoglobins, das grüne Billiverdin hin. Da die Stickstofffixierung ohne Leghämoglobin nicht stattfinden kann, ist eine Grünfärbung von Knöllchengewebe ein Zeichen für inaktives Gewebe.

Grünes Knöllchengewebe zeigt somit das Fehlen von Leghämoglobin an und ist dadurch ein eindeutiges Zeichen für inaktives Knöllchengewebe.

Im Folgenden soll die N_2-Fixierung hinsichtlich der metabolischen Umsatzprozesse in einer infizierten Zelle und den darin befindlichen Kompartimenten dargestellt werden (Abb. 3). Über das Phloem wird Photosyntheseenergie in Form von Saccharose (Kouchi & Yoneyama, 1984) in die infizierten Zellen geliefert. Im Zytosol der Zellen findet der glycolytische Abbau vornehmlich zu Malat statt (Udvardi & Day, 1997). Malat wird in die Bakteroide transportiert und ist dort die Hauptenergiequelle (Driscoll & Finan, 1993). Über den Zitronensäurezyklus führt Malat zur Bildung von $NADH+H^+$ und $FADH_2$, welche dann zum einen über die auf der Bakteroidmembran befindliche Elektronentransportket-

te die notwendige Energie in Form von ATP bereitstellt oder selber als Reduktionsäquivalent an der Nitrogenase fungieren. Der Energiebedarf und Bedarf an Reduktionsäquivalenten für die N_2-Fixierung ist erheblich, was aus der Summenformel ersichtlich ist:



$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \longrightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i$$

Pro fixiertem N_2 werden also 16 ATP und 8e$^-$ benötigt. Gleichzeitig entsteht pro Mol fixiertem N_2 auch ein Mol H_2. Diese Wasserstoffreduktion ist, bedingt durch die Funktionsweise der Nitrogenase, obligat (Ogo et al., 2004). Einige Rhizobienstämme können jedoch die durch H^+-Reduktion verlorene Energie durch eine Aufnahmehydrogenase (hup+) wieder zurückgewinnen, allerdings bleibt der theoretisch errechnete Mindestenergieverbrauch, ausgedrückt in respiratorischen C-Kosten, bei 1,5 mg C je mg fixiertem N (Schulze et al., 1994). Der tatsächliche gemessene C-Verbrauch der N_2-Fixierung scheint jedoch mit 6 mg C je mg fixiertem N deutlich höher zu liegen (Schubert, 1986; Vance & Heichel, 1991). Oftmals wurde in solchen Messungen auch der Kohlenstoffverbrauch für die Erhaltung miteinbezogen, und genaue Messungen der rein für den N_2-Fixierungsprozess verbrauchten C-Kosten kamen mit 2 bis 3 mg C je mg fixiertem N dem errechneten Wert sehr nahe (Schulze et al., 1999). Dennoch bleibt festzuhalten, dass die Bereitstellung der Energie für die N_2-Fixierung einen hohen Kohlenstoffverbrauch bedeutet.

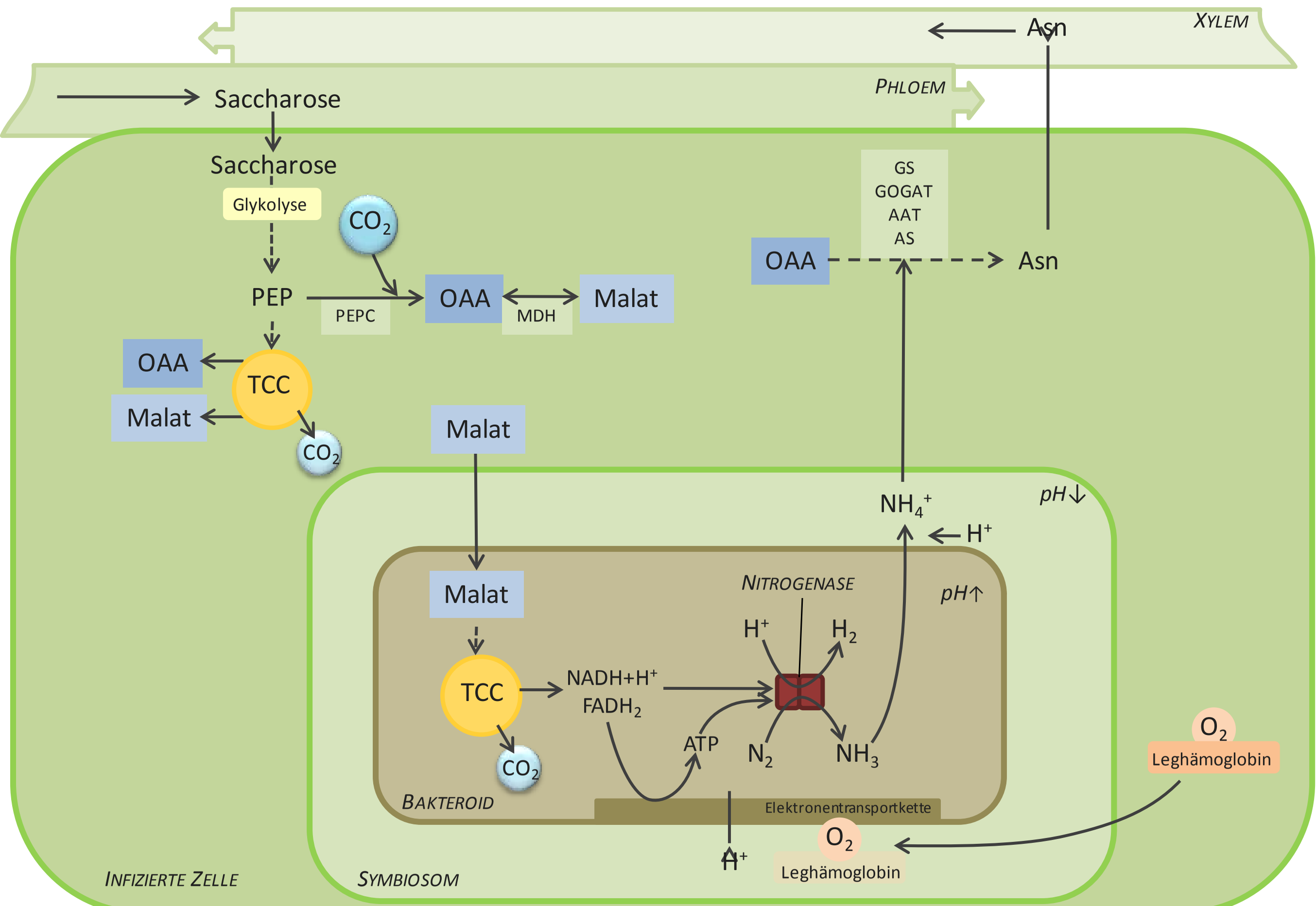


Abbildung 3: **Infizierte Zelle eines indeterminierten Knöllchens. Dargestellt ist der Vorgang der N_2-Fixierung im Kontext der metabolischen Prozesse der Energiebereitstellung und des N-Einbaus und N-Abtransports.**

Die von der Pflanze gelieferten Assimilate in Form von Saccharose werden glykolytisch abgebaut. Aus dem Phosphoenolpyruvat (PEP) kann über den Umbau im Zitronensäurezyklus (TCC) das für die Energiebelieferung des Bakteroiden notwendige Malat oder die für die Assimilation des NH_4^+ notwendige Oxalacetat abgezweigt werden. Der Zitronensäurezyklus ist allerdings auch mit CO_2-Freisetzung verbunden. Vom PEP ausgehend kann alternativ ein anaplerotischer Stoffwechselweg über die CO_2-Fixierung an der Phosphoenolpyruvat-carboxylase (PEPC, EC 4.1.1.31) eingeschlagen werden, um Oxalacetat (OAA) zu bilden. Aus OAA kann über die Malatdehydrogenase (MDH, EC 1.1.37) Malat gebildet werden. Malat gelangt in den Symbiosomen und dient dort der Energiebereitstellung für die an der Nitrogenase ablaufenden Prosesse. Der an der Nitrogenase gebundene Stickstoff in Form von NH_3 wird nach Protonierung im Zytosol des Symbiosoms als NH_4^+ im Zytosol der infizierten Zelle über Transaminierungsschritte an Oxalacetat gebunden und als Asparagin (Asn) ins Xylem der Pflanze abgegeben.

Involvierte Enzyme sind neben den bereits erwähnten: GS, Glutaminsynthetase (EC 6.3.1.2); GOGAT, Glutamat-Synthase (NADH- EC 1.4.1.14 bzw. FAD-EC 1.4.7.1); AAT, Aspartat Aminotransferase (EC 2.6.1.1); AS, Asparagin Synthetase (EC 6.3.1.4). Gestrichelte Linien symbolisieren unvollständig dargestellte Stoffwechselwege.

Das Primärprodukt der N_2-Fixierung ist NH_3. Bereits 1967 entdeckten Bergersen und Turner, dass bei Inkubation von isolierten Bakteroiden aus Sojabohnenknöllchen in $^{15}N_2$-Atmosphäre $^{15}NH_3$ aus den Bakteroiden ausgeschieden wurde. Aktuelle Arbeiten bestätigen mittels NMR-Spektroskopie diesen Befund (Scharff et al., 2003). Als Nebenprodukt fällt an der Nitrogenase Wasserstoff an, der gemessen werden kann und so als indirektes Maß für die N_2-Fixierung vor allem für den methodischen Ansatz dieser Arbeit eine Bedeutung hat.

Im Zytosol des Symbiosoms herrscht ein sehr niedriger pH, was zu einer Protonierung des NH_3 zu NH_4^+ führt (Day et al., 2001). Das NH_4^+ gelangt dann durch Diffusion durch die Symbiosommembran in das Zytosol der Zelle (Streeter, 1989). Im Zytosol der Zelle wird das NH_4^+ sofort über mehrere Transaminierungsschritte auf das Oxalacetat übertragen und als Asparagin aus der Zelle ins Xylem transportiert (Lodwig et al., 2003). Der unmittelbare Einbau des NH_4^+ ist von zentraler Bedeutung für eine funktionierende N_2-Fixierung; Cordoba et al. (2003) konnten an Luzernepflanzen zeigen, dass eine durch Antisensetechnik verhinderte Expression der Glutamat Synthase (GOGAT, EC 1.4.1.14) im Knöllchen zu einer Verminderung der N_2-Fixierung um bis zu 70% führte und die Pflanzen aufgrund von N-Mangel chlorotische Schäden aufwiesen. N_2-Fixierung ist somit auf die ausreichende Bereitstellung von Oxalacetat als Kohlenstoffgerüst für den N-Einbau angewiesen. Für je zwei Mol gebundenen Stickstoffs muss also ein Mol Oxalacetat zum Einbau bereit gestellt werden.

Ableitung der Fragestellungen und methodischer Ansätze der Arbeit

Aus dieser kurzen Darstellung der N_2-Fixierungsvorgänge ist zusammenfassend festzuhalten, dass organische Säuren, vor allem Malat und Oxalacetat, in zwei Punkten eine zentrale Rolle im N_2-Fixierungsprozess in den infizierten Zellen spielen.

1. Durch den Abbau von Malat über den Zitronensäurezyklus in den Bakteroiden sind sie die Energiequelle für den N_2-Fixierungsprozess. Verringert sich der Malatfluss in die Bakteroide, kommt die N_2-Fixierung zum erliegen. Die N_2-Fixierungsaktivität hängt daher von der Malatverfügbarkeit im Bakteroiden ab.

2. In der Funktion als Kohlenstoffgerüst dient Oxalacetat dem N-Einbau und dem N-Transport in die Pflanze. Wird der N-Einbau gebremst, so wird die N_2-Fixierung entsprechend herabgesetzt.

Der Kohlenstoffverbrauch der N_2-Fixierung stellt in landwirtschaftlichen Produktionssystemen immer auch eine Konkurrenz zur Biomasseproduktion dar. Daher ist es von Interesse, einen in den Knöllchen etablierten Mechanismus zu untersuchen, der einen erheblichen Einfluss auf den Netto-C-Verbrauch der N_2-Fixierung hat: die Knöllchen-CO_2-Fixierung an der Phosphoenolpyruvatcarboxylase (PEPC, EC 4.1.1.31) im Zytosol der Wirtszelle (Abb.3).

Die Bedeutung der PEPC im Knöllchen wurde bereits vielfach untersucht, so kommen spezifische, nur im Knöllchen exprimierte PEPC Formen (nePEPC) vor (Vance & Stade, 1984). Ein direkter Zusammenhang zwischen N_2-Fixierung und der PEPC-Expression konnte aufgezeigt werden. Beispielsweise führt eine Überexpression von PEPC zu einer erhöhten N_2-Fixierleistung (Schulze et al., 2000), wohingegen eine verminderte PEPC-Expression zu einer verringerten N_2-Fixierung führte; dadurch wurden typische N-Mangelsymptome bei den Pflanzen hervorgerufen (Schulze et al., 1998; Nomura et al., 2006). Die CO_2-Fixierung ist demnach ein für die N_2-Fixierung bedeutsamer Stoffwechselvorgang. Die Fixierung von CO_2 im Knöllchen ist eine Möglichkeit, die Kohlenstoffbilanz des Knöllchens zu verbessern. Wird das Oxalacetat (bzw. Malat) auf diesem anaplerotischen Stoffwechselweg gebildet, anstatt aus dem Zitronensäurezyklus abgezogen zu werden, wird Kohlenstoff gespart. Mit dem Zitronensäurezyklus ist eine CO_2-Freisetzung verbunden. Bei der Bildung von Oxalacetat (bzw. Malat) über PEPC wird hingegen CO_2 fixiert. In Anbetracht des hohen Kohlenstoffbedarfs der N_2-Fixierung stellte sich die Frage nach der möglichen Bedeutung dieses Prozesses für die N_2-Fixierung in Zuständen angespannter C-Versorgungslage der Leguminose.

In der Hülsenfüllungsphase der Erbsen werden große Mengen an Kohlenstoff und Stickstoff in den wachsenden Hülsen festgelegt (Salon et al., 2001). Dieser Umstand bedeutet also einen erhöhten Bedarf an Stickstoff, verbunden mit einer eher angespannten C-Versorgungslage der Knöllchen. Die Frage war also, inwieweit die CO_2-Fixierung als interner Mechanismus in Leguminosenknöllchen etabliert ist, um in Zuständen angespannter C-Versorgungslage der Knöllchen bei tendenziell erhöhter N-Nachfrage die

C-Effizienz zu erhöhen, um somit die N_2-Fixierung im ausreichenden Maß realisieren zu können (siehe Kapitel 1).

Weiterhin wurde der Frage nachgegangen, welchen Einfluss eine modifizierte CO_2-Konzentration im Wurzelraum auf die CO_2-Fixierleistung hat, und ob sich dadurch möglicherweise die N_2-Fixierleistung der Knöllchen beinflussen lässt. Dazu wurde ein System entwickelt, in dem die Gaszusammensetzung im Wurzelraum unabhängig von der Zusammensetzung um den Spross variiert werden kann. Es wurden Luzernepflanzen mit ausschließlich im Wurzelraum modifiziertem CO_2-Gehalt (ca. 100 ppm versus 2000 ppm CO_2) angezogen, und der Einfluss auf die N_2-Fixierung ermittelt (siehe Kapitel 2).

Um diese Fragestellungen bearbeiten zu können, musste zunächst ein System etabliert werden, mit welchem der Wurzelraum der Pflanzen mit Gasgemischen unterschiedlicher Zusammensetzung belüftet werden konnte, ohne dass eine Kontamination des Sprosses geschah. Dieses so etablierte System sollte weiterhin auch Untersuchungen mit Hilfe von $^{13}CO_2$ im Wurzelraum ermöglichen, ohne dass die Gefahr einer direkten Assimilation über den Spross bestand.

Darüber hinaus sollte mit diesem System eine Erfassung der H_2-Freisetzung im offenen Durchfluss ermöglicht werden, da der an der Nitrogenase freiwerdende Wasserstoff als Maß für die Nitrogenaseaktivität ein für die N_2-Fixierungsforschung besonders wichtiger Parameter ist. Da die Messung der H_2-Freisetzung ein indirektes Maß und mit einigen analytischen Problemen verbunden sein kann (bspw. ‚Argon induced decline'), sollte die Methode durch Applikation von $^{15}N_2$ verifiziert werden (siehe Kapitel 3).

Kapitel 1: Ontogenetisch bedingt erhöhte N_2-Fixierung bei Erbsen geht mit erhöhter Knöllchen CO_2-Fixierung einher.

Running head: Efficiency of N_2 fixation in pea plants during ontogeny

Plants Interacting with Other Organisms

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The Importance of Nodule CO_2 Fixation for the Efficiency of Symbiotic Nitrogen Fixation in Pea Plants before and during Pod-Filling

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Footnotes

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Abstract

Nodule CO_2 fixation is of pivotal importance for nitrogen fixation (N_2 fixation). The process provides malate for bacteroids and oxaloacetate for nitrogen assimilation. The hypothesis of the present paper was that increased nodule CO_2 fixation would contribute to a more efficient N_2 fixation at pod-filling in grain legumes. Pod-filling is connected with increased nitrogen demand and lower assimilate availability. We studied growth, N_2 fixation and nodule composition before flowering and at early pod-filling in pea plants (*Pisum sativum* L.). In parallel experiments, $^{15}N_2$ and $^{13}CO_2$ uptake along with nodule hydrogen and CO_2 release was measured. Plants at pod-filling showed higher growth rates and N_2 fixation per plant. Specific activity of active nodules was about 50% higher at pod-filling. The higher nodule activity was accompanied by higher amino acid concentration in nodules and xylem sap with a higher share of asparagine. Nodule $^{13}CO_2$ fixation was increased at pod-filling, both per plant and per $^{15}N_2$ fixed unit. However, malate concentration in nodules was only 40% of that before flowering and succinate was not anymore detectable. The data indicate that increased N_2 fixation at pod-filling is connected with strongly increased nodule CO_2 fixation. Nodule capacity for CO_2 fixation and organic acid formation appears to be overstretched while assimilate supply remains sufficient. It is concluded that strategies to improve the capability of nodules to fix CO_2 and form organic acids might prolong intensive N_2 fixation into the later stages of pod-filling in grain legumes.

Introduction

Legume nodules fix substantial amounts of CO_2 largely through the combined activity of carbonic anhydrase (EC 4.2.1.1) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) resulting in carboxylation of phosphoenolpyruvate (PEP) (Vance et al., 1983; King et al., 1986; Rosendahl et al., 1990). Nodule CO_2 fixation rates are difficult to measure since any applied labeled CO_2 is diluted by simultaneous intensive nodule respiration. Measurements thus tend to underestimate nodule CO_2 fixation rates, as more as a large proportion of the fixed CO_2 is rapidly respired. Nevertheless, estimates reveal that nodule CO_2 fixation rates per protein unit can reach those of young maize leaves (Miller et al., 1987). In fact, PEPC levels in nodules are in the range of those in leaves of C4 plants, and PEPC is expressed in alfalfa nodules about 10- to 15-fold greater than in other organs and can comprise up to 2% of the soluble protein in the nodules (Vance and Stade, 1984; Vance et al., 1994). Several nodule-enhanced forms of carbonic anhydrase, PEPC and malate dehydrogenase (EC 1.1.1.37), have been identified from legume nodules (de la Pena et al., 1997; Miller et al., 1998; Atkins et al., 2001). The fixed carbon in nodules is channeled into malate formation. Malate is taken up by the symbiosome and drives nitrogen fixation. In addition, a substantial portion of the fixed carbon is used for nitrogen assimilation into aspartate and asparagine. Moreover, a possible involvement of malate in the functioning of a putative nodule oxygen diffusion barrier has been proposed (Vance and Heichel, 1991; Galvez et al., 2000), although the precise mechanism is not yet defined.

Nodule CO_2 fixation is apparently tightly bound to nitrogenase activity. In developing nodules PEPC protein occurs alongside nitrogenase protein (Suganuma et al., 1997). A down-regulation of PEPC in nodules impairs nitrogen fixation (Schulze et al., 1998b; Nomura et al., 2006). Taking the whole plant into consideration, nodule CO_2 fixation constitutes a carbon-saving mechanism when the fixed carbon is used for N assimilation and N transport to shoots. While any malate channeled into the symbiosome is rapidly respired, the use of oxaloacetate for N assimilation is connected with carboxylation of PEP. In turn, any use of carbon skeletons from the tricarboxylic acid cycle (TCA cycle) for N assimilation is connected with a preceding decarboxylation of PEP. Oxaloacetate from N

transport may then be reduced to malate in shoots, coupled with photosynthesis. On root/nodule basis this kind of pathway of carbon supply for N transport is connected to a certain energy gain from glycolysis together with CO_2 fixation rather than CO_2 loss.

