Diss. ETH No. 12912

The effect of elevated atmospheric p_{CO2} on phosphorus nutrition of white clover (*Trifolium repens* L.)

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY
ZÜRICH

for the degree of
DOCTOR OF NATURAL SCIENCES

presented by

José Pedro P. F. Almeida
M.Sc. Dipl. Ing. Zoot. U. T. Lisbon (Portugal)

born August 27, 1960
Lisbon, Portugal

accepted on the recommendation of

PROF. DR. J. NÖSBERGER
Examiner

PROF. DR. E. FROSSARD & DR. A. LÜSCHER
Co-examiners

1998

When with some reason or
None, on the soul's wide fear
The shadow of death comes,
The spirit is seeing clear
-A clearness without calm-
How life passing is shadow,
How life ceasing is sorrow,
And loves life more.
(Fernando Pessoa, 10.2.33)

In memory of my Mother

TABLE OF CONTENTS

TABLE OF CONTENTS i
LIST OF ABBREVIATIONS iii

I SUMMARY 1

II ZUSAMMENFASSUNG 4

III GENERAL INTRODUCTION 7

1. THE EFFECT OF P NUTRITION ON THE PLANT BIOMASS RESPONSE TO ELEVATED p_{CO2} 8
1.1. The effect of elevated p_{CO2} on plant growth 8
1.2. P uptake, long distance transport and P distribution in shoots 9
1.3. Photosynthesis and shoot to root balance 10

2. THE EFFECT OF P ON THE RESPONSE OF SYMBIOTIC N_2 FIXATION TO ELEVATED p_{CO2} 11

3. OBJECTIVES OF THIS STUDY 12

IV ELEVATED p_{CO2} AFFECTS P PARTITIONING AND ROOT ACID PHOSPHATASE ACTIVITY 13

1. ABSTRACT 13

2. INTRODUCTION 14

3. MATERIALS AND METHODS 15
3.1. Plant materials and growth conditions 15
3.2. Plant sampling 16
3.3. Leaf gas exchange 17
3.4. Estimated maximum rate of net shoot P uptake 17
3.5. Non structural carbohydrates 18
3.6. Phosphorus 18
3.7. Nitrogen 18
3.8. Root acid phosphatase activity 18
3.9. Statistical analysis 20

4. RESULTS 20

5. DISCUSSION 28
5.1. Transpiration rate and Pi partitioning 28
5.2. Leaf photosynthesis 31
5.3. Source-sink balance and biomass accumulation 32

6. CONCLUSIONS 33

V P SUPPLY AFFECTS PHOTOSYNTHESIS AND CARBON PARTITIONING BUT DOES NOT PRIMARILY CONTROL N_2 FIXATION 34

1. ABSTRACT 34

2. INTRODUCTION 35

3. MATERIALS AND METHODS 37
3.1. Plant materials and growth conditions 37
3.2. Plant sampling 38
3.3. Chemical analyses 38
3.4. Calculation of symbiotic N_2 fixation 39
3.5. Leaf gas exchange 39
3.6. Statistical analysis 40

4. RESULTS 40
4.1. Nodule mass and plant growth 40
4.2. $\%N_{sym}$ and N partitioning 41
4.3. Photosynthesis 43
4.4. Carbohydrate partitioning 45

5. DISCUSSION 47
5.1. Nodulation and nodule growth 47
5.2. Symbiotic N_2 fixation ($\%N_{sym}$) is reduced at low P supply 48
5.3. Photosynthesis and carbon partitioning are in balance with plant N demand 48
5.4. Which mechanisms inhibit nodulation, nodule growth and $\%N_{sym}$ under low P? 50
5.5. Specific N_2 fixation partly compensated low nodule growth 52

VI GENERAL DISCUSSION 54

1. THE EFFECT OF ELEVATED p_{CO2} ON TRANSPIRATION RATE HAD ASSOCIATED EFFECTS ON WHITE CLOVER NUTRITION 55

2. DOES ELEVATED p_{CO2} INDUCE A REDUCTION OF FLOWS IN THE LONG DISTANCE TRANSPORT? 57

3. DOES ELEVATED p_{CO2} AFFECTS LEAF SENESCENCE? 58
3.1. Leaf physiological age 58
3.2. Field research 59

VII LITERATURE CITED 60

CURRICULUM VITAE 72

ACKNOWLEDGEMENTS 73

LIST OF ABBREVIATIONS

A	net leaf rate of CO_2 uptake per unit leaf area
C_i	intercellular concentration of CO_2
DAP	days after plantation
DW	dry weight
J_{max}	apparent potential rate of electron transport at saturated light
N_{sym}	total quantity of symbiotically fixed N per plant
$\%N_{sym}$	relative contribution of symbiotically fixed N to the total plant N
Pa	Pascal
p_{CO2}	partial pressure of CO_2
Pi	soluble orthophosphate, extracted with 2% (v/v) acetic acid
Rubisco	ribulose 1,5-biphosphate carboxylase/oxigenase
RubP	ribulose 1,5-biphosphate
S.e.	Standard error
$V_{c,max}$	apparent maximum rate of carboxylation with non limiting ribulose-1,5-biphosphate
WSC	water-soluble carbohydrates

I SUMMARY

White clover (*Trifolium repens* L.) and its symbiosis with *Rhizobium* are important components of temperate and mediterranean grassland ecosystems. From the factors affecting white clover growth and its symbiosis, the P nutrition is particularly important.

The actual atmospheric carbon dioxide partial pressure (p_{CO2}) of 35 Pa is predicted to double within the next century. This increase may result in increases of plant biomass dependent, however, from nutrient availability. Earlier studies showed that under nitrogen (N) and phosphorus (P) deficiency the p_{CO2} stimulation of plant growth was reduced. In contrast, under field conditions in the Swiss Free Air Carbon dioxide Enrichment experiment, under a high P availability and a low N fertilisation, white clover had a positive yield response to elevated p_{CO2}, associated with an increase of N derived from the symbiosis (N_{sym}). Therefore a question arises about the possible effect of P deficiency on the response of white clover and its symbiosis to elevated p_{CO2}.

The reduction of leaf stomatal conductance under elevated p_{CO2} results in a decrease of leaf transpiration rate. Therefore, we expected that (1) this decrease of leaf transpiration would affect the transport flow of P from root to the shoot, changing the P partitioning. At the level of symbiosis, in contrast to earlier reports on P deficiency, investigations from other stress factors concluded that the C supply from the shoot to the nodules did not regulate nodule N_2 fixation. Alternatively the plant N demand was suggested to be the regulatory factor for the N_{sym}. Therefore we expected that (2) the decline of N_2 fixation under P deficiency would result from an adaptation to a lower white clover N demand, irrespective of the C availability from the shoot.

To test these two hypotheses a study was undertaken with white clover under controlled environment on quartz sand as growth substrate with a low N (1.5 m*M* N) nutrient solution. In a first experiment, two atmospheric p_{CO2} (35 and 70 Pa) were combined with four levels of P supply (0.0027; 0.075; 0.67 and 2 m*M*) aiming to assess the physiological processes at the whole plant level. A

second experiment was conducted in a similar system to assess the combined effects of two atmospheric p_{CO2} (35 and 70 Pa) and two levels of P supply (0.0027 and 0.075 m*M*) on nodulation of white clover.

Our results showed that the response of white clover to P nutrition and elevated p_{CO2} consists of several mechanisms. At low P, the low rates of leaf transpiration led to low rates of shoot P uptake per plant, irrespective of p_{CO2}. The higher leaf photosynthetic rates (measured on the young leaves) under elevated p_{CO2} appeared to be enabled by an increase of the Pi remobilization from leaf structural P fraction and from an increased senescence of old leaves. With increasing P supply, the estimated shoot P uptake rate per plant increased; however, it was reduced in parallel to a reduced transpiration rate under elevated as compared to ambient p_{CO2}. This p_{CO2}-induced decline of shoot P uptake was associated with an absolute change of P partitioning in favour to the root, while the total P content per plant was not affected by p_{CO2}. The increased P content of roots resulted in a marked reduction of root acid phosphatase activity under elevated p_{CO2}.

Elevated p_{CO2} did not affect nodulation, nodule growth and percentage N from symbiosis (%N_{sym}). P deficiency prevented nodulation or stopped nodule growth when applied to nodulated plants and strongly reduced plant growth. Even though N_2 fixation was clearly reduced under P deficiency these plants were not N limited since N concentration was significantly higher as compared with the other P levels. The combined N supply (mineral and symbiotic) covered the low demand of these P deficient plants. With increasing P supply nodule growth and %N_{sym} increased. The lack of an effect of the increased rate of photosynthesis on N_2 fixation under elevated p_{CO2} and the high WSC contents in roots and nodules indicated that C supply did not limit the N_2 fixation, irrespective of P supply. Alternatively, a systemic effect appeared to be involved in the response of the N_2 fixation process, most likely triggered by white clover N demand. The balance between N demand and N fixation was attained by a partial compensation of nodule function (specific N_2 fixation) to nodule mass. Apparently, this balance would not change under elevated p_{CO2} since nodule growth and N_{sym} were not affected by p_{CO2}. However, the N efficiency was

higher under elevated p_{CO2} (higher plant dry mass at lower N concentration), most likely due to a different N allocation within the plant (between sinks). Questions arising from these results are discussed in view of developing further investigations.

II ZUSAMMENFASSUNG

Weissklee (*Trifolium repens* L.) und seine Symbiose mit *Rhizobium* sind wichtige Komponenten der Grasland-Ökosysteme der gemässigten und mediterranen Klimate. Von den Faktoren, die das Wachstum des Weissklees und dessen Symbiose beeinflussen, ist die Phosphor- (P-)Ernährung von besonderer Bedeutung.

Der aktuelle Kohlendioxid-Partialdruck (p_{CO_2}) von 35 Pa wird sich im nächsten Jahrhundert voraussichtlich verdoppeln. Diese Zunahme beeinflusst verschiedene physiologische Prozesse in Pflanzen, die zu einer Zunahme an Pflanzenbiomasse führen, wenn genügend Nährstoffe verfügbar sind. Frühere Studien zeigten, dass Stickstoff- (N-) und P-Mangel die p_{CO_2}-Stimulierung des Pflanzenwachstums verminderten. Hingegen wurde in einem Freilandversuch mit CO_2-Anreicherung (Swiss Free Air Carbon dioxide Enrichment experiment) gezeigt, dass Weissklee bei hoher P- und niedriger N-Düngung ertragsmässig positiv auf erhöhtes p_{CO_2} reagierte, verbunden mit einer Zunahme des Anteils aus der symbiotischen N_2-Fixierung stammenden N (%N_{sym}). Infolgedessen bleibt die Frage über eine mögliche Wirkung von P-Mangel auf die Reaktion des Weissklees und seiner Symbiose auf erhöhtes p_{CO_2} offen.

Die Reduktion der stomatären Leitfähigkeit unter erhöhtem p_{CO_2} führt zu einer Verminderung der Transpirationsrate. Infolgedessen erwarteten wir, dass (1) diese Verminderung der Blatttranspiration den P-Transportfluss von der Wurzel zum Spross reduziert, und deshalb die P-Verteilung ändert. Im Gegensatz zu früheren Arbeiten über P-Mangel kamen Untersuchungen über die Wirkung anderer Stressfaktoren auf die Symbiose zum Schluss, dass die C-Zufuhr des Sprosses zu den Wurzeln nicht die N_2-Fixierung der Knöllchen reguliert. Es wurde postuliert, dass der N-Bedarf der Pflanze die N_2-Fixierung reguliert. Demzufolge erwarteten wir, dass (2) die Abnahme der Symbiose (%N_{sym}) bei P-Mangel von einer Anpassung an den niedrigeren N-Bedarf des Weissklees herrührt, unabhängig von der C-Zufuhr des Sprosses.

Um diese beiden Hypothesen zu prüfen, wurde ein Versuch mit Weissklee in Wachstumskammern mit Quarzsand als Substrat durchgeführt. Zwei p_{CO_2}-Stufen (35 und 70 Pa) wurden mit vier P-Angeboten (0.0027; 0.075; 0.67 und 2 m*M*) bei einem tiefen Angebot von mineralischem N (1.5 m*M*) kombiniert, um die Auswirkungen auf die physiologischen Prozesse zu messen. In einem zweiten Versuch wurden zwei p_{CO_2}-Stufen (35 und 70 Pa) mit zwei P-Angeboten (0.0027 und 0.075 m*M*) kombiniert, um die Reaktion der Knöllchenbildung von Weissklee zu erfasssen.

Unsere Ergebnisse zeigten, dass die Reaktion des Weissklees auf das P-Angebot und den Anstieg von p_{CO_2} aus mehreren Mechanismen besteht. Bei tiefem P-Angebot führten die niedrigen Blatttranspirationsraten zu tiefen P-Aufnahmeraten pro Pflanze, unabhängig von p_{CO_2}. Jedoch schien die erhöhte Photosyntheserate von jungen Blättern bei erhöhtem p_{CO_2} durch grössere P-Mobilisierung von strukturgebundenem P in den Blättern und einer rascheren Seneszenz der alten Blätter unterstützt zu werden. Bei höherem P-Angebot verringerte sich die P-Aufnahmerate des Sprosses unter 70 Pa p_{CO_2} im Vergleich zu 35 Pa p_{CO_2} parallel zu einer Abnahme der Transpirationsrate. Diese CO_2-bedingte Verminderung war verbunden mit einer auffälligen Änderung der P-Verteilung zugunsten der Wurzel, während die Gesamtmenge an P in der Pflanze von p_{CO_2} unbeeinflusst blieb. Der erhöhte P-Gehalt in den Wurzeln unter erhöhtem p_{CO_2} hatte eine deutliche Reduktion der sauren Phosphatase-Aktivität in der Wurzel zur Folge.

Erhöhtes p_{CO_2} hatte keinen Einfluss auf Knöllchenbildung, Knöllchenwachstum und %N_{sym}. Bei tiefem P-Angebot wurde die Knöllchenbildung verhindert oder das Knöllchenwachstum bei nodulierten Pflanzen gestoppt, während das Pflanzenwachstum reduziert war. Bei höherem P-Angebot stiegen das Knöllchenwachstum und %N_{sym} an. Obwohl unter P-Mangel eine reduzierte N_2-Fixierung beobachtet wurde, war das Wachstum dieser Pflanzen nie N-limitiert, denn sie zeigten eine höhere N-Konzentration als Pflanzen, die bei höherem P-Angebot gewachsen waren. Die kombinierte N-Ernährung mit N_{sym} und mineralischem N (N_{min}) deckte den tiefen N-Bedarf dieser Pflanzen bei weitem. Der gänzlich fehlende Effekt der unter erhöhtem p_{CO_2} gesteigerten

Photosyntheserate auf die N_2-Fixierung, wie auch die hohe Konzentration an wasserlöslichen Kohlehydraten in den Knöllchen und Wurzeln zeigten, dass die C-Versorgung der Knöllchen durch den Spross, unabhängig von der P-Versorgung, für die N_2-Fixierung nicht limitierend war. Andererseits schien ein systemischer Effekt in der Regulierung des N_2-Fixierungsprozesses involviert zu sein, der wahrscheinlich durch den N-Bedarf des Weissklees ausgelöst worden war. Eine scheinbare Balance zwischen N-Bedarf und N-Assimilation wurde durch eine teilweise Kompensierung der Knöllchenaktivität (fixierte N-Menge pro Knöllchenmasse) zur Knöllchenmasse erzielt. Diese Balance änderte sich nicht unter erhöhtem p_{CO2}, da die Knöllchenmasse und $\%N_{sym}$ unverändert blieben. Unter erhöhtem p_{CO2} war der N-Nutzungskoeffizient grösser (grössere Pflanzenmasse bei tieferer N-Konzentration), meist bedingt durch eine andere N-Verteilung in der Pflanze. Fragen, die sich aus den Ergebnissen ergeben, werden diskutiert und darauf aufbauend weitere Forschungperspektiven entwickelt.

III GENERAL INTRODUCTION

White clover (*Trifolium repens* L.) is an important legume in temperate and irrigated mediterranean grasslands. The inclusion of this species facilitates the production of forage with a high protein and metabolic energy content. In addition, symbiosis of white clover with *Rhizobium leguminosarum* bv. *trifolii* allows an input of nitrogen from the atmosphere into the ecosystems (Boller and Nösberger 1987).

Many factors affect white clover growth and development, like temperature, irradiance, water, nutrients and defoliation (the physiological effects were reviewed by Hart 1987). In respect to mineral nutrition, phosphorus (P) is regarded as a crucial element for white clover nutrition and for the symbiosis.

P is a macronutrient component of molecules such as nucleic acids, phospholipids and ATP; it is therefore essential for plant growth. Those molecules together with orthophosphate are involved in the basic processes of energy transfer, control of enzymatic reactions and control of metabolic pathways. In addition, the relevance of P availability for white clover growth is based on the comparison with temperate grasses used in the same swards. White clover has a lower efficiency for P uptake as consequence of its less extensive root system (Caradus 1980; Caradus 1990).

