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Regulation of phosphate deficiency-induced carboxylate exudation in cluster roots of white lupin (*Lupinus albus* L.)

Dissertation

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Table of contents

Page

General introduction 1-10
Phosphorus availability in soils 1
Mechanisms of P acquisition in higher plants 1
Perspectives to improve P nutrition in crop plants 6
Fertilization management and cropping systems 6
Selection and breeding of P-efficient plants 7
Lupinus albus – a model plant to study chemical P acquisition in plants 9

General Methods 11-13
Plant cultivation and harvest 11
Determination of organic acids by HPLC 12
Root tissue concentrations 12
Root exudate collection 12
Conditions for HPLC 12
Statistics 13

Chapter 1: Phosphorus deficiency-induced alterations of organic acid metabolism during cluster root development

Introduction 14-21
Phosphorus deficiency-induced biosynthesis of carboxylates 14
Phosphorus deficiency-induced inhibition of citrate turnover 17
Inhibition of aconitase 17
Reduced assimilation of nitrate with 2-oxoglutarate as N acceptor 17
ATP-citrate lyase 18
Reduced respiration 19

Materials and methods 22-34
Enzyme assays 22
PEP-C immunodetection 22
Pyruvate determination 22
Malic enzyme (ME) activity determination 24
Aconitase and $NADP^+$-Isocitrate-Dehydrogenase (NADP-ICDH) activity assay 25
Hydrogen-peroxide (H_2O_2) determination 26
Malondialdehyde determination 27
Histology of dehydrogenase activities 28
Respiration 28
Root O_2 uptake 28
AOX Western Blot analysis 29
ATP-citrate lyase assay 32
Inhibitor treatments 33

Results 35-59
Phosphorus deficiency-induced biosynthesis of carboxylates 35
Carboxylate accumulation and metabolic activity status in different developmental stages of cluster roots 35
Malic enzyme activity 37
Pyruvate concentrations 38
Phosphorus deficiency-induced inhibition of citrate turnover 39
Inhibition of Aconitase and NADP-dependent isocitrate-dehydrogenase (NADP-ICDH) 39
H_2O_2 – a natural inhibitor of aconitase in P-deficient white lupin ? 40
Peroxide (H_2O_2) concentrations 40
Malondialdehyde (MDA) concentrations 41
Effects of the external application of aconitase inhibitors on accumulation and root exudation of citrate 42
Inhibition of the enzyme aconitase by hydrogen peroxide (H_2O_2) 42
Inhibition of the enzyme aconitase by monofluoroacetate (MFA) and its influence on carboxylate accumulation in young cluster roots 44
Inhibition of the enzyme aconitase by monofluoroacetate (MFA) and its influence on carboxylate exudation from young cluster roots 45
Inhibition of the enzyme aconitase by monofluoroacetate (MFA) and its influence on carboxylate exudation from P-sufficient seedling root tips 46
Inhibition of the enzyme aconitase by monofluoroacetate (MFA) and its influence on aconitase and NADP-ICDH activities 47
In situ activity of dehydrogenases involved in citrate turnover 49
Reduced respiration 51
Respiration rates and cytochrome and alternative oxidase (AOX) capacities 51
Western Blot analysis 52
Uncoupling of respiration 53
Oxidative damage of the respiratory chain ? 54
Partial root incubation with respiration inhibitors 55
Reduced assimilation of nitrate into 2-oxoglutarate as N acceptor ? 56
ATP-citrate lyase (ACL) activity 58
Inhibition of the citrate cleaving enzyme ATP-citrate lyase (ACL) 59

Discussion 60-82
Increased biosynthesis of organic acids under P-deficient conditions 60
Phospho*enol*pyruvate carboxylase (PEP-C) activity 60
Citrate synthase (CS) activity 61
Pyruvate concentration and malic enzyme (ME) activity 61
Reduced turnover of citrate under P-deficient conditions 62
Reduced aconitase activity 63
Reduced activity of dehydrogenases in the TCA cycle 64
NADP-dependent isocitrate dehydrogenase (NADP-ICDH) activity 64
In-situ staining of dehydrogenase (DH) activities 65
Reduced respiration 66
Reduced nitrate assimilation 68
Reduced ATP-citrate lyase activity 69