Although it is commonly accepted that under most conditions nodules are sufficiently supplied with assimilates, various stress conditions affecting photosynthesis (drought, phosphorus deficiency,) might alter the situation (Galvez et al., 2005; Schulze et al., 2006). Moreover, during ontogeny the onset of pod formation profoundly changes source-sink relations within plants, in particular in grain legumes (Salon et al., 2001). Growing pods not only attract a considerably higher amount of nitrogen than emerging leaves, but significant amounts of carbon as well. Thus nitrogen is in increasing demand from nodules during pod-filling, which at the same time most probably find themselves in a position of more unreliable assimilate supply. If nodules cannot meet the pods' N requirements, additional nitrogen is attracted from older leaves, inducing progressive senescence (Schiltz et al., 2005). This, in turn, reduces the overall photosynthetic capacity of the plant. Eventually, these interconnections, at least in part, determine the rapidity of ripening and extent of nitrogen yield in pods (Schulze et al., 1998a). This might be critical, in particular in a grain legume like semi-leafless pea varieties. In such varieties breeding efforts for agronomical benefits were connected with a considerable reduction in photosynthetic capacity.

The hypothesis of the present paper was that nitrogen fixation during pod-filling versus vegetative growth would be supported through increased nodule CO_2 fixation supplying organic acids and carbon skeletons for N assimilation, thereby achieving a more efficient nitrogen fixation. We choose a semi-leafless variety of pea plants for this study. Growth, N assimilation, nodule amino acid (AA), organic acids (OA) and free sugar composition were compared between a 14-day growth interval before flowering, and one during pod-filling. Moreover, in additional experiments root/nodule fixation of $^{15}N_2$ and $^{13}CO_2$, along with evolution of H_2 and CO_2 was measured at both stages of ontogeny.

Results

Growth and nitrogenase activity

Pea plants during pod-filling showed higher dry matter formation and N assimilation than pea plants before flowering (Table I). N assimilation was measured as the total N increment of plants during the growth intervals. Since the plants received no combined nitrogen, N assimilation corresponded to N_2 fixation.

Table I: Dry matter increment, N accumulation, total and active nodule dry matter and specific N_2 fixation of pea plants during a 14-day growth interval before flowering or at pod-filling. Values in parenthesis are in % of the values before flowering.
Data are means of four replicates. * indicates a statistically significant difference to the growth interval before flowering (t-test, P≤0.05).

parameter	unit	growth interval	
		before flowering	pod-filling
Δ dry matter	g 14 d^{-1} $plant^{-1}$	0.94	1.50* (160)
Δ N	mg 14 d^{-1} $plant^{-1}$	26	46* (177)
Δ N/Δ dry matter	mg g^{-1}	28	31 (110)
Total nodule dry matter	mg $plant^{-1}$	54	97 (179)
Active nodule dry matter	mg $plant^{-1}$	54	66 (122)
Share of senescent nodules	%	0	32
Specific N_2 fixation of active nodules	µg N mg dm^{-1} h^{-1}	1.43	2.07* (145)

Total nodule dry matter per plant during pod-filling was about 180% of that before flowering. However, about one third of the nodules at pod-filling showed clear

signs of senescence, being either greenish or brownish and soft. The higher plant productivity during pod-filling coincided with an about 50% higher specific activity of nodules. The more efficient nitrogen fixation resulted in a constant ΔN/Δdm ratio.

Amino acid concentrations in active nodules and xylem sap

The higher specific activity of nodules during pod-filling is reflected in a higher total concentration of AA in xylem sap and nodule tissue when compared to plants before flowering (Fig. 1 and 2). Additionally, in both nodules and xylem sap the increased total AA concentration was largely a result of more asparagine. Apart from changes in asparagine concentration in xylem sap and nodules, no significant shifts in the concentration of any of the other detected AAs were found.

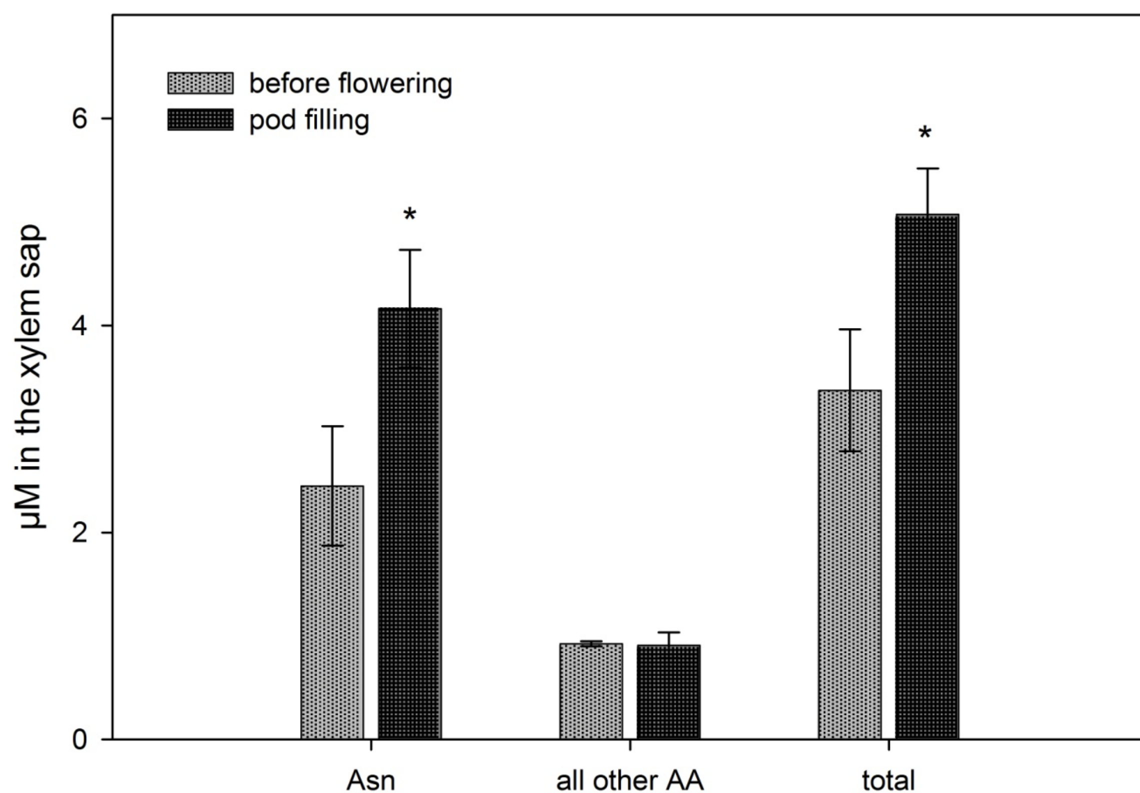

Figure 1: Xylem sap AA concentration of plants before flowering and at pod-filling. Further detected AAs were Tyr, Ser, Asp, Gly, Val, Thr, Gln, and Glu. Data are means of 4 or 2 replicates before flowering or pod-filling respectively. Error bars

represent standard deviation. * indicates a statistically significant difference between the two ontogeny stages (t-test, P≤0.05).

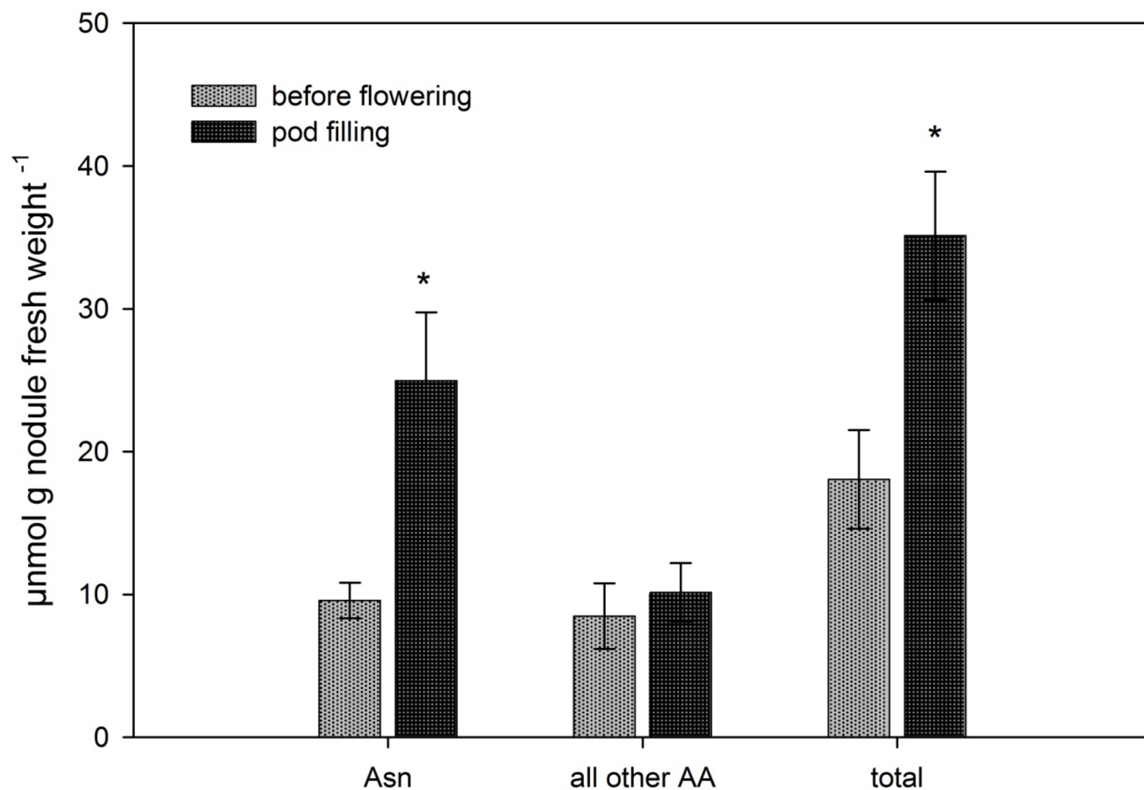

Figure 2: Nodule AA concentration of plants before flowering and at pod-filling. Further detected AAs were Tyr, Ala, Ser, Leu, Asp, Gly, Val, Arg, His, Phe, Ile, Thr, Gln, Glu, Met and Lys. Data are means of 4 replicates. Error bars represent standard deviation. * indicates a statistically significant difference between the two ontogeny stages (t-test, P≤0.05).

Free sugar and organic acid concentrations in active nodules

Total sugar concentration in active nodules showed a tendency to be higher at pod-filling, but at statistically non-significant levels (Fig. 3). In addition to sucrose and galactose, significant concentrations of fructose in active nodules were detected during pod-filling. Overall the detected concentrations in free sugars varied strongly between replicates within both growth intervals.

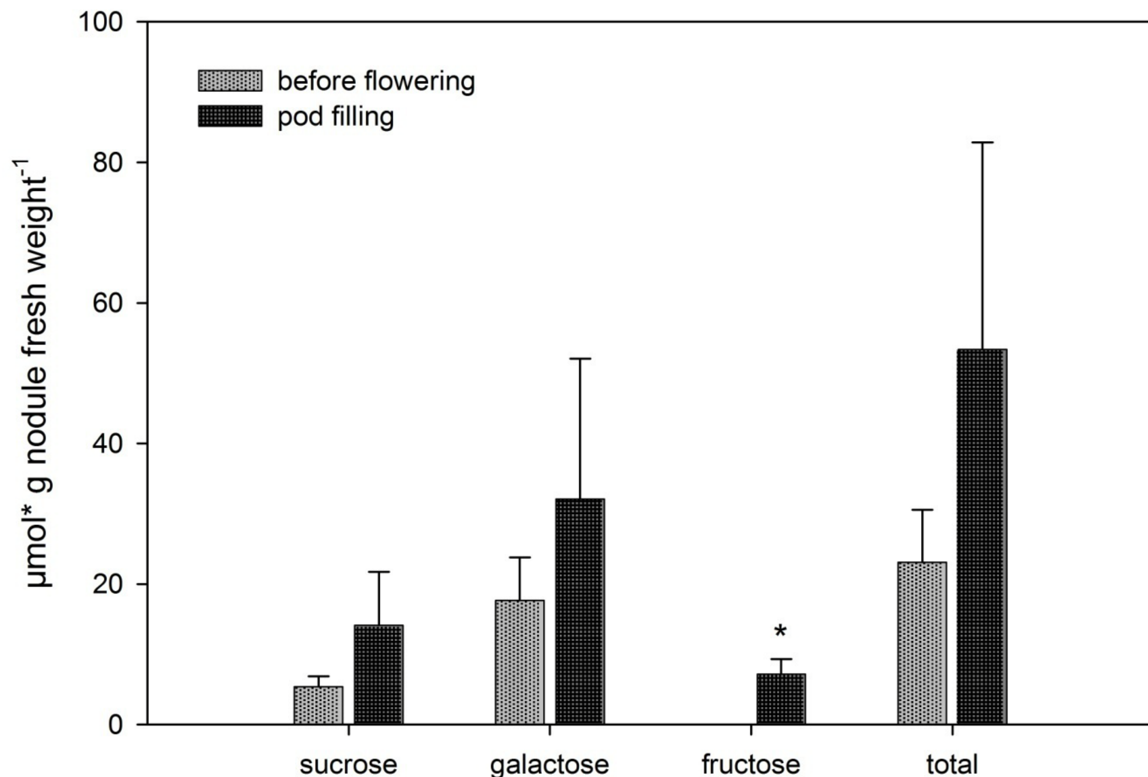

Figure 3: Nodule sugar concentration of plants before flowering and at pod-filling. Data are means of 4 replicates. Error bars represent standard deviation. * indicates a statistically significant difference between the two ontogeny stages (t-test, P≤0.05).

By contrast, OA concentration was significantly higher in nodules before flowering (Fig. 4). This was a result of more malate and succinate, both known to be taken up by the symbiosome and to support N_2 fixation (Vance and Heichel, 1991; White et al., 2007).In fact, succinate was not detectable in nodules of plants at the end of the growth interval during pod-filling. The levels of tartrate in nodules were equal at both growth intervals. Fumarate was only detected in negligible concentrations.

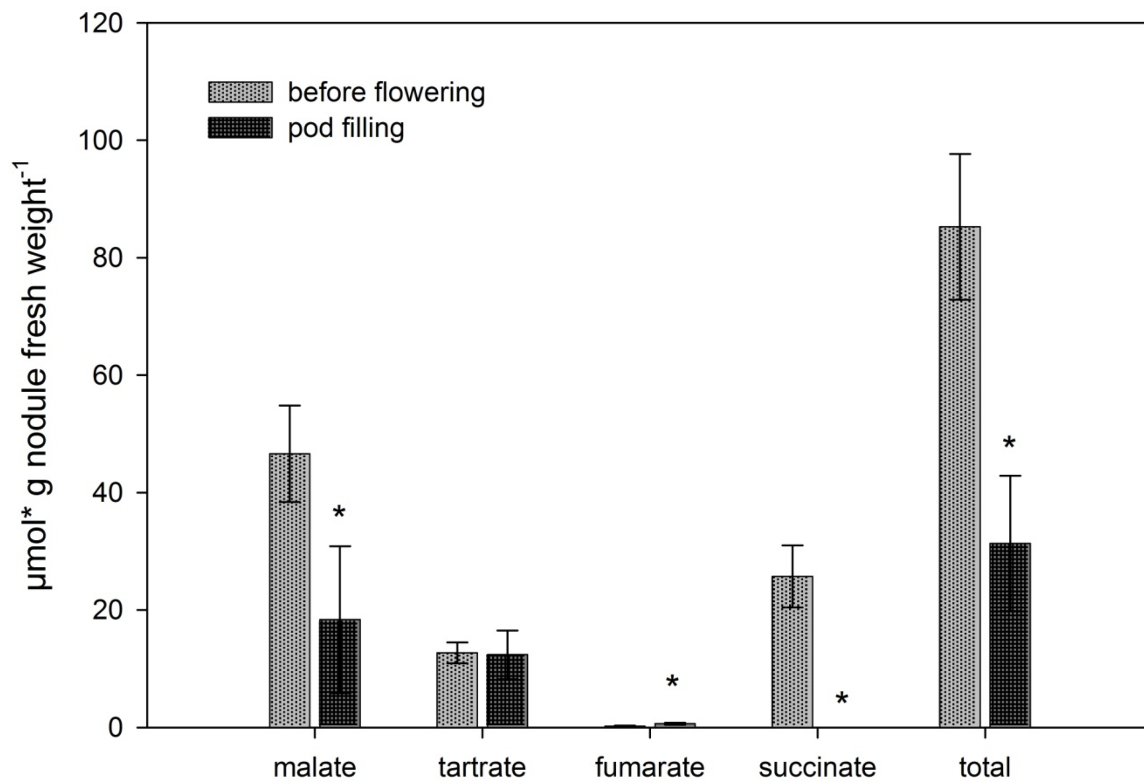

Figure 4: Nodule OA concentration of plants before flowering and at pod-filling. Data are means of 4 replicates. Error bars represent standard deviation. * indicates a statistically significant difference between the two ontogeny stages (t-test, P≤0.05).

O_2 and CO_2 exchange of nodulated roots

Net CO_2 release per unit root/nodule dry matter was much lower at pod-filling (Table II). CO_2 release per unit of reduced nitrogen was equal between the growth intervals, while the O_2 uptake per unit of reduced nitrogen was about 50% higher at pod-filling, which resulted in a significantly lower respiratory coefficient of nodulated roots. Thus lower CO_2 release per unit of nodulated root, in addition to higher oxygen uptake per unit of fixed nitrogen and a significantly lower apparent respiratory coefficient, coincides with nodules of higher specific activity and increased N_2 fixation per plant.

Table II: CO_2 release and O_2 consumption of roots and nodules per fixed N and apparent root/nodule respiratory coefficient.
Data are means of four replicates. * indicates a statistically significant difference compared to state before flowering (t-test, P≤0.05).

parameter	unit	growth interval	
		before flowering	pod-filling
CO_2 release of nodulated roots per N fixed	mol CO_2 mol N^{-1}	9.1	9.6
Net CO_2 release of nodulated roots	nmol CO_2 mg dm^{-1} h^{-1}	253.4	152.1*
O_2 uptake of nodulated roots per N fixed	mol O_2 mol N^{-1}	10.6	15.4*
Apparent respiratory quotient of nodulated roots	mol CO_2 mol O_2^{-1}	0.86	0.62*

Nodule PEPC and AAT activity

Nodule phosphoenole pyruvate carboxylase (PEPC, EC 4.1.1.31) and aspartate aminotransferase (AAT, EC 2.6.1.1) activity was about one third higher during pod-filling when compared to nodules from plants before flowering (Fig. 5).

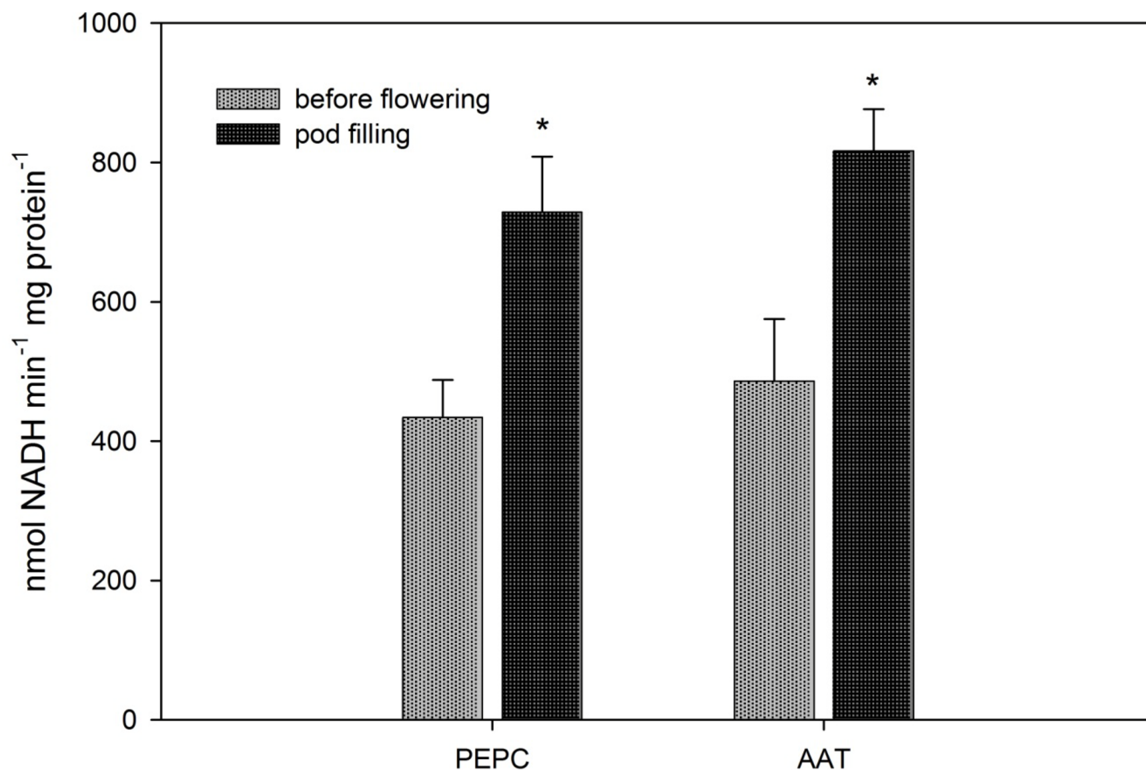

Figure 5: Nodule PEPC and AAT activity of plants before flowering and at pod-filling. Data are means of 6 replicates with standard deviations as error bars. * indicates a statistically significant difference between the two ontogeny stages (t-test, P≤0.05).