A major expectation for the next century is the doubling of the actual atmospheric carbon dioxide partial pressure (p_{CO2}) (Bowes 1993). The increase in p_{CO2} affects several physiological processes, which may result in increased yields with simultaneous changes in the species proportion of swards (Lüscher et al. 1996; Hebeisen et al. 1997; Zanetti et al. 1997). However, P and N availability are expected to modulate the p_{CO2} response in both individual plants and swards (Goudrian and De Ruiter 1983; Rogers et al. 1993; Fischer et al. 1997). In white clover swards on a fertile soil, Zanetti et al. (1996) concluded that the input of symbiotic N_2 into the ecosystem was increased and that this could be determinant of the positive biomass response to elevated p_{CO2}.

However, elevated p_{CO_2} did not increase *per se* the percentage of total N derived from the symbiosis (Zanetti et al. 1996). Such an increased percentage N derived from the symbiosis most likely would be a consequence from a limited N availability from the soil (Diaz et al. 1993; Hartwig et al. 1996) associated with changes in the microbial community in the rhizosphere of white clover (Schortemeyer et al. 1996).

Therefore two important questions arise from these studies:

1) Which are the P dependent mechanisms that govern the response of white clover to elevated p_{CO_2} ?

2) How is the effect of P nutrition on the N_2 fixation process of white clover symbiosis modulated by carbon availability to the host plant?

1. THE EFFECT OF P NUTRITION ON THE PLANT BIOMASS RESPONSE TO ELEVATED p_{CO_2}

1.1. The effect of elevated p_{CO_2} on plant growth

The actual atmospheric p_{CO_2} (35 Pa) is predicted to double within the next century, due to the burning of fossil fuels, industrialisation, intensification of agriculture and deforestation (Bowes 1993). This rise of atmospheric p_{CO_2} is expected to affect plant growth. From a compilation of 770 experimental observations on 56 species, Kimball (1983) concluded that the doubling of p_{CO_2} would result in an overall average yield increase of 32%. Such effect of p_{CO_2} on plant yield is likely to result from increased CO_2 assimilation rates at a lower stomatal conductivity (Morison 1985). Assuming no limitation by nutrients, a stimulation of leaf photosynthesis could be maximised by an increase of ribulose 1,5-biphosphate (*RubP*) regeneration under elevated as compared to ambient p_{CO_2} (Long and Drake 1992). However, in conditions of resource limitation, the acclimation of leaf photosynthesis to a long-term exposure to elevated p_{CO_2} may force the source activity to balance the sink capacity by decreasing the initial values of photosynthetic stimulation (Stitt 1991; Long and Drake 1992; Paul and Stitt 1993). Such acclimation of leaf photosynthesis is generally associated with a re-allocation of N, mainly from ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco) towards other physiological processes (Stitt 1991; Long and Drake 1992; Paul and Stitt 1993). This mechanism is suggested to occur in situations where the N availability limits growth and when the source capacity exceeds the sink activity (Farage et al. 1998). However, when plants are growing in N limiting conditions but the balance between source and sink at the whole plant level is maintained, then the *rubisco* concentration is not affected, irrespective the decline in N concentration, and acclimation of leaf photosynthesis does not occur (Farage et al. 1998).

1.2. P uptake, long distance transport, and P distribution in shoots

Although the total amount of P in the soil may be high, the major fraction is often present in complex mineral and soil organic compounds; these P forms are unsuitable for plant uptake (Schachtman et al. 1998). However, some mineral P-compounds may be solubilised by organic acids produced by roots (Johnson et al. 1996) while the organic P-compounds may be hydrolysed by phosphate mobilising enzymes produced by roots or soil microbes (Panara et al. 1990; Jungk 1996; Bosse and Köck 1998).

The movement of P from soil to the root surface is mainly a diffusion process. Generally, the rate of plant P uptake is higher than the rate of diffusion in the soil, thus depleting the assimilable P forms around the roots (Jungk 1996; Schachtman et al. 1998). Therefore, in contrast to N, the uptake of P by fine root interception assumes a significant importance (Gahoonia and Nielsen 1998).

The transport of P across the root surface is driven by specialised transport mechanisms and it seems to be coupled to a proton motive force, dependent on the membrane electrochemical potential (Dunlop and Bowling 1978). However, the precise mechanisms governing P transport across the root surface and its regulation are still not clear (Schachtman et al. 1998).

Although leaf transpiration is partly involved in the transport of P across the root surface, its role in this process may be only minor (Marschner 1995). However, leaf transpiration together with the *root pressure* determines the rate of water flux, which is a mass flow process, in the long-distance transport of nutrients within the plant (Jeschke et al. 1996). In addition, leaf transpiration is suggested to determine the distribution of nutrients within the shoot, depending on the rate and duration of transpiration of the organ (Marschner 1995).

Under elevated p_{CO2} the decrease in stomatal conductivity leads to an increase in water use efficiency (defined as dry matter produced per unit water lost or as carbon net uptake per unit water lost) (Morison 1985; Woodward and Bazzaz 1988). However, the decrease of leaf transpiration rates (Morison 1985) under elevated p_{CO2} may decrease the long distance mass flow of nutrients, affecting their distribution among the sink sites. If the distribution of nutrients among sinks is affected, the balance between root and shoot could be altered under elevated p_{CO2}. Yet, reports do not present the effect of elevated p_{CO2} on leaf stomatal conductivity and transpiration rate in relation to nutrient partitioning.

1.3. Photosynthesis and shoot to root balance

A decrease in P supply is suggested to limit leaf photosynthesis by a combination of three mechanisms: (*i*) an increase in stomatal resistance (Terry and Ulrich 1973); (*ii*) a decrease in carboxylation activity (Brooks 1986, Paul and Stitt 1993); (*iii*) and a decrease in *RubP* regeneration (Brooks 1986; Rao 1997). The increase in stomatal resistance is suggested to be due to an increase in leaf ABA concentration (Marschner 1995). The other two mechanisms are suggested to result from a decrease on the amount and/or activity of Rubisco and on the rate at which Calvin cycle intermediates are regenerated (Lauer et al. 1989; Rao, 1977). However, several reports show that the reduction of photosynthesis due to P deficiency do not limit carbohydrate supply to the roots, in contrast it is even enhanced (Freeden et al. 1989; Cakmak et al. 1994a, 1994b). By this mechanism, root growth is preserved as a plant strategy aiming to overcome the nutrient deficiency situation. As a result, such a change of assimilates allocation leads to the frequently observed increases in the root/shoot ratio (Marschner et al. 1997). Root and shoot growth is thus kept in a functional balance reflecting the plant's adaptability to P availability.

2. THE EFFECT OF P ON THE RESPONSE OF SYMBIOTIC N_2 FIXATION TO ELEVATED p_{CO2}

P is considered to have a direct effect on symbiotic N_2 fixation (Cadisch et al. 1989; Cadisch et al. 1993). The reduction of N_2 fixation due to P deficiency on several plant species has been interpreted has a consequence of the P limitation on host plant and nodule growth with no direct effect on nodule function (Robson et al. 1981; Pereira and Bliss, 1987; Adu-Gyamfi et al. 1989; Cadisch et al. 1993). In contrast, in young pea plants and soybean, P deficiency had a direct effect on nodule function (Jakobsen 1985; Israel 1987; Sa and Israel 1991). Although, P deficiency induced a reduction on N_2 fixation in soybean via both effects simultaneously that is reducing plant and nodule growth and nodule function (Israel 1993).

The control of symbiotic N_2 fixation is suggested to be mediated by the C supply from shoot to nodules (Jakobsen 1985) and by a direct limitation of P to nodule function (Sa and Israel 1991). This would cause a decline in the N reduction process in situations of P deficiency (Sa and Israel 1991). In those reports the results were discussed based on the assumption that bacteroid P metabolism would depend directly on the supply of this nutrient from the host plant. However, recent observations report that bacteroides have a high capacity for direct P uptake and even increase the uptake rates strongly in conditions of P deficiency (Al-Niemi et al. 1997; Al-Niemi et al. 1998). In addition, several other reports showed that (*i*) under stress conditions like shading or defoliation, N_2 fixation was not dependent on carbohydrates supply to nodules (Hartwig et al. 1990; Weisbach et al. 1996), (*ii*) enhanced carbon availability to the host plant did not result in higher N_2 fixation rates (Finn and Brun 1982; Williams et al. 1982; Zanetti et al. 1998) and that (*iii*) N sink demand would be involved in the control of N_2 fixation (Parsons et al. 1993; Heim et al. 1993; Hartwig et al. 1994). In view of these reports the N_2 fixation dependence on P supply clearly needs to be re-evaluated.

3. OBJECTIVES OF THIS STUDY

The objective of this study was to investigate the interactive effects of P supply and atmospheric p_{CO2} on the growth and physiology of white clover (*Trifolium repens* L.). Two main features were investigated:

1) The influence on (a) leaf transpiration rate and on (b) the associated changes of P partitioning and root acid phosphatase activity.

2) The influence on (a) photosynthesis and carbohydrates partitioning and on (b) the associated changes of nodulation, nodule growth and symbiotic N_2 fixation.

IV ELEVATED p_{CO2} AFFECTS P PARTIONING AND ROOT ACID PHOSPHATASE ACTIVITY

1. ABSTRACT

The transport of phosphorus (P) from root to shoot and P distribution among sink sites depend on mass flow and thus on the rate of transpiration. A decrease in transpiration rate due to elevated p_{CO2} could thus reduce P transport to shoot and change partitioning among root and shoot. To test this hypothesis, four levels of phosphorus combined with two levels of p_{CO2} were applied to nodulated white clover (*Trifolium repens* L.) plants. Transpiration rate declined at elevated p_{CO2} and reduced the estimated P flow to the shoot. However total P content per plant was not affected by p_{CO2}. In contrast, elevated p_{CO2} decreased leaf Pi concentration and increased root Pi concentration suggesting a strong change in Pi partitioning among these pools. Associated with these changes, root acid phosphatase activity declined under elevated p_{CO2}. In P deficient white clover the dead leaf fraction increased by 77% under elevated as compared to ambient p_{CO2}. Plant growth, photosynthesis, and the source-sink balance were affected by P supply and p_{CO2} in a close association with changes in Pi partitioning.

2. INTRODUCTION

Phosphorus (P) is a macronutrient essential for plant growth. The movement of P from bulk soil to the root surface is mainly a diffusion process; therefore, P uptake by the plant causes a depletion zone around the roots (Schachtman, et al. 1998). In view of this depletion, the uptake of P by fine root interception is particularly important (Gahoonia and Nielsen 1998). White clover (*Trifolium repens* L.) has a less extensive root system compared to temperate grasses; this is likely the cause of its relatively low capacity and efficiency for soil P uptake (Caradus 1990). Such a difference in P uptake efficiency between white clover and temperate grasses calls for detailed studies on white clover P nutrition.

Root pressure and leaf transpiration determine the xylem mass flow rate in the transport of water and P from the root towards the shoot (Marschner 1995; Jeschke et al. 1996). Phloem transports photosynthates, amino acids and other nutrients from shoot to root sink sites; part of these solutes again enter the xylem stream to be cycled back to the shoot and its sinks (Cakmak et al. 1994a; Jeschke et al. 1997). The cycling of potassium and nitrogen provide the driving forces for xylem volume flow - referred above as "root pressure" (Marschner et al. 1996). This cycling of water and solutes in the plant provide the nutrients to sinks needed for growth. The pattern of nutrient allocation between sinks determines the ability of plants to capture the resources in the root (water and nutrients) and shoot zones (CO_2, light) (Freeden et al. 1989; Cakmak et al. 1994a; Cakmak et al. 1994b). P deficiency causes an increase in the root/shoot ratio due to an increase in the relative allocation of photosynthates and nutrients to the root (Cakmak et al. 1994a; Cakmak et al. 1994b). In addition, under P deficiency the activity of white clover root acid phosphatase increases (Caradus and Snaydon 1987), as a mechanism aiming at hydrolysing soil organic P compounds (Marschner 1995).

The predicted increase of the actual (35 Pa) atmospheric CO_2 partial pressure (p_{CO_2}) is expected to result in increased plant biomass due to higher photosynthetic rates, lower stomatal conductance, thus resulting in higher values of water use efficiency (Bowes 1993). However, the yield increase at elevated p_{CO_2} of grasslands on a fertile soil depended upon plant species (Lüscher et al. 1998), N supply and cutting frequency (Fischer et al. 1997; Hebeisen et al. 1997). Further, P supply is also important for the response of plants to elevated p_{CO_2} since grassland and agricultural crops species show only a limited positive yield response to a rise in p_{CO_2} when P deficient conditions are applied (Goudrian and De Ruiter 1983). In *Trifolium subterraneum* L., elevated p_{CO_2} caused a reduction of leaf Pi concentration; at 0.05 m*M* P supply elevated p_{CO_2} caused a decline of carbon uptake rates that could be offset by increasing P supply to 2 m*M* P (Duchein et al. 1993). It was proposed that elevated p_{CO_2} would cause an impairment of P uptake rather than a sink limitation of growth (Morin et al. 1992; Duchein et al. 1993). However, the cause for the reduction of leaf Pi concentration under elevated p_{CO_2} remains an open question. Moreover, the mechanism(s) responsible for the interaction between P supply and p_{CO_2} at the whole plant level are not clear.

The present study was undertaken to examine the interactive effects of P supply and p_{CO_2} on white clover. We aimed to test the hypothesis that lower transpiration rates under elevated p_{CO_2} would reduce mass flow in the long distance transport and thus P transport to sinks. We expected that a lower transpiration rate due to elevated p_{CO_2} would affect P partitioning between root and shoot and reflect associated changes in root acid phosphatase activity. The following results are relevant to understand the interactive effects of the predicted increase in atmospheric p_{CO_2} and white clover P nutrition.

3. MATERIALS AND METHODS

3.1. Plant materials and growth conditions

White clover (*Trifolium repens* L. cv. Milkanova) cuttings were grown for 10 days in controlled environment chambers (Conviron, Type PGV-36) in trays with quartz-sand. Then, (0 DAP, Days After Planting) uniform plants were transplanted in pairs into individual pots (40 cm x 17.5 cm x 12.5 cm deep) with quartz-sand (0.7-1.2 mm), and grown for another 25 days (25 DAP). Between the 0 DAP and 25 DAP, the p_{CO2} was 35 Pa, the day/night temperatures were 18/13 °C and relative humidity was 75%. The light/dark periods were 16/8 hours with stepwise increasing light irradiance from 150 μmol m^{-2} s^{-1} PAR at day 1 to 550 μmol m^{-2} s^{-1} at 25 DAP. A nutrient solution similar to that of Hammer et al. (1978) with modified phosphorus (0.075 m*M* KH_2PO_4) and nitrogen (1.5 m*M* N) concentrations was supplied twice daily. The plants were inoculated three times with *Rhizobium leguminosarum* bv. *trifolii* (strain RBL 5020, Leiden, The Netherlands); details of inoculation procedures are given in materials and methods from chapter V.

At 25 DAP, the experimental treatments were applied: two levels of p_{CO2} - 35 Pa (ambient) and 70 Pa (elevated) - were combined with four levels of KH_2PO_4 - 0.0027 m*M* (low P), 0.075 m*M* (medium P), 0.67 m*M* (high P) and 2 m*M* (very high P). N concentration in the nutrient solution was kept at 1.5 m*M* and the concentrations of other nutrients were as in Hammer et al. (1978); the pH of the nutrient solution was adjusted to 6.0 by addition of KOH. The day/night temperatures, relative humidity and light/dark period was maintained as in the previous period. The experiment was conducted in a Randomised Complete Block Design with 4 replicates (1 pot with 2 plants per replicate and treatment) and lasted 30 days (55 DAP). At seven-day intervals, the plants and p_{CO2} levels were rotated between and within chambers to exclude chamber-effects. The levels of p_{CO2} were monitored and controlled by infrared gas analysers (WMA-2, PP-system).

3.2. Plant sampling

After 30 days of exposure to p_{CO2} and P treatments (55 DAP), plants were harvested destructively and dissected into the following fractions: leaf laminae, petioles plus stolons and roots. The leaf lamina fraction was subdivided into 5 categories according to leaf position behind the apex and age: (1) first to third unfolded leaf on the main stolon (young), (2) fourth and fifth unfolded leaf on the main stolon (middle), (3) sixth to eighth unfolded leaf on the main stolon (old), (4) all leaves from lateral branches and (5) dead leaves.

Leaf area of all plants was determined with a leaf area meter (LI-COR, model LI-3000 a, Lincoln, NE, USA). The samples of the first harvested plant of a pot were placed immediately in dry ice and stored at -20° C until lyophilisation. After lyophilisation, the roots were cleaned and nodules separated. The samples of the second harvested plant of the same pot were oven-dried at 65°C for 48 hours. The DW of all fractions was determined and samples finely ground with a ball mill (MM2, Retsch, Arlesheim, Switzerland). The lyophilised samples from leaf and root fractions were used for analysis of non-structural carbohydrates, total P concentration, Pi concentration and determination of root acid phosphatase activity. The oven-dried samples from each plant were pooled and analysed for whole plant N and P concentrations.

3.3. Leaf gas exchange

The rates of photosynthesis and of transpiration were determined with an infrared gas analyser (Ciras-1, PP-Systems) and a Parkinson leaf cuvette (type Broad leaf, PP-Systems). Leaf gas exchange rates were measured between the 50 and 55 DAP. The measurements were made on the first fully unfolded leaf of the primary main stolon, at growth p_{CO2} and saturating light (870 μmol m^{-2} s^{-1} PAR).