Factors determining reduced citrate turnover 71
Reduced activity of aconitase 72
Inhibition of aconitase by H_2O_2 production ?.......... 72
Nitric oxide (NO) as an aconitase inhibitor ? 73
Substrate limitation of respiration ? 74
P deficiency-induced oxidative damage ? 75
Citrate accumulation as related to artificial inhibition of selected metabolic sequences 77
Influence of an inhibited respiration by azide or SHAM on citrate tissue concentrations 77
Inhibition of the aconitase enzyme by external application of MFA 78
Inhibition of nitrate reductase (NR) by tungstate 79
Inhibition of the ATP-citrate lyase by external application of hydroxycitrate 80
Conclusions and outlook 80

Chapter 2: Mechanisms of citrate export in cluster roots

Introduction 82-85

Materials and methods 86-95
Vesicle isolation and characterization.......... 86
Citrate transport across vesicle membranes 91
Acidification of the cytosol 92
Isolation of protoplasts from mature cluster roots and root tips 93
Isolation of root hair protoplasts from seedlings and cluster roots 94

Results 96-108
Purification of plasma membrane vesicles 96
Characterization of the plasma membrane H^+-ATPase in roots of white lupin as related to the P-nutritional status 97
Alterations of plasma membrane H^+-ATPase activity during cluster root development 97
ATP hydrolysis 99
pH optimum 100
Plasma membrane H^+-ATPase activity as affected by carboxylates 101
Proton transport across membranes 102
^{14}C citrate transport into inside-out vesicles 103
Carboxylate exudation and intracellular pH 104

Protoplast isolation 106
Protoplast isolation from mature cluster root laterals 106
Protoplast isolation from root tips 107
Protoplast isolation from root hairs 107

Discussion 109-119
Plasma membrane H^+-ATPase characterization 109
Characterization of the root H^+-ATPase as related to the P-nutritional status 109
Plasma membrane H^+-ATPase activity in different cluster root segments . 110
Plasma membrane H^+-ATPase activity depending on the external pH........ 111
Plasma membrane H^+-ATPase activity as affected by carboxylates 112
Citrate transport across the plasma membrane 114
Protoplast isolation from mature cluster root laterals 115
Regulation of the citrate release mechanism in cluster roots of P-deficient white lupin 116
Conclusions 119

Chapter 3: Role of modifications of atmospheric CO_2 concentrations on root exudation and rhizosphere processes in cluster roots

Introduction 120-122

Materials and methods 123-127
Plant cultivation and harvest 123
Root exudate collection and determination of organic acids 124
Plants cultivated in nutrient solution 124
Determination of ortho-phosphate in root tissue and shoot tips 124
Plants cultivated in rhizoboxes 125
Enzymatic citrate determination 125
Determination of total phosphate in root and shoot tissue 126
Determination of acid and alkaline phosphatase activities in rhizosphere soil 127

Results 128-134
Plants grown in nutrient solution 128
Plant growth and development 128
Cluster root development 130
Cluster root function 132
Plants grown in rhizoboxes 135
Plant growth and development 135
Cluster root development 138
Cluster root function 138
Citrate exudation 138
Phosphatase activity 139
Phosphate concentrations and contents 140

Discussion 141-150
Plant growth and development 141
Cluster root development 143
Function of cluster roots 143
Activity of phosphatases 145
P acquisition and P nutritional status of the plants 146
Microbial diversity 150