Nodule CO_2 fixation

Nodule CO_2 fixation was directly measured through $^{13}CO_2$ application. Nodule CO_2 fixation per plant was significantly increased at pod-filling (Table III). While nodule CO_2 fixation per plant was increased thirty-fold compared to before flowering, nodule CO_2 fixation per nodule dry matter was about four times higher. Thus the higher CO_2 fixation per plant during pod-filling was a result of both, increased nodule mass and a higher specific CO_2 fixation activity. Alongside measurements

of CO_2 fixation, nodule N_2 reduction was determined through $^{15}N_2$ application. There was a slight non-significant tendency towards more specific N_2 fixation during pod-filling. However, no distinction between active and inactive nodules was made in this experiment. When nodule CO_2 fixation was related to the amount of fixed nitrogen, the calculated value was increased about three times at pod-filling when compared to nodules before flowering.

Table III: Nodule CO_2 fixation per plant, specific N_2, CO_2 fixation per nodule mass and specific CO_2 fixation per N fixed.
Data are means of four or two replicates from plants before flowering and during pod-filling, respectively. * indicates a statistically significant difference compared to state before flowering (t-test, $P \leq 0.05$).

parameter	unit	growth interval	
		before flowering	pod-filling
^{13}C fixation through nodules	$\mu g\ h^{-1}\ plant^{-1}$	0.5	14.6*
nodule specific ^{13}C fixation	μg C g nodule $dm^{-1}\ h^{-1}$	33	120*
nodule specific $^{15}N_2$ fixation	μg N g nodule $dm^{-1}\ h^{-1}$	1.45	1.88
CO_2 fixation per N_2 reduced	g C g N^{-1}	0.04	0.11*

Nitrogenase efficiency

A measurement of nodule activity in terms of higher H_2 evolution confirmed a higher N_2 fixation at pod-filling measured by N accumulation and $^{15}N_2$ application (Table IV). However, the electron allocation coefficient (EAC) was significantly lower at pod-filling, indicating an increased share of electron flow onto H^+ vs. N_2, and thus a decreased relative efficiency of nitrogenase.

Table IV: Nodule H_2 evolution in an N_2/O_2 mixture (80/20, v/v) and an Ar/O_2 (80/20, v/v) before flowering and at pod-filling.
Data are means of four replicates. * indicates a statistically significant difference compared to state before flowering (t-test, P≤0.05).

parameter	unit	growth interval	
		before flowering	pod-filling
ANA	μmol H_2 plant^{-1} h^{-1}	5.4	13.8*
TNA	μmol H_2 plant^{-1} h^{-1}	17.2	33.7*
EAC		0.69	0.59*

Discussion

Our data clearly demonstrate a more intensive nitrogen fixation in pea plants at pod-filling in comparison to vegetative growth. Nitrogen fixation peaks during that period, presumably due to newly developing nitrogen attraction throughout pod-formation and pod-filling (Peat et al., 1981). Pea pods grow quickly and have a high tissue N concentration (Salon et al., 2001). In our experiment the increasing N requirements of the growing pods are met by higher N_2 fixation per plant. The higher nitrogen fixation rate per plant was in part the result of more nodules; however, in addition the active share of nodules had a much higher specific activity. A significant amount of nodules already showed clear signs of senescence at pod-filling. With regard to the physiological background of more intensive nitrogen fixation at pod-filling we have extended the knowledge in the following points.

1. While nodules at pod-filling had still sufficient sugars available, the reserves of organic acids were depleted: in other words, the steady state of organic acid formation and use had reached a low level. This applied in particular to malate and succinate, both known to energetically support N_2 fixation and, in the case of malate, to additionally feed carbon skeletons into N assimilation and N transport.

2. More intensive nitrogen fixation is connected with strongly increased nodule CO_2 fixation both per plant and per unit of reduced nitrogen. Nodule CO_2 fixation feeds nodule malate supply; the low malate level at pod-filling therefore indicates that the nodule's ability to transform sugars into malate is overstretched by the simultaneous intensive use of the product to support N_2 fixation.

3. Intensive CO_2 fixation at pod-filling results in clearly lower net CO_2 release per unit roots and nodules. This is even more surprising as nodule O_2 uptake and thus respiration was increased by more than 50%. Both facts resulted in an unusually low apparent respiratory quotient of roots and nodules at pod-filling.

4. Intensive N_2 fixation at pod-filling was combined with a lower relative efficiency of nitrogenase. This may be explained by the possible insufficient availability of carbon skeletons for nitrogen assimilation, to which the enzyme might react with

increased electron allocation to H^+, thereby avoiding excessive ammonium accumulation. When nodule malate formation is suppressed through a PEPC antisense construct, transgenic plants react with a clearly lower EAC compared to untransformed plants (Schulze et al., 1998b).

There are several reports that nitrogen fixation in grain legumes peaks at early pod-filling and shows a subsequent occasionally steep decline (Peat et al., 1981; Jensen, 1987; Imsande, 1989; Vikman and Vessey, 1993a). This decline is often connected to the briskly progressing senescence of leaves. Under optimal experimental conditions, i.e. sufficient water, nutrients and light, intensive nitrogen fixation often remains stable into very late pod-filling (Vikman and Vessey, 1993b). This was the case in our experiments, which lasted about two weeks into pod-filling. During that time plants kept green leaves and almost no leaf senescence was visible at the end of the experimental growth interval. Contrary to this, a significant share of nodules showed clear signs of senescence indicated by a greenish color. Higher nitrogen fixation of the active nodule share was related with increased amino acid concentrations in nodules and xylem sap. The progressing nodule senescence might be connected to the measured increased oxygen uptake of the nodules and the presumably related oxidative stress (Puppo et al., 2005).

In our experiment increased nitrogen fixation at pod-filling was clearly connected with higher CO_2 fixation and improved use of refixed carbon for nitrogen assimilation. This is supported by the measured $^{13}CO_2$ uptake in addition to increased *in vitro* activity of PEPC and AAT and the larger share of asparagine in nodules and xylem sap. These data suggest that at pod-filling more N assimilation is supported through carbon skeletons from PEP carboxylation. Figure 6 demonstrates that N assimilation and N transport to shoots on the basis of carbon skeletons from that source versus drainage of ketoacids from the TCA cycle (α-ketoglutarate) constitutes a carbon-saving mechanism for roots and nodules. In case of PEP carboxylation one atom of carbon is gained for N transport while organic acids from the TCA cycle are eventually formed combined with a previous loss of carbon from PEP. In this way increased CO_2 fixation improves the overall root/nodule carbon balance for N turnover and N transport to shoots. In fact, various data achieved on different grain legumes yielded consistently lower

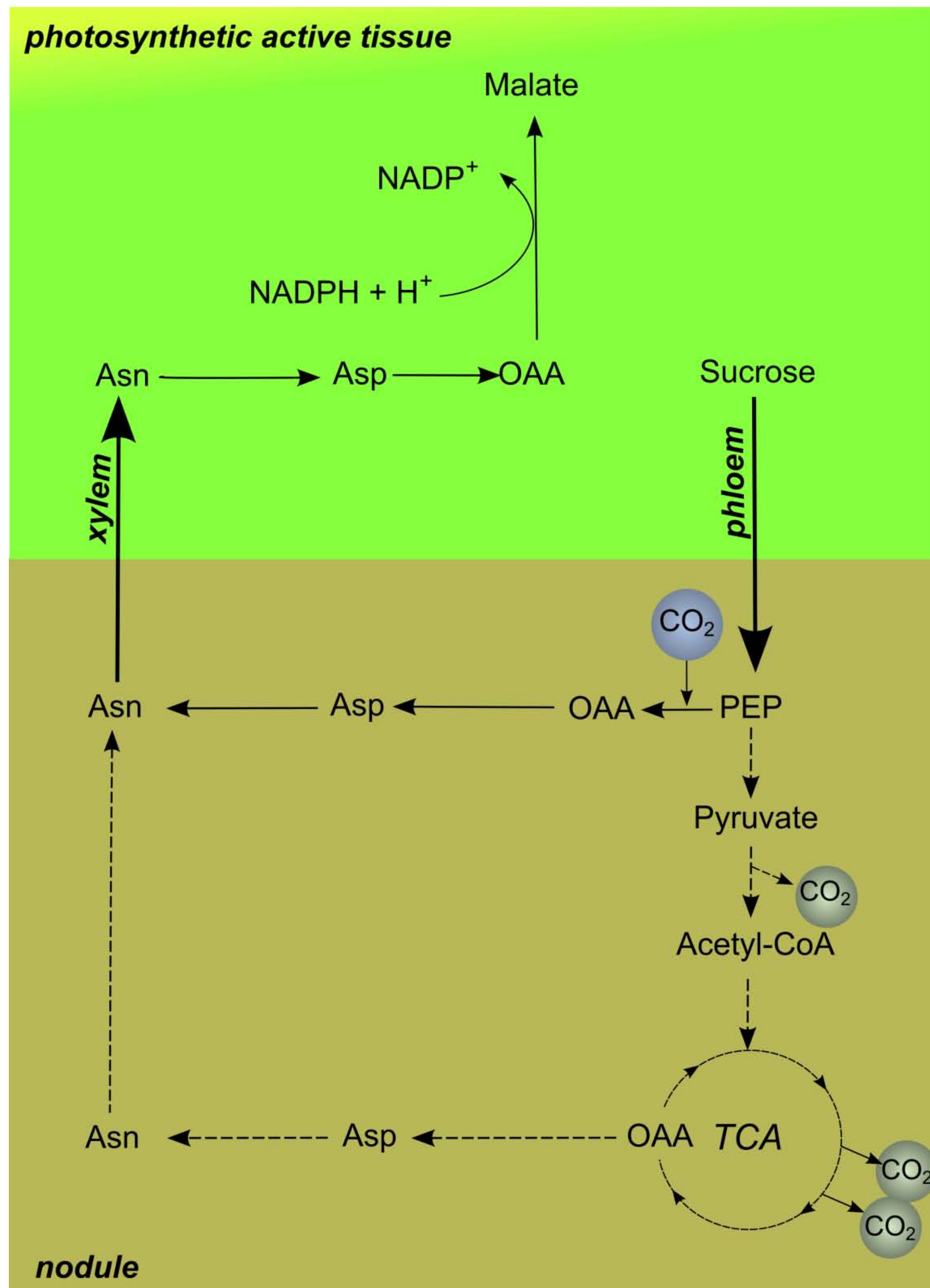

Figure 6: PEP in nodules can either deliver oxaloactetate (OAA) as carbon skeleton via the TCA cycle, which is connected with CO_2 loss or become carboxylated and serve directly as carbon skeleton for N assimilation and N transport to the shoot. Carboxylation of PEP is therefore a carbon-saving anaplerotic method for the efficient deliverance of C skeletons for N assimilation in nodules.

specific root/nodule carbon loss for driving N_2 fixation during pod-filling versus vegetative growth (Schulze et al., 2000; Adgo and Schulze, 2002). The data in this report suggest that nodule CO_2 fixation and use of the resulting carbon skeletons for N transport is a major mechanism for carbon-efficient N_2 fixation. We could not detect assimilate shortage in active nodules during pod-filling. However, it cannot be ruled out that a certain depletion of background reserves like starch or poly-hydroxybutyrate had already occurred and would result in unstable assimilate availability during pod-filling. The high variability of our data with respect to nodule sugar concentration might be an indication of this. Various reports indicate that although assimilate supply does not usually limit nodule activity (Vance and Heichel, 1991; Schulze, 2004), it might be critical during pod-filling when growing pods not only induce high activity in nodules but in addition attract huge amounts of carbon. For example, the use of hup^+ versus hup^- *Rhizobium* strains show effects on nitrogen fixation and growth not before the onset of pod growth (Bergersen et al., 1995). Provision of additional assimilates through sugar spraying on leaves had no effect on pea plants during vegetative growth while it significantly improved nitrogen fixation at pod-filling (Schulze et al., 1994). The interdependence between pea plant photosynthetic capacity and assimilate supply of nodules during pod-filling might be particularly critical in semi-leafless varieties like the one used in our experiments.

Stronger nodule CO_2 fixation is also connected with emerging P deficiency in legumes (Schulze et al., 2006). P deficiency impairs photosynthetic activity and assimilate supply to nodules. Consequently a carbon-saving mechanism like CO_2 fixation might be of vital importance for adaptation to low P availability. Christeller (1977) suggested that nodule N assimilation had to be almost completely supported by oxaloacetate from PEPC activity, a fact that would result in an up to 25% proportion of nodule fixed carbon in N transport. Otherwise, a significant drainage of ketoacids out of the TCA cycle pool would impair acetic acid influx into the cycle due to insufficient oxaloacetate availability (Walker, 1962). Overall our data support this hypothesis, in that the ability to convert sugar into organic acids was limited or at least on the edge of limitation at pod-filling. In particular, the virtual 'disappearance' of succinate in nodules at pod-filling suggests that the TCA cycle is significantly drained for N assimilation. This might constitute an additional

factor in overstretching nodule activity and adding to emerging nodule senescence at pod-filling, as the gap between increasing energy demand and impaired ability for energy provision widens.

In conclusion, the capacity of nodules to fix CO_2 is of crucial importance for their efficiency. Nodule CO_2 fixation provides a mechanism for saving carbon when the fixed carbon is used for N assimilation and N transport. The ability of nodules to fix CO_2 can be influenced through the use of agronomic measures, breeding and genetic techniques. High CO_2 around nodules apparently support nitrogen fixation (Yamakawa et al., 2004). There are reports that straw application positively affects nitrogen fixation in a subsequent crop (Shivashankar and Vlassak, 1978; Evans et al., 1997). This might be the result of higher CO_2 concentration in the soil atmosphere. Selection for high PEPC activity in nodules of alfalfa plants improved their growth performance. Various nodule-enhanced forms of key enzymes of the biochemical pathways have been identified (Suganuma et al., 1997; Miller et al., 1998; Fedorova et al., 1999). While down-regulating of e.g. PEPC reduces nitrogen fixation (Nomura et al., 2006), over-expression of MDH increases nodule specific activity (Schulze et al., 2002). Our data suggest that an improvement of nodule capability to channel assimilates into oxaloacetate and malate formation through CO_2 fixation might prolong intensive nitrogen fixation in grain legumes into the later stages of ontogeny.

Materials and Methods

Pot experiment – growth and harvest

Pisum sativum L. cv. ´Erbi` plants were grown in glass pots (Ø=100mm, h=200mm) on C and N free fine quartz sand amended with basic fertilization as previously described (Adgo and Schulze, 2002). Plants were inoculated with 1 mL of *Rhizobium leguminosarum* (E163) grown on YEM to an approximate cell density of 10^9 mL^{-1}. The cell suspension was applied to the sand at the base of the stem of each plant at day three and six after emergence. Inoculation led to effective nodulation and N_2 fixation while an uninoculated control died due to N starvation. Plants were kept under controlled conditions with a 14/10 h day/night cycle at 24/16°C and a photon flux density of 450 µmol m^{-2} s^{-1} during light periods. The plants were watered daily with deionized water to 75% of the maximum water holding capacity of the sand. The maximum water holding capacity of the sand was 21% of its weight.

Ten days after emergence (DAE), four replicates were harvested and separated into shoots, roots, active (reddish) and inactive (greenish or brownish) nodules (Baudouin et al., 2004). A second harvest of further four replicates was carried out at 24 DAE (BBCH 36). At harvest the intact root/nodule system was submerged in liquid nitrogen. Frozen nodules were detached and separated into active and inactive. Active nodules were subjected to analyses of free AAs, OAs and sugars. The 14 days between first and second harvest are referred to as 'the growth interval before flowering'. At the second harvest nodule *in vitro* PEPC and AAT activity was determined on active nodules of plants from six parallel pots.

A further experiment following the same experimental scheme was made with a growth interval from 28 to 42 DAE (early pod-filling, BBCH 71). This growth interval is subsequently referred to as 'the growth interval during pod-filling'.

Gas exchange experiments – growth and harvest

In a second set of experiments root nodule gas exchange was measured on plants in aeroponic culture (Schulze and Drevon, 2005). The measurements were performed on plants corresponding in age and developmental stage to the end of

the growth intervals before flowering and during pod-filling in the pot experiment. Root/nodule net CO_2 release, O_2 uptake, H_2 evolution in air (apparent nitrogenase activity, ANA) and under argon (total nitrogenase activity, TNA), $^{15}N_2$ uptake and $^{13}CO_2$ net uptake were measured. Inoculated plants were grown for three weeks in N free sand culture as described above and transferred to an aeroponic system at 14 DAE. The system was designed to allow more detailed root nodule gas exchange measurements. Plant roots were transferred into darkened glass cylinders (Ø=20 mm, h=600 mm) with rubber stoppers at both ends. The stem base of each plant was carefully placed through a hole (Ø=5 mm) in the upper rubber stopper. The cylinder contained 250 mL of the following nutrient solution (mM): KH_2PO_4, (0.06), K_2SO_4, (0.7); $MgSO_4$, (0.5); $CaCl_2$, (0.8); and micronutrients (µM): H_3BO_3, (4.0); Na_2MoO_4, (0.1); $ZnSO_4$, (1.0); $MnCl_2$, (2.0); $Co(NO_3)_2$, (0.2); $CuCl_2$, (1.0) and FeNaEDTA, (10). The pH was buffered with MES (0.25 mM) and adjusted to 6.5 in the nutrient solution by applying KOH. Plants were held at their stem bases with sterilized sponge leaving roots in the nutrient solution. The solution was intensely aerated by a flow of normal air of about 1 vol. min^{-1}. The nutrient solution was renewed daily. Solution that had evaporated or passed through the plant was replaced by deionized water one to three times a day. Root/nodule gas exchange measurements were performed at 20 to 24 DAE (end of growth interval before flowering) or 38 to 42 DAE (end of growth interval during early pod-filling). The experiments were carried out under controlled conditions in a climate chamber with a 16/8h day/night cycle at temperatures of approximately 25/18°C and relative humidity of about 70%. Light intensity at plant height was approximately 360 µmol m^{-2} s^{-1}.

Dry matter and N concentration

Plant material was dried at 60°C to a constant weight. Dry shoot, root or nodule material was weighed and ground to a fine powder. N concentration was determined by means of an elementary analyzer (Vario EL, Elementar Analysen GmbH, Hanau, Germany). All samples were measured in duplicate.

Nodule amino acid, organic acid and sugar concentration

For the analysis of free AAs and OAs in nodules, nodules were extracted as previously described (Fischinger et al., 2006). Amino acids were analyzed by

reverse phase HPLC in the ion suppression mode by precolumn derivatization through ortho-phthaldialdehyde (Chen et al., 1979). OAs were separated through HPLC and were detected by a photodiode array detector. For sugar analysis water extracts were used. Separation was achieved by reverse HPLC and detected by a refractometer. For analytical details see Keutgen and Pawelzik (2008).

Nodule in vitro PEPC and AAT activity

To measure *in vitro* nodule PEPC and AAT activity, 100 mg of freshly detached nodules were ground in extraction buffer (100mM MES-NaOH [pH 6.8], 100 mM sucrose, 2% v/v 2-mercaptoethanol, 15% v/v ethylene glycol, 2 mm PMSF) and centrifuged 15 min at 10 000 g to obtain the soluble protein fraction. Protein content was measured using Bradford´s reagent (Bradford, 1976). PEPC and AAT activity were measured in a coupled or direct assay, respectively, monitoring the disappearance of NADH at A_{340} and 20°C using protocols described in Egli et al. (1989). Enzyme activities were measured in active nodules at 28 and 44 DAE on six replicates. All samples were measured in triplicate.

Xylem sap amino acid concentration

For xylem sap collection the shoot was cut directly under the cotyledons. To avoid any contamination, closing the phloem and removing the cell bleeding sap was achieved by rinsing the cut surface of the root part for about 15 sec with 1M $CaCL_2$ solution. The root was then placed in a pressure chamber (Model 600 Pressure Chamber Instrument, PMS Instrument Co, Corvallis, Oregon, USA), where it was subjected to a 300 MPa overpressure. The xylem sap was collected for a period of 10 min. During the whole procedure the xylem sap was kept on ice and was then frozen immediately (-20°C). AAs were analyzed as described above.