3.4. Estimated maximum rate of net shoot P uptake

The maximum rate of net shoot P uptake per plant due to mass flow in the long-distance transport pathway was estimated using the transpiration rate as obtained from the leaf gas-exchange measurement, the total leaf area per plant and the concentrations of P in the nutrient solution.

3.5. Non structural carbohydrates

The water soluble carbohydrate fraction (WSC) was extracted from 10 mg of ground sample material twice with 1 cm^3 80% (v/v) ethanol at 80°C for 30 min.

The WSC in the combined supernatants and the insoluble starch in the pellets were determined as described by Fischer et al. (1997).

3.6. Phosphorus

Total P was extracted, after combusting 50 mg of ground sample material at 545°C for 6 hours, with 20% (w/v) HCl. Soluble phosphorus (Pi) was extracted from 20 mg of lyophilised ground sample material with 10 cm^3 2% (v/v) acetic acid as described by Freeden et al. (1989). The determination of extracted P (total P and Pi) was by the colorimetric method as described by Tiessen and Moir (1993). Structural P was estimated as the difference between total P and Pi. The results were expressed on the basis of DW minus starch mass (structural DW).

3.7. Nitrogen

Total N was determined from 50 mg oven-dried ground whole plant samples, using an elemental analyser (LECO CHN-1000, LECO corp., St. Joseph, MI).

3.8. Root acid phosphatase activity

Acid phosphatase was extracted from 50 mg of lyophilised ground sample material with 10 cm^3 of extraction buffer. The activity was assayed using 0.2 ml of 45 m*M* disodium p-nitrophenyl phosphate (Sigma Nr.104) as substrate in a total volume of 4 ml, at pH 4.5 and 30°C for 10 minutes as described by Rao et al. (1990). Activity was expressed in μmol p-nitrophenol produced per g structural DW per minute.

Table 1. Dry matter partitioning, root:shoot ratio and the allometric constant k [ln(root) = b + k ln(shoot)] of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply.

P supply (m*M*)	0.0027		0.075		0.67		2		S.e.[1]	p [2]		
p_{CO2} (Pa)	35	70	35	70	35	70	35	70		p_{CO2}	P	p_{CO2} x P
Leaf laminae (g·plant^{-1})	1.18	1.02	3.23	3.42	5.59	7.15	5.30	7.97	0.69	**	***	***
Dead Leaves (g·plant^{-1})	0.44	0.78	0.16	0.16	0.07	0.07	0.30	0.15	0.11	*NS*	***	*
Petiole, stolons (g·plant^{-1})	1.72	1.66	4.80	5.22	5.47	6.45	5.77	7.32	0.74	**	***	*
Root (g·plant^{-1})	1.20	1.01	3.02	2.70	2.91	3.44	3.11	3.34	0.43	*NS*	***	*NS*
Total plant (g·plant^{-1})	4.54	4.47	11.21	11.50	14.04	17.10	14.48	18.78	2.44	*	***	***
Root:shoot ratio	0.35	0.30	0.37	0.31	0.26	0.25	0.27	0.22	0.03	*	**	***
k^3	0.50	0.68	1.01	1.44	1.15	1.00	1.19	1.22				
S.e. for *k* values	(0.11)	(0.06)	(0.19)	(0.43)	(0.20)	(0.29)	(0.29)	(0.17)				

[1] Standard errors of means (n=4)

[2] Significance from the ANOVA - *NS* not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

[3] Values calculated by individual linear regressions. All regressions were significant at $p<0.05$.

3.9. Statistical analysis

Analyses of variance were carried out to test main effects, interactions and their orthogonal polynomial components, using the SAS statistical analysis package (SAS Institute, Cary, NC, USA). Non-normal distributions and heterogeneity of variance of data were corrected for analysis by ln transformation. The calculation of orthogonal polynomials aimed to study the functional response to P treatments, covering the whole range within the tested P levels.

The allometric relationship between the relative growth of root and shoot, i.e. the slope *k* in ln(*root*)= *b* + *k* ln(*shoot*), was calculated by linear regression of ln(DW), as described by Farrar and Williams (1991).

4. RESULTS

The effects of p_{CO_2} on shoot biomass of white clover after 30 days of growth depended strongly on P supply (CO_2 x P significant at 0.1%). The maximum DW in ambient p_{CO_2} was attained at 0.67 m*M* P supply (11.13 g) and in elevated p_{CO_2} at 2 m*M* P supply (15.44 g; from Table 1). This interaction was strongest for the leaf DW where the p_{CO_2} effect varied from -14% at 0.0027 m*M* P to +50% at 2m*M* P supply (from Table 1). The interaction was similar in the petiole and stolon fraction. The negative p_{CO_2} effect on the leaf DW at 0.0027 m*M* P supply was due to a 77% increase in dead leaves under elevated p_{CO_2} that reached 43% of the whole leaf fraction (Table 1). In all the other levels of P supply the dead leaf DW was very low and not affected by p_{CO_2} (Table 1). Root DW was not changed by p_{CO_2}. Therefore, the root:shoot ratios indicate a declining trend with elevated p_{CO_2} and with increasing P supply (Table 1). More important, the allometric coefficient *k* for ln(*root*) versus ln(*shoot*) was not affected by p_{CO_2}. However, it was affected by P supply, being lowest at 0.0027 m*M* P (k<1) (Table 1).

Total P content per plant was not affected by p_{CO_2} between 0.0027 m*M* P and 0.67 m*M* P supply; at 2 m*M* P supply, however, under elevated p_{CO_2} plants

Table 2. Total N content per plant, total P content per plant and N/P mass ratio of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO_2} combined with four levels of P supply.

DAP (days)	25 [1]	55 [2]										
P supply (m*M*)	0.075	0.0027		0.075		0.67		2		p [1]		
p_{CO_2} (Pa)	35	35	70	35	70	35	70	35	70	p_{CO_2}	P	p_{CO_2} x P
N (mg·plant^{-1})	25.4 ± 4.9	107.6^a	117.5^a	237.7^b	225.6^b	367.2^c	323.8^c	381.9^c	427.5^c	*NS*	***	*NS*
P (mg·plant^{-1})	1.9 ± 0.3	3.5^a	3.0^a	13.5^b	15.1^b	75.3^c	73.0^c	91.3^c	128.1^d	*NS*	***	*
N/P	13.5 ± 0.6	30.6^a	32.6^a	17.6^b	14.9^b	4.9^c	4.4^{cd}	2.6^e	3.3^{de}	*NS*	***	*

[1] Means from 4 replicates ± S.e.
[2] Retransformed means from 4 replicates. Values with the same letter are not significantly different at p <0.05. Significance from the ANOVA - *NS* not significant, * p<0.05, ** p<0.01, *** p<0.001.

Table 3. Photosynthetic rate and carbohydrate content of leaves and roots from white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO_2} combined with four levels of P supply.

P supply (m*M*)	0.0027		0.075		0.67		2		S.e.[1]	p[2]		
p_{CO_2} (Pa)	35	70	35	70	35	70	35	70		p_{CO_2}	P	p_{CO_2} x P
CO_2 exchange rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$):												
-Morning	2.9	5.0	7.3	12.9	13.9	18.6	10.0	18.8	1.5	***	***	*
-Afternoon	3.8	4.7	[4]	[4]	13.7	12.5	11.5	16.9	1.6	*NS*	***	*
Starch content ($mg \cdot cm^{-2}$)												
-Young leaves	1.58^{a}	1.91^{b}	1.15^{a}	1.80^{b}	1.40^{b}	2.46^{c}	1.44^{b}	2.2^{b}	[5]	***	*NS*	*
-Middle leaves	0.61^{a}	1.07^{b}	0.32^{a}	1.95^{b}	1.51^{b}	3.34^{c}	1.21^{b}	3.02^{c}	[5]	***	*NS*	***
-Old leaves	0.12^{a}	0.96^{b}	0.02^{a}	0.57^{b}	0.92^{b}	2.10^{c}	0.75^{b}	1.28^{bc}	[5]	***	*NS*	***
Root carbohydrate content:												
-Starch ($mg \cdot g^{-1}$ DW)	80.9	76.1	68.5	81.0	56.4	77.6	57.1	59.4	7.4	*NS*	*NS*	*NS*
-WSC ($mg \cdot g^{-1}$ DW) gluc.eq.[3]	87.8	90.1	94.0	87.7	92.1	84.1	90.9	85.0	5.2	*NS*	*NS*	*NS*

[1] Standard errors of means (n=4)

[2] Significance from the ANOVA - *NS* not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[3] gluc. eq., glucose equivalent.

[4] Not available

[5] Retransformed means. Values within leaf class with the same letter are not significantly different $p < 0.05$.

---

showed a higher total P content (Table 2). This difference was due to the higher biomass accumulation since whole plant P concentration was not affected by p_{CO_2} (data not shown). Total N content per plant was not affected by p_{CO_2} (Table 2). The N/P mass ratio between total N per plant and total P per plant was not affected by the p_{CO_2} but by the P treatments (Table 2). As suggested by Dunlop and Hart (1987) for white clover, ratios of N/P above 18 would be an indication of P deficiency. Accordingly, our results indicated a strong P deficiency at 0.0027 m*M*, the limit of deficiency around 0.075 m*M* P and no deficiency at 0.67 m*M* P and 2 m*M* P supply.

In the beginning of the light period, elevated p_{CO_2} enhanced photosynthesis at all levels of P supply. At the end of the light period, except at 2 m*M* P supply, photosynthesis was no longer enhanced by p_{CO_2} (Table 3). Starch contents of leaves increased significantly at elevated p_{CO_2}; the maximum starch content at elevated p_{CO_2} was observed at 0.67 m*M* P supply in all leaf classes (Table 3). In contrast to the leaves, WSC and starch contents in the root were not affected by p_{CO_2} or P supply.

Leaf transpiration rate reached the highest treatment mean value at 0.67 m*M* P or at around 1 m*M* P supply as predicted from the orthogonal polynomial regression (Fig. 1A). Elevated p_{CO_2} decreased the leaf transpiration rate and the largest reduction coincided with the maximum transpiration rates mentioned. Similarly, the estimated shoot P uptake rate per plant at 0.67 m*M* P supply was lower under elevated p_{CO_2}; the orthogonal polynomial regressions indicated a significant decrease between 0.3 m*M* P and 1.9 m*M* P supplies (Fig. 1B). The estimated shoot P uptake rate per plant for maximum DW yield in ambient p_{CO_2} (calculated at 0.6 m*M* P supply from the orthogonal polynomial regression) was 25 $nmol \cdot plant^{-1} \cdot min^{-1}$. To attain a similar value of shoot P uptake under elevated p_{CO_2} the P concentration in the nutrient solution needed to be increased to 0.9 m*M*.

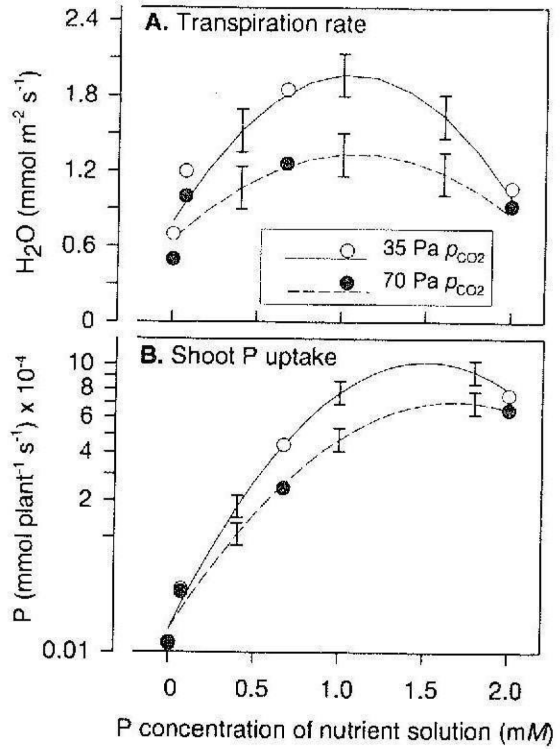

Figure 1. Leaf transpiration rate (A) and estimated maximum rate of shoot P uptake per plant (B) based on transpiration of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply. Lines are response curves predicted by orthogonal polynomial regressions (A, R^2=0.96, n=32; B, R^2=0.95, n=32; ± S.e. of prediction); symbols are treatment means (n=4).

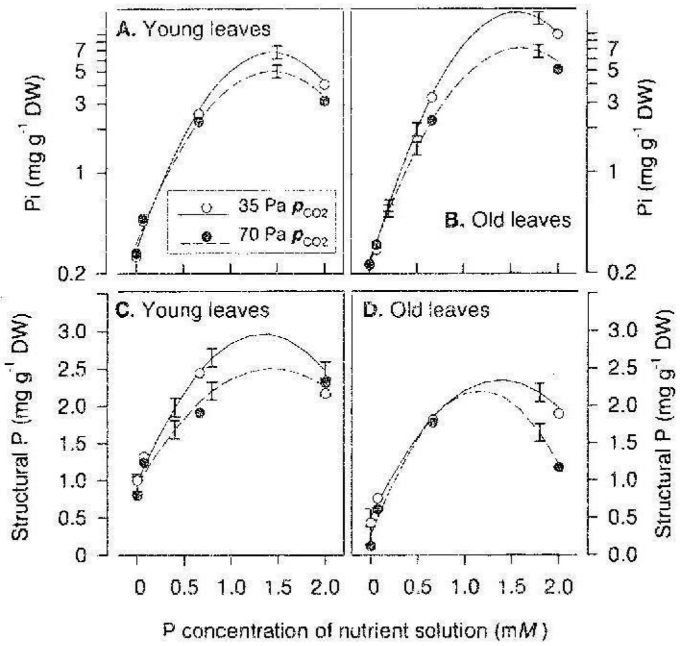

Figure 2. Leaf Pi concentration (A, B) and leaf structural P content (C, D) according to leaf age (A, C - young; B, D - old) of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply. Lines are response curves predicted by orthogonal polynomial regressions (A and B, R^2=0.98, n=64; C and D, R^2=0.90, n=64; ± S.e. of predictions); symbols are treatment means (n=4).

Pi and structural P concentrations of leaf laminae increased with P supply irrespective of leaf classes (Fig. 2). Pi concentration was not changed by p_{CO2} between 0.0027 m*M* and 0.67 m*M* P supplies. At the 2 m*M* P supply, however, elevated p_{CO2} induced lower Pi concentration irrespective of leaf age. As predicted from orthogonal polynomial regressions the reduction of leaf Pi concentration in the young leaf fraction occurred between 0.67 m*M* P and 2 m*M*

P supply; in the old leaf fraction the reduction was more pronounced and occurred between 0.5 m*M* P and 2 m*M* P supply (Fig. 2A and 2B). Structural P concentration was reduced by elevated p_{CO2} at 0.67 m*M* P supply in the young leaves; as predicted by the orthogonal polynomials this reduction occurred between 0.5 m*M* and 1.8 m*M* P supply (Fig. 2C). In the old leaves, structural P concentration was significantly reduced at 0.0027 m*M* and 2 m*M* P supply (Fig. 2D).

In contrast to the results from leaves, Pi concentration in the root was higher under elevated p_{CO2} at 0.67 m*M* P supply. As predicted by the orthogonal polynomial regressions, the increase in root Pi concentration occurred between 0.3 m*M* P and 1.6 m*M* P supply, reaching a maximum at 1.5 m*M* under elevated p_{CO2} and at 2 m*M* P supply under ambient p_{CO2} (Fig. 3A). Root acid phosphatase activity was affected by P supply and p_{CO2} but in an opposite way to that of root Pi concentration. Increasing P supply or p_{CO2} levels reduced root acid phosphatase activity consistently (Table 4). The reduction associated with the increase of p_{CO2} was largest at 0.075 m*M* and 0.67 m*M* P supplies. Root P concentration is involved in the control of phosphatase activity (Noat et al. 1980; Dracup et al. 1984); our results showed a significant overall negative correlation between root acid phosphatase activity and root Pi concentration. The fitted curve (Fig. 3B) suggests a threshold Pi concentration around 2 - 3 mg·g^{-1} DW, below that the activity increased sharply.

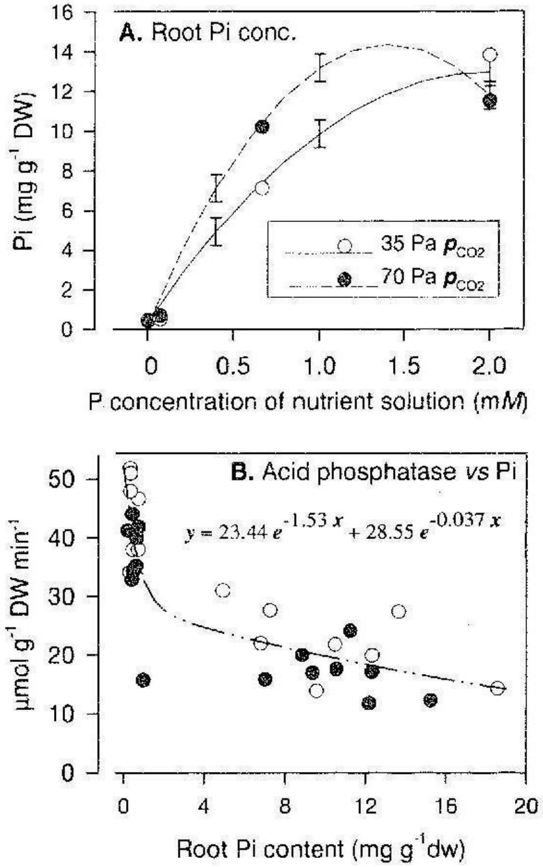

Figure 3 Root Pi content (A) and its effect on root acid phosphatase activity (B) of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply. A: Lines are response curves predicted by orthogonal polynomial regressions (R^2=0.96, n=32, ± S.e. of prediction; symbols are treatment means, n=4). B: Fitted regression curve over both p_{CO2} levels (R^2=0.73, n=32).