General conclusions and outlook 151-155

Abstract 156-160
Abstract (German) 161-165

References 166-187

List of figures shown

Figure No.	Title	page
1	Models for morphological and root-induced chemical phosphate mobilization in the rhizosphere	3
2	Schematic representation of P-deficiency-induced metabolic processes, as indicated by heavy arrows, that may circumvent P-dependent basic reactions of carboxylate metabolism, resulting in carboxylate production	16
3	Schematic representation suggesting metabolic processes being inhibited under P deficiency, as indicated by „ = “, promoting a reduced turnover of citrate	18
4	Schematic representations of P-deficiency-induced modifications of respiration with potential impact on citrate accumulation (a) reduced respiration leading to a feedback inhibition of the TCA cycle by overreduction of the reduction equivalents or (b) increased production of H_2O_2, caused by an impaired respiration, leading to inhibition of the aconitase enzyme	20
5	Incubation of part of the root system with metabolic inhibitors in a separate jar	34
6	Immunoblot analysis of PEP-C in different white lupin root segments	36
7	Malic enzyme (ME) activity in different white lupin root segments	37
8	Pyruvate tissue concentrations in different white lupin root segments	38
9	aconitase and NADP-dependent ICDH activity in different white lupin root segments	40
10	Peroxide concentrations in different white lupin root segments	41
11	Malondialdehyde (MDA) concentrations in different white lupin root segments	42
12	Malate and citrate concentrations and citrate/malate tissue concentration ratios in young cluster root segments after incubation with the aconitase inhibitor H_2O_2, applied at the concentrations of 10 m*M* and 5 m*M* for 1.5 h, 3 h and 5 h.	43
13	Malate and citrate tissue concentrations and citrate/malate tissue concentration ratios in young and mature cluster root segments after incubation with 10 m*M* the aconitase inhibitor monofluoroacetate (MFA) for 8 h.	44
14	Malate (left) and citrate (middle) exudation rates and citrate/malate exudation ratios (right) in young and mature cluster root segments after incubation with 10 m*M* the aconitase inhibitor monofluoroacetate (MFA) for 8 h	45
15	Organic acid exudation rates in seedling root tips after incubation with 10 m*M* the aconitase inhibitor monofluoroacetate (MFA), 20 *µM* Al, and a combination of both (Al+MFA) for 12 h, followed by a localized root exudate collection for 2 h	47
16	aconitase and NADP-dependent ICDH activity in young and mature white lupin root segments after incubation with 10 m*M* MFA for 8 h	48
17	Histological formazan staining of dehydrogenase activities in different root zones of P-deficient white lupin with citrate and succinate as substrates	50
18	Respiration, measured as O_2 depletion, in different white lupin root segments	52
19	Western blot analysis of alternative oxidase protein in different white lupin root segments	53

20	The effect of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) with the concentrations of 0; 0.2; 1; 10, and 20 *µM* on respiration of different white lupin root segments	54
21	The effect of partial root incubation with the respiration inhibitors azide (1 mM) and SHAM (7.5 m*M*) for 4 h and 8 h on malate and citrate concentrations in young (-P y) and mature (-P m) cluster roots of white lupin	56
22	Malate (left) and citrate (middle) concentrations and citrate/malate tissue concentration ratios (right) in young cluster root segments after incubation with the nitrate reductase inhibitor Na_2WO_4, applied at the concentrations of 300 *µM*, 600 *µM* and 1000 *µM* for 16 h	57
23	reaction scheme of the enzyme ATP-citrate lyase	58
24	**A**: malate and citrate root segment tissue concentrations. **B**: in vitro activities of ATP-citrate lyase in different white lupin root segments; **C**: transcript levels of ACL	58
25	Malate and citrate concentrations and citrate/malate tissue concentration ratios in young cluster root segments after incubation with the citrate lyase inhibitor hydroxycitrate (HC), applied at the concentrations of 5 m*M* for 12 h and of 100 m*M* for 8 h	59
26	Spatial variation of pH, plasma membrane H^+-ATPase activity, and exudation of citrate along cluster roots of P-deficient white lupin	98
27	Development of citrate exudation rate per plant and pH in the nutrient solution in +P control plants and P-deficient plants during plant growth	98
28	Vanadate sensitive PM H^+-ATPase hydrolytic activity measured as phosphate release by ATP cleavage in PM vesicles derived from different segments of cluster roots of P deficient plants. A: H^+-ATPase activity per cluster; B: per cluster fresh weight; C: per protein.	99
29	PM H^+-ATPase hydrolytic activity measured as phosphate release by ATP cleavage in PM vesicles derived from roots of two to five weeks old P sufficient (+P) or P deficient (-P) plants	100
30	PM H^+-ATPase hydrolytic activity measured as phosphate release by ATP cleavage in PM vesicles derived from roots of five weeks old P sufficient (+P) or P deficient (-P) plants at different pH values in the assay solution. Maximum activity for +P at pH 6.50; for -P at pH 6.35	100
31	PM H^+-ATPasehydrolytic activity measured as phosphate release by ATP cleavage in PM vesicles derived from -P and +P control plant roots dependent on citrate (A) and malate (B) concentrations in the assay solution.	101
32	Effect of NO_3 (100 m*M*), vanadate (0.1 m*M*), malate (7.5 m*M*) and citrate (5 m*M*) on the pH gradient formation in PM vesicles, determined by the absorbance change of acridine orange at λ = 492 nm. Vesicles were isolated from P deficient roots. The acidification was stopped by the addition of 10 m*M* EDTA-BTP	102
33	Immunodetection of the H^+-ATPase protein by Western Blotting of PM vesicles isolated from roots of +P control plants or of P-deficient (-P) cluster-containing plants	103
34	Citrate and malate concentrations in different root segments of white lupin as affected by buffering the external pH and application of propionate	105
35	Protoplasts isolated from A+B: mature cluster root laterals. Red colouration inside the protoplasts means low viability. C: from cotyledons. D-G: root hair protoplasts isolated according to the methods of Gassmann and Schroeder (1994) and Cocking (1985)	108