Root nodule H_2, CO_2 and O_2 exchange

For the H_2 and CO_2 evolution measurement, roots and nodules had to be enclosed under airtight conditions in gas cylinders allowing the application of a regulated amount of air and to measure the composition of the outflowing gas. For that purpose, the hole in the upper rubber stopper was sealed with a plasticine material with high beeswax content. The beeswax gave the material a soft, pliable consistence that ensured a tight adherence to the rubber stopper, tubing, and

plant stem. The material is non-toxic to plants. Before sealing, stiff tubing to act as an inflow and outflow of the sealed root/nodule compartment was laid through the hole in the upper rubber stopper and also sealed with the plasticine material. The inflow tubing reached down to the lower end of the glass cylinder while the outflow was put above any nodules on the lower side of the upper rubber stopper.

The sealed root/nodule compartment was connected to an open flow gas exchange measurement system that allowed to apply a mixture of N_2/O_2 (80/20, v/v). For measurements, the nutrient solution level was lowered to about one third of the glass cylinder, leaving the lower virtually nodule-free part of the root system in the solution. An airflow of 200mL min^{-1} (about 1.2 volumes min^{-1}) was applied to the root compartment. A sub-sample (100mL min^{-1}) of the outflowing gas was taken, dried (ice trap and $MgClO_4$) and passed through an H_2 analyzer and a CO_2 analyzer (S121 Hydrogen Analyzer, S161 infrared CO_2 analyzer Quibit Systems, Canada). When a stable H_2 and CO_2 outflow from the root/nodule compartment was reached, the value was taken as ANA and root nodule net CO_2 release. Subsequently the inflow air composition was switched to Ar/O_2 (80/20, v/v). Argon is inert to nitrogenase and thus the whole electron flow is diverted to H^+. Consequently H_2 evolution under argon represents total enzyme activity (TNA). The peak value taken approximately 5 min after switching to Ar/O_2 was taken as TNA value. The electron allocation coefficient (EAC) of nitrogenase activity was calculated as 1–(ANA/TNA). N_2 fixation in nitrogen per time was calculated from the ANA and TNA values according to Schulze et al. (2006). O_2 uptake was measured on parallel plants grown in glass containers with a volume of 150 mL. The open flow measurement of O_2 uptake was done as described in Schulze and Drevon (2005). For measurements the flow through the root/nodule compartment was lowered to 30 mL min^{-1}. The oxygen concentration in the outflowing gas was measured with an Oxynos 100 (Rosamount, Germany).

Nodule $^{13}CO_2$ and $^{15}N_2$ fixation

For determination of N_2 and CO_2 fixation, nodules of pea plants were exposed to $^{15}N_2$ and $^{13}CO_2$. For $^{15}N_2$ application, the whole tube was filled with nutrient solution and the nutrient solution was subsequently replaced by a $^{15}N_2$ (99 atom%$^{15}N_{exc}$)/O_2 (80/20) mixture. Roots and nodules were exposed to the $^{15}N_2/O_2$

mixture for 30 min. Subsequently the root/nodule compartment was flushed with an N_2/O_2 mixture enriched with 2000 µL L^{-1} $^{13}CO_2$ (99 atom%) for 15 min in an open flow-through system to avoid a significant dilution of $^{13}CO_2$ by root respiration. The O_2 concentration during $^{15}N_2/O_2$ incubation did not decline below 18%.

Immediately after label application plants were fixed in liquid nitrogen and separated into roots, shoots and nodules. The plant material was dried at 60°C to a constant weight. The dried shoot and root fractions were weighed and milled using a pebble mill. Nodules were ground with mortar and pestle.

For ^{15}N and ^{13}C analysis, sub-samples of the dried plant material were measured with a combination of a C/N analyser (Porapak PQS) and a mass spectrometer (Finnigan MAT, model 252). The $^{13}CO_2$ uptake was determined by multiplying the C content of a fraction with the ^{13}C excess of this fraction over the ^{13}C% of an unlabeled reference group:

$$^{13}C_{fixed}[g] = \frac{C[g] * \left(^{13}C_{treatment}\% - {}^{13}C_{reference}\% \right)}{100}$$

Acknowledgement

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Kapitel 2: Erhöhte CO_2-Konzentration im Wurzelraum von Luzerne führt zu erhöhter N_2-Fixierung.

Elevated CO_2 concentration around alfalfa nodules increases N_2 fixation

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Running title: legume nodule CO_2 fixation

Tables: 4
Figures: 9
Date of submission:

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Abstract

Nodule CO_2 fixation provides malate for bacteroids and oxaloacetate for N assimilation. The process is thus of central importance for efficient nitrogen fixation. Nodule CO_2 fixation is known to depend on external CO_2 concentration. The hypothesis of the present paper was that nitrogen fixation in alfalfa plants is enhanced when the CO_2 concentration around nodules is kept at levels common to the soil atmosphere (2500 µL L^{-1}, $+CO_2$ treatment) versus CO_2 free air ($-CO_2$ treatment). Nodulated plants of alfalfa were grown in nutrient solution in a system that allowed a separate aeration of the root/nodule compartment avoiding any additional CO_2 feeding to shoots. Nitrogen fixation and growth was strongly increased in the $+CO_2$ treatment in a three-week experimental period. Plants in the $+CO_2$ treatment tended to develop fewer and bigger nodules with a much higher %N concentration and individual activity. More intensive nitrogen fixation coincided with increased nodule CO_2 fixation and higher per plant amounts of amino acids and organic acids in nodules. Moreover, the concentration of amino acids, in particular that of asparagine, was increased in nodules and the xylem sap. In a parallel experiment on plants with inefficient nodules the $+CO_2$ treatment remained without effect. Our data support the thesis that nodule CO_2 fixation is pivotal for efficient nitrogen fixation. It is concluded that strategies which enhance nodule CO_2 fixation will improve nitrogen fixation and nodule formation. Moreover, sufficient CO_2 application to roots and nodules is necessary for growth and for the making of accurate measurements on legume nodules in aeroponic growth systems.

Key words: alfalfa, $^{13}CO_2$, H_2 evolution, *Medicago sativa*, N_2 fixation, nitrogen fixation, nodule CO_2 fixation, PEPC, xylem sap, amino acid.

Introduction

Numerous studies have shown that legumes react to increased CO_2 concentrations around shoots with an orchestrated increase in root and nodule growth (Aranjuelo *et al.*, 2009; Murphy, 1986; Phillips *et al.*, 1976). With only few contradictory reports, nodule specific activity remains unchanged (Cabrerizo *et al.*, 2001) and the higher N demand of the more intensely growing shoot at high CO_2 concentrations is eventually met by improved per plant nitrogen fixation capacity in more and bigger nodules. Improved assimilate supply to nodules has no short term effect on nodule-specific activity (Vance and Heichel, 1991) and in turn, erratic assimilate supply to nodules is buffered through nodule carbon pools like starch, α-polyhydroxybutyrate and glycogen (Wang *et al.*, 2007). A decline in nodule activity at night is apparently more a reaction to lower temperatures than to less assimilate supply (Schweitzer and Harper, 1980). Rather than falling short of sugar availability, nodule-specific activity appears to be limited by the nodule ability to form organic acids (Vance, 1998; Wang *et al.*, 2007). In particular malate formation is important, since malate is the principal source for the energetic feeding of the bacteroids (Driscoll and Finan, 1993), and at the same time functions as carbon skeleton for N assimilation after reconversion to oxaloacetate (Rosendahl *et al.*, 1990). Moreover, malate might be involved in a putative osmoregulatory functioning of the nodule oxygen diffusion barrier that controls microaerobic conditions in the nodule infected zone (Minchin, 1997). The microaerobic conditions inside the nodule are part of the reason that nodule carbon metabolism is shunted towards organic acid, namely malate, formation. Phosphoenolpyruvate (PEP) rather than being decarboxylated, is transformed into oxaloacetate and malate by the combined activity of carbonic anhydrase (CA) (Atkins *et al.*, 2001), phosphoenolpyruvate carboxylase (PEPC) (Vance *et al.*, 1994) and malate dehydrogenase (MDH) (Schulze *et al.*, 2002). For PEPC and MDH nodule-enhanced forms are described (Miller *et al.*, 1998; Suganuma *et al.*, 1997) and CA shows a nodule-specific expression in various legumes (Atkins *et al.*, 2001; de la Pena *et al.*, 1997). In fact, overexpression of neMDH in alfalfa nodules did not only increase per plant nitrogen fixation but in addition the specific activity of individual nodules (Denton *et al.*, 2002). The importance of the biochemical pathway towards malate in nodules is highlighted as well by the fact that PEPC and MDH activity occurs alongside nitrogenase expression and activity

in emerging nodules (Egli *et al.*, 1989; Vance *et al.*, 1983). Studies with labeled CO_2 reveal that nodules indeed have considerable CO_2 fixation rates (Warembourg and Roumet, 1989) and down-regulation of PEPC activity in nodules through an antisense strategy impairs nitrogen fixation (Schulze *et al.*, 1998). Although leaf PEPC has a low K_m for CO_2 concentration, *in situ* saturation of the enzyme capacity might strongly depend on the ongoing drainage of its products (Kromer *et al.*, 1996; Willmer *et al.*, 1990). PEPC is tightly regulated in part by the nodule malate concentration (Zhang *et al.*, 1995). Christeller *et al.* (1977) have shown that nodule CO_2 fixation in lupin is a function of external CO_2 concentration. The apparent saturation is reached between 20 to 40 mL L^{-1} CO_2 in the air around nodules. However these measurements were made on excised nodules, in which the use of malate might progressively decline due to less N_2 fixation and N assimilation. CO_2 concentration in the soil gaseous phase is high, depending strongly on microbial activity. Concentrations of up to 5000 µL L^{-1} are reported (Buyanovsky and Wagner, 1983). In experimental systems with sand culture but in particular in aeroponic systems, CO_2 concentrations around roots and nodules is often very low since the systems need to be intensely aerated to secure oxygen availability for nodules and roots. This aeration is usually made with ambient air (around 360 µL L^{-1} CO_2) and in particular roots of young plants do not amend significant additional CO_2 from respiration. There are some scattered reports that nodule activity is increased through long term high CO_2 concentrations around roots and nodules (Grobbelaar *et al.*, 1971; Mulder and Van Veen, 1960; Yamakawa *et al.*, 2004; Yamakawa *et al.*, 1997). Such experiments, however, necessitate a strict separation of shoots and a root/nodule compartment to avoid CO_2 feeding of leaves and thus a mixture of shoot and root effects. The hypothesis of the present paper was that long-term high CO_2 concentration around roots and nodules (2500 µL L^{-1} versus zero µL L^{-1} + root/nodule respiration) would improve nitrogen fixation of young alfalfa plants due to increased CO_2 fixation, resulting in better provision of organic acids for driving N_2 fixation and supporting N assimilation in nodules. Particular emphasis was put on the avoidance of any cross-over effect through accidental additional CO_2 feeding of the shoots.

Materials and Methods

Plant growth

Cuttings of alfalfa plants (*Medicago sativa* L.) cv ´Saranac` and ´Insaranac` were made from approximately four-week old plants grown in nutrient solution. ´Insaranac` forms ineffective nodules (Barnes *et al.*, 1990; Viands *et al.*, 1979). Cuttings were treated with rooting hormone mix, planted in containers with fine quartz sand and maintained in a controlled environment chamber with a 16/8h day/night cycle at temperatures of approximately 25/18°C and a relative humidity of about 70%. The light intensity was 360 µmol m^{-2} s^{-1}. The quartz sand was kept at about 70% of its maximum water holding capacity (21% of its weight) by the addition of N free nutrient solution of the following composition: macronutrients, (mM): K_2SO_4, 0.7; $MgSO_4$, 0.5; $CaCl_2$, 0.8; KH_2PO_4, 0.015, and micronutrients, (µM): H_3BO_3, 4.0; Na_2MoO_4, 0.1; $ZnSO_4$, 1.0; $MnCl_2$, 2.0; $Co(NO_3)_2$, 0.2; $CuCl_2$, 1.0 and FeNaEDTA 10. The pH was buffered with 0.25 mM MES and adjusted to 6.5 by applying KOH. In addition, at seven and 14 days after planting each tray received 5 µmol P as KH_2PO_4. After rooting, cuttings were inoculated with *Sinorhizobium meliloti* strain 102F51. Nodules appeared six to seven days after inoculation. Three weeks after inoculation 12 cuttings of even size were selected and carefully transferred to glass tubes (h=600 mm, inner diameter=20 mm) with nutrient solution. The tubes were closed with a rubber stopper at the lower side. Plants were put through a hole in a rubber stopper at the upper side of the tube and hold at their stem with sponge. The aeroponic cultivation of alfalfa plants in the glass tubes is described in Schulze and Drevon (2005). The glass tubes were filled with the nutrient solution described above except for phosphorus. Each plant received 3 or 7.5 µmol P as KH_2PO_4 per day during the first week or second week after transplanting, respectively. Subsequently the P application was increased to 15 µmol P per plant and day. This P supply resulted in a P concentration in the nutrient solution of 12, 30 or 60 µM, respectively. The solution was changed daily and aerated with ambient air at a flow rate of about 1.2 volumes min^{-1}.

Application of different CO_2 concentrations to the root/nodule compartment

Plants were kept in the glass tubes for four days and aerated with ambient air to allow them to adapt. At day five the root/nodule compartment was sealed for H_2 and CO_2 evolution measurement and the long-term application of air with different CO_2 concentrations. For that purpose the hole in the upper rubber stopper was sealed with a plasticine material with high beeswax content. The beeswax gave the material a soft and pliable consistence that ensured a tight adherence to the rubber stopper and the plant stem. The material is non plant-toxic. Before sealing stiff tubing as inflow and outflow of the sealed root/nodule compartment was laid through the hole in the upper rubber stopper and also sealed with the material. The inflow tubing reached to the lower end of the glass cylinder while the outflow was put above any nodules on the lower side of the upper rubber stopper. The inflow and outflow of the glass tubes were subsequently connected to a gas flow (N_2/O_2, 79/21, v/v) with either zero or 2500 µL L^{-1} CO_2 concentration (Fig. 1). The respective air flows were sucked through the sealed root nodule compartment with a flow rate of 200 mL min^{-1} and directed outside of the growth chamber. Any possible leakage in the system would result in a dilution of the applied air. No CO_2 enriched air was able to reach the shoots. Repeated measurements of the CO_2 concentration around shoots showed ambient CO_2 concentrations.

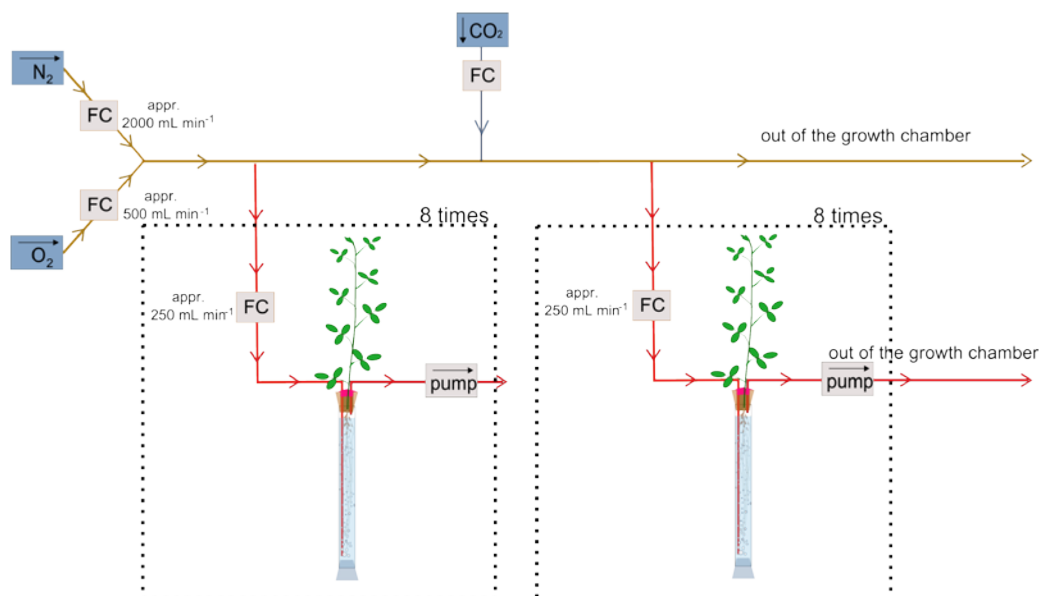

Figure 1. Experimental setup for long-term application of air with different CO_2 concentration to root/nodule compartments. The parts enclosed in dotted lines are repeated eight times each. The gas input comes from pressurized gas bottles. The N_2 and O_2 gas was free of any CO_2 or H_2 contamination.

H_2 evolution measurements

For the H_2 evolution measurement, the sealed root/nodule compartment was connected to an open-flow gas exchange measurement system that allowed the application of a mixture of N_2/O_2 (79/21, v/v) to the root/nodule compartment. For the measurements the nutrient solution level was lowered to about 1/3 of the glass cylinder, leaving the lower virtually nodule-free part of the root system in the solution. An airflow of 200mL min^{-1} (about 1.2 volumes min^{-1}) was applied to the root compartment. A subsample (100mL min^{-1}) of the outflowing gas was taken, dried (ice trap and $MgClO_4$) and passed through an H_2 analyser (Quibit Systems, Canada). When a stable H_2 outflow from the root/nodule compartment was reached, this value was taken as apparent nitrogenase activity (ANA). Subsequently the air composition in the inflowing airstream was changed to Ar/O_2 (80/20, v/v). Argon is inert to nitrogenase and thus the whole electron flow is diverted to H^+. Consequently H_2 evolution under argon represents total enzyme activity (total nitrogenase activity, TNA). The peak value taken three to five minutes after switching to Ar/O_2 was regarded as the TNA value. The electron allocation coefficient (EAC) of nitrogenase activity was calculated as 1–(ANA/TNA). ANA, TNA and the EAC were measured before the introduction of the treatments, two days after treatment introduction and at the end (after 3 weeks) of the experimental period.

Xylem sap harvest

For xylem sap collection the shoot was cut directly under the cotyledons. To avoid any contamination, closing of the phloem and removing the cell bleeding sap was achieved by rinsing the cut surface of the root part for about 15 sec with 1M $CaCL_2$ solution. The root was subsequently placed in a pressure chamber (Model 600 Pressure Chamber Instrument, PMS Instrument Co, Corvallis, Oregon, USA), and subjected to 300 MPa pressure. The xylem sap was collected for a period of 10 min. During the whole procedure the xylem sap was kept on ice and was then frozen immediately (-20°C).

$^{13}CO_2$ application

In a second experiment a set of ´Saranac` plants was treated in the same way as in the first experiment. After three weeks of growth at different CO_2 concentrations an airstream containing 2500 µL L^{-1} $^{13}CO_2$ (98Vol $\%_{exc.}$) was applied to each root compartment for 15 min. The application was made to alternate plants from the $+CO_2$ and the $–CO_2$ treatment. The airstream was set up in the same way as to the CO_2 feeding system, i.e. the $^{13}CO_2$ enriched air was sucked through the root/nodule compartment to avoid accidental feeding of the shoots. Immediately at the end of the labeling period the root/nodule compartment was flushed with ambient air and the plants were rapidly taken out of the tubes and submerged in liquid nitrogen. The plants were subsequently divided into shoots, roots and nodules and vacuum-dried. Reference plants were harvested in a growth chamber separated from the $^{13}CO_2$ application.

Nodule amino acid and organic acid concentration

The nodules were picked from the intact plant; directly frozen in liquid nitrogen and stored at –20°C until analyses. For analyses of free amino acids (AA) nodules were homogenized with mortar and pestle in liquid N_2. Subsequently, 0.5 mg of the material was extracted with 3mL of 50% ethanol (v/v) in a 40 °C water bath for 20 min. The solution was centrifuged for 30 min at 8000g and 4 °C. The supernatant was immediately used for HPLC analyses after filtration (0.45 mm). AA were detected with a fluorescence detector after precolumn derivatization by orthophthaldialdehyde (Chen *et al.*, 1979).

Dry matter, N, C, ^{13}C concentration

The plants were divided in shoots, roots and nodules. The fractions were dried to a constant weight at 60°C. Dried material from experiments one and two was grounded to a fine powder with a pebble mill. The powdered material was subsequently analysed with a combination of a C/N analyser (Porapak PQS) and a mass spectrometer (Finnigan MAT, model 252).