Table 4. Acid phosphatase activity ($\mu mol \cdot g^{-1}$ $DW \cdot min^{-1}$) of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply. roots. The initial value before imposing treatments (35 Pa p_{CO2} and 0.075 m*M* P) was 18.8 ±1.6 $\mu mol \cdot g^{-1} DW \cdot min^{-1}$.

	P supply (m*M*)			
	0.0027	0.075	0.67	2
35 Pa p_{CO2}	45.1	40.4	23.6	20.9
70 Pa p_{CO2}	40.4	31.0	15.4	14.5
S.e.[1]	3.6			
p	p_{CO2} <0.01; P <0.05; p_{CO2}xP <0.01			

[1] Standard error of means (n=4)

5. DISCUSSION

5.1. Transpiration rate and Pi partitioning

The results clearly present for the first time, that P allocation between leaves and root was changed at elevated p_{CO2}. Associated with these changes, the rate of leaf transpiration declined under elevated p_{CO2} (Fig. 1) due to a reduction in stomatal conductivity similar to other experiments (Long and Drake 1992). The decrease in leaf transpiration rate under elevated p_{CO2} led to a significant reduction in the estimated rate of shoot P uptake between 0.3 m*M* and 1.9 m*M* P supply (Fig. 1B). Within this range of P supply, leaf Pi as well as leaf structural P concentrations decreased under elevated p_{CO2} (Fig. 2) while root Pi concentration increased (Fig. 3A). A similar p_{CO2} effect on leaf P and acid-soluble esterified P concentrations was also observed in *Trifolium subterraneum*, where the decline occurred at 0.05 m*M*, 0.5 m*M* and 2 m*M* P supplies (Morin et al. 1992; Duchein et al. 1993) but no data was given for other plant organs.

Increasing P supply level enhanced stomatal conductivity as observed in other reports (Morison and Batten 1986). However, stomatal conductivity was reduced from 0.67 m*M* P to 2 m*M* P supply at ambient p_{CO2} resulting in a decline of leaf transpiration rate (Fig.1). Indeed the decline of leaf photosynthesis under ambient p_{CO2} from 0.67 m*M* to 2 m*M* P supply (Table 3) was caused by such an increase in stomata limitation of photosynthesis (chapter V). Most likely, supraoptimal P supply might inhibit CO_2 assimilation partly by stomatal closure and by causing an extra transport of triose-phosphate out of the chloroplast and thereby declining the pentose-phosphate pathway cycle of intermediates (Flügge et al. 1980; Usuda and Edwards 1982; Morison and Batten 1986). Further, a limitation to root growth by pot size would cause an increase in ABA which would eventually lead to a stomatal closure (Marschner 1995). However, maximum root dry mass was attained at 0.67 m*M* P supply under elevated p_{CO2} (Table 1) where no appreciable stomatal closure was evident.

Under P deficiency (0.0027 m*M* P supply), in contrast to the other levels of P supply, leaf and root Pi concentrations and the estimated shoot P uptake rate were low and not affected by p_{CO2}. However, at 0.0027 m*M* P supply under elevated p_{CO2} the dead leaf fraction was increased (Table 1) and structural P concentration in the old leaf fraction was reduced (Fig. 2D). Thus, the internal remobilization of P under elevated p_{CO2} and 0.0027 m*M* P supply appears increased. Such increase in P remobilization from older leaves would allow more P being available to active leaves. Most probably, at 0.0027 m*M* P supply, the trend for higher photosynthetic rates under elevated p_{CO2} (Table 3) indicate that the low estimated rate of shoot P uptake is complemented by a higher leaf P turnover and remobilization from older leaves.

Our results suggest that the pattern of allocation and P partitioning be related to the apparent flow rate of solutes in the transpiration stream to the shoot. The reduction in the estimated shoot P uptake rate and in leaf Pi and structural P concentrations under elevated p_{CO2} resembles the situation of P deficiency: in *Ricinus communis* under ambient p_{CO2}, P deficiency reduced the transpiration rate and water flow in the xylem (Jeschke et al. 1996), decreased P, K, Mg and amino acid concentrations in the xylem sap and decreased root Pi concentration (Jeschke et al. 1997). In our experiment, however, root Pi concentration was increased at 0.67 m*M* P supply under elevated p_{CO_2} (predicted range 0.3-1.6 m*M* P supply; Fig. 3A). This fact does not suggest an increased P deficiency under elevated p_{CO_2}. Further, in addition, total P content per plant (Table 2) was not affected by p_{CO_2} in the range of 0.0027-0.67 m*M* P supply. Therefore, the altered P allocation between leaves and root resulted most likely from the lower net solute flow rate from root to shoot under elevated p_{CO_2}. These results suggest a rather direct effect of elevated p_{CO_2} on P partitioning between shoot and root pools, since the transpiration rate affects the transport from the root to the shoot and the pattern of P distribution among shoot sinks more than the uptake of P from the soil into the root (Marschner 1995).

The changes in P partitioning seem to be associated with changes in the activity of root acid phosphatase (Fig. 3B). In the range of 0.075 m*M* P to 0.67 m*M* P supply, the increased root Pi concentration under elevated p_{CO_2} (Fig. 3A) appears to have induced a decrease in root acid phosphatase activity (Table 4). In contrast to our results, wheat seedlings show a decreased root P concentration under elevated p_{CO_2} that apparently causes an increase in root acid phosphatase activity (Barret et al. 1998). The effect of p_{CO_2} on seedlings is known to be profuse and different from that on other stages of plant development because seedlings are normally source-limited (Stitt 1991). Therefore, in the case of wheat seedlings, the decrease of root P concentration reflects most likely an enhancement of initial development by elevated p_{CO_2} rather than an effect of elevated p_{CO_2} on P partitioning. An increase of root acid phosphatase activity is a mechanism to counteract P deficiency by aiming at hydrolysing complex P ester from soil components (Marschner 1995). Therefore, in white clover a p_{CO_2} induced reduction of root acid phosphatase activity might be particularly disadvantageous for its growth, particularly in view of its inherently low efficiency for soil P uptake in comparison to its usual companion grasses (Caradus 1980).

5.2. Leaf photosynthesis

Leaf photosynthesis as well as leaf Pi concentration declined with decreasing P supply (Table 3; Fig. 2A and 2B). At 0.0027 m*M* P supply leaf Pi concentration reached critical concentrations similar to those of P deficient soybean leaves (Freeden et al. 1989) or even below those of P-starved spinach leaves (Brooks 1986). It is likely that the limitation of leaf photosynthetic rates imposed by the lower P supply treatments was due to a combination of a decrease in carboxylase activity and a limitation in the regeneration of ribulose-1,5-biphosphate (Brooks 1986; Freeden et al. 1989). The morning rates of leaf photosynthesis were stimulated by elevated p_{CO_2} to similar levels as reported by Long and Drake (1992). In contrast, in the end of the light period the p_{CO_2} stimulation effect on leaf photosynthetic rates was lost at or below 0.67 m*M* P supply (Table 3). Associated with this, the starch concentration of leaves was increased under elevated p_{CO_2} and was highest at 0.67 m*M* P supply. This effect could be due to a limitation of the orthophosphate pool at the cellular level (Morin et al. 1992; Duchein et al. 1993), or to a sink limitation in the use of photosynthates (Sharkey 1985; Rao 1997). Both mechanisms would result in starch accumulation in the leaves. In contrast to 0.67 m*M* P supply, at 2 m*M* P supply in the end of the light period the p_{CO_2} stimulation of photosynthetic rates was not lost; from 0.67 m*M* P to 2 m*M* P supply starch concentration decreased in young and middle leaves (Table 3). These observations together with the increase in leaf Pi concentration at the highest P supply levels (Fig. 2A and 2B) support the hypothesis of a limitation in leaf orthophosphate availability under elevated p_{CO_2}. However, plant biomass accumulation was higher at 2 m*M* than at 0.67 m*M* P supply, indicating an increased sink capacity, thus also supporting the idea of an alleviation of sink limitation at ample P supply. Our results do not allow conclusively explain the reason of the decrease in the leaf photosynthetic rate at the end of the light period at 0.67 m*M* P and elevated p_{CO_2}.

5.3. Source-sink balance and biomass accumulation

The allometric constant *k* in the ln(*root*) *vs.* ln(*shoot*) relation did not depend on p_{CO_2}; however, plant DW was increased after growth at elevated p_{CO_2}. These suggest an ontogenetic effect of elevated p_{CO2} by increasing the growth rate of the plants (Farrar and Williams 1991). However, *k* was smaller than 1 at 0.0027 m*M* P supply indicating a P dependent effect in dry matter partitioning. This agrees with a higher allocation of carbohydrates towards the root at 0.0027 m*M* P supply. The shift of carbon partitioning in favour of root growth to possibly offset the P deficient situation (Cakmak et al. 1994b; Jeschke et al. 1996) seems therefore to remain also under elevated p_{CO2}. Nevertheless, above the 0.075 m*M* P supply level, the apparently direct effect of elevated p_{CO2} on Pi partitioning among sinks combined with the higher accumulation of starch in the leaves suggests a change in the source-sink balance at elevated p_{CO2}. Indeed, the positive yield response of white clover to p_{CO2} increased with increasing P supply.

Under elevated p_{CO2} the maximum leaf laminae DW was observed at 2 m*M* P supply, whereas under ambient p_{CO2} it was maximal at 0.67 m*M* P supply. Such a response pattern was also observed in other species (Rogers et al. 1993). In a Free Air Carbon Dioxide Enrichment (FACE) experiment on a fertile soil with high levels of P and K fertilisation, harvestable biomass of white clover showed a strong positive yield response to elevated p_{CO2} (Hebeisen et al. 1997; Lüscher et al. 1998). In our experiment at 2 m*M* P supply, total P concentration of leaves ranged from 4.4 to 6.6 $mg \cdot g^{-1}$ DW which is similar to the total P concentration we measured in leaves from the FACE experiment (4.3 ± 0.3 $mg \cdot g^{-1}$ DW). These results support clearly the hypothesis presented by Lüscher et al. (1996) that a high P availability is a pre-requisite for the potential maximum yield response of white clover to elevated p_{CO2}.

6. CONCLUSIONS

The low leaf transpiration rate under elevated p_{CO2} induced apparently a change in Pi partitioning between shoot and root, with a consequent decrease of root acid phosphatase activity.

These results indicate that in situations of limited P availability, the response of white clover to elevated p_{CO2} might be impaired. Further investigations are needed to test this hypothesis under field conditions.

V P SUPPLY AFFECTS PHOTOSYNTHESIS AND CARBON PARTITIONING BUT DOES NOT PRIMARILY CONTROL N_2 FIXATION

1. ABSTRACT

Under P deficiency, the decrease of photosynthesis, and the limitation of P availability on the host plant growth and on nodule function is suggested to determine the level of symbiotic nitrogen (N_2) fixation. However, no concomitant measures of photosynthesis, carbohydrate concentrations and N_2 fixation under P deficiency were reported. A study was undertaken with white clover, to test the hypotheses that (*i*) under P deficiency the decline of N_2 fixation is the result of an adaptation to the low N demand for growth and (*ii*) the changes in leaf photosynthetic capacity would balance C source and C sink without limiting *per se* the N_2 fixation process.

Severe P deficiency prevented nodulation or stopped nodule the increase of nodule mass when deficiency was applied to previously nodulated white clover plants. The specific N_2 fixation increased and partly compensated poor nodulation at P deficiency. As a result of this partly compensation, nitrogen assimilation did not limit the growth of white clover. Although $\%N_{sym}$ was more inhibited by low P than plant growth itself, plant N concentration increased due to mineral uptake from nutrient solution. Leaf photosynthesis declined under P deficiency due to reductions of $V_{c,max}$ and J_{max}. This adaptation of photosynthetic apparatus indicated a balance between C source and C sink and is not considered to cause a C limitation to nodule growth or function. Our results support the view that N_2 fixation is rather regulated by an N feedback mechanism than by the shoot photosynthetic capacity.

2. INTRODUCTION

Phosphorus (P) is often suggested to determine the level of symbiotic nitrogen (N_2) fixation by limiting host plant growth, nodule growth and function (Robson et al. 1981; Jakobsen 1985; Israel 1987; Sa and Israel 1991; Israel 1993). However, some of these suggestions were based on results from experiments under field conditions or using pots with soil (Robson et al. 1981; Bonetti et al. 1984; Jakobsen 1985; Pereira and Bliss 1987; Adu-Gyamfi et al. 1989). Therefore the effect of mineral P addition on N_2 fixation could not be excluded from the soil P mobilisation. In addition, all these experiments assess the N_2 fixation indirectly through an estimation of nitrogenase activity (acetylene reducing assays). Only two studies reported the P effect on N_2 fixation using the ^{15}N-isotope dilution procedure, but in soil and field conditions with tropical legumes (Cadisch et al. 1989; Cadisch et al. 1993). Thereby, the effect of P supply on symbiotic N_2 fixation is not clear. Despite these restrictions, early studies suggested that low P reduced the shoot growth and, therefore, the consequent decline of photosynthesis would reduce the carbon supply to the nodules (Jakobsen 1985). Reduced C supply together with a low orthophosphate supply from host to nodules would inhibit the oxidative phosphorylation and ATP-dependent reactions in the plant-cell fraction of nodules, thereby decreasing the N reduction (Israel 1987; Sa and Israel 1991; Israel 1993).

The N reduction process requires about 2.9 mg C mg^{-1} N_2 fixed, that could account for 32% of plant photosynthates under certain conditions (Minchin and Pate 1973; Warembourg and Roumet 1989). Indeed, the above mentioned studies assign to the supply of photosynthates from shoots to nodules a crucial key of N_2 fixation under P deficiency. However, the concomitant measurement of photosynthesis, carbohydrates, and N_2 fixation, as affected by the P supply was not reported. Whether the proportional decline of photosynthesis and nodule mass due to P deficiency results also in a decreased carbohydrate concentration of nodules, remains an open question. Reports on the effect of other stress factors suggested that the carbohydrate supply to nodules do not exert a primarily control on N_2 fixation (Hartwig et al.

1990; Hartwig et al. 1994; Weisbach et al. 1996; Gordon et al. 1997). Alternatively, the effect of N demand on the control of N_2 fixation is being clearly identified (Heim et al. 1993; Parsons et al. 1993; Hartwig et al. 1994; Neo and Layzell 1997). In agreement with this hypothesis, the reports about the effect of elevated p_{CO2} on N_2 fixation showed that an increase of shoot C availability does not increase *per se* neither the specific nitrogenase activity of nodules (Finn and Brun 1982; Williams et al. 1982) nor the relative contribution of symbiotically fixed N to the total plant N ($\%N_{sym}$) (Zanetti et al. 1998).

P deficiency decreases plant growth and thus the N demand of plants (Rufty et al. 1990; Rufty et al. 1993; Jeschke et al. 1996; Jeschke et al. 1997); several reports concluded that P deficient plants, fully dependent on N_2 fixation, were not N limited (Robson et al. 1981; Jakobsen 1985; Israel 1987). Results from other stress factors, where nodule growth was impaired, suggested that specific N_2 fixation would increase in response to enhanced plant N demand (Sangakkara et al. 1996a; Zanetti et al. 1998). These conclusions together are clearly indicative of an apparent balance between legume N sink (demand) and N source. If such a concept would apply also for P deficiency then, (1) the decline of N_2 fixation would be the result of an adaptation to the low N demand and (2) an effect of P nutrition on photosynthesis would bring the C source and C sink into balance but would not primarily control the nodule growth and function. A study was therefore undertaken to test these two hypotheses with white clover (*Trifolium repens* L.) in a controlled environment. A wide range of P supply levels were combined with two atmospheric CO_2 partial pressures (p_{CO2}), aiming to assess the involvement of photosynthesis in the control on N_2 fixation. To avoid the interaction with soil P mobilisation, quartz-sand was used as substrate for plant growth. So far, this is the first report on photosynthesis, carbon partitioning and host plant-N_2 fixation as affected by long term phosphorus nutrition and carbon availability.

3. MATERIALS AND METHODS

From the experiment described in the chapter IV, we present here the results from the leaf limitation factors on photosynthesis, C and N partitioning, N derived from symbiosis and nodule growth.

A second experiment, here described, was carried out to investigate the effect of P and p_{CO2} on nodulation.