36	Shoot and root fresh weight of P deficient white lupin plants under ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing)	128
37	P deficient white lupin plants cultivated at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations after 35 days of growth. below: shoots and roots of P-deficient white lupin plants cultivated at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations 29 days after sowing	129
38	Root/shoot mass ratio of P deficient white lupin plants under ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing)	130
39	Total number of cluster roots per plant of P-deficient white lupin plants at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing)	130
40	Total number of cluster roots per root fresh biomass of P deficient white lupin plants at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing)	131
41	Distribution of cluster roots of different developmental stages depending on plant age and atmospheric CO_2 concentrations	131
42	Malate and citrate exudation rates in different white lupin cluster root segments at six different days after sowing at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) CO_2 concentrations	132
43	P_i concentrations per root biomass in different cluster root segments at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing)	133
44	P_i concentrations per shoot biomass in the shoot tip at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing)	134
45	Shoot and root dry weight and total plant dry weight of white lupin plants grown in rhizoboxes at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations with sufficient (+P) and without (-P) external P supply, 35 days after sowing	136
46	Distribution of cluster roots in different developmental stages (four harvest dates) of white lupin plants grown in rhizoboxes, at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations grown with sufficient (+P) and without (-P) external P supply (data from J. Wasaki and A. Rothe)	137
47	Citrate exudation from root segments of +P and P-deficient plants 35 days after sowing at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations (data from G. Neumann)	138
48	Rhizosphere acid and alkaline phosphatase activity [nmol substrate turnover h^{-1} g^{-1} rhizosphere soil] of white lupin plants grown in rhizoboxes at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations grown with sufficient (+P) and without (-P) external P supply 35 days after sowing (data from J. Wasaki and A. Rothe)	139
49	Shoot and root total P concentration per dry weight and P root and shoot contents of white lupin plants grown in rhizoboxes at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations grown with sufficient (+P) and without (-P) external P supply 35 days after sowing. (data from J. Wasaki and A. Rothe)	140