Results

Growth, nodulation and %N

Growth of nodulated plants with effective nodules was significantly increased in the +CO_2 treatment (Tab. 1, Fig. 2). Nodules of ´Saranac` plants were bigger and appeared pink while nodules of ´Insaranac` plants were white. Total dry matter formation in Saranac was increased by 250% through the application of CO_2 to the root nodule compartment while this treatment was without any effect on Insaranac plants, neither on total dry matter formation nor on any particular plant organ. In relative terms the increase in shoot and roots of ´Saranac` plants was about equal but stronger when compared to nodules. Plants with CO_2 application tended to form less and bigger nodules. There was a large variability in nodule number, nodule per plant dry matter and nodule individual dry matter. The mean value for the nodule individual dry matter was about fivefold higher in the +CO_2 treatment, statistically significantly different with $P \leq 0.1$. CO_2 application had no effect on the inefficient nodules of ´Insaranac` plants. `Saranac` plants in the CO_2 treatment achieved the growth advantage by progressively more leaf and branch formation during the experimental period (Fig. 3 and 4). Between 14 to 20 days after introduction of the CO_2 treatments both parameters were significantly different. At the end of the experimental period %N concentration and C/N ratios showed no significant difference in shoots or roots between the treatments either in ´Saranac` or ´Insaranac` plants. However, nodules of +CO_2 ´Saranac` plants had about 180% N concentration when compared to nodules of the –$CO_2$ plants. The high %N value in the +$CO_2$ plants resulted in a significantly lower nodule C/N ratio. Nodule %N and C/N ratio were not affected by $CO_2$ application in ´Insaranac` plants.

Table 1. Dry matter, nodule number, and nodule individual weight of +CO2 and -CO2 alfalfa plants.
Plants were grown for two weeks with different levels of CO_2 concentration in the root/nodule compartment. Data are means of four replicates. * indicates a statistically significant difference from the $+CO_2$ treatment of the same alfalfa line (t-test, $P \leq 0.05$). No statistical comparison between ´Saranac` and ´Insaranac` has been found.

Parameter	Treatments			
	Saranac		Insaranac	
	$+CO_2$	$-CO_2$	$+CO_2$	$-CO_2$
Shoot dry matter (mg plant^{-1})	373	143*	243	253
Root dry matter (mg plant^{-1})	153	61*	109	114
Nodule dry matter (mg plant^{-1})	24.3	12.1	3.7	4.6
Total dry matter (mg plant^{-1})	550	216*	355	370
Nodule number	24	40	10	10
Nodule individual dry weight (mg nodule^{-1})	1.60	0.31	0.11	0.12

Figure 2. Nodulated alfalfa plants grown for three weeks with either –CO_2 (left) or +CO_2 (right) application to a separated root/nodule compartment, Nodules of plants from both treatments are shown below the plants.

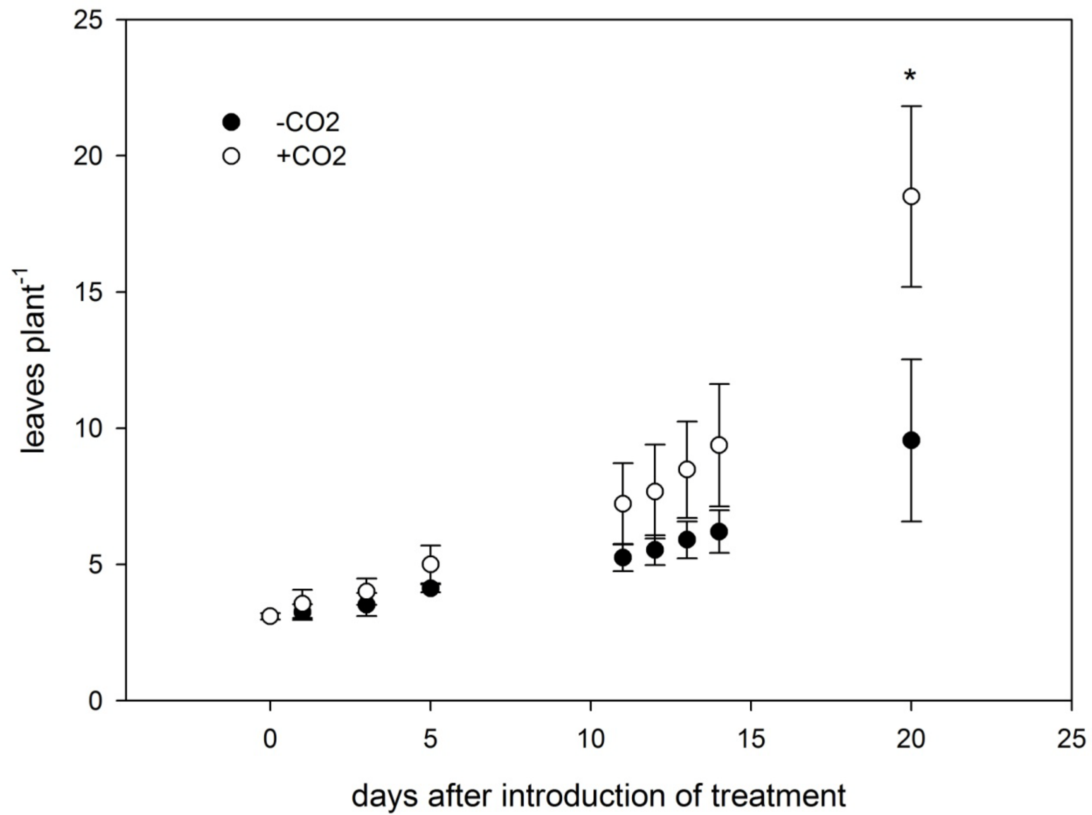

Figure 3. Development of leaf number per plant (´Saranac`) during the course of a three-week experimental period with application of an N_2/O_2 mixture (80/20; v/v) with either zero (-CO_2) or 2500 µL L^{-1} CO_2 (+CO_2) to the root/nodule compartment. Data are means of four replicates. Bars represent standard deviation. *indicates a statistically significant difference from the +CO_2 treatment (t-test, $P \leq 0.05$).

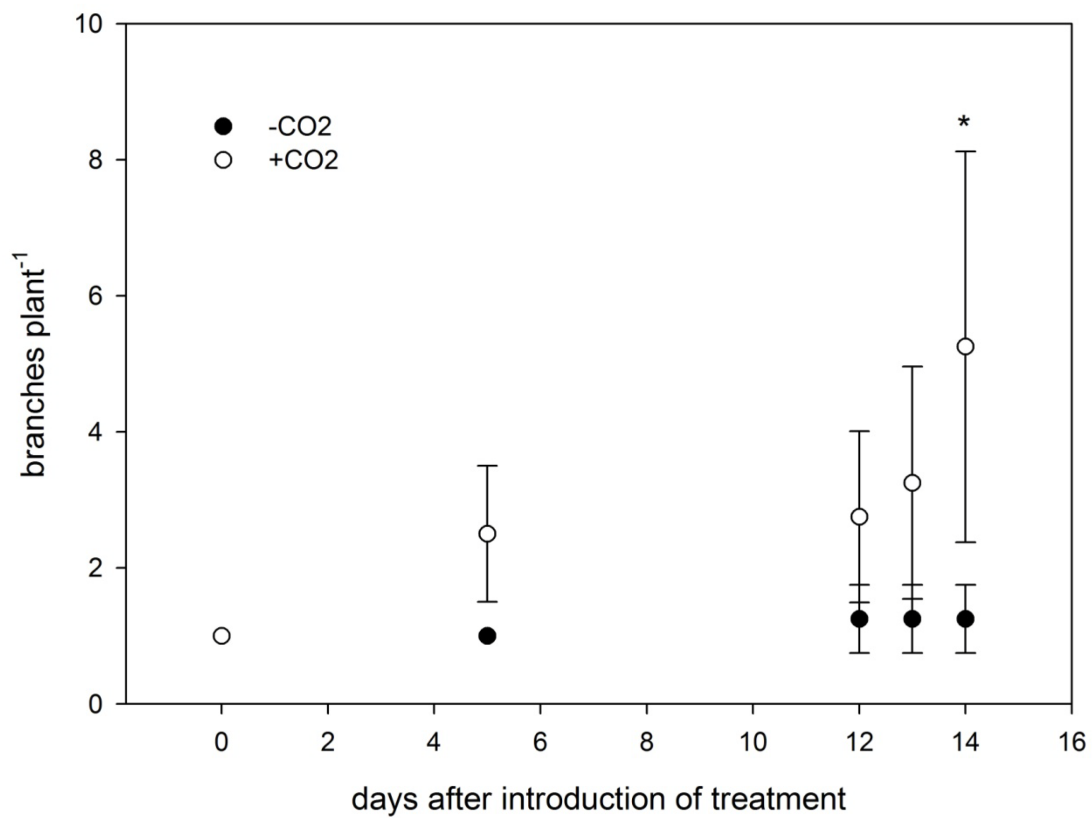

Figure 4. Development of branch number per plant (´Saranac`) during the course of a three-week experimental period with application of an N_2/O_2 mixture (80/20; v/v) with either zero (-CO_2) or 2500 µL L^{-1} CO_2 (+CO_2) to the root/nodule compartment. Data are means of four replicates. Bars represent standard deviation. *indicates a statistically significant difference from the +CO_2 treatment (t-test, $P \leq 0.05$).

Nitrogen fixation

At 2 to 3 days after the introduction of the CO_2 treatment a visible change in plant appearance occurred in that the +CO_2 plants formed more new leaves and developed a lighter leaf green when compared to the –CO_2 plants. The differentiation in green intensity evened out during the experimental period, which was in accordance with an equal or tendentially even higher N concentration in the +CO_2 plants at the end of the experimental period (Tab. 2). N_2 fixation measured on the basis of H_2 evolution did not differ between treated plants before introduction of the different CO_2 application (Tab. 3). ´Insaranac` plants showed no measureable $H_2$ evolution during the course of the experiment. At 2 days after introduction of the $CO_2$ treatments, a significant differentiation in $N_2$ fixation of the ´Saranac` plants occurred (Tab. 3). Nitrogen fixation in the +CO_2 plants was about 225% of that in the –CO_2 plants. The differentiation in N_2 fixation did not show significantly further widening until the end of the experimental period, but remained at the approximate relation measured at day 2 after beginning of the CO_2 treatment.

Amino acids in nodule and xylem sap

The higher nitrogen fixation activity of plants in the +CO_2 treatment is supported through a tendency towards higher concentration of asparagine in nodules. Figure 5 shows the proportion of asparagine among the five most abundant amino acids measured. The amount of amino acids in nodules per plant is significantly increased in the + CO_2 treatment (Fig. 6). These facts resulted in a higher total concentration of amino acids in the xylem sap (Fig. 7). This higher total concentration was a result of particular increases in asparagine, glutamine, and aspartate.

Table 2. %N concentration and C/N ratio in shoots, roots and nodules of +CO_2 and -CO_2 alfalfa plants.

Plants were grown for two weeks with different levels of CO_2 concentration in the root/nodule compartment. Data are means of four replicates. * indicates a statistically significant difference from the +CO_2 treatment (t-test, P≤0.05).

Parameter	Treatments			
	Saranac		Insaranac	
	+CO_2	-CO_2	+CO_2	-CO_2
Shoot N concentration (% N)	2.3	2.0	2.3	2.4
Root N concentration (% N)	2.8	2.7	2.8	3.1
Nodule N concentration (% N)	9.2	5.1**	4.8	5.2
C/N shoot (g C g N^{-1})	19	29	19	18
C/N root (g C g N^{-1})	15.3	16	16	14
C/N nodule (g C g N^{-1})	5.5	7.3*	8.1	8.2

Table 3. N_2 fixation of alfalfa plants (Saranac) before and during application of different levels of CO_2 concentration to the root/nodule compartment.

Plants were grown for two weeks with different levels of CO_2 concentration in the root/nodule compartment. Data are means of four replicates. * indicates a statistically significant difference from the $+CO_2$ treatment (t-test, P≤0.05).

Parameter	Treatments	
	$+CO_2$	$-CO_2$
Total N_2 fixation activity before introduction of treatments (mg N d^{-1} $plant^{-1}$)	192	171
Total N_2 fixation activity 2 days after introduction of treatments (mg N d^{-1} $plant^{-1}$)	283	126*
Total N_2 fixation activity 21 days after introduction of treatments (mg N d^{-1} $plant^{-1}$)	965	415*
EAC 21 days after introduction of treatments	0.59	0.61
Specific N_2 fixation 21 days after introduction of treatments (mg N g nodule dry $matter^{-1}$ d^{-1})	43	39
N_2 fixation activity of an individual nodule 21 days after introduction of treatments (µg N $nodule^{-1}$ d^{-1})	41	11*

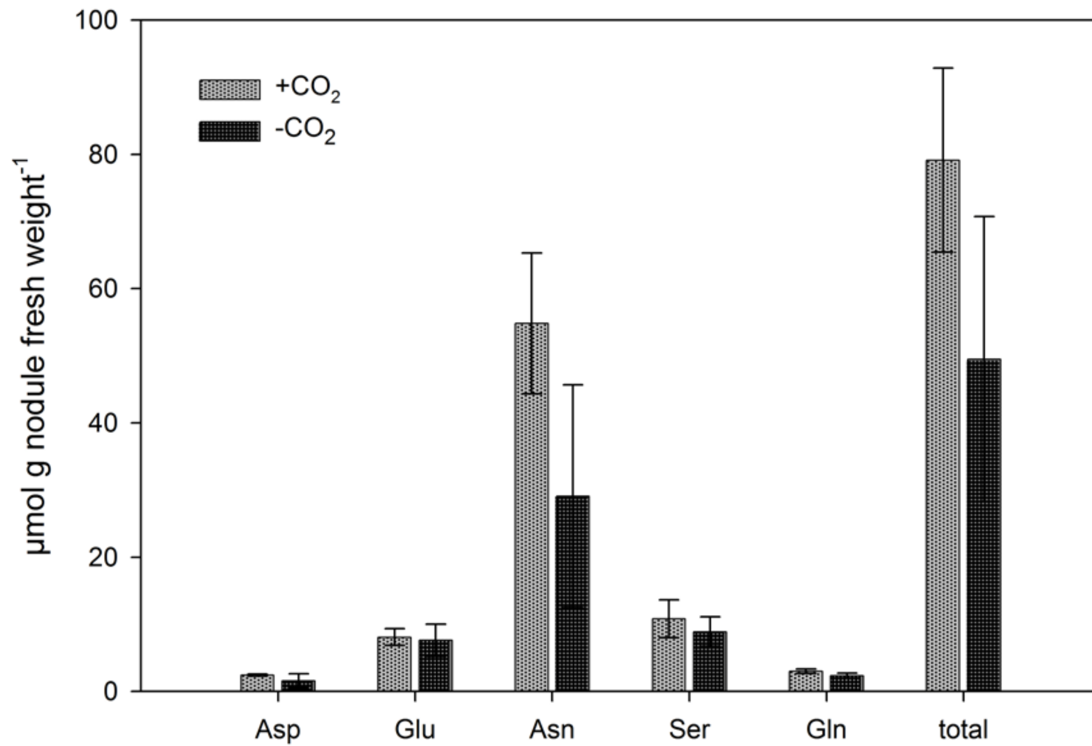

Figure 5. Concentration of the five most abundant amino acids in nodules. Data are means of four replicates. Bars represent standard deviation. *indicates a statistically significant difference from the $+CO_2$ treatment (Tukey-test, $P \leq 0.05$). In addition to the amino acids shown, Ala, Gaba, Tyr, Arg, Try, Lys, Val, Thr, Leu, His, Ile, Gly and Prol. were also detected in concentrations below 0.5 µmol g nodule fresh weight^{-1}.

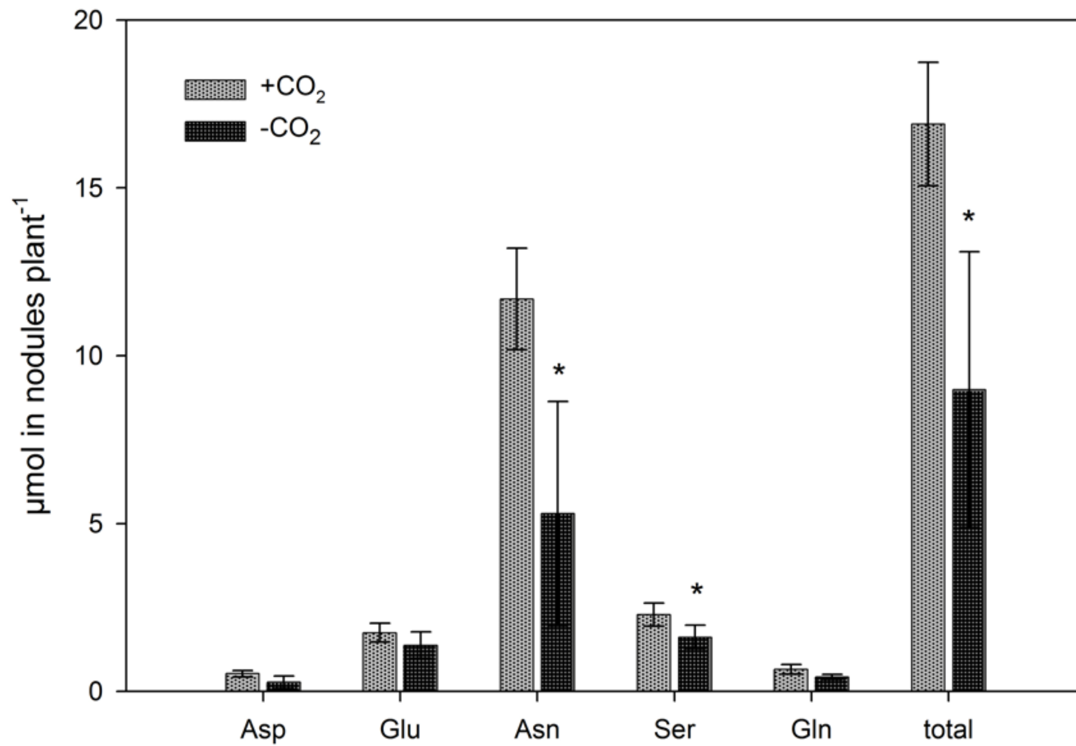

Figure 6. Total amount in nodules per plant of the five most abundant nodule amino acids. Data are means of four replicates. Bars represent standard deviation. *indicates a statistically significant difference from the $+CO_2$ treatment (Tukey-test, $P \leq 0.05$).

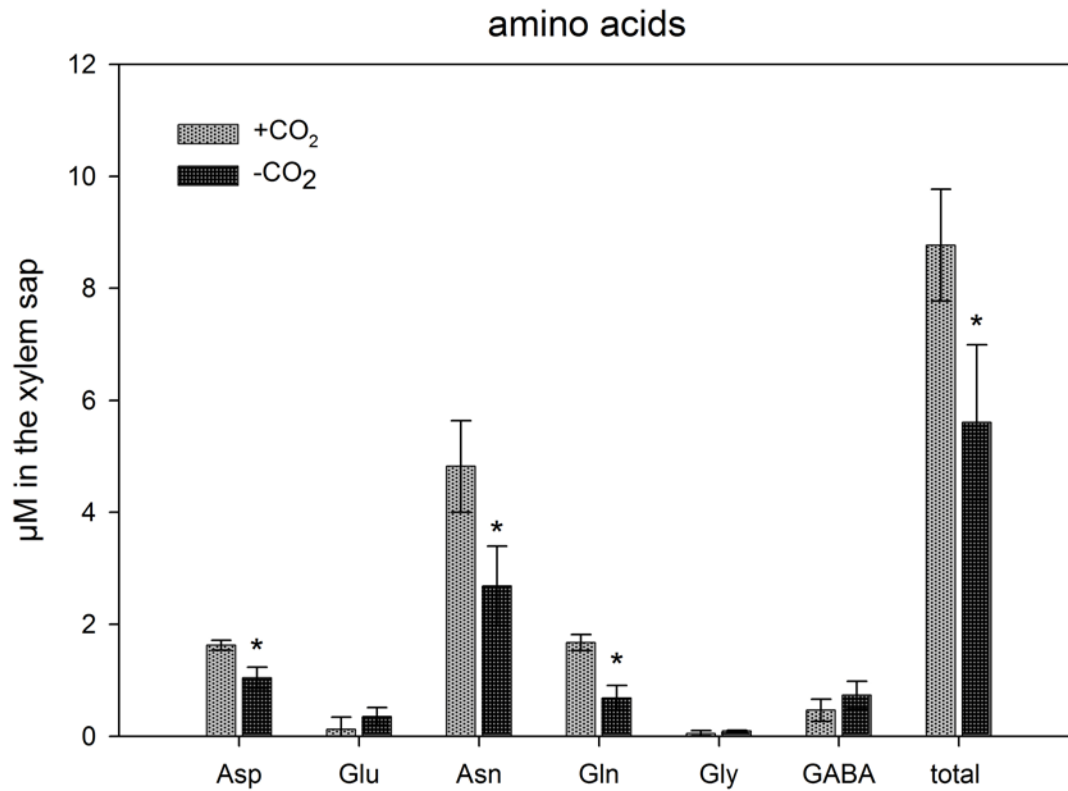

Figure 7. Concentration of amino acids found in the xylem sap in concentrations above 0.1 µM. Data are means of four replicates. Bars represent standard deviation. *indicates a statistically significant difference from the +CO_2 treatment (Tukey-test, $P \leq 0.05$).