3.1. Plant material and growth conditions

The outline of the first experiment was described before (page 15). Briefly, white clover (*Trifolium repens* L. cv. Milkanova) cuttings were grown for 10 days in trays with quartz-sand. At the 11th day (0 DAP, Days After Planting) two plants were transplanted into individual pots and grown for another 25 days (25 DAP) with 0.075 m*M* KH_2PO_4 in the nutrient solution and an atmospheric p_{CO2} of 35 Pa.

The plants were inoculated under 0.075 m*M* P supply with *Rhizobium leguminosarum* bv. *trifolii* (strain RBL 5020, Leiden, The Netherlands) at 0 DAP, 6 DAP and 17 DAP by supplying 25 ml, 40 ml and 50 ml of inoculum culture per plant respectively, with a concentration of $2x10^8$ cells per ml.

At 25 DAP, four levels of KH_2PO_4 (0.0027 m*M*, 0.075 m*M*, 0.67 m*M* and 2 m*M*) were applied in combination with two levels of atmospheric p_{CO2} - 35 Pa (ambient) and 70 Pa (elevated) - for 30 days (until 55 DAP). From that time on (25 DAP) the 1.5 m*M* N in the nutrient solution was labelled with 0.9% ^{15}N-enriched NH_4NO_3 in which ammonium and nitrate were equally labelled (Isotec, Mimisburg, OH, and Matheson, Secaucus, NJ, USA). Once per week the pots were flushed abundantly with de-ionised water, to prevent any possible accumulation of nutrients.

For the nodulation experiment white clover cuttings were grown in a similar way as in the first experiment, using the same conditions. However, from the very early beginning, that is the time of transplantation (0 DAP), two levels of KH_2PO_4 (0.0027 m*M* and 0.075 m*M*) in combination with two levels of atmospheric p_{CO2} (35 Pa and 70 Pa) were applied for 44 days. The other nutrients in nutrient solution, pH, day/night temperatures, relative humidity, light/dark periods and light irradiance were kept as in the first experiment (page 14). The plants were inoculated in both P treatments at 0 DAP, 7 DAP and 14 DAP using the same procedure as before. The experiment was conducted in a Randomised Complete Block Design with 4 replicates (1 plant per replicate and treatment).

3.2. Plant sampling

Plants were harvested at 25 and 55 DAP in the first experiment and at 0, 22 and 44 DAP in the second experiment. At each harvest plants were separated into root and shoot fractions. The shoots were divided into stolons, petioles and leaf laminae. The details on dissection procedure, leaf area measurement, and preparation of sample materials were mentioned before (page 16). The samples of the first harvested plant of a pot were placed immediately in dry ice and stored at -20° C until lyophilisation. After lyophilisation, nodules were removed from roots and cleaned under the microscope. The samples of the second harvested plant of the same pot were oven-dried at 65°C for 48 hours.

The lyophilised samples were used for analysis of non-structural carbohydrates, total P and N content. The oven-dried samples from the second harvested plant were pooled and analysed for whole plant N, ^{15}N and P contents.

3.3. Chemical analyses

The water-soluble carbohydrate (WSC), starch and total P content were determined as described in chapter IV (page 18).

Total N and ^{15}N contents were determined from 1 mg ground sample material by a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK) in the University of Saskatchewan, Saskatoon, Canada. N

contents in the leaves, roots and nodules are presented on a dry weight corrected for starch content basis (structural DW).

3.4. Calculation of symbiotic N$_2$ fixation

In the first experiment, the relative contribution of symbiotically fixed N to the total plant N (%N$_{sym}$) was determined according to the ^{15}N-isotope-dilution method as described by Zanetti et al. (1998), integrated for the period 25-55 DAP.

3.5. Leaf gas exchange

Leaf gas exchange was determined with an infrared gas analyser (Ciras-1, PP systems) and a Parkinson leaf cuvette (type Broad leaf, PP-systems) at 25 DAP and 55 DAP, on the first unfolded leaf at light saturation (870 µmol m^{-2} s^{-1} PAR). Leaf internal p_{CO2} concentration (C_i) was varied by varying external leaf p_{CO2} in the leaf cuvette (5 Pa, 10 Pa, 15 Pa, 25 Pa, 35 Pa, 70 Pa, 90 Pa, 110 Pa, and 150 Pa); corresponding photosynthetic rates (A) were recorded.

The apparent maximum rate of carboxylation with non limiting ribulose-1,5-biphosphate ($V_{c,max}$) and the apparent potential rate of electron transport at light saturation (J_{max}) were calculated for each leaf from the fit of the A/C_i values to the biochemical model of Farquhar et al. (1980) and Farquhar and Caemerer (1982) as described by Kirschbaum and Farquhar (1984). Percent stomatal limitation of photosynthesis (l) was calculated using the photosynthetic rate (A) recorded at the growing p_{CO2} regime (C_a), and the photosynthetic rate (A_0) estimated from the A/C_i response at $C_i = C_a$ according to:

$$l = 100 \times (A_0 - A) / A_0 \qquad \text{(Long and Drake 1992)}$$

3.6. Statistical data analysis

Statistical analyses were carried out using the general linear model procedure of the SAS statistical analysis package (SAS Institute, Cary, NC, USA). Data were transformed to correct the effects of non-homogeneity of variance and response scaling. In all tests 95% confidence level was used.

Table 5. Nodule and plant mass of white clover plants grown for 44 days (0 DAP to 44 DAP) at two levels of p_{CO_2} and of P supply and inoculated with *Rhizobium leguminosarum* bv. *trifolii* at 0, 7 and 14 DAP.

P (m*M*)	p_{CO_2} (Pa)	Nodules[1] (mg DW)	Plant[1] (g DW)
0.0027	35	0^{a}	0.3^{a}
	70	0^{a}	0.3^{a}
0.075	35	50.9^{b}	3.7^{b}
	70	34.6^{b}	5.1^{c}
p	p_{CO_2}	*NS*	<0.05
	P	<0.001	<0.001
	p_{CO_2} x P	*NS*	<0.05

[1] Values are retransformed means (n=4); when referenced with the same letter did not reach significant differences at $p<0.05$ by Tukey's-test.

4. RESULTS

4.1. Nodule mass and plant growth

Low P supply (0.0027 m*M* P) impaired strongly nodulation and nodule growth (increase in nodule DW) under both p_{CO_2}. When inoculation occurred under 0.0027 m*M* P supply no nodules were observed in contrast to the nodulation from plants grown at 0.075 m*M* P (Table 5). At 0.075 m*M* P as compared with 0.0027 m*M* P, plant DW was strongly increased (Table 5). After allowing plants to nodulate at 0.075 m*M* P supply, the reduction of P supply to 0.0027 m*M* (at 25 DAP) stopped completely the increase in nodule mass for the next 30 days of the experiment (55 DAP, Fig. 4A). At 0.075 m*M* P nodule continued to increase DW between 25 DAP and 55 DAP. Increasing P supply levels above 0.075 m*M* P resulted in a further increase of nodule mass. The atmospheric p_{CO_2} had no effect on nodule mass (Fig. 4A).

As expected, plant dry mass increased strongly with increasing P supply. The response of plant dry mass to elevated p_{CO_2} was however, dependent on P supply (Table 1, page 19). Nodule mass response to P supply between 25 DAP and 55 DAP, was stronger as compared with plant mass response; as a result the ratio total nodule mass/total plant mass increased with increasing P supply but it was not affected by p_{CO_2} growth regime (Fig. 4B). At 0.075 m*M* P supply, this ratio was not affected by plant age (25 DAP *vs* 55 DAP). The reduction of P supply from 0.075 m*M* to 0.0027 m*M* resulted in a strong decline of the ratio total nodule mass/total plant mass (Fig. 4B).

4.2. %N_{sym} and N partitioning

The %N_{sym} integrated for the period 25-55 DAP increased with increasing P supply and was not affected by p_{CO_2} regime (Fig. 4C). In contrast, specific N_2 fixation (mg $N_{sym}\cdot mg^{-1}$ nodule DW) declined with increasing P supply (Fig. 4D). As for the %N_{sym} and nodule mass, no p_{CO_2} effect was observed on specific N_2 fixation.

Total N content per plant, at 55 DAP, increased with P supply (Table 2, page 21). The reduction of P supply to 0.0027 m*M* P for 30 days, did not impair N uptake (25 DAP *vs* 55 DAP) and increased the N content per plant by a factor of 4.7 (Table 2 page 21). In contrast to plant N content, plant N concentration and leaf N concentration were higher at 0.0027 m*M* P (Fig. 5A and 5B). With increasing P supply, leaf N concentration declined in both p_{CO_2}; however, the reduction was faster under elevated p_{CO_2}. As a result of this stronger decline, at 0.075 m*M* P and 0.67 m*M* P supply, leaf N concentrations were lower under elevated as compared to ambient p_{CO_2} (Fig. 5B). In contrast, root N concentration was not affected by P supply or by p_{CO_2} (Fig. 5C). Nodule N concentration was reduced at 0.0027 m*M* P supply as compared with the other

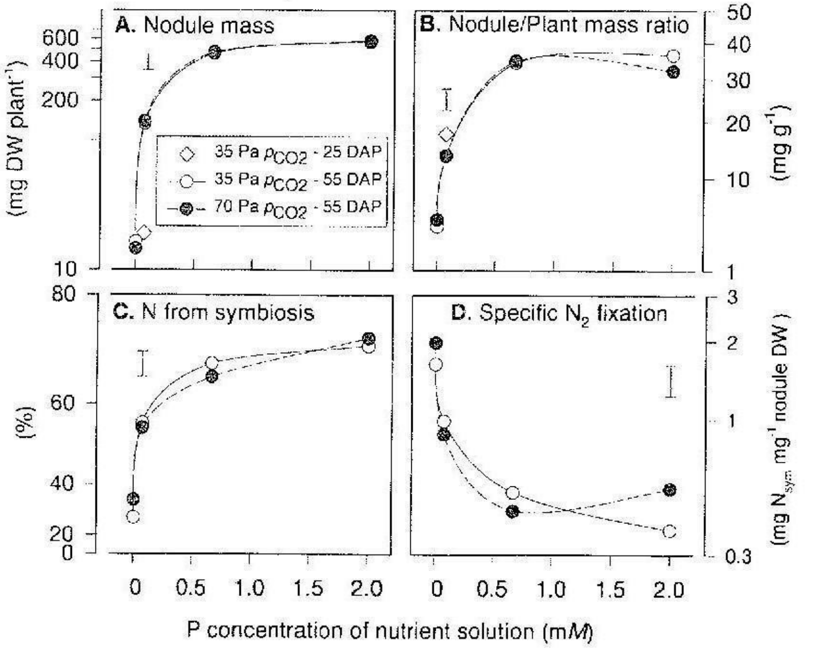

Figure 4. Nodule mass per plant (A), total nodule mass/total plant mass ratio (B), percentage N derived from symbiosis (C) and Specific N_2 fixation (D) of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply. Symbols are treatment means (n=4) and bars represent 2 times S.e. of means.

P levels (Fig. 5D). At 0.0027 m*M* P, nodules showed a declining trend in the N concentration at ambient as compared to elevated p_{CO2} (p<0.07).

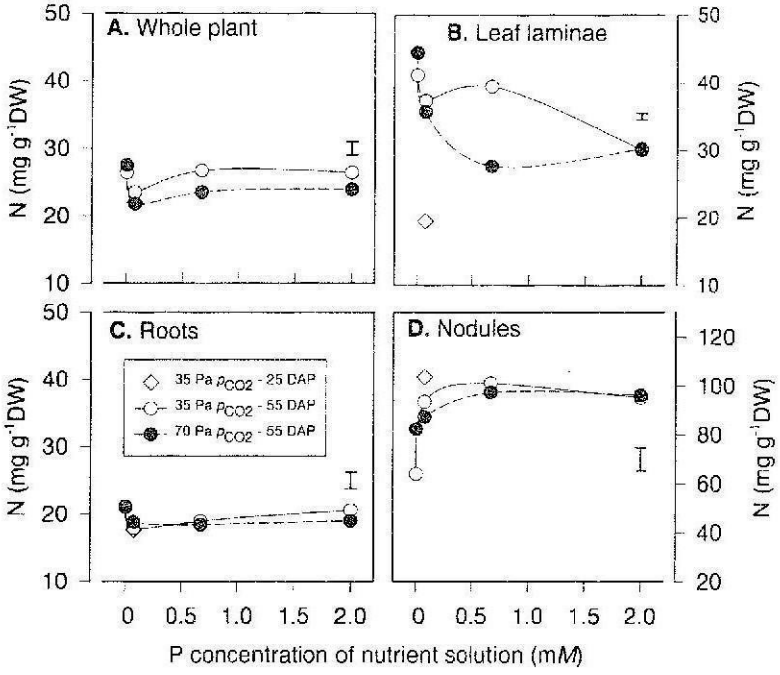

Figure 5. Whole plant N concentration (A) and N concentrations of the leaf laminae (B), of the roots (C) and of the nodules (D) in a structural DW basis of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply. Symbols are treatment means (n=4) and bars represent 2 times S.e. of means.

4.3. Photosynthesis

P supply and elevated p_{CO2} stimulated the leaf photosynthesis (Table 3, page 22). However, under 35 Pa p_{CO2} photosynthesis was highest at 0.67 m*M* P supply declining significantly at 2 m*M* P supply (Table 3, page 22).

The effect of P supply on enhancing leaf photosynthesis was associated with an increase of $V_{c,max}$ and J_{max} (Table 6). However, under elevated as compared with ambient p_{CO2}, white clover leaves had a lower $V_{c,max}$ between 0.075 m*M* P and 2 m*M* P supply. The increase of plant age (25 DAP *vs* 55 DAP) as well, was associated with a reduction of $V_{c,max}$ and J_{max}.

Table 6. Apparent maximum rate of carboxylation with non limiting *RubP* ($V_{c,max}$), apparent potential rate of electron transport at saturated light (J_{max}) and stomatal limitation on leaf photosynthesis (*l*) of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply.

DAP	P (m*M*)	p_{CO2} (Pa)	$V_{c,max}$ (µmol CO$_2$ m^{-2} s^{-1})	J_{max}	*l* (%)
25[1]	0.075	35	93.4 ±7.1	248.1 ±29.5	31.9 ±5.1
55[2]	0.0027	35	36.6^{a}	95.9^{a}	31.9^{ab}
		70	39.2^{a}	119.5^{ab}	26.7^{ab}
	0.075	35	64.1^{b}	153.0^{bc}	37.9^{a}
		70	53.4^{ab}	149.0^{b}	19.6^{bc}
	0.67	35	117.7^{c}	221.4^{d}	34.0^{ab}
		70	82.5^{bd}	192.3^{cd}	14.3^{c}
	2	35	129.5^{c}	218.2^{d}	48.0^{a}
		70	104.6^{cd}	214.4^{d}	12.4^{c}
p	p_{CO2}		<0.001	*NS*	<0.001
	P		<0.001	<0.001	*NS*
	p_{CO2} x P		<0.05	<0.05	<0.01

[1] Mean values (n=4) ±S.e.

[2] Results are re-transformed means (n=4). When referenced with the same letter did not reach significant differences at $p<0.05$ by Tukey's test.

Under ambient p_{CO2} the stomatal limitation of photosynthesis (l) was not affected by P supply, despite a strong trend for a higher limitation at 2 mM P supply (Table 6). Under elevated p_{CO2}, however, the stomatal limitation of photosynthesis was lower than at ambient p_{CO2} and declined with increasing P supply (Table 6).

Table 7. Leaf starch concentration (all leaves in the main stolons) of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply.

DAP	p_{CO2} (Pa)	P (mM) 0.0027	0.075	0.67	2
		(mg glucose equivalent g^{-1} DW)			
25[1]	35	276.3 ± 18.7			
55[2]	35	190.7^{a}	147.1^{a}	311.7^{b}	241.2^{c}
	70	367.8^{bd}	299.0^{bc}	425.2^{d}	371.8^{bd}
	p	p_{CO2} <0.001; P<0.001; p_{CO2} x P<0.01			

[1] Mean values (n=4) ±S.e.

[2] Results are re-transformed means (n=4); when referenced with the same letter did not reach significant differences at p<0.05 by Tukey's test.

4.4. Carbohydrate partitioning

Doubling p_{CO2} increased leaf starch concentration irrespective of P supply (Table 7). In contrast, root starch concentration was not affected by p_{CO2} or by P supply (Table 3, page 22). The response of nodule starch concentration to the treatments differed from that of leaves and roots: At 0.0027 mM P nodule starch concentration was lower than at higher P supply levels. Under ambient p_{CO2} this decline was more pronounced as compared to elevated p_{CO2} (Fig. 6A).

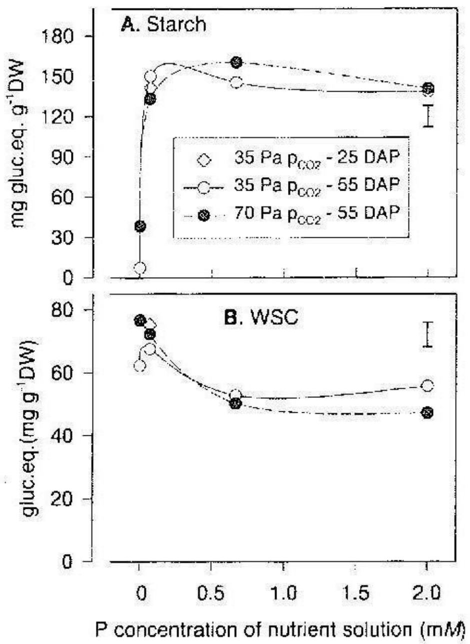

Figure 6. Nodule carbohydrate concentrations: starch (A) and WSC (B) of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply. Symbols are treatment means (n=4) and bars represent 2 times S.e. of means.