List of tables shown

Table No.	Title	page
1	Characteristics of different developmental stages of cluster roots I	35
2	Characteristics of different developmental stages of cluster roots II	36
3	The effect of partial root incubation with the respiration inhibitors azide (1 m*M*) and SHAM (7.5 m*M*) for 4 h and 8 h on citrate/malate tissue concentration ratios in young (-P y) cluster roots of white lupin	56
4	Homogenization stock solution (MO) for vesicle isolation	87
5	GS stock solution	87
6	MR stock solution (resuspension medium)	87
7	Homogenization solution	87
8	Gradient Solution (GS)	88
9	Different inhibitor treatments to determine the activities of the different ATPases in the vesicle suspension derived from different subcellular membrane fractions	89
10	Solution 1 to stop the ATPase reaction	90
11	Solution 2 for the colour reaction with P_i	90
12	Vanadate-sensitive and –insensitive plasma membrane ATPase reaction medium	90
13	pH-dependent vanadate-sensitive and –insensitive plasma membrane ATPase reaction medium	91
14	Incubation solution for ^{14}C-citrate transport determination	92
15	Washing solution	92
16	Solutions used for protoplast isolation	94
17	Solutions used protoplast isolation from root hairs	95
18	Purity of vesicle preparations characterized by inhibition of marker enzymes (ATPases).	97
19	Uptake of ^{14}C labelled citrate and H^+ pumping activity by addition of citrate alone or citrate + Mg-ATP for energetization in PM vesicles derived from P-deficient (-P) and +P control plants.	104
20	Preparation of the mixed reagent for P_i determination	125
21	Preparation of acids and molybdate-vanadate solution to determine Phosphate-P	126
22	Number of cluster roots of white lupin plants removed for further investigations at different harvest times. Plants were grown in rhizoboxes at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations grown with sufficient (+P) and without (-P) external P supply (data from Wasaki and Rothe)	137

Abbreviations

ACL	ATP-citrate lyase
AOX	alternative oxidase
A-9-C	anthracene-9-carboxylic acid
BSA	bovine serum albumin
BHT	butylated hydroxytoluene
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CoA	Co-Enzyme A
DAS	days after sowing
DH	dehydrogenase
DMAB	3-dimethylaminobenzoic acid
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
ICDH	isocitrate-dehydrogenase
ID	inner diameter
IRE	iron-responsive elements
IRP	iron-regulatory protein
Gln	glutamine
Glu	glutamate
HC	hydroxycitrate
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethansulfonic acid
MBTH	3-methyl-2-benzothiazolinone
MDA	malon dialdehyde (indicator of lipid peroxidation)
MDH	malate dehydrogenase
ME	malic enzyme
MES	β-morpholino-ethanesulfonic acid
MFA	monofluoroacetate (inhibitor of aconitase)
min	minute
NAD(H)	Nicotinic-acid-amide-adenine-dinucleotide
NADP(H)	Nicotinic-acid-amide-adenine-dinucleotide-phosphate
NBT	Nitro blue tetrazolium: 2,2‘-Di-p-nitrophenyl-5,5‘-diphenyl-3,3‘-[3,3‘-dimethoxy-4,4‘-diphenylene]ditetrazoliumchloride
NO	nitric oxide
NR	nitrate reductase
2-OG	2-oxoglutarate
P	phosphate
PEP-C	phosphoenolpyruvate-carboxylase
PK	pyruvate kinase
PM	plasma membrane
PMSF	phenylmethylsulfonylfluoride
POX	horseradish peroxidase
PVP-40	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SHAM	salicylhydroxamic acid

TBA	thiobarbituric acid
TCA	trichloro-acetic acid or tricarboxylic acid cycle
TEMED	N,N,N‘,N‘-Tetramethylethylendiamin
TEP	1,1,3,3-tetraethoxypropan
Trizma	Tris (hydroxymethyl) aminomethane

General introduction

Phosphorus availability in soils

In many tropical and subtropical areas crop production is severely limited by phosphorus (P) deficiency. In most cases it is the P available for plant uptake that is limiting rather than the total amount of P *per se*. The amount of ortho-phosphate (P_i) in the soil solution, the only P form which can be taken up by plants, is already low in fertile soils, where available P seldom exceeds 10 μM (Raghothama, 1999). In most soils, the concentration of available P_i (approx. 2 μM) in soil solution is several orders of magnitude lower than that in plant tissues (5-20 m*M*) and generally below that of many other micronutrients (Raghothama, 1999). Phosphorus is considered to be the most limiting nutrient for growth of leguminous crops in tropical and subtropical regions. At the same time the total amount of P, comprising Fe-/Al phosphates or phosphates bound to Fe- or Al-oxides or -hydroxides in more acidic soils, or consisting of complex structures of Ca-phosphates with limited solubility in more calcareous and alkaline soils, lies between 0.02 and 0.15 % (Amberger, 1988). Depending on the content of soil organic matter, up to 30-80 % of the total P can be bound in complex forms of organic P esters, with phytates frequently forming the dominant fraction (Neumann and Römheld, 2001).