Root/nodule CO_2 fixation

Root and nodule CO_2 fixation was measured on a further set of ´Saranac` plants given the same treatment as in the growth experiment. Apparent CO_2 fixation per plant was increased in roots and nodules of the + CO_2 treatment approximately three- and four-fold, respectively (Tab. 4).

Table 4. CO_2 fixation capacity of alfalfa (Saranac) nodules after two weeks` growth at different CO_2 concentrations in the root/nodule compartment.

Data are means of three replicates. * indicates a statistically significant difference compared to the +CO_2 treatment (t-test, P≤0.05).

Parameter	Treatments	
	+CO_2	-CO_2
Root CO_2 fixation (µg C g root dry matter^{-1} h^{-1})	42	14
Nodule CO_2 fixation (µg C g nod dry matter^{-1} h^{-1})	82	22*
Nodule fixation per N_2 reduced (mg C g N^{-1})	88.4	34.5

Nodule organic acid composition

Organic acid formation per plant was increased in the $+CO_2$ plants (Fig. 8) by approximately %. This was a result of more nodule fresh weight per plant while the concentration of organic acids in nodules was not increased (Fig. 9). Among the organic acid detected, fumarate, malate and tartrate were the most abundant, while succinate and citrate were only found in low concentrations.

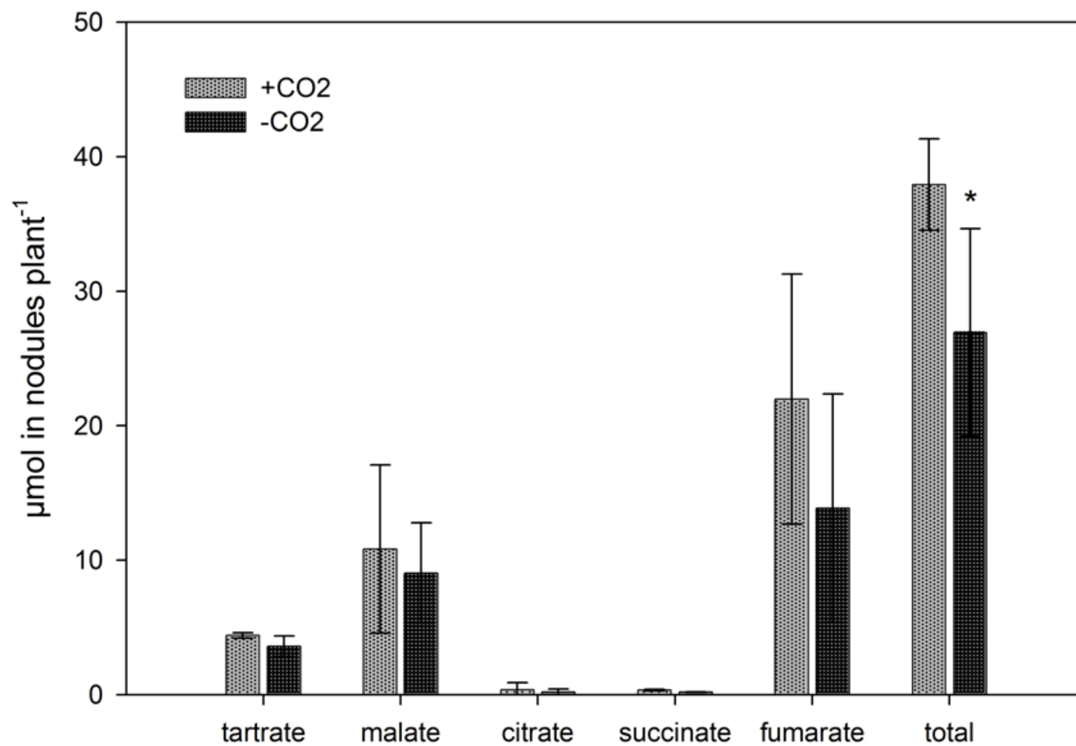

Figure 8. Total amount in nodules per plant of the organic acids detected in nodules. Data are means of four replicates. Bars represent standard deviation. *indicates a statistically significant difference from the $+CO_2$ treatment (Tukey-test, $P \leq 0.05$).

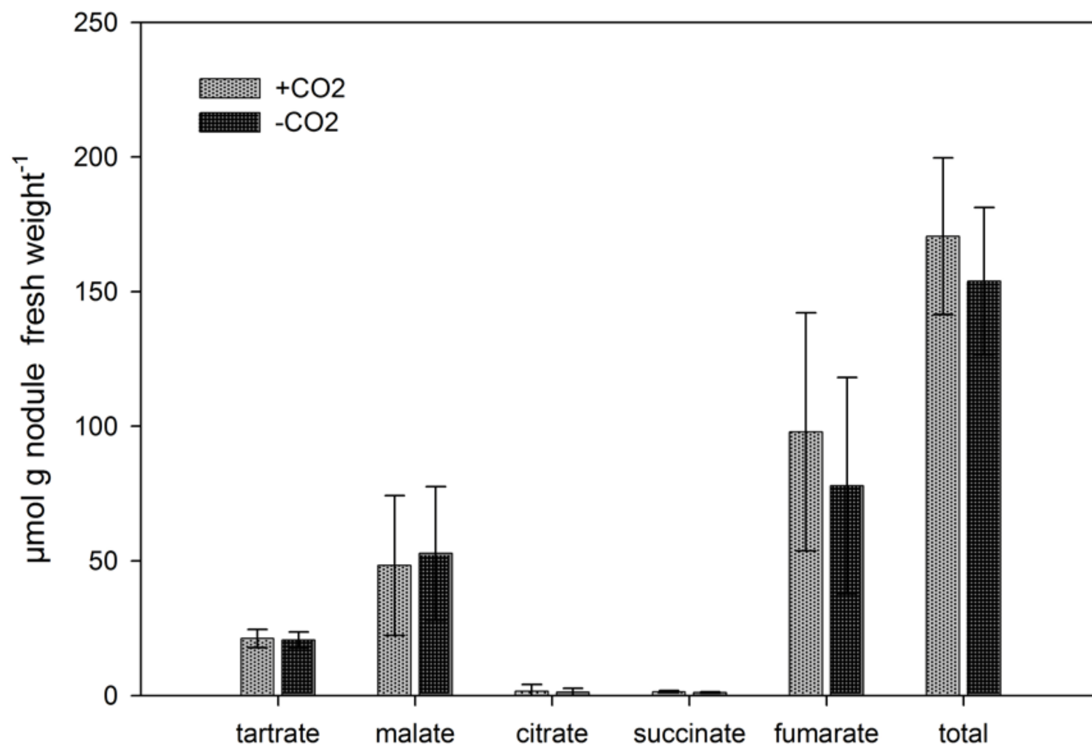

Figure 9. Concentration of detected organic acids in nodules. Data are means of four replicates. Bars represent standard deviation. *indicates a statistically significant difference from the +CO_2 treatment (Tukey-test, $P \leq 0.05$).

Discussion

The results of our study highlight the importance of nodule CO_2 fixation for nitrogen fixation and growth of legumes. Nodule CO_2 fixation is known to be tightly coupled to N_2 fixation, as is proven for example by the concomitant expression of PEPC in nodules and emerging nitrogenase activity (Vance *et al.*, 1983) or through decreased N_2 fixation as a result of decreased PEPC expression in response to transformation with an antisense PEPC construct (Nomura *et al.*, 2006). The extent of nodule CO_2 fixation of lupin roots depends on external CO_2 concentration with an apparent saturation at 2 to 4% CO_2 in the soil atmosphere (Christeller *et al.*, 1977). We found increased nitrogen fixation and growth at CO_2 concentrations of about 2500 µL L^{-1} versus zero to 100 µL L^{-1} around nodules and roots in alfalfa plants. Thus, although the CO_2 concentration in the +CO_2 treatment is close to that found in the soil atmosphere, it might still not have been saturating for nodule CO_2 fixation. Effects of high CO_2 concentrations around nodules are reported for soybean, pea and common bean (Grobbelaar *et al.*, 1971; Yamakawa *et al.*, 2004). These reports found not only a consistent effect on N_2 fixation per plant but also on nodulation and nodule size. However, in most of these experiments a certain concomitant CO_2 feeding of shoots and thus a mix of effects on nodule and shoot CO_2 fixation cannot be completely ruled out. Our experimental setup meant that any additional CO_2 from the root/nodule compartment reaching the shoots could be avoided. Sucking the CO_2 enriched air through the root/nodule compartment rather than pressing it would have led to a CO_2 dilution in the airstream in the event of any possible leakage. Repeated measurements of the CO_2 concentration in the outflowing air from the root/nodule compartments in addition to measurements of CO_2 concentration around shoots and around the whole experimental setup proved the validity and viability of the system with respect to specific CO_2 feeding of roots and nodules. Moreover, the observed effects were restricted to plants with efficient nodules, while plants nourished with nitrate displayed no effect on growth. In addition, we observed a more or less equal increase in shoot and root growth through CO_2 feeding of roots and nodules, while CO_2 feeding of the shoots tends to preferentially supports root growth (Schulze *et al.*, 1999). A distinctly higher N_2 fixation per plant in the +CO_2 treatment is evidenced by H_2 evolution measurements and also by significantly

higher amino acid content in the total of nodules and higher amino acid concentrations in the xylem sap. At the end of the experimental period better nitrogen fixation per plant in the +CO_2 treatment was largely a result of bigger nodules with a higher individual efficiency. Although we did not determine nodule number or dry weight at two days after introduction of the treatments, H_2 evolution measurements at this point in time indicate an increased specific nodule activity. Nitrogen fixation per plant in the +CO_2 treatment was strongly increased in comparison to measurements before CO_2 application while it was more or less constant in the –CO_2 treatment. Active nodules take at least six to seven days to emerge in alfalfa; thus it is unlikely that significantly more active nodules had been formed at the time of the H_2 evolution measurement two days after introduction of the treatments. It is conceivable that CO_2 feeding had accelerated the development of young nodules already established at the point in time when the CO_2 treatment commenced. However, this is not consistent with the fact that we noted a tendency towards lower numbers of effective nodules in the +CO_2 treatment at the end of the experimental period. In our experiment we found a most noticeable initial increase in nodule specific activity initially, and a further formation of increasingly larger nodules with a higher per nodule activity. By contrast, shoot CO_2 feeding in most reported experiments shows neither a short nor a long term-effect on nodule specific activity (Cabrerizo *et al.*, 2001; Vance and Heichel, 1991).

The observed increase in growth and nitrogen fixation at the beginning of the experiment was connected with the development of more new leaves with a lighter green color when compared to those of the –CO_2 treatment. Moreover, the plants developed additional branches. Such reactions might be connected with hormone effects induced by improved N nutrition. Nitrogen fixation in the –CO_2 treatment could obviously not fully support the growth potential of the alfalfa plants. Better legume growth with nitrate nutrition as opposed to exclusive nitrogen fixation has been repeatedly reported (Herrmann *et al.*, 2001). Under natural soil conditions, a mixed supply of nitrogen from nodules and soil solution is normal and apparently the optimal way to meet the plants` nitrogen requirements (Lamb *et al.*, 1995). At the end of our experimental period the nitrogen concentration in the leaves of the +CO_2 treatment tend to surpass that in the –CO_2 treatment, and leaf color was comparable and appeared healthy in both treatments.

Increased nitrogen fixation in the +CO_2 treatment was accompanied by a higher asparagine content in nodules per plant and an increased asparagine concentration in the xylem sap. However, nodule concentration in the detected organic acids or in particular in malate was not improved through the +CO_2 treatment. For analysis, the nodules had been fixed in liquid nitrogen while adhering to the roots and subsequently not been allowed to melt before extraction. Thus organic acid analysis and in particular that for malate, does allow a one-off insight into a steady-state turnover in which the organic acids are intensely drained through uptake by the symbiosome and respiration and also through carbon skeleton provision for the increasingly available ammonium. Consequently the equal concentrations in nodules with strongly different concurrent malate use do indicate improved malate production brought about by nodule CO_2 fixation. In fact, we found that organic acid concentration is higher in senescent nodules compared to active ones and also in nodules left detached yet otherwise intact for a certain period of time (unpublished data). Both observations indicate that a decrease in nodule nitrogen fixation activity is connected with an organic acid accumulation.

Our measurements on root/nodule CO_2 fixation not only show that it was higher in the +CO_2 treatment, thus supporting the thesis of improved organic acid formation. In addition, the long term +CO_2 treatment also improved root/nodule CO_2 fixation capacity, since the measurements were made with equal $^{13}CO_2$ concentration in both treatments (2500 µL L^{-1}). Thus sufficient CO_2 around nodules apparently contributes to the emergence of efficient nodules.

In conclusion, our results support the thesis that short and long term CO_2 concentration around nodules is of importance for nitrogen fixation activity and the formation of efficient nodules in alfalfa. This has implications for experimental procedures measuring nodule gas exchange, in particular in aeroponic systems. Measurements using pure N_2/O_2 mixtures or ambient air might underestimate nitrogen fixation. Moreover, long-term aeroponic growth with aeration of the nutrient solution with ambient air might impair the formation of optimally efficient nodules, in particular in young plants when root/nodule respiration does not sufficiently increase nodule internal and external CO_2 concentration. The biochemical pathway leading from nodule CO_2 fixation to malate production and use can be influenced through breeding and techniques of plant genetic transformation. Both strategies might improve nitrogen fixation activity in particular

in the early stages of growth in alfalfa plants. Moreover, agronomic measures improving soil respiration and thus CO_2 concentration in the soil atmosphere might contribute to more efficient legume growth.

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Kapitel 3: Methoden der Gaswechselmessung im Wurzelraum von Leguminosen – Etablierung und Verifizierung

Argon effects on legume nodule H_2 evolution and O_2 uptake

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Running title: legume nodule activity under argon

Tables: 2

Figures: 3

Date of submission:

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Abstract

Replacement of N_2 by argon in the air around nodules directs nitrogenase electron flow in its total onto H^+ resulting in increased nodule H_2 evolution (total nitrogenase activity = TNA). However, argon application induces a so-called argon-induced decline in nitrogenase activity (Ar-ID) connected with decreased nodule oxygen permeability. Consequently, TNA measurements tend to underestimate total nitrogenase activity. The objective of the present work was to study the extent of the Ar-ID under conditions of different activity in nodules of pea and alfalfa plants. In addition, the time sequence of a decline in nodule H_2 evolution and O_2 uptake after argon application was determined. TNA measurements in an optimized gas exchange measurement system yielded reliable results proven by parallel determination of $^{15}N_2$ uptake. A comparatively little Ar-ID occurred within two hours. With down-regulated nodules only a slight leveling-off of TNA was measured while at P deficiency an actual slight increase occurred. Parallel experiment on control plants revealed that a decrease in nodule oxygen uptake began several minutes after the onset of the decline in H_2 evolution. It is concluded that the Ar-ID strongly depends on the physiological status of the nodule. The primary effect of the replacement of N_2 by argon is apparently different from oxygen diffusion control. A gas exchange system allowing a quick measurement of TNA yields reliable results and does not disturb nodule activity. Gas exchange measurements provide a powerful tool for studying nodule physiology and should be combined with material from molecular studies.

Key words: alfalfa, argon-induced decline, legumes, nitrogen fixation, nodule,

nodule oxygen uptake, N_2 fixation, pea.

Introduction

When N_2 in the air around nodules is replaced by argon (Ar), the electron flow through nitrogenase is in its total directed onto H^+. The resulting H_2 evolution is a comparatively simple and non-destructive way to measure nitrogenase total activity (TNA = total nitrogenase activity). In conjunction with H_2 evolution in ambient air (ANA = apparent nitrogenase activity), the relative efficiency of nitrogenase in terms of electron allocation can be calculated as 1-ANA/TNA (EAC = electron allocation coefficient) (Hunt and Layzell, 1993).

However, there are various reports that find an underestimation of actual N_2 fixation through measurements of TNA (Imsande, 1991; Schulze, 2004). This underestimation might be connected to the so-called argon-induced decline (Ar-ID) in nitrogenase activity (King and Layzell, 1991). After a few minutes of argon application, an occasionally steep decline resulting in a more or less constant H_2 evolution baseline occurs. During the decline and the subsequent low baseline of H_2 evolution an increase in O_2 concentration around nodules results in a transient recovery of nitrogenase activity (King and Layzell, 1991). This observation is the basis for the assumption that the Ar-ID is a phenomenon induced by tightening of nodule oxygen diffusion resistance after application of argon. However, the immediate effect of argon application on a nodule is a cessation of the ammonia/ammonium flow into in the symbiosome space, most probably inducing a rapid acidification (Day *et al.*, 2001). Moreover, any downstream biochemical pathway connected to N assimilation will be slowed down or interrupted. For the current understanding it is unclear whether the Ar-ID is initially induced by the impairment of ammonia production and subsequent N assimilation followed by an exclusion of excessive oxygen by the nodule, or, in turn, an initial closure of the oxygen diffusion barrier results in lower nitrogenase activity.

The Ar-ID is connected with further unexplained phenomena, for example a transient recovery of nitrogenase activity often observed after several minutes (10-20) of argon application (Hunt and Layzell, 1993). Moreover, it is reported that nitrogenase activity shows a permanent partial recovery after two to three hours of argon application (Drevon and Hartwig, 1997). Finally, there are several reports that no Ar-ID was found even during prolonged application. As far as available data tell there is no restriction of the phenomena to any particular legume or

nodule type nor is it clear whether any particular physiological condition of the nodule is related to reactions of nodules to argon application.

A prerequisite for the study of the phenomena related to the Ar-ID is an efficient set-up of the gas exchange measurement system. In particular, the rapid replacement of N_2 by Ar in the air around nodules is important together with low overall total gas volume. For the present study we designed a gas exchange measurement system that allowed a separate aeration of a root/nodule compartment. The plants were grown in nutrient solution and the small root/nodule compartment allowed a rapid replacement of N_2 by Ar. Moreover, nitrogenase activity could be tested in parallel by application of $^{15}N_2$.

Through the use of the experimental setup we planned to address the following hypotheses.

1. A precise measurement of TNA is possible when the total replacement of N_2 by Argon and the measurement of a peak H_2 evolution value (TNA) is made within a five minute period. Previous experiments had shown that longer argon application was connected to lower subsequent ANA values when compared to the measurement of nodule activity before argon application.

2. The beginning of the Ar-ID would not coincide with a change in nodule O_2 uptake.

3. The occurrence of an Ar-ID decline would depend on nodule activity.

To achieve these goals we performed root/nodule gas exchange measurements on pea (*Pisum sativum* L.) and alfalfa (*Medicago sativa* L.) plants. In a first experiment we determined N_2 fixation through H_2 evolution in an N_2/O_2 and subsequently an Ar/O_2 mixture. The calculated N_2 fixation was compared to $^{15}N_2$ uptake of the same set of plants. The Ar-ID was measured on pea plants under phosphorus stress and after shoot darkening. Moreover, on alfalfa plants the sequence of occurrence of a decrease in H_2 evolution and O_2 uptake was determined after Ar/O_2 application.

Materials and Methods

Plant growth

Pea *(Pisum sativum* L.) cv. ´Davina` and alfalfa (*Medicago sativa* L) cv. ´Saranac` plants were grown in black plastic pots (Ø=200mm, h=100mm) on C- and N-free fine quartz sand amended with basic fertilization as previously described (Adgo and Schulze, 2002). Plants were inoculated with 1 mL of *Rhizobium leguminosarum* grown on YEM to an approximate cell density of 10^9 mL^{-1}. At 14 days after emergence (DAE) plants were transferred to an aeroponic system. The system was designed to allow more detailed root nodule gas exchange measurements (Schulze and Drevon, 2005). Plant roots were transferred into darkened glass cylinders (Ø=20 mm, h=600 mm) with rubber stoppers at both ends. The stem base of each plant was carefully placed through a hole (Ø=5 mm) in the upper rubber stopper. The cylinder contained 250 mL of the following nutrient solution (mM): KH_2PO_4, (0.06), K_2SO_4, (0.7); $MgSO_4$, (0.5); $CaCl_2$, (0.8); and micronutrients (µM): H_3BO_3, (4.0); Na_2MoO_4, (0.1); $ZnSO_4$, (1.0); $MnCl_2$, (2.0); $Co(NO_3)_2$, (0.2); $CuCl_2$, (1.0) and FeNaEDTA (ferric monosodium salt of ethylenediamine tetraacetic acid), (10). The pH was buffered with MES [2-(*N*-morpholino) ethane-sulfonic acid] (0.25 mM) and adjusted to 6.5 in the nutrient solution by applying KOH. Plants were held at their stem bases with sterilized sponge leaving roots in the nutrient solution. The solution was intensely aerated by a flow of normal air of about 1 vol. min^{-1}. The nutrient solution was renewed daily. Solution that had evaporated or passed through the plant was replaced by deionized water one to three times a day. The experiments were carried out under controlled conditions in a climate chamber with a 16/8h day/night cycle at temperatures of approximately 25/18°C and relative humidity of about 70%. Light intensity at plant height was approximately 360 µmol m^{-2} s^{-1}.