Root WSC concentration was neither affected by p_{CO2} or by P supply (Table 3, page 22). In contrast, nodule WSC concentration was higher at the two lowest levels of P supply, i.e. 0.0027 mM and 0.075 mM P (Fig. 6B). Moreover, nodule WSC concentration was always lower than the correspondent root WSC concentration (Fig. 6B and Table 3 page 22).

The different responses of nodule starch and WSC fractions to the treatments resulted in a change of carbohydrate fractionating. At 25 DAP and 0.075 mM P, WSC represented 35% of the total carbohydrates. This proportion remained unchanged until the end of the experiment (55 DAP, 36% at 0.075

m*M* P) and was not affected by p_{CO2} or P levels above 0.075 m*M*. The reduction to 0.0027 m*M* P however, increased the proportion of WSC at 55 DAP to 91% and 68% under 35 Pa and 70 Pa p_{CO2}, respectively.

5. DISCUSSION

5.1. Nodulation and nodule growth

Nodulation was prevented when infection occurred under P deficiency on small plants, irrespective of atmospheric p_{CO2} level (Table 5). So far, this is the first time that such an absolute response of nodulation to P deficiency is reported. The strong extent of this effect might be due to the low P concentration we were able to supply, without interference of soil P assimilation as most likely occurred in experiments where soil was used as substrate (Robson et al. 1981; Bonetti et al. 1984; Jakbsen 1985; Pereira and Bliss 1987; Adu-Gyamfi et al. 1989). However, such a distinct effect of adverse environmental conditions is not a new phenomenon, and it was demonstrated under K deficiency, drought (Sangakkara et al. 1996ab), high soil temperature (Purwantari et al. 1995) and salt stress (Banet et al. 1996). Whether it occurs in nature, indicating a narrower ecological range of adaptation for nodulation as compared to plant growth is not clear.

In line with the above observation (Table 5), when P deficiency was applied to previously nodulated plants, nodule growth stopped irrespective of atmospheric p_{CO2} (Fig. 4A). The decrease in nodule N concentration at 0.0027 m*M* P supply (Fig. 5D) suggests, either a decline of protein synthesis reinforcing the view of an impairment of nodule growth, or a reduced metabolic potential of these nodules.

5.2. Symbiotic N_2 fixation ($\%N_{sym}$) is reduced at low P supply

In contrast to the complete cessation of nodule growth at 0.0027 m*M* P supply between 25 and 55 DAP (Fig. 4A), N_2 fixation still contributed with aprox. 30% of the total new N assimilated by the plant (Fig. 4C). Therefore the low growth of P deficient white clover was still supported by the combination of symbiotic N and the mineral N supply. With increasing P supply, $\%N_{sym}$ was increased.

Whole plant, leaf and root N concentrations (Fig. 5A, 5B and 5C) reached the maximum values at 0.0027 m*M* P supply. These results reflect the effect of combining the supply of N_2 with a low level of mineral N and indicate that growth was not limited by N assimilation under P deficiency. The same conclusion was presented by Robson et al. (1981), Jakobsen (1985) and Israel (1987). Alternatively, such an increased N concentration could also be interpreted as a result of a lower plant size (Farrar and Williams 1991) under P deficiency. However, in our experiment at the lowest P supply the N assimilation exceed plant dry matter increment between 25 DAP and 55 DAP, leading to an increased N concentration at 55 DAP as compared to 25 DAP. Therefore, these results most likely indicate a limitation in the use of the total assimilated N at 0.0027 m*M* P supply. Therefore the limitation of P supply on plant growth was rather a direct effect of P than an indirect P induced N-limitation. Indeed, the high N/P mass ratio at 0.0027 m*M* P supply (Table 2, page 21) supports this view.

5.3. Photosynthesis and carbon partitioning are in balance with plant N demand

Leaf photosynthetic rates, at 55 DAP, declined with decreasing P supply (Table 3, page 22). This decline of leaf photosynthesis was associated with decreases of $V_{c,max}$ and of J_{max} (Table 6). At 0.0027 m*M* P supply, the lower value of $V_{c,max}$ was in contrast to the highest leaf N concentration on a structural DW basis (Fig. 5B) and on a leaf area basis (data not shown). Elevated p_{CO2} stimulated photosynthetic rates in all levels of P supply (Table 3, page 22); however, between 0.075 m*M* and 2 m*M* P supply $V_{c,max}$ was lower under elevated as compared to ambient p_{CO2} (Table 6), in association with a lower leaf N concentration under elevated p_{CO2} (Fig. 5B).

This results clearly show that the decrease of leaf photosynthetic rates with decreasing P supply was due to a decrease in carboxylase activity (decline of $V_{c,max}$) and a simultaneous limitation in the regeneration of *RubP* (decline of

J_{max}). Most likely, the decline of carboxylase activity was rather due to a decrease in activation state of Rubisco than a decline on its concentration, since N concentration in the leaves increased with decreasing P supply. Indeed, as our results suggested, P deficient tobacco did not show any reduction in protein or Rubisco concentration (Paul and Stitt 1993). Which mechanisms trigger the reduction of $V_{c,max}$ are unclear, but low P limits growth directly, and therefore photosynthetic enzyme gene regulation do not control N allocation as in low N conditions (Paul and Stitt 1993).

Between 0.075m*M* and 2 m*M* P supply, the decline of $V_{c,max}$ under elevated p_{CO2} suggests an adaptive response of photosynthetic apparatus. Likely, the balance between C source and C sink was maintained while N would be allocated towards other process, as the decline in N leaf concentration (Fig. 5B) suggests (Stit 1991; Long and Drake 1992). In addition, the similar values of the young leaf Pi concentration under both p_{CO2}, at 0.0027 m*M* and 0.075 m*M* P supply (Fig. 2A, page 25) would suggest that this adaptive response would not be due to a limitation by leaf Pi concentration. Such adaptation is suggested to decrease the source C capacity, avoiding an excess of photosynthates (Stitt 1991).

Taking into account (*i*) the leaf acclimation response to P and p_{CO2}, (*ii*) the leaf N concentration (Fig. 5B) and (*iii*) the leaf Pi concentration (Fig. 2A, page 24), it appears that the photosynthetic apparatus would keep the potential for further re-adjustments to C demand. Whether an increase in nodule function or growth and thus an increase in C demand would trigger the re-adjustment of leaf photosynthetic apparatus, need further investigations.

At 0.0027 m*M* P supply, from 25 to 55 DAP the starch concentration in the nodules decreased (Fig. 6A). In contrast, at 55 DAP and 0.0027 m*M* P supply, nodule WSC concentration was the highest (Fig. 6B). In addition, root WSC concentration was always higher than the correspondent nodule WSC concentration (Table 3, page 22; Fig. 6B). Assuming that the root is a source of WSC to nodules, these results suggest that at 0.0027 m*M* P supply as compared to higher P levels, nodules were the weakest sink for WSC. Increasing P supply, nodule WSC concentration decreased indicating a stronger sink that result in the maintenance of the initial value (25 DAP) of nodule's starch concentration. However, it will remain unclear whether the maintenance of the 0.0027 m*M* P supply for a longer time would result in the senescence of nodules, as the decrease of starch concentration seems to indicate.

5.4. Which mechanisms inhibit nodulation, nodule growth and %N_{sym} under low P?

As in our experiment (Table 5), a complete failure of nodulation was also observed under K deficiency (Sangakkara et al. 1996a). This effect was suggested to result from the reduction of root hair density and percentage of the taproot covered with root hairs (Sangakkara et al. 1996a). However, this may not be the case in our study since P deficiency does not affect white clover root morphology that could prevent nodulation (Haynes and Ludecke 1981). Therefore we assume that nodulation was impaired by other factors, either in the earlier stages of nodulation or in the initial stages of nodule development. The process of root infection is mediated through the balance between stimulatory and inhibitory compounds that affect the induction of *nod* gene in free-living bacteria; these compounds are secreted from white clover root tip cells (Djordjevic et al. 1987). The level of the inhibitory compound secretion is regulated by a signal from the phloem (Djordjevic et al. 1987).

When P deficiency was imposed to nodulated plants, nodule growth was inhibited (Fig. 4A). With increasing P supply, nodule and plant growth were strongly stimulated. One tempting interpretation would be that nodule growth depended directly on the P supply from the host plant; therefore, at low P, nodules would be strongly P deficient and its growth would be impaired. However, nodule P concentration is often reported to be higher than host plant (Jakbsen 1985; Israel 1987; Pereira and Bliss 1987; Adu-Gyamfi et al. 1989; Sa and Israel 1991; Israel 1993; Yahiya et al. 1995). Indeed, recent studies show that the bacteroides assimilate P by the direct contact of nodules with the nutrient solution and increase strongly P uptake rates at P deficiency, maintaining therefore a high P concentration (Al-Niemi et al. 1997; Al-Niemi et al. 1998). In view of this it is unlikely that nodule P concentration would limit nodule growth. Earlier reports give another possible view of interpretation, based on a limited C supply from the shoot to nodules. However, (*i*) the increased photosynthesis at elevated p_{CO2} had no effect on nodule growth and function, (*ii*) carbohydrate accumulated in the leaves under elevated p_{CO2}, (*iii*) the WSC concentration was higher in the root than in the nodules and (*iv*) the WSC concentration of nodules was higher at low P as compared with higher levels of P supply. Therefore, these results do not support the view of nodules C limitation. Further, these results are in contrast to earlier suggestions which tried to explain the effect of P supply on N_2 fixation by a limitation of C supply to nodules (Bonetti et al. 1984; Jakbsen 1985; Pereira and Bliss 1987; Adu-Gyamfi et al. 1989).

Alternatively, our results suggest most likely a systemic effect on nodule growth under an internal control from the host plant (Kosslak and Bohlool 1984; Olsson et al. 1989). Also nodulation was suggested to be dependent from such a similar effect (Djordjevic et al. 1987).

With increasing P supply, the mass ratio between N and P contents per plant (N/P) declined (Table 2, page 21) suggesting an increase in the N demand. Indeed, simultaneously, the N content per plant increased (Table 2, page 21) as well the $\%N_{sym}$ (Fig. 4C). In parallel to the concept of mineral N assimilation regulation (Rufty et al. 1990; Rufty et al. 1993; Marschner et al. 1996; Jeschke et al. 1997), the N_2 fixation is suggested to be regulated by a N feedback mechanism; thereby it is suggested that the increase of an N compound concentration in the phloem (and in the root) induces a decrease of N_2 fixation (Parsons et al. 1993; Heim et al. 1993; Hartwig et al. 1994; Neo and Layzell 1997). In agreement with this concept, the root N:shoot N ratio showed a significant negative correlation with $\%N_{sym}$ (Fig. 7) supporting the view of a N feedback mechanism on the N_2 fixation. Therefore, low P supply would reduce the N demand; thereby, a feedback mechanism would reduce the N assimilation, resulting in a balance between N source and N sink. Taking into account this mechanism, the inhibition of N assimilation in early stages of the symbiosis establishment would also prevent the infection and thus nodulation. The suggested increase of an N compound could then act as the control signal

leading to the increase of *nod* gene inhibitory compounds secretion by the root.

5.5. Specific N_2 fixation partly compensated low nodule growth

Elevated p_{CO2} had no effect on specific N_2 fixation (Fig. 4D). In contrast to $\%N_{sym}$ (Fig. 4C), specific N_2 fixation decline with increasing P supply. The highest value was 2.1 mg N per mg nodule DW (0.0027 m*M* P supply; Fig. 4D). This value is below the highest specific N_2 fixation reported by Zanetti et al. (1998) (3.91 mg N per mg nodule DW) in white clover nodules, with the same plant cultivar and *Rhizobium* strain. Therefore, we assume that the values obtained at 0.0027 m*M* P supply were below the maximum limit of nodules function. Since N did not limit white clover growth at 0.0027 m*M* P supply, we suggest that the increase of specific N_2 fixation in relation to the N demand, compensated the inhibition of nodule growth at low P supply. Similar increases

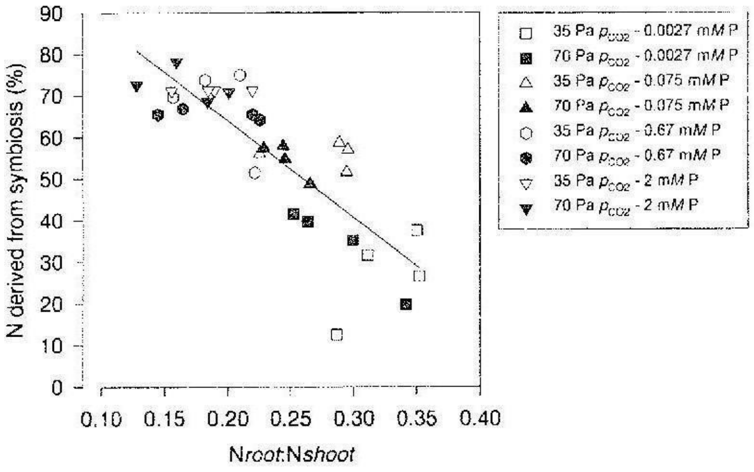

Figure 7. Correlation between root N:shoot N ratio and percentage N derived from symbiosis ($\%N_{sym}$) of white clover plants grown for 30 days (25 DAP to 55 DAP) at four different levels of P supply combined with two atmospheric p_{CO2} levels. R^2=-0.83 significant at p<0.01.

in specific N_2 fixation associated with a low nodule growth were reported in white clover following high mineral N supply (Zanetti et al. 1998) and in faba bean and common bean at K deficiency (Sangakkara et al. 1996a). However, $\%N_{sym}$ was still low at 0.0027 m*M* P supply (Fig. 4C). Therefore in view of the $\%N_{sym}$ response, specific N_2 fixation only partly compensated the low nodule growth in contrast to the fully compensation reported at K deficiency (Sangakkara et al. 1996a). This contrast between the effect of P deficiency and K deficiency on $\%N_{sym}$ was also reported by Cadisch et al. (1993) and likely derived from the different role of P and K on plant nutrition.

VI GENERAL DISCUSSION

The objective of this study was to investigate the interactive effect of P supply and atmospheric p_{CO2} on the growth of white clover, in two aspects: (*i*) the effect of the reduction of leaf transpiration rate, expected to occur under elevated p_{CO2}, on P partitioning; and (*ii*) the effect on the N_2 fixation process as affected by the increase of shoot C availability under elevated p_{CO2}.

We showed, for the first time, that the response of white clover P nutrition to elevated p_{CO2} is determined by whole plant mechanisms. This was based on the following observations:

1) Elevated p_{CO2} stimulated leaf photosynthesis at all levels of P supply (Table 3, page 22). Simultaneously, leaf transpiration rate declined under elevated p_{CO2} (Fig. 1A, page 24). This reduction in leaf transpiration caused an apparent decrease in the shoot P uptake by a decrease in the long-distance P transport rate (Fig. 1B, page 24), resulting in a change of P partitioning. Elevated p_{CO2} reduced shoot P concentration (Fig. 2, page 25), in contrast to the increased concentration of root (Fig. 3A, page 27). These changes resulted in a decline of root acid phosphatase activity under elevated p_{CO2} (Table 4, page 28).

2) The increased shoot carbon availability under elevated p_{CO2} did not affect *per se* the percent N derived from symbiosis at any level of P supply (Fig. 4C, page 42). White clover nodule function (specific N_2 fixation) showed an adjustment to nodule growth (Fig. 4A and 4D, page 42), in an apparent but clear response to plant N demand (Table 2, page 21). This effect would result in a balance between N source to N sink irrespective of P supply. Therefore, in contrast to reports presented until now, we suggest that N_2 fixation of P deficient white clover nodules is not directly limited by leaf photosynthetic rates. Alternatively, our results support the hypothesis of a N feedback mechanisms on N_2 fixation irrespective of P supply.

In addition to those conclusions, the adaptation of leaf photosynthetic apparatus to P and p_{CO2} (Table 6, page 44) bring in balance C source to C sink;

however, at low P supply white clover leaves kept the potential for re-adaptation to new situations, as an increase in the C demand.