Intensive P fertilization as a widespread practice in many industrial countries does not provide an adequate sustainable solution to this problem due to the poor affordability of P fertilizers, as well as unfavourable soil chemistry. Up to 80 % of the applied P_i may be fixed in the soil (Raghothama, 1999; Lambers et al., 2003). At the current rate of usage of mineral P fertilizers, readily available sources of high quality phosphate rocks with low heavy metal contaminations are unrenewable and will be depleted within the next 60 to 90 years (Raghothama, 1999). Additionally, a high mineral P input supports surface runoff, and P might be lost by soil erosion or leaching (Lægreid et al., 1999), which is a waste of the limited P resources and results in eutrophication of rivers, lakes and natural habitats.

Mechanisms of P acquisition in higher plants

Plants that naturally grow on P-deficient soils are well adapted to low P soil concentrations to cope with this situation. They show a broad range of different mechanisms to overcome P deficiency and may therefore serve as model plants to investigate adaptations typical for P efficiency.

Mechanisms that determine P efficiency comprise (1) development of a more extended root system under P-deficient conditions with longer and/or more and thinner lateral roots and root hairs (Neumann and Römheld, 2002), (2) symbiosis with mycorrhizae (e.g. Jacobsen et al., 1992; Read, 2003), (3) a high internal P use efficiency, determined by a generally lower internal P demand, slow growth rates, efficient P remobilization from older tissues, and (4) chemical mobilization of the sparingly soluble soil P fraction in the rhizosphere by root exudation of organic metal chelators, secretion of phosphohydrolases and modifications of rhizosphere pH and redox potential (Marschner, 1995; Neumann and Römheld, 2001; Neumann and Römheld, 2002) (Fig. 1). Plant species differ in their distribution and amount of P-mobilizing root exudates released under P deficiency as well as in the root zones the exudation occurs (reviewed e.g. by Crowley and Rengel, 1999; Dakora and Phillips, 2002). In general, dicots, particularly legumes, are more efficient than monocots to produce and exude carboxylates (especially citrate and malate) into the rhizosphere (Jones, 1998; Raghothama, 1999).

A habitat of plant species with extraordinarily high expression of adaptive responses for chemical P mobilization in the rhizosphere are the sand plains in south-west Australia, which are characterized by extremely low availability of nutrients, in particular phosphate and micronutrients. On these heavily leached soils, Proteaceae are a significant component of the biodiversity and biomass (Roelofs et al., 2001). These Proteaceae, trees and shrubs mainly distributed in the native vegetation of Australia and South Africa (Lamont, 1983), are characterized by development of so-called cluster roots, bottlebrush-like clusters of short (5-10 mm) rootlets of determinate growth covered with a dense mat of root hairs. Cluster roots are formed along secondary lateral roots with ten or more meristems per cm (Gardner et al., 1982; Lamont, 1983; Dinkelaker et al., 1989, 1995; Johnson et al., 1996b; Keerthisinghe et al., 1998), and occur in most species of Proteaceae, but also in other plant genera such as Casuarinaceae, Betulaceae, Myricaceae, Eleagnaceae or Fabaceae, e.g. in *Lupinus albus* or *L. consentinii* (Dinkelaker et al., 1995; Gilbert et al., 1998; Neumann and Martinoia, 2002). Formation of cluster roots is mainly induced under P deficiency (Lamont, 1982), but can also be found under Fe deficiency (Arahou and Diem, 1997; Hagström et al., 2001). In white lupin, light microscopy showed that both, cluster root and normal lateral root primordia, arise opposite xylem poles in the pericycle (Johnson et al., 1996b), although a triarch stele resulting in the formation of three longitudinal rows of rootlets was also observed (Peek et al., 2003). Unlike typical lateral roots which emerge at random along the axis of primary and secondary roots, cluster rootlet meristems emerge from every protoxylem pole within an axis (Gilbert et al., 2000). A greater number of protoxylem poles leads to greater rootlet density (Lambers et al., 2003).