System for gas exchange measurement and application of labelled gases of variable composure

Figure 1 shows the setup for long-term application of gases in variable composure to a separated root/nodule compartment. The detailed procedure for aeroponic

growth, allowing a separate gas application to a root/nodule compartment is described in Schulze and Drevon (2005). The system allows application of gas mixtures with a variable proportion of zero to 100% of N_2, Ar, O_2, CO_2, $^{13}CO_2$ and $^{15}N_2$. 16 plants can be connected to the system treated simultaneously and individually.

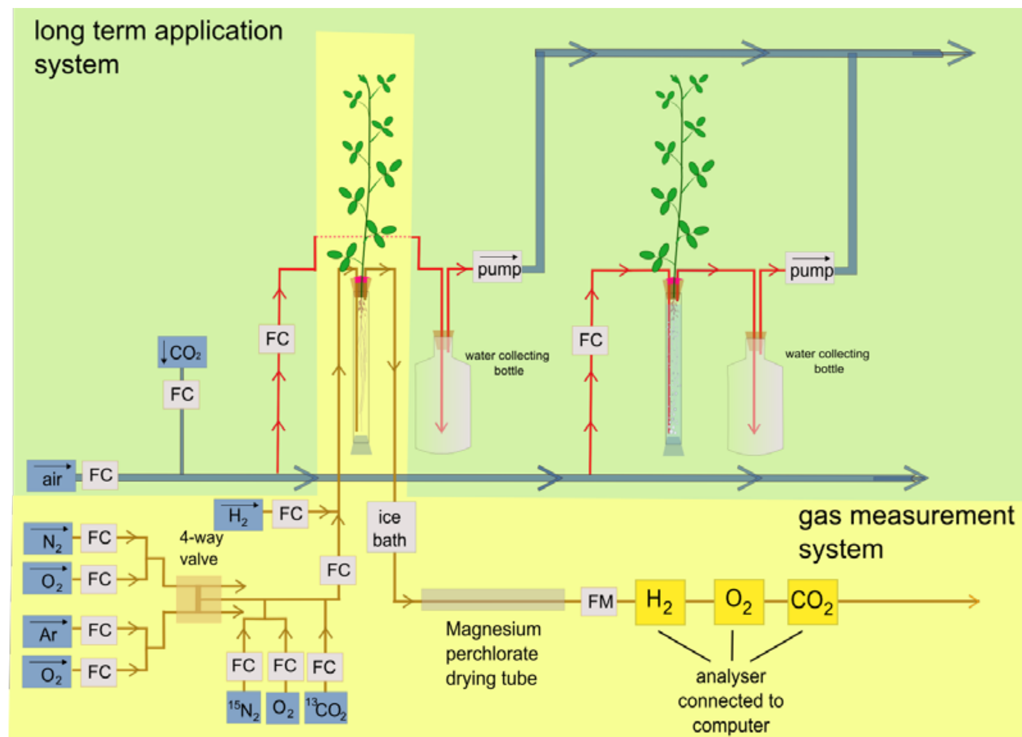

Figure 1: Setup of system for long-term application of gas of variable composure to root/nodule compartments and gas exchange measurements
The system allows the separate long-term application of gas of variable composure to individual root/nodule compartments. In the gas flow a variable proportion of zero to 100% of N_2, Ar, O_2, CO_2, $^{13}CO_2$ and $^{15}N_2$ can be adjusted. Measurements of root/nodule H_2 and CO_2 evolution as well as O_2 uptake are possible.

Moreover, measurements of root/nodule H_2 and CO_2 evolution are possible. The O_2 concentration can be ascertained in the outflowing air in an open flow system. Minimization of flow rates and size of the root/nodule compartment allows a direct measurement of root/nodule O_2 uptake in an open flow system. Moreover, measurements of O_2 uptake in a closed circuit as described by Ribet and Drevon (1995) and Bacanamwo *et al.* (1997) are possible. Gas flow rates are governed by electronic flow controlers (MKS instruments, Munich, Germany). Data on flow

rates, H_2, CO_2 and O_2 content in the gas streams are collected continuously by a computer system. For the present report the system was used for measurements of H_2 evolution in N_2/O_2 (79/21, v/v) and after switching to Ar/O_2 (79/21, v/v). N_2 fixation rates calculated from these data (Schulze *et al.*, 2006) were compared to measurements of $^{15}N_2$ uptake of the same plants. In addition, the Ar-ID was studied with parallel measurements of O_2 uptake in open flow and under low P or after prolonged darkening of the shoots (two days). Darkening of the shoots was achieved through enwrapping with aluminum foil and aeration of the enclosure with ambient air. For the low P treatment, plants were left without further P application beginning two days after the transfer to the nutrient solution.

H_2 evolution measurements

For the H_2 evolution measurement, the sealed root/nodule compartment was connected to an open flow gas exchange measurement system that allowed the application of a mixture of N_2/O_2 (79/21, v/v) and of Ar/O_2 (79/21, v/v) to the part of the root system in the solution. An airflow of 200mL min^{-1} (about 1.2 volumes min^{-1}) was applied to the root compartment. A subsample (100mL min^{-1}) of the outflowing gas was taken, dried (ice trap and $MgClO_4$) and passed through an H_2 analyser (Quibit Systems, Canada). When a stable H_2 outflow from the root/nodule compartment was reached, this value was taken as apparent nitrogenase activity (ANA). Subsequently the air composition in the inflowing airstream was changed to Ar/O_2 (80/20, v/v). Argon is inert to nitrogenase and thus the whole electron flow is diverted to H^+. Consequently H_2 evolution under argon represents total enzyme activity (total nitrogenase activity, TNA). The peak value taken three to five minutes after switching to Ar/O_2 was regarded as TNA value. The electron allocation coefficient (EAC) of nitrogenase activity was calculated as 1–(ANA/TNA). ANA, TNA and the EAC were measured before the introduction of the treatments, two days after treatment introduction and at the end (after 3 weeks) of the experimental period.

$^{15}N_2$ application

For the measurement of nodule $^{15}N_2$ uptake plants were grown in the aeroponic system as described above. Prior to $^{15}N_2$ application the whole glass cylinder was filled with nutrient solution and a slow gas stream of $^{15}N_2/O_2$ (80 [98vol.%$_{exc.}$]/20,

v/v) was applied to the inlet directly beneath the upper rubber stopper (outlet for gas exchange measurements). When 2/3 of the nutrient solution was replaced, the root/nodule compartment was sealed for one hour by connecting in- and outlet. After the application periods, $^{15}N_2$ was replaced through refilling of the glass cylinder with nutrient solution and intensive bubbling with $^{15}N_2$ free air. Plants were immediately removed from the solution, fixed in liquid nitrogen and separated into shoots, roots and nodules. The plant material was dried at 60°C to a constant weight.

Ar-ID and O_2 uptake

For measuring the O_2 uptake during the Ar-ID in a flow through system, the total flow was lowered to 40 mL L^{-1}. At this flow rate a clear difference in oxygen concentration of inflowing and outflowing air from the root nodule compartment could be measured.

Results

Comparison between H_2 evolution and $^{15}N_2$ uptake

H_2 evolution in air yielded a constant ANA value (Fig. 2).

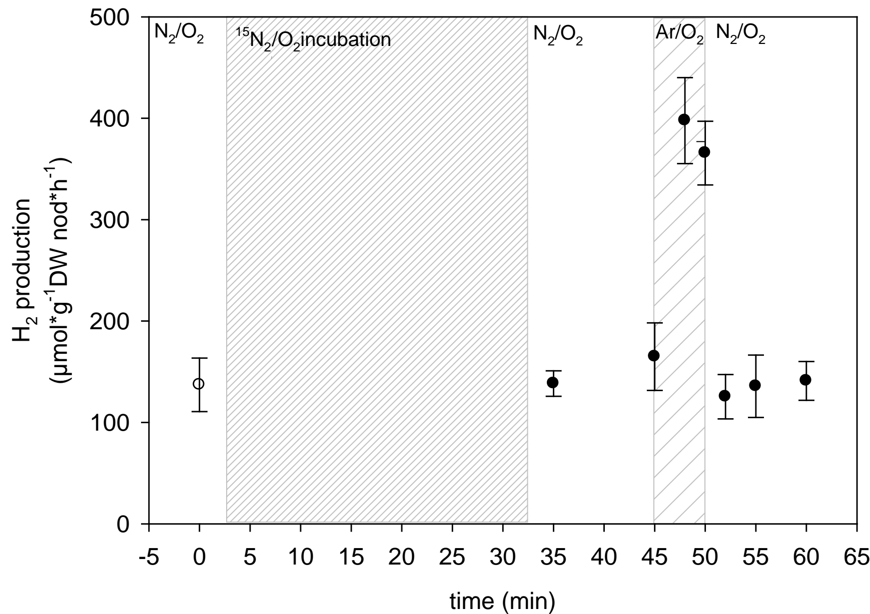

Figure 2: H_2 evolution in N_2/O_2 and Ar/O_2. The figure shows the H_2 evolution of alfalfa plants, in N_2/O_2 (ANA) and Ar/O_2 (TNA). Data are means of four replicates.

After switching to argon, a higher level of H_2 evolution was reached after about 2 min and taken as TNA value. The TNA remained constant until 5 min after switching to argon. After reapplication of an N_2/O_2 mixture, the ANA value was not different from that before Ar/O_2 application. Depending of age and activity of nodules, a clearly decreasing effect was detected on plants after 10 to 20 min of Ar/O_2 treatment (data not shown). $^{15}N_2$ uptake measured on the same plants was not different from N_2 fixation values calculated from ANA and TNA measurements (Tab. 1).

Table 1: N_2 fixation based on $^{15}N_2$ uptake and H_2 evolution gas measurement. Data show N_2 fixation activity of 70 day old alfalfa plants ± standard deviation. Measurements were made on the same plants consecutively. The values are means of four replicates.

	N fixed (µg N*plant^{-1}*h^{-1})	EAC
Based on $^{15}N_2$ uptake	115± 9 (100)	0,61± 0,04 (100)
Based on H_2 evolution	107± 13 (93)	0,59± 0,05 (92)

O_2 uptake after application of Ar/O_2

Oxygen uptake of the plants and the time frame of occurrence of a decline in H_2 evolution and O_2 uptake are shown in Table 2. A measurement of O_2 uptake yielded a value of 46.7 or 63 µmol O_2 plant^{-1} and h^{-}1 for alfalfa and pea plants, respectively. After switching to Ar/O_2, the Ar-ID began in alfalfa and pea plants began 4.2 and 6.4 min later, respectively. After equilibration of the system, the value for O_2 uptake in Ar/O_2 was measureable beginning at about 2 min after switching to Ar/O_2 and was not significantly different from the O_2 uptake before the switch (42.1 and 54.3 µmol O_2 plant^{-1} and h^{-1} for alfalfa and pea, respectively). The O_2 uptake remained constant for 12.4 min in alfalfa plants and 9.2 min in pea plants. A subsequent decline in O_2 uptake (increase in O_2 concentration of the outflowing Ar/O_2) lasted for about 10-15 min, after which root/nodule O_2 uptake was not anymore detectable in the flow-through system in both plant species.

Table 2: Root nodule O_2 uptake in N_2/O_2 and 4 min after Ar/O_2 application and point in time of a decline of H_2 evolution and root/nodule O_2 uptake after application of Ar/O_2 in *Pisum sativum* L. and *Medicago sativa* L.
Data are means of three and six replicates for *Pisum sativum* L. and *Medicago sativa* L., respectively. Measurements were made on plants at an age of 25 (*Pisum sativum* L.) and 72 (*Medicago sativa* L.) days after emergence. (O_2 uptake in Ar/O_2 was measured 4 min after argon application. The difference in O_2 uptake in Ar/O_2 4 min after argon application was not significantly different to the O_2 uptake in N_2/O_2 (t-test, $P \leq 0.05$). ± represents standard deviation.

	Medicago sativa L.	*Pisum sativum L.*
O_2 uptake in N_2/O_2 (µmol O_2 plant^{-1} h^{-1})	46.7 ±2.3	63 ±3.3
O_2 uptake in Ar/O_2 (µmol O_2 plant^{-1} h^{-1})	42.1 ± 3.4	54.3 ±7.3
Time until the beginning of an Ar-ID (min)	4.2 ±0.3	6.3 ±2.1
Time until the beginning of a decrease in root/nodule O_2 uptake after Ar/O_2 application (min)	12.4 ±1.2	9.2 ±0.9

Ar-ID after leaf darkening and under P-deficiency

Figure 3 shows the Ar-ID in untreated pea plants in comparison to plants under P deficiency and such in which the shoots had been darkened before measurements. While control plants showed a clear Ar-ID decline and a transient recovery beginning about 10-15 min after Ar/O_2 application, darkened plants displayed much less decline in the TNA value. In P deficient plants we detected a slow increase in H_2 evolution.

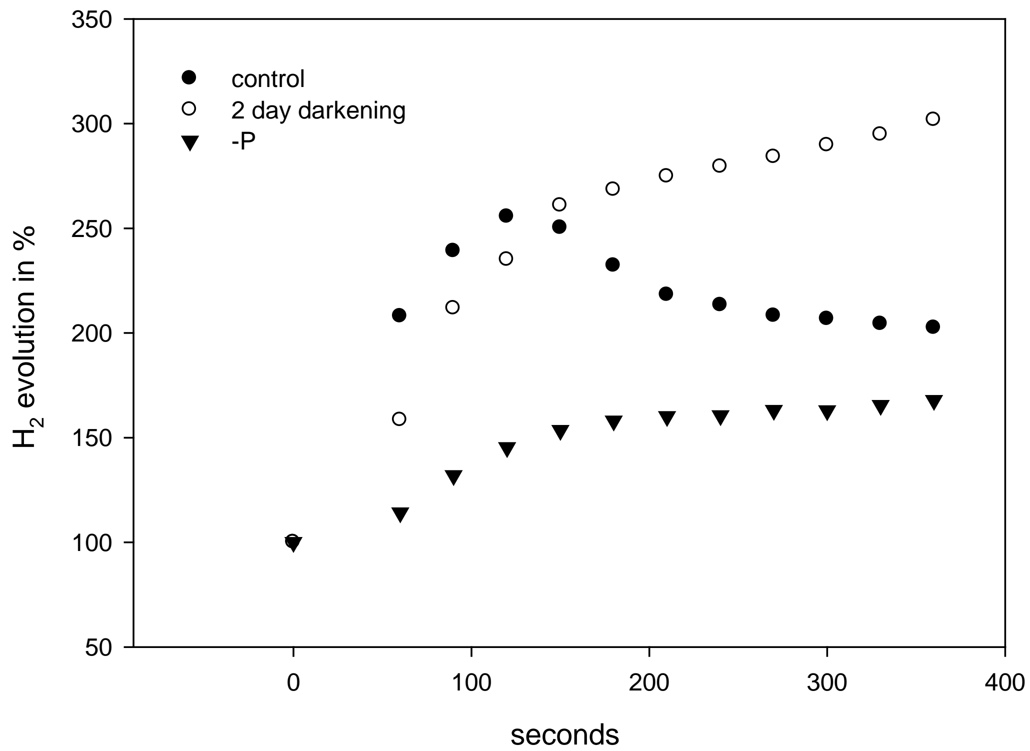

Figure 3: H_2 evolution after application of Ar/O_2 to the root/nodule compartment in control plants and plants with two day darkened leaves or under P deficiency. Data are means of three replicates.

Discussion

The Ar-ID is connected to several unexplained phenomena, for example the transient or partial recoveries of nitrogenase activity after a certain time of argon application. The effects of replacement of N_2 by Ar in the air around nodules are profound and a long-term application reduces nodule activity from transient to permanent, depending on the length of exposure. Permanent argon application induces nodule senescence. With respect to short term effects in particular the influence of argon on nodule oxygen permeability has been studied. It was clearly shown that a short-term increase in oxygen concentration around nodules induces a transient recovery of nodule activity during the Ar-ID. Moreover, stepwise increases of the oxygen concentration (O_2 ramping) are connected to stepwise increases in nitrogenase activity (King and Layzell, 1991). However, comparable phenomena, although to a lesser extent, can be seen on undisturbed nodules in an N_2/O_2 mixture when the oxygen concentration is altered (Hunt *et al.*, 1989). Consequently, nodule reaction to increase oxygen concentration during the Ar-ID is only indirect evidence that a tightening in nodule oxygen diffusion control, e.g. through the variable oxygen diffusion barrier in the nodule cortex is the primary effect reducing nitrogenase activity. In fact, our results reveal that decreasing nodule oxygen uptake follows a few minutes after the Ar-ID had already commenced. During the first minutes of argon application the oxygen uptake did not significantly differ from the O_2 uptake in N_2/O_2. The following steep decrease in oxygen uptake resulted in levels that were not anymore measureable in the used flow-through system that has to detect small changes in O_2 concentration against a huge O_2 background. Overall these data support the hypothesis that the initial effect of argon application is different from restriction in oxygen diffusion. The primary event in the nodule is a cessation of ammonia production. A resulting acidification of the symbiosome space does impair symbiosome malate uptake and malate might be involved in osmocontractile mechanisms of oxygen diffusion control. Thus less malate use due to less N_2 fixation and N assimilation might decrease nodule oxygen permeability. The progressing decrease in oxygen permeability in nodules during the Ar-ID does most probably result in the almost total shutdown of activity during longer periods of application.

The study of the extent of the Ar-ID at different nodule activity showed unexpected results in that we did not find a significant Ar-ID on plants with downregulated nodule activity (P-deficiency and darkened leaves). Our data do not support any immediate explanation. However, while nodule specific activity and oxygen permeability in P deficient plants is increased (Schulze and Drevon, 2005), shoot darkening decreases the specific activity of nodules (Fischinger *et al.*, 2006). These facts further support the thesis that oxygen diffusion restriction is not the primary effect on nodules after argon application.

Apart from the long term effects our data show that taking a TNA value is not connected to permanent or even short-term decrease in nitrogenase activity. A procedure that allows to restrict the effective time at which nodules are subjected to Ar/O_2 necessitates a setup for measurement with short gas flow ways and a small and well aerateable root/nodule compartment. Our data show that in that case the quickly taken TNA value yields reliable data for total N_2 fixation as evidenced by the parallel measurements of $^{15}N_2$ uptake on the very same plants.

Overall the gas exchange setup allows a precise study of nodule N_2 fixation and short and long term effects of argon application. Gas exchange of the nodules is essential for the functioning of the N_2 fixation process. N_2 fixation consumes high amounts of ATP and reductants and thus strongly depends on O_2 deliverance for bacteroids respiration. On the other hand, nitrogenase expression and functioning depends on microaerobic conditions. A putative O_2 diffusion barrier in nodules as a regulatory mechanism for nodule oxygen conductance is under discussion (Serraj and Sinclair, 1996; van Heerden *et al.*, 2008; Vessey *et al.*, 1988). However, also other gases around the nodules may influence N_2 fixation activity. Mulder and Van Veen (1960) described an influence of modified CO_2 concentration around the roots and nodules on the N_2 fixation activity in white clover. Moreover, recent studies on the nodule CO_2 fixation revealed a close connection between phosphoenolpyruvate carboxylase (PEPC) expression level and a functioning nitrogenase activity (Nomura *et al.*, 2006; Rosendahl *et al.*, 1990; Yamakawa *et al.*, 2004).

In addition to these physiological aspects of nodule gas exchange, the measurement of nodule H_2 evolution constitutes the only reliable and non destructive method for measuring N_2 fixation. H_2 evolution is as well an indicator

for electron allocation at nitrogenase and thus the variable relative efficiency of nitrogenase while acetylene reduction relies on estimates on that respect.

During the last decades remarkable progress was achieved in understanding aspects of the molecular biology of nitrogen fixation. For example considerable advances are reached in understanding the metabolic pathway that supports bacteroid malate supply and the provision of carbon skeletons for N assimilation (Vance and Gantt, 1992). So-called nodule enhanced or at least preferentially in nodule expressed forms of carbonic anhydrase (CA), PEPC and malate dehydrogenase (MDH) have been found for alfalfa (Pathirana *et al.*, 1992), pea (Fedorova *et al.*, 1999), soybean (Xu *et al.*, 2003) which are crucial for the mentioned metabolic pathway. This metabolic pathways can be specifically influenced through genetic techniques (Schulze *et al.*, 1998), and legume transformation is becoming a readily tool for research on most legume species (Somers *et al.*, 2003). Genome sequencing efforts for the model legumes *Medicago truncatula* (Rose, 2008) and *Lotus japonicus* (Sato *et al.*, 2008) are close to completion and a huge variety of mutants are available.