1. THE EFFECT OF ELEVATED p_{CO2} ON TRANSPIRATION RATE HAD ASSOCIATED EFFECTS ON WHITE CLOVER NUTRITION

Our results showed that the reduction of leaf transpiration rate, in response to elevated p_{CO2} had other effects beside the generally reported increase in water use efficiency (Morison, 1985; Woodward and Bazzaz, 1988). We showed an evidence of an apparent reduction in the P long distance transport rate from root to shoot. Most likely this reduction affected not only P but also the other nutrients in the solution of the xylem stream, as it would be the case of N. However, plant growth was stimulated by p_{CO2} (Table 1, page 19). Percentage N derived from symbiosis was not affected by p_{CO2} (Fig. 4C, page 42). As a result, the whole plant N concentration declined significantly under elevated p_{CO2} (Fig. 5A, page 43) irrespective of P supply. Leaf N concentration also showed a decline under elevated as compared to ambient p_{CO2} (Fig. 5B, page 43). This averaged decline of N concentration under elevated p_{CO2} was, however, dependent from the P supply and varied with leaf age. In contrast to the leaf Pi concentration (Fig. 2, page 25), the N concentration in the youngest leaf fraction was not affect by P supply or p_{CO2} (Fig. 8A, next page); in the old leaf fraction, N concentration declined under elevated p_{CO2} and with increasing P supply (Fig. 8B). These observations, which result from the different nutrient availability to the plant (limitation of P in contrast to non-limitation of N), indicate:

1) Plant N demand seems to decrease under elevated p_{CO2} (a higher growth at a lower N concentration) as suggested by Conroy and Hocking (1993);

2) As a response to elevated p_{CO2} white clover rather changed N allocation than increase the percentage N derived from symbiosis;

3) The change of N allocation as a response of white clover to elevated p_{CO2}, seems complex:

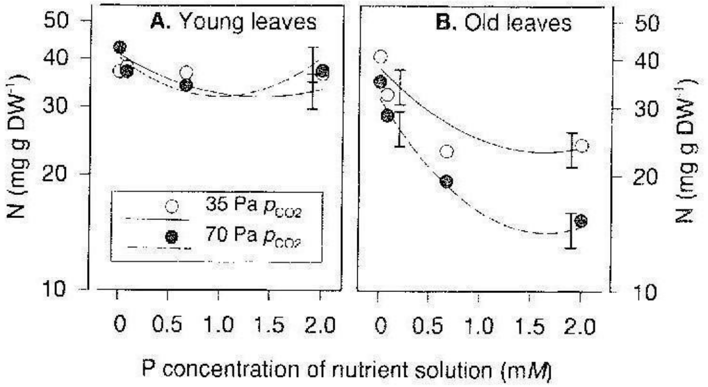

Figure 8. Leaf N concentration (on the basis of structural DW) according to leaf age (A - young; B - old; as described in chapter IV) of white clover grown for 30 days (25 DAP to 55 DAP) at 35 Pa and 70 Pa p_{CO2} combined with four levels of P supply. Lines are response curves predicted by orthogonal polynomial regressions (A and B, R^2=0.92, n=64; ± S.e. of predictions); symbols are treatment means (n=4).

a) in young leaves, N appears to be allocated from Rubisco towards other proteins within the leaf, since N concentration did not changed under elevated p_{CO2} (Fig. 8A). This would agree with the suggestions of Stitt (1991), Tissue et al. (1993) and Riviere-Rolland et al. (1996);

b) in the old leaves the decline of N (Fig. 8B) and the increase in dead leaves at low P under elevated p_{CO2} (Table 1, page 19) appears to result from an N allocation towards other sinks.

These conclusions show that different compensation mechanisms of Pi and N are operating to force the balance between the respective sources and sinks under elevated p_{CO2}. In relation to Pi, the compensation was attained by an apparent increase in P remobilization as suggested by the decrease in structural P concentration and by the increase of leaf senescing at low P (as suggested by the increase of dead leaves) under elevated p_{CO2} (Table 1, page 19). In contrast to P, N assimilation was kept at higher levels irrespective P supply but a clear N re-allocation was observed without any compensation by the symbiotic N_2 fixation.

These results suggest that under elevated p_{CO2}, nodulated white clover adapt the balance between source and sinks. This suggestion would not apply only for C, as concluded by Stitt (1991), Long and Drake (1992) and by Paul and Stitt (1993), but also for P and N, despite the different compensation mechanisms of each nutrient. If this effect would be species-specific, this could explain further the reported differences in the p_{CO2} response between white clover and other grassland species (Zanetti et al. 1996; Hebeisen et al. 1997; Lüscher et al. 1998). These results rise other questions that claim for further investigation. In the following paragraphs we will present two direct questions arising from these discussion of results.

2. DOES ELEVATED p_{CO2} INDUCE A REDUCTION OF FLOWS IN THE LONG DISTANCE TRANSPORT?

The primary effect of elevated p_{CO2} on white clover was a decrease in stomatal conductivity and a concomitant reduction of leaf transpiration rate. This response would cause a decline in the long-distance flow from root to shoot. In addition, except at very high P supply, the afternoon values of leaf photosynthesis, under elevated p_{CO2}, lost the morning stimulation (Table 3, page 22). In the discussion (page 31) one open question remained about a possible limitation in the export of photosynthates from the leaf. Taking these results together, one possible view is that the apparent reduction in the transport flow does not match with the increased demand of photosynthate export from the leaf. For a better understanding of this mechanism further investigations are necessary:

1) To assess directly the transport flows. This could be achieved by the use of isotopes, i.e. ^{33}P or ^{32}P and ^{14}C, in relation to a non-mobile nutrient. Extensive studies in the transport of solutes were presented by Pate et al. (1979), Cakmak et al. (1994a), Cakmak et al. (1994b), Jeschke et al. (1996) and Jeschke et al. (1997)

2) To assess the effect of the K supply level on the diurnal rate of CO_2 uptake associated with export rates of photosynthates from the leaf. We assume that, beside other factors, K is an important factor for phloem transport flow (Cakmak et al. 1994a; 1994b). The effect of K in the transport, photosynthesis and osmoregulation was extensively reviewed by Marschner (1995).

The results of these investigations are important for further understanding the response of white clover to elevated p_{CO2}. The interactive effect of K supply and p_{CO2} was never reported. The results of these studies may be particularly relevant at the field level because of the limited K availability in many soils, its role in plant nutrition, particularly in the transport process, and its interaction with other nutrients.

3. DOES ELEVATED p_{CO2} AFFECTS LEAF SENESCENCE?

3.1. Leaf physiological age

Under elevated p_{CO2}, Pi and N concentrations were strongly reduced particulary in the old leaf fractions. This effect was also reported for other *Trifolium* species in relation to P (Morin et al. 1992; Duchein et al. 1993). In relation to N compounds, the decrease of Rubisco concentration under elevated p_{CO2} is a general feature reported for several species and mainly under conditions of nutrient limitation (reviewed by Bowes, 1991). We discussed the reduction of N and P leaf concentrations in view of a compensation for the reduced transport flows. However, in an earlier study, Omer and Horvath (1983) reported a reduction in the cycle length of several annual species under elevated p_{CO2}, and suggested that this effect would result from earlier plant senescence under elevated p_{CO2}. Beside, it was reported that the decrease of Rubisco under elevated p_{CO2} was stronger with increasing leaf and plant development (Nie et al. 1995; Hibberd et al. 1996). In addition to this decrease of Rubisco during leaf development, thylakoid proteins were reduced under elevated p_{CO2} in the fully mature leaves (Van Oosten and Besford 1995). Indeed, these results are indicative of earlier leaf senescence under elevated p_{CO_2} (Nie et al. 1995).

The Pi and N old leaf concentrations could agree with this suggestion. Therefore, this effect claims for further investigation. Under field conditions this effect may have particular importance, so we will develop further this view in the next paragraph.

3.2. Field research

At P deficiency we reported an increase of dead leaves under elevated p_{CO_2}, representing 43% of total leaf dry mass in the end of 30 days of growth (Table 1, page 19). Under field conditions, such an increase in death tissue may be relevant because it may represent an important loss of harvestable biomass in terms of quantity and quality, since leaves are the most valuable fraction. In addition, the effects of P partitioning on root acid phosphatase activity may be important in conditions of limited soil P availability. Therefore, these effects need to be evaluated under field conditions and perhaps it would allow to explain some of the different responses to elevated p_{CO_2} between "nutrient rich" *vs* "nutrient poor" grassland ecosystems (Zanetti et al. 1996; Hebeisen at al. 1997; Stöcklin et al. 1998).

The results of this investigation are relevant at the field level and they would contribute to the increase of our understanding about the impact of elevated p_{CO_2} on grassland ecosystems.

VII LITERATURE CITED

Adu-Gyamfi JJ, Fujita K and Ogata S (1989) Phosphorus absortion and utilization efficiency of pigeon pea (*Cajanus cajan* (L) Millsp.) in relation to dry matter production and dinitrogen fixation. Plant and Soil 119, 315-324

Al-Niemi TS, Kahn ML and McDermott TR (1997) P metabolism in the Bean-*Rhizobium tropici* Symbiosis. Plant Physiology 113, 1233-1242

Al-Niemi TS, Kahn ML and McDermott TR (1998) Phosphorus uptake by bean nodules. Plant and Soil 198, 71-78

Banet G, Wininger S, Badani H, Ben-Dor B, Friedman Y and Kapulnik Y (1996) Toxic and osmotic effects of salinity on growth and nodulation of *Medicago sativa*. Symbiosis 21, 209-222.

Barret DJ, Richardson AE and Gifford RM (1998) Elevated atmospheric CO_2 concentrations increase wheat root phosphatase activity when growth is limited by phosphorus. Australian Journal of Plant Physiology 25, 87-93.

Boller BC and Nösberger J (1987) Symbiotically fixed nitrogen from field-grown white clover and red clover mixed with ryegrasses at low levels of ^{15}N-fertilization. Plant and Soil 104, 219-226.

Bonetti R, Montanheiro MNS and Saito SMT (1984) The effects of phosphate and soil moisture on the nodulation and growth of *Phaseolus vulgaris*. Journal of Agricultural Science 103, 95-102.

Bosse D and Köck M (1998) Influence of phosphate starvation on phosphohydrolases during development of tomato seedlings. Plant, Cell and Environment 21, 325-332.

Bowes G (1991) Growth at elevated CO_2: photosynthetic responses mediated through rubisco. Plant Cell and Environment 14, 795-806.

Bowes G (1993) Facing the inevitable: Plants and increasing atmospheric CO_2. Annual Review of Plant Physiology and Plant Molecular Biology 44, 309-332.

Brooks A (1986) Effects of phosphorus nutrition on ribulose-1,5-biphosphate carboxylase activation, photosynthetic quantum yield and amounts of some Calvin-cycle metabolites in spinach leaves. Australian Journal of Plant Physiology 13, 221-237.

Cadisch G, Sylvester-Bradley R and Nösberger J (1989) ^{15}N-Based estimation of nitrogen fixation by eight tropical forage-legumes at two levels of P:K supply. Field Crops Research 22, 181-194.

Cadisch G, Sylvester-Bradley R, Boller BC and Nösberger J (1993) Effect of phosphorus and potassium on N$_2$ fixation (^{15}N-dilution) of field-grown *Centrosema acutifolium* and *C. macrocarpum*. Field Crops Research 31, 329-340.

Cakmak I, Hengeler C and Marschner H (1994a) Changes in phloem export of sucrose in leaves in response to phosphorus, potassium and magnesium deficiency in bean plants. Journal of Experimental Botany 45, 1251-1257.

Cakmak I, Hengeler C and Marschner H (1994b) Partitioning of shoot and root dry matter and carbohydrates in bean plants suffering from phosphorus, potassium and magnesium deficiency. Journal of Experimental Botany 45, 1245-1250.

Caradus JR (1980) Distinguishing between grass and legume species for efficiency of phosphorus use. New Zealand Journal of Agricultural Research 23, 75-81.

Caradus JR (1990) The structure and function of white clover root systems. Advances in Agronomy 43, 1-46.

Caradus JR and Snaydon RW (1987) Aspects of the phosphorus nutrition of white clover populations. II Root exocellular acid phosphatase activity. Journal of Plant Nutrition 10, 287-301.

Conroy J and Hocking P (1993) Nitrogen nutrition of C$_3$ plants at elevated atmospheric CO$_2$ concentrations. Physiologia Plantarum 89, 570-576.

Diaz S, Grime JP, Harris J and McPherson E (1993) Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. Nature 364, 616-617.

Djordjevic MA, Redmond JW, Batley M and Rolfe BG (1987) Clover secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii*. The EMBO journal 5, 1173-1179.

Dracup MNH, Barret-Lennard EG, Greenway H and Robson AD (1984) Effect of phosphorus deficiency on phosphatase activity of cell walls from roots of *subterranean clover*. Journal of Experimental Botany 35, 466-480.

Duchein M, Bonicel A and Betsche T (1993) Photosynthetic net CO$_2$ uptake and leaf phosphate concentrations on CO$_2$ enriched clover (*Trifolium subterraneum* L.) at three levels of phosphate nutrition. Journal of Experimental Botany 44, 17-22.

Dunlop J and Bowling DJF (1978) Uptake of phosphate by white clover. II The effect of pH on the electrogenic phosphate pump. Journal of Experimental Botany 29, 1147-1153.

Dunlop J and Hart AD (1987) Mineral nutrition. *In*.: MJ Baker and WM Williams, eds, White Clover. CAB International, Wallingford, Oxon, UK, pp 1-30.

Farage PK, McKee IF and Long SP (1998) Does a low nitrogen supply necessarily lead to acclimation of photosynthesis to elevated CO$_2$? Plant Physiology 118, 573-580.

Farquhar GD, Caemmerer S and Berry JA (1980). A biochemical model of photosynthetic CO$_2$ assimilation in leaves of C3 species. Planta 149, 78-90.

Farquhar GD and Caemmerer S (1982) Modelling of photosynthetic response to environmental conditions. *In:* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology II. Water Relations and Carbon Assimilation. Spring-Verlag, Berlin, D, pp 549-588.

Farrar JF and Williams ML (1991) The effect of increased carbon dioxide and temperature on carbon partitioning, source-sink relations and respiration. Plant, Cell and Environment 14, 819-830.

Finn GA and Brun WA (1982) Effect of atmospheric CO$_2$ enrichment on growth, nonstructural carbohydrate content, and root nodule activity in soybean. Plant Physiology 69, 327-331.

Fischer BU, Frehner M, Hebeisen T, Zanetti S, Stadelman F, Lüscher A, Hartwig UA, Hendrey GR, Blum H and Nösberger J (1997) Source-sink relations in *Lolium perenne* L. as reflected by carbohydrate concentrations in leaves and pseudo-stems during regrowth in a free air carbon dioxide enrichment (FACE) experiment. Plant, Cell and Environment 20, 945-952.

Flügge UI, Freisl M and Heldt HW (1980) Balance between metabolite accumulation and transport in relation to photosynthesis by isolated spinach chloroplasts. Plant Physiology 65, 574-577.

Freeden AL, Rao IM and Terry N (1989) Influence of phosphorus nutrition on growth and carbon partitioning in *Glycine max*. Plant Physiology 89, 225-230.

Gahoonia TS and Nielsen NE (1998) Direct evidence on participation of root hairs in phosphorus (^{32}P) uptake from soil. Plant and Soil 198, 147-152.

Gordon AJ, Minchin FR, Skot L. and James CL (1997) Stressed-induced decline in soybean N$_2$ fixation are related to nodule sucrose synthase activity. Plant Physiology 114, 937-946.

Goudrian J and De Ruiter HE (1983) Plant growth in response to CO$_2$ enrichment, at two levels of nitrogen and phosphorus supply. 1. Dry matter, leaf area and development. Netherlands Journal of Agricultural Science 31, 157-169.

Hammer PA, Tibbits TW, Langhans RW and McFarlane JC (1978) Base-line Growth Studies of "Grand Rapids" Lettuce in Controlled Environments. Journal of the American Society for Horticultural Science 103, 649-655.

Hart AD (1987) Physiology. *In:* MJ Baker and WM Williams, eds, White Clover. CAB International, Wallingford, Oxon, UK. pp 125-147.

Hartwig UA, Boller BC, Baur-Höch B and Nösberger J (1990) The influence of carbohydrates reserves on the response of nodulated white clover to defoliation. Annals of Botany 65, 97-105.

Hartwig UA, Heim I, Lüscher A and Nösberger J (1994) The nitrogen-sink is involved in the regulation of nitrogenase activity in white clover after defoliation. Physiologia Plantarum 92, 375-382.

Hartwig UA, Zanetti S, Hebeisen T, Lüscher A, Frehner M, Fischer BU, Van Kessel C, Hendrey R, Blum H and Nösberger J (1996) Symbiotic nitrogen fixation: one key to understand the response of temperate grassland ecosystems to elevated CO$_2$. *In:* C Köner and FA Bazzaz, eds, Carbon dioxide, populations, and communities. Academic Press, London, UK, pp 253-264.

Haynes RJ and Ludecke TE (1981) Yield, morphology and chemical composition of two pasture legumes as affected by lime and phosphorus applications to an acid soil. Plant and Soil 62, 241-254.

Hebeisen T, Lüscher A, Zanetti S, Fischer BU, Hartwig UA, Frehner M, Hendrey R, Blum H and Nösberger J (1997) Growth response of *Trifolium repens* L. and *Lolium perenne* L. as monocultures and bi-species mixture to free air CO$_2$ enrichment and management. Global Change Biology 3, 149-160.

Heim I, Hartwig UA and Nösberger J (1993) Current nitrogen fixation is involved in the regulation of nitrogenase activity in white clover (*Trifolium repens* L). Plant Physiology 103, 1009-1014.