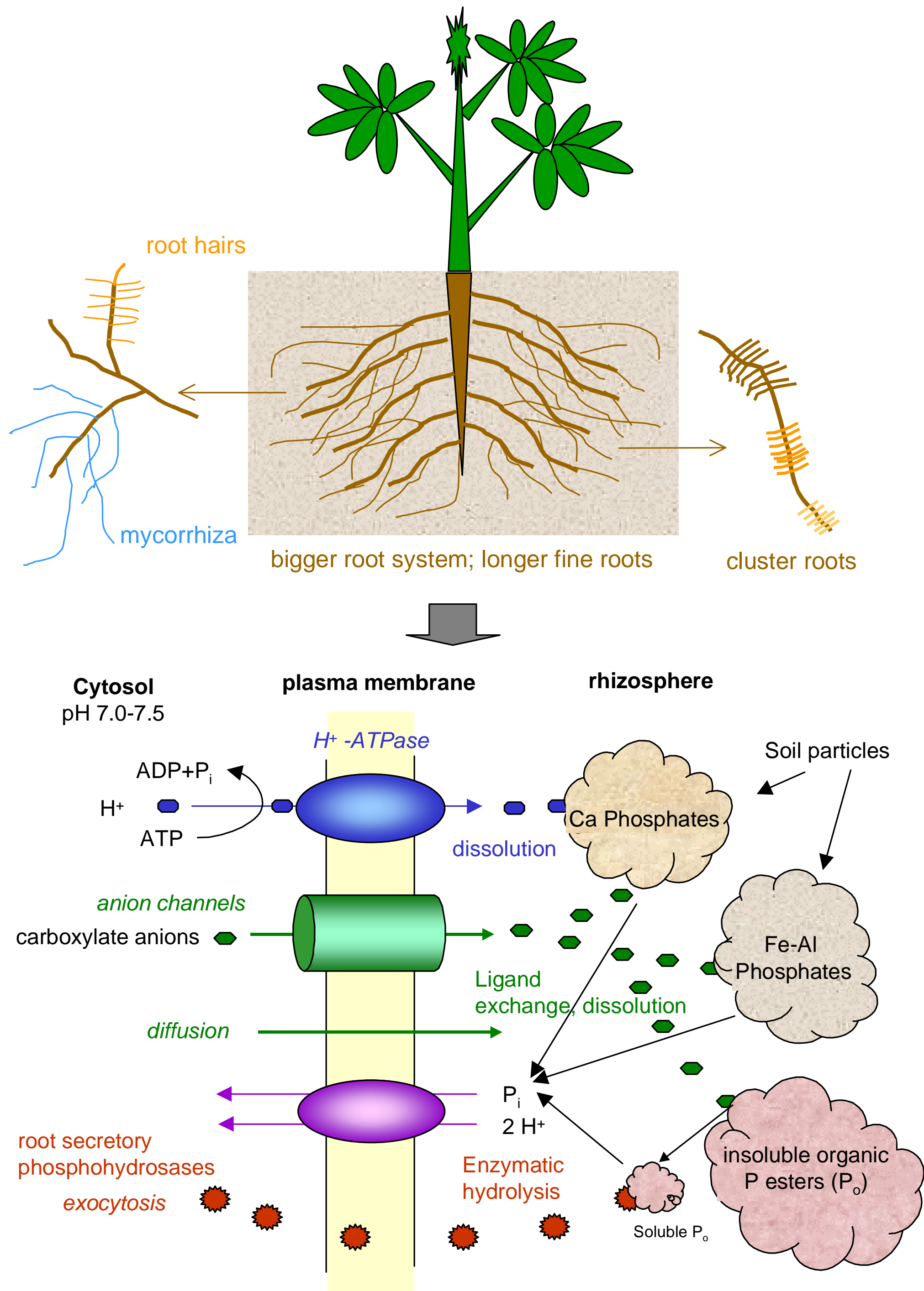


Fig. 1: Models for (above) P deficiency-induced morphological adaptations of root growth and (below) root-induced chemical phosphate mobilization in the rhizosphere by exudation of carboxylates, protons, and root secretory phosphatases (adapted from Neumann and Martinoia, 2002).