Advances in genomics and molecular biology of nitrogen fixation and the availability of a wide variety of mutants and transformed material necessitate the advancement of studies that combine these achievements with precise advanced techniques for physiological measurements. Various questions concerning physiological aspects of legume nodule N_2 fixation are of growing interest (Lodwig *et al.*, 2003; White *et al.*, 2007). Measurement of nodule gas exchange provides such a tool.

In conclusion, the time frame of the onset of the Ar-ID in nitrogenase activity and the decrease in nodule oxygen uptake suggest that the primary event inducing the Ar-ID is different from oxygen diffusion restriction into the nodule. The subsequent almost total shutdown of nodule activity is connected to a very strong decline in nodule oxygen uptake. Apart from these effects a quick taking of a TNA value (within five minutes of argon application) yields reliably values as evidence by $^{15}N_2$ uptake measurements and apparently remain without effect on nodules, at least with respect to H_2 evolution in air.

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Zusammenfassende Diskussion und Schlussfolgerung

Voraussetzung für die Bearbeitung der die Knöllchen-N_2-Fixierung betreffenden Fragestellungen war es, ein System zu etablieren, mit dem relevante Gaswechselvorgänge im Knöllchen-/Wurzelraum erfasst werden konnten. Außerdem sollten Behandlungen mit unterschiedlichen Gaszusammensetzungen im Wurzelraum ermöglicht werden. Besonders wichtig war die Messung der Wasserstofffreisetzung als indirektes Maß für die N_2-Fixierung der Nitrogenase.

Wie in Kapitel 3 dargestellt, gelang es, ein Gaswechselmesssystem zu etablieren, welches diesen Anforderungen entsprach. Dazu wurden die Pflanzen in eigens dafür konzipierten Nährlösungsröhren mit kleinen Gasvolumina kultiviert und im offenen Durchfluss die Wasserstofffreisetzung in N_2/O_2 (ANA) und in Ar/O_2 (TNA) im Wurzelraum gemessen. Die kleinen Gasvolumina in den Pflanzenanzuchtsystemen sowie in den gesamten Gasflusswegen führten zu einer hohen Genauigkeit in der Messung, wie der Vergleich mit der tatsächlichen N_2-Fixierung mittels $^{15}N_2$-Applikation gezeigt hat. Mit dem etablierten System wurden 93% der tatsächlichen N_2-Fixierung erfasst.

Dieses Ergebnis zeigt eine deutliche Verbesserung der Methode im Vergleich zu früheren Arbeiten, in denen von einer Unterschätzung der tatsächlichen N_2-Fixierung um 20% berichtet wird (Schulze, 2004). Diese hohe Genauigkeit mag vor allem auf die Optimierung des Systems im Hinblick auf kleine Gasvolumina zurückzuführen sein, da somit die Reaktion im Wurzelraum unmittelbar im Analysator aufgezeigt werden kann. Einen weiteren Vorteil bieten diese kleinen Volumina auch im Hinblick auf die schwierige Bestimmung der Sauerstoffaufnahme der Knöllchen. Die Schwierigkeit, die geringen Veränderungen in der Sauerstoffaufnahme der Knöllchen im offenen Durchfluss zu ermitteln, wurde dadurch versucht zu umgehen, dass die Sauerstoffaufnahme in einem zum Kreislauf geschlossenen System gemessen wurde (Aydi et al., 2004; Schulze & Drevon, 2005). Dieses System führt allerdings zu dem Problem, dass eine zeitgleiche Ermittlung der H_2-Freisetzung erheblich erschwert ist. Die Messungen zeigen, dass durch den Einsatz adäquater Analysatoren und entsprechend kleiner Gasflüsse und Gasvolumina im Wurzelraum die Detektion veränderter O_2-Aufnahme (veränderte O_2-Konzentrationen im offenen Durchfluss) möglich ist.

Für die Fragestellungen hinsichtlich der CO_2-Fixierung war es darüberhinaus von zentraler Wichtigkeit, das System zur Begasung des Wurzelraumes mit unterschiedlichen CO_2-Konzentrationen dahingehend zu verbessern, dass eine Kontamination des Spross-Gasraumes durch die Gase des Wurzelraumes auszuschließen wäre. Dem wurde durch den Ansatz Rechnung getragen, die Gasgemische durch die Nährlösungsröhren zu saugen, anstatt zu pressen. Eine Undichtigkeit würde so zu einer Verdünnung der durch den Wurzelraum gesaugten Luft führen, doch kann ein Austritt des Gases ausgeschlossen werden. Dies wurde durch Messungen der Gaszusammensetzung um den Spross bestätigt. Es wurde also ein System etabliert, welches die zur Bearbeitung der Fragestellungen notwendigen Voraussetzungen erfüllte.

Im Hinblick auf die Relevanz der Knöllchen-CO_2-Fixierung für die N_2-Fixierung wurden zwei experimentelle Ansätze gewählt. Zum einen wurde untersucht, inwieweit die CO_2-Fixierung ein interner Machanismus ist, um einen Mangel an organischen Säuren in den Knöllchen zu kompensieren (Kapitel 1), zum anderen, ob sich durch erhöhte CO_2-Fixierung die N_2-Fixierleistung erhöhen lässt (Kapitel 2).

In der Hülsenfüllungsphase der Erbsen besteht im Vergleich zu vegetativen Wachstumsphasen ein erhöhter N-Bedarf durch das Hülsenwachstum. Dieser erhöhte N-Bedarf wurde in den vorliegenden Arbeiten durch erhöhte N_2-Fixierungsleistung der Knöllchen gedeckt. Dieser unter optimalen Bedingungen typische Verlauf einer bis in die späte Hülsenfüllung ansteigenden N_2-Fixierung führte zu einem gegenüber dem vegetativen Stadium erhöhten N_2-Fixierleistung pro Pflanze und pro Knöllchentrockenmasse. Die Erbsen in der Hülsenfüllungsphase können als ein System betrachtet werden, in dem, angetrieben durch internen N-Bedarf (Hülsenfüllung), die N_2-Fixierung hochreguliert ist, und am Maximum läuft. Dieses Hochregulieren der N_2-Fixierung ging auch mit einer Hochregulation der CO_2-Fixierung einher. So fanden sich in der Hülsenfüllungsphase eine in vitro erhöhte PEPC-Aktivität, die aus einer erhöhten gemessenen CO_2-Fixierung pro Pflanze resultierte. Diese erhöhte CO_2-Fixierung mag eine Reaktion auf die zu diesem Zeitpunkt verringerten Gehalte an organischen Säuren in den Knöllchen sein. Die PEPC-Aktivität ist an die Malatkonzentration im Knöllchen gekoppelt, was dadurch nachgewiesen wurde, dass eine erhöhte Malatkonzentration die PEPC-Aktivität unterdrückt (Woo & Xu, 1996). Es ist

denkbar, dass eine sinkende Malatkonzentration im Knöllchen gewissermaßen die Notwendigkeit anzeigt, den Prozess der CO_2-Fixierung in Gang zu setzen.

In den Versuchen mit zwei CO_2-Stufen im Wurzelraum (Kapitel 2) wurde der Ansatz gewählt, die CO_2-Fixierungsaktivität der Knöllchen durch die Außenkonzentration im Wurzelraum zu manipulieren. Christeller et al. (1977) zeigten, dass die CO_2-Aufnahme der Knöllchen einer Sättingungsfunktion der Außenkonzentration um die Knöllchen entspricht. In unseren Versuchen wurde die $^{(13)}CO_2$-Fixierung durch eine erhöhte CO_2-Außenkonzentration signifikant erhöht. Da sowohl den mit $-CO_2$ als auch den mit $+CO_2$ vorbehandelten Pflanzen zum Zeitpunkt der $^{13}CO_2$-Applikation eine gleich hohe $^{13}CO_2$-Konzentration angeboten wurde, beruhte die in der $+CO_2$-Variante erhöhte CO_2-Aufnahme in die Knöllchen vermutlich auf einer erhöhten Expression der CO_2-Fixierungssysteme (PEPC, AAT). Eine weitere Bearbeitung dieser Fragestellung beispielsweise durch Genexpressionsanalyse wäre wünschenswert.

Bereits nach 2 Tagen führte eine erhöhte CO_2-Konzentration im Wurzelraum zu einer Erhöhung der N_2-Fixierleistung. Dieser kurzfristig einsetzende Effekt weist auf eine durch erhöhte CO_2-Konzentrationen im Wurzelraum induzierte spezifisch erhöhte N_2-Fixierleistung hin. Gegen Ende des Versuchszeitraumes allerdings beruhte die erhöhte N_2-Fixierleistung pro Pflanze auf einer erhöhten Knöllchentrockenmasse. Die erhöhte CO_2-Fixierung schien also neben der anfangs spezifischen Erhöhung der N_2-Fixierung die N_2-Fixierleistung auf Ganzpflanzenebene durch eine verbesserte Knöllchenentwicklung zu steigern. Dieses verbesserte Knöllchenwachstum mag auf eine entspanntere C-Versorgungslage der Knöllchen hinweisen, wohingegen die erhöhte Anzahl kleinerer Knöllchen auf einen erhöhten Neuansatz schließen läßt. Da die Knöllcheninitiation über den Spross moderiert wird (Downie & Parniske, 2002) und vom N-Ernährungstatus des Sprosses abhängt, mag die hohe Anzahl neuer Knöllchen eine Reaktion auf die schlechtere N-Versorgungslage des Sprosses sein.

Die erhöhte CO_2-Fixierung führte zu gegen Ende der Versuchsperiode deutlich erhöhten Sprossgewichten. Dieser Effekt trat nur bei den Stickstoff fixierenden Pflanzen auf. Bei nitraternährten Pflanzen zeigte sich hingegen kein Effekt unterschiedlicher CO_2-Behandlungen auf das Pflanzenwachstum. Dies zeigt, dass die durch den erhöhten

CO_2-Gehalt erhöhte Trockenmassebildung vermutlich ein Resultat erhöhter N_2-Fixierung ist.

Zusammenfassend lassen sich aus den Ergebnissen folgende Aussagen ableiten:

1. Die CO_2-Fixierung und die N_2-Fixierung sind in ihrer Intensität eng aneinander gekoppelt.
2. Die CO_2-Fixierung ist in Phasen erhöhter N_2-Fixierungsaktivität ein intern etablierter Mechanismus, der helfen kann, den erhöhten Bedarf an organischen Säuren im Knöllchen zu decken.
3. Die N_2-Fixierung lässt sich in ihrer Intensität durch erhöhte CO_2-Konzentration im Wurzelraum positiv beeinflussen. Und kann so zu einer erhöhten N-Fixierung führen. Eine CO_2-Verarmung des Wurzelraums hingegen führt zu einer herabregulierten N_2-Fixierung.

Diese Ergebnisse haben zum einen eine Bedeutung für experimentelle Verfahren, zum anderen für die N_2-Fixierung in der landwirtschaftlichen Anwendung.

Oftmals wird die N_2-Fixierung experimentell bestimmt, indem die H_2-Freisetzung in Luft (360 ppm CO_2) oder reinem N_2/O_2 gemessen wird. Vor allem bei jungen Pflanzen mit geringer Wurzelrespiration kann dies bedeuten, dass die Messungen an Pflanzen mit einer durch geringe CO_2-Konzentrationen im Wurzelraum herabregulierten N_2-Fixierleistung durchgeführt werden. Zudem kann auch eine Beeinflussung des Knöllchenansatzes und der Knöllchenentwicklung nicht ausgeschlossen werden. Besonders bei experimentellen Arbeiten zur N_2-Fixierung wäre es angeraten, den Einfluss der CO_2-Konzentration um die Knöllchen zu berücksichtigen.

Für die Landwirtschaft spielt vor allem die züchterische Bearbeitung der CO_2-Fixierungssysteme eine Rolle. Hinsichtlich der CO_2-Fixierungskapazität wurden deutliche Sortenunterschiede gefunden. So zeigten Versuche an Soja- und Vignabohnen sehr unterschiedliche Reaktionen der N_2-Fixierung auf eine erhöhte CO_2-Konzentration in der Rhizosphäre (Yamakawa et al., 2004). Der züchterische Ansatz muss aber sowohl die CO_2-Fixierung als auch den daran anschließenden N-Einbau berücksichtigen. Jessen et al. (1988) zeigten bereits die Schwierigkeiten einer züchterischen Bearbeitung der Knöllchen-

CO_2-Fixierung. So führte die Selektion auf erhöhte PEPC Aktivität zu einer signifikant erhöhten N_2-Fixierung, die jedoch aufgrund von unzureichendem N-Export aus dem Knöllchen in den Spross nicht im vollen Maße der N-Versorgung des Sprosses zugutekam.

Eine weiterer Ansatz für eine Verbesserung der N_2-Fixierung geht über die Beeinflussung der CO_2-Konzentration im Wurzelraum. Christeller et al. (1977) beschreiben, dass die Sättigung der CO_2-Fixierung des Knöllchens erst bei einer Außenkonzentration von $20*10^3$ ppm erreicht ist, wohingegen die Konzentration im Boden lediglich bei ca. $5*10^3$ ppm liegt (Buyanovsky & Wagner, 1983). Daher ist es denkbar, dass Bodenbearbeitungsmaßnahmen, die zu einer Erhöhung der Boden-CO_2-Konzentration führen, einen positiven Effekt auf die N_2-Fixierung haben. Arbeiten, welche beispielsweise den positiven Effekt einer Strohdüngung auf die N_2-Fixierleistung der Leguminosen beobachteten (Evans et al., 1997), könnten ein Hinweis auf solche Effekte sein.

Vor dem Hintergrund wachsender Nahrungsmittelnachfrage und steigender Energiepreise kann die symbiontische N_2-Fixierung, quasi als solarbetriebener N_2-Fixierungsprozess, zunehmend Bedeutung erlangen. Um einen größeren Beitrag zum N-Eintrag in landwirtschaftliche Produktionssysteme leisten zu können, ist eine Steigerung der N_2-Fixierungseffizienz anzustreben. Die Arbeiten haben gezeigt, dass die Knöllchen-CO_2-Fixierung einen möglichen Ansatzpunkt darstellt, die N_2-Fixierleistung der Leguminosen zu steigern.

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Zusammenfassung

In den vorliegenden Arbeiten wurden Versuche an Leguminosen (*Pisum sativum* L. und *Medicago sativa* L.) hinsichtlich des Einflusses der Knöllchen-CO_2-Fixierung auf die N_2-Fixierung durchgeführt. Es wurde gezeigt, dass die CO_2-Fixierung von zentraler Wichtigkeit für die N_2-Fixierung ist.

Methodische Grundlage für diese Arbeiten war die Etablierung einer Gaswechselmessanlage, die relevante Gaswechselprozesse im Knöllchen-/Wurzelraum erfasst und vom Spross unabhängige Behandlungen des Wurzelraumes mit verschiedenen Gasgemischen ermöglicht. Das etablierte System mit folgenden Anwendungsmöglichkeiten wird beschrieben: (i) Messung der H_2-Freisetzung als indirektes Maß für die N_2-Fixierung, (ii) Messung der Sauerstoffaufnahme und CO_2-Freisetzung der Wurzeln und Knöllchen im offenen Durchfluss, (iii) Langzeitapplikation von Gasen unterschiedlicher Zusammensetzung (Bsp. erhöhte CO_2-Konzentration) zum Wurzelraum, (iv) Applikation von Isotopengasen ($^{15}N_2$ und $^{13}CO_2$) in den Wurzelraum. Mittels $^{15}N_2$-Applikation wurde die Wasserstofffreisetzungsmethode zur Bestimmung der N_2-Fixierung verifiziert. Mit 93% der tatsächlichen N_2-Fixierung zeigte das Verfahren eine sehr hohe Genauigkeit.

Unter der Knöllchen-CO_2-Fixierung versteht man die Bindung von CO_2 an Phosphoenolpyruvat an der Phosphoenolpyruvatcarboxylase (EC 4.1.1.31) im Zytosol von infizierten Zellen des Knöllchens. Das Resultat dieser Reaktion ist Oxalacetat, welches im Stoffwechsel des Knöllchens zwei wichtige Funktionen hinsichtlich der N_2-Fixierung hat. Oxalacetat kann als Kohlenstoffgerüst für den N-Einbau fungieren und zudem nach dem Umbau zu Malat als Energiequelle für den N_2-Fixierungsprozess an der Nitrogenase dienen.

Es konnte in dieser Arbeit gezeigt werden, dass zwischen der CO_2-Fixierung der Knöllchen und der N_2-Fixierung ein enger Zusammenhang bestand. Regulationszustände erhöhter N_2-Fixierung gingen mit einer erhöhten CO_2-Fixierung einher.

Außerdem wurde deutlich, dass die CO_2-Fixierung ein interner Mechanismus sein kann, um in Phasen erhöhter N_2-Fixierleistung (Hülsenfüllungsphase der Erbsen) den relativen Mangel an organischen Säuren zu kompensieren.

Darüber hinaus wurde gezeigt, dass eine erhöhte CO_2-Konzentration im Wurzelraum von Luzerne (Belüftung mit 2500 ppm versus Belüftung mit CO_2-freier Luft) eine erhöhte CO_2-Fixierung in den Knöllchen induziert. Diese erhöhte CO_2-Fixierleistung resultierte in einer erhöhten N_2-Fixierung der Pflanze, verbunden mit einem erhöhten Transport von Aminosäuren in den Spross. Eine erhöhte CO_2-Konzentration im Wurzelraum zeigte weiterhin einen Einfluss auf die Knöllchenentwicklung: sie ging einher mit der Bildung von weniger aber größeren Knöllchen.

Die mögliche Bedeutung dieser Ergebnisse für experimentelle Verfahren der Gaswechselmessungen im Kontext der N_2-Fixierung sowie die Verbesserung der N_2-Fixierungsleistung von Leguminosen für die landwirtschaftliche Nutzung werden diskutiert.

Summary

The present work reports results from experiments on the influence of CO_2 fixation for the N_2 fixation in root nodules of legumes (*Pisum sativum* L. and *Medicago sativa* L.). It was shown that the CO_2 fixation is of key importance to the N_2 fixation.

The methodological basis for these studies was the establishment of a gas exchange measuring system that records the relevant gas exchange processes in the root nodule space/rhizosphere and that allows the treatment of the rhizosphere with various gas mixtures, without influencing the gas composition around the shoot. The established system with the following possible applications is described: (i) determination of the nitrogenase activity via H_2 evolution measurement (ii) measurement of oxygen uptake and CO_2 release by the roots and root nodules in an open flow, (iii) long term treatment with gases of mixed composition (for example increased CO_2 concentration) compared with the rhizosphere, (iv) application of isotope gases ($^{15}N_2$ and $^{13}CO_2$) into the rhizosphere. The hydrogen release method used to determine N_2 fixation rates was verified with the application of $^{15}N_2$. The method showed a very high precision, with measuring 93% of the actual N_2 fixation.

During CO_2 fixation in the root nodules, CO_2 is fixed at phosphoenolpyruvate at the phosphoenolpyruvat carboxylase (EC 4.1.1.31) in the cytosol of infected root nodule cells. The result of this reaction is oxaloacetate, which fulfils two important metabolic functions in the context of N_2 fixation. Oxaloacetate may function as a carbon backbone for the N assimilation, or, after conversion to malate, it may serve as an energy source for the N_2 fixation process at the nitrogenase.

It could be shown here that there is a close relationship between CO_2 fixation and N_2 fixation of the root nodules. States of upregulated N_2 fixation were coupled with states of increased CO_2 fixation. It was found that CO_2 fixation can be an internal mechanism that allows a compensation of a relative lack of organic acids in periods of increased N_2 fixation activity (pod filling of pea plants).

Furthermore, it was demonstrated that an increased CO_2 concentration in the rhizosphere of alfalfa (aeration with 2500 ppm versus with CO_2 free air) induces an increased CO_2 fixation in the root nodules.

This increased CO_2 fixation activity resulted in an increased N_2 fixation in the plant coupled with an increased transport of amino acids into the shoot. An increased CO_2 concentration in the rhizosphere showed an influence on root nodule development: it induced the development of fewer but larger nodules.

The potential significance of these results for experimental techniques of gas change measurement in the context of N_2 fixation, as well as for improvements of N_2 fixation performance of legumes for the agricultural practice are discussed.

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- Fischinger S.A. and Schulze J.: Argon effects on legume nodule H_2 evolution and O_2 uptake (in Vorbereitung)
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