Hibberd JM, Richardson P, Whitbread R and Farrar JF (1996) Effects of leaf age, basal meristem and infection with powdery mildew on photosynthesis in barley grown in 700 μmol mol^{-1} CO$_2$. New Phytologist 134, 317-325.

Ingestad T (1982) Relative addition rate and external concentration: driving variables used in plant nutrition research. Plant, Cell and Environment 5, 443-453.

Israel DW (1987) Investigation of the Role of Phosphorus in Symbiotic Dinitrogen Fixation. Plant Physiology 84, 835-840.

Israel DW (1993) Symbiotic dinitrogen fixation and host-plant growth during development of and recovery from phosphorus deficiency. Physiologia Plantarum 88, 294-300.

Jakobsen I (1985) The role of phosphorus in the nitrogen fixation by young pea plants (*Pisum sativum*). Physiologia Plantarum 64, 190-196.

Jeschke DW, Peuke A, Kirkby A, Pate JS and Hartung W (1996) Effects of P deficiency on the uptake, flows of C, N and H_2O within intact plants of *Ricinus communis* L. Journal of Experimental Botany 47, 1737-1754.

Jeschke DW, Kirkby A, Peuke A, Pate JS and Hartung W (1997) Effects of P deficiency on assimilation and transport of nitrate and phosphate in intact plants of castor bean (*Ricinus communis* L.). Journal of Experimental Botany 48, 75-91.

Johnson JF, Vance CP and Allan D (1996) Phosphorus deficiency in *Lupinus albus*: Altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. Plant Physioloy 112, 31-41.

Junk AO (1996) Dynamics of nutrient movement at the soil-root interface. *In:* Y Waisel, A Eshel and U Kafkafi, eds, Plant Roots. The hidden half.. Marcel Dekker, Inc., New York, USA, pp 529-556.

Kimbal B (1983) Carbon dioxide and agricultural yield: an assemblage and analysis of 430 prior observations. Agronomy Journal 75, 779-788.

Kirschbaum MUF and Farquhar GD (1984) Temperature dependence of whole-leaf photosynthesis in *Eucalyptus pauciflora* Sieb. ex Spreng. Australian Journal of Plant Physiology 11, 519-538.

Kossiak RM and Bohlool BB (1984) Supression of nodule development of one side of split-root system of soybeans caused by prior inoculation of the other side. Plant Physiology 75, 125-130.

Lauer MJ, Pallardy SG, Blevins DG and Randall DD (1989) Whole leaf carbon exchange characteristics of phosphate deficient soybeans (*Glycine max* L.). Plant Physioloy 91, 848-854.

Long SP and Drake BG (1992) Photosynthetic CO_2 assimilation and rising atmospheric CO_2 concentrations. *In:* NR Baker and H Thomas, eds, Crop Photosynthesis: Spatial and Temporal Determinants. Elsevier Science Publishers, Amsterdam, NL, pp 69-103.

Lüscher A, Hebeisen T, Zanetti S, Hartwig UA, Blum H, Hendrey GR, and Nösberger J (1996) Differences between legumes and nonlegumes of permanent grasslands in their responses to free air CO_2 enrichment: Its effect on competition in a multispecies mixture. *In:* C Köner and FA Bazzaz, eds, Carbon dioxide, populations, and communities. Academic Press, London. UK, pp 287-300.

Lüscher A, Hendrey GR and Nösberger J (1998) Long-term responsiveness to free air CO_2 enrichment of functional types, species and genotypes of plants from fertile permanent grassland. Oecologia 113, 37-45.

Marschner H (1995) Mineral Nutrition of Higher Plants. 2nd ed. Academic Press, London, UK. 889 p.

Marschner H, Kirkby EA and Cakmak I (1996) Effect of mineral nutritional status on shoot-root partitioning of photoassimilates and cycling of mineral nutrients. Journal of Experimental Botany 47, 1255-1263.

Marschner H, Kirkby EA and Engels C (1997) Importance of Cycling and Recycling of Mineral Nutrients within Plants for Growth and Development. Botanica Acta 110, 265-273.

Minchin FR and Pate JS (1973) The carbon balance of a legume and the functional economy of its root nodules. Journal of Experimental Botany 24, 259-271.

Morin F, André M and Betsche T (1992) Growth kinetics, carbohydrate, and leaf phosphate content of clover (*Trifolium subterraneum* L.) after transfer to a high CO_2 atmosphere or to high light and ambient air. Plant Physiology 99, 89-95.

Morison JIL (1985) Sensitivity of stomata and water use efficiency to high CO_2. Plant, Cell and Environment 8, 467-474.

Morison JIL and Batten GD (1986) Regulation of mesophyl photosynthesis in intact wheat leaves by cytoplasmic phosphate concentrations. Planta 168, 200-206.

Neo HH and Layzell DB (1997) Phloem glutamine and the regulation of O_2 diffusion in legume nodules. Plant Physiology 113, 259-267.

Nie GY, Hendrix DL, Webber AN, Kimbal BA, and Long SP (1995) Increased accumulation of carbohydrates and decreased photosynthetic gene transcript levels in wheat grown at an elevated CO_2 concentration in the field. Plant Physiology 108, 975-983.

Nie GY, Long SP, Garcia RL, Kimball BA, Lamorte RL, Pinter PJ Jr, Wall GW and Webber AN (1995) Effects of free-air CO_2 enrichment on the development of the photosynthetic apparatus in wheat, as indicated by changes in leaf proteins. Plant Cell and Environment 18, 855-864.

Noat G, Crasnier M and Ricard J (1980) Ionic control of acid phosphatase activity in plant cell walls. Plant, Cell and Environment 3, 225-229.

Olsson JE, Nakao P, Bohlool BB and Gresshoff PM (1989) Lack of Systematic Supression of Nodulation in Split Root Systems of Supernodulating Soybean (*Glycine Max* (L.) Merr.) Mutants. Plant Physiology 90, 1347-1352.

Omer LS and Horvath SM (1983) Elevated carbon dioxide concentrations and whole plant senescence. Ecology 64, 1311-1314.

Panara F, Pasqualini S and Anonielli M (1990) Multiple forms of barley root acid phosphatase: purification ans some characteristics of the major cytoplasmic isoenzyme. Biochimica et Biophysica Acta 1037, 73-80.

Parsons R, Stanforth A, Raven JA and Sprent JI (1993) Nodule growth and activity may be regulated by a feedback mechanism involving phloem nitrogen. Plant Cell and Environment 16, 125-136.

Pate JS, Atkins CA, Hamel K, McNeil DL and Layzell DB (1979) Transport of organic solutes in phloem and xylem of a nodulated legume. Plant Physiology 63, 1082-1088.

Paul MJ and Stitt M (1993) Effects of nitrogen and phosphorus deficiencies on levels of carbohydrates, respiratory enzymes and metabolites in seedlings of tobacco and their response to exogenous sucrose. Plant Cell and Environment 16, 1047-1057.

Pereira PAA and Bliss FA (1987) Nitrogen fixation and plant growth of common bean (*Phaseolus vulgaris* L.) at different levels of phosphorus availability. Plant and Soil 104, 79-84.

Purwantari ND, Date RA and Dart PJ (1995) Nodulation and N_2 fixation by *Calliandra calothyrsus* and *Sesbania sesban* grown at different root temperatures. Soil Biology and Biochemistry 27, 421-425.

Rao IM, Freeden AL and Terry N (1990) Leaf Phosphate Status, Photosynthesis, and carbon partitioning in Sugar Beet. III. Diurnal changes in carbon partitioning and carbon export. Plant Physiology 92, 29-36.

Rao IM (1997) The role of phosphorus in photosynthesis. *In:* M Pessarakli, eds, Handbook of Photosynthesis. Marcel Dekker Inc., New York, US, pp 173-194.

Richardson AE, Djordjevic MA, Rolfe BG and Simpson RJ (1988) Effects of pH, Ca and Al on the exudation from clover seedlings of compounds that induce the expression of nodulation genes in *Rhizobium trifolii*. Plant and Soil 109, 37-47.

Riviere-Rolland H, Contard P and Betsche T (1996) Adaptation of pea to elevated atmospheric CO_2: Rubisco, phosphoenolpyruvate carboxylase and chloroplast phosphate translocator at different levels of nitrogen and phosphorus nutrition. Plant Cell and Environment 19, 109-117.

Robson AD, O'Hara GW and Abbott LK (1981) Involvement of phosphorus in nitrogen fixation by subterranean clover (*Trifolium subterraneum* L.). Australian Journal of Plant Physiology 8, 427-436.

Rogers G, Payne L, Milham PJ and Conroy JP (1993) Nitrogen and phosphorus requirements of cotton and wheat under changing atmospheric concentrations. Plant and Soil 155/156, 231-234.

Rufty TW Jr, MacKown CT and Israel DW (1990) Phosphorus Stress Effects on Assimilation of Nitrate. Plant Physiology 94, 328-333.

Rufty TW Jr, Israel DW, Volk RJ, Qiu J and Sa TM (1993) Phosphate regulation of nitrate assimilation in soybean. Journal of Experimental Botany 44, 879-891.

Sa TM and Israel DW (1991) Energy Status and Functioning of Phosphorus-Deficient Soybean Nodules. Plant Physiology 97, 928-935.

Sangakkara UR, Hartwig UA and Nösberger J (1996a) Soil moisture and potassium affect the performance of symbiotic nitrogen fixation in faba bean and common bean. Plant and Soil 184, 123-130.

Sangakkara U R, Hartwig U A and Nösberger J (1996b) Growth and symbiotic nitrogen fixation of *Vicia faba* and *Phaseolus vulgaris* as affected by fertilizer potassium and temperature. Journal of the Science of Food and Agriculture 70, 315-320.

Schachtman D , Reid RJ and Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant Physiology 116, 447-453.

Schortemeyer M, Hartwig UA, Hendrey GR and Sadowsky MJ (1996). Microbial community changes in the rhizospheres of white clover and perennial ryegrass exposed to free air carbon dioxide enrichment (FACE). Soil Biology and Biochemistry 28, 1717-1724.

Sharkey TD (1985) O_2-insensitive photosynthesis in C3 plants. Its occurrence and a possible explanation. Plant Physiology 78, 71-75.

Stitt M (1991) Rising CO_2 levels and their potential significance for carbon flow in photosynthetic cells. Plant, Cell and Environment 14, 741-762.

Stöcklin J, Schweizer K and Körner C (1998) Effects of elevated CO_2 and phosphorus addition on productivity and community composition of intact monoliths from calcareous grassland. Oecologia 116, 50-56.

Terry N and Ulrich A (1973) Effects of Phosphorus Deficiency on the Photosynthesis and Respiration of Leaves of Sugar Beet. Plant Physiology 51, 43-47.

Tiessen H and Moir JO (1993) Characterization of available P by sequential extraction. *In:* MR Carter, ed, Soil Sampling and Methods of Analysis. Lewis Publishers, Boca Raton, USA, pp 75-86.

Tissue DT, Thomas RB and Strain BR (1993) Long-term effects of elevated CO_2 on photosynthesis and rubisco in loblolly pine seedlings. Plant Cell and Environment 16, 859-865.

Usuda H and Edwards GE (1982) Influence of varying CO_2 and orthophosphate concentrations on rates of photosynthesis, and synthesis of glycolate and dihydroxyacetone phosphate by wheat chloroplasts. Plant Physiology 69, 469-473.

Van Oosten JJ and Besford RT (1995) Some relationships between the gas exchange, biochemistry and molecular biology of photosynthesis during leaf development of tomato plants after transfer to different carbon dioxide concentrations. Plant Cell and Environment 18, 1253-1266.

Warembourg FR and Roumet C (1989) Why and how to estimate the cost of symbiotic N_2 fixation? A progressive approach based on the use of ^{14}C and ^{15}N isotopes. Plant and Soil 115, 167-177.

Weisbach C, Hartwig UA, Heim I and Nösberger J (1996) Whole nodule carbon metabolites are not involved in the regulation of the oxygen permeability and nitrogenase activity in white clover nodules. Plant Physiology 110, 539-545.

Williams LE, DeJong TM and Phillips DA (1982) Effect of Changes in Soot Carbon-Exchange Rate on Soybean Root Nodule Activity. Plant Physiology 69, 432-436.

Woodward FI and Bazzaz FA (1988) The responses of stomatal density to CO_2 partial pressure. Journal of Experimental Botany 39, 1771-1781.

Yahiya M, Samiullah and Fatma A (1995) Influence of phosphorus on nitrogen fixation in chikpea cultivars. Journal of Plant Nutrition 18, 719-727.

Zanetti S, Hartwig UA, Lüscher A, Hebeisen T, Frehner M, Fischer BU, Hendrey GR, Blum H and Nösberger J (1996) Stimulation of symbiotic N_2 fixation in

Trifolium repens L. under elevated atmospheric pCO_2 in a grassland ecosystem. Plant Physiology 112, 575-583.

Zanetti S, Hartwig UA, vanKessel C, Lüscher A, Hebeisen T, Frehner M, Fischer BU, Hendrey GR, Blum H and Nösberger J (1997) Does nitrogen nutrition restrict the CO_2 response of fertile grassland lacking legumes? Oecologia 112, 17-22.

Zanetti S, Hartwig UA and Nösberger J (1998) Elevated atmospheric CO_2 does not affect *per se* the preference for symbiotic nitrogen as opposed to mineral nitrogen of *Trifolium repens* L. Plant Cell and Environment 21, 623-630.

CURRICULUM VITAE

27 August 1960	Born in Lisboa, Portugal, son of Maria do Rosário and João Manuel Fragoso de Almeida
1966-1970	Primary school in Lisboa, Externato Luso-Britânico
1970-1978	Secondary school in Lisboa, Escola Preparatória Eugénio dos Santos and Liceu Padre António Vieira
1978-1983	Dipl. Animal Sciences (Eng. Zootécnica) University of Évora (Portugal)
1983-1984	Research Assistant in the Department of Pastures and Forages from the National Station of Plant Breeding (ENMP, Elvas, Portugal)
since 1984	Assistant for Pastures and Forage crops, in the Escola Superior Agrária from the Instituto Politécnico of Castelo Branco (Portugal)
1989	M.Sc. in Animal Production, Faculty of Veterinary from The University of Technology (Lisboa, Portugal)
since 1990	Senior Lecturer for Pastures and Forage crops, in the Escola Superior Agrária from the Instituto Politécnico of Castelo Branco (Portugal)
since 1995	Ph.D. studies at the Swiss Federal Institute of Technology, Zürich, Institute of Plant Sciences.

ACKNOWLEDGEMENTS

My sincere gratitude and acknowledgements to Prof. Dr. Josef Nösberger, for giving me the chance to work in his group, for his invaluable professional and scientific guidance, for his tolerance and for the good motivations during this time. Thank you for all.

Through Prof. João Pedro Várzea, my Director and old friend, I would like to convey my gratitude to ESACB for giving me the chance to study in Switzerland for these three years. In the bad times your motivations were always a strong reason for continuing. O meu Bem-Hajas, João Pedro.

To my colleagues and dear friends, Prof. Luis Peças, Prof. Filipe Carreiro, Prof. Carlos Andrade, and Eng. António Galvão for replacing me in my lectures and tasks for all this time. It was a very nice example of friendship. To my friend Eng. Rui T. Monteiro, for the printing and layout of the tesis. Also, I must thank all the other colleagues from ESACB for the encouragement. O meu Bem-Hajam a todos.

My sincere gratitude to the Fundação Calouste Gulbenkian, Lisboa, Portugal, for the grant to study at the ETH. Thank you very much.

I am sincerely grateful to Prof. Dr. Emmanuel Frossard, for consenting to be my co-examiner, for allowing me to work in his group, for his concern, discussions and for "einen Hauch Mittelmeer..."! Through Prof. Frossard I would like to convey my gratitude to all friends from "Pflanzenernährung" group. Specially thanks to Dr. Astrid Oberson and to Monica Langmeyer, Oula Trouré and Alain Gaume, my lab-companions.

Thanks a lot, Dr. Marco Frehner, for the constant support during my lab work. You had two difficult tasks: the "Ciras-sing" and the "Skiing"... I am sorry to disappoint you with the second one!

Thanks to Dr. Ueli A. Hartwig for the nice discussions on BNF.

Thanks to Dr. Andreas Lüscher for all the support.

I am sincerely grateful to "my Prof. title I left on the toilet of the airport" Ravi Sangakkara, for all discussions, criticism, corrections and especially, above that, for your friendship!

To my friends. A very special and warm "Obrigado" to the crew of "TLOTS" (do you still remember what that means?): Satya Pasumarty, Mischi-Muschick, Paola Curioni, Hubert Isopp and Brenda Lauterbach. We sure did a good effort by "diluting" someone's complains in a couple of beers, talks and laughs...! A special thanks for Werner Wild; I think you cope well in this group also!

To the FACE friends, Markus Daepp, Dani Suter and Kaspar Rüegg, for all the "CO_2 effects"...! To Bernt Fischer, for so many nice discussions help and friendship. To Irmi Kuhn, Jeanette Habenczius and to Anni Dürsteler. To all members of "Nö" group, **MANY THANKS**!

Last, but not least... to my wife Jana and to my very best friends David and Jan! I have no words to thank your "paciência", tolerance and Love! To you I owe so much of this... "Bem-Hajam"!