Cluster roots were first described for Proteaceae and therefore originally were called ‘proteoid roots‘ (Purnell, 1960). Meanwhile ‘proteoid‘ roots have been described in a wide range of species and genera, and the more appropriate term ‘cluster‘ roots has been introduced (Roelofs et al., 2001).

Almost all cluster root-forming species are non-mycorrhizal (Dinkelaker et al., 1995; Skene, 1998), which might be an adaptation to their area of distribution, where a short season of rainfall does not support the time-consuming development of symbiosis with mycorrhizal fungi (Lamont, 1982; Gilbert et al., 1998).

Cluster roots exhibit intense expression of root-induced chemical changes by exudation of substances which improve nutrient availability in the rhizosphere, such as organic metal chelators (carboxylates, phenolics), protons which acidify the rhizosphere, or phosphatases to hydrolyse organically bound P. Additionally, in cluster roots a higher reducing capacity was found (Neumann et al., 2000). Among the carboxylates, citrate and malate are generally the main compounds (Roelofs et al., 2001), but also fumarate, cis- or trans-aconitate, malonate, maleinate, succinate, or lactate were found in the root exudates (Lambers et al., 2000). The distribution and the amount of carboxylates released depend on the plant species and the developmental age of the cluster roots (Johnson et al., 1996b; Keerthisinghe et al., 1998; Neumann et al., 1999; Kamh et al., 1999).

Citrate has frequently been reported to be the most efficient carboxylate anion to solubilize sparingly soluble P, followed by oxalate. Malate or even acetate have much lower solubilizing effects (Jones, 1998). More generally, the number of carboxylic groups and their arrangement relative to other carboxyl and hydroxyl moieties determine the stability of the organic ligand-metal complexes and, therefore, their potential to release P_i into the soil solution. For example, tricarboxylates chelate Fe more strongly than dicarboxylates, or even monocarboxylates (Ryan, 2003). Several mechanisms for this solubilization process have been described: Citrate dissolves sparingly soluble phosphates such as calcium-phosphates or Fe- and Al-phosphates by chelation of Fe, Al and Ca by citrate-forming metallo-organic complexes, whereby the phosphate anion is released. Under acidic conditions Ca-P has a higher solubility and citrate-metal complexes are more stable.

Another mechanism for P_i release into the soil solution is the desorption of P from Fe- and Al-oxides, -hydroxides, and oxihydroxides by anion exchange. Additionally, P bound to humic-Fe/Al-complexes can be liberated by carboxylates. Carboxylates can also counteract P fixation by blocking P sorption sites in the soil matrix (e.g. Dinkelaker et al., 1989; Gerke, 1992; Gerke and Hermann, 1992; Jones, 1998; Dakora and Phillips, 2002), and they can serve as substrates for bacteria which degrade organically bound P (Ryan, 2003).

Whether the organic acids released by roots of P-deficient plants are really able to mobilize sufficient amounts of P to alleviate P deficiency in plants is still a matter of debate (Jones, 1998; Jones et al., 2003). Many mobilization studies revealed that high concentrations of carboyxlates (> 100 μM for citrate, > 1 mM for oxalate, malate or tartrate) are required to mobilize significant quantities of P into the soil solution (Jones, 1998). Below a concentration of 10 μmol citrate or oxalate g^{-1} soil, the P mobilization is negligible or small (Ammann and Amberger, 1988; Gerke et al., 2000). Dinkelaker et al. (1989) and Gerke et al. (1994) found around 50 μmol citrate g^{-1} soil in the cluster root rhizosphere of white lupin under non-sterile conditions, which was sufficient to explain the high efficiency of this plant species for acquisition of sparingly soluble soil P sources (Gerke et al., 2000). Additionally, Roelofs et al. (2001) reported that the amount of carboxylates exuded by Proteaceae may certainly influence P availability, as well as the availability of other nutrients, and suggested that carboxylates, at least malonate and citrate, are important for nutrient acquisition by Proteaceae. On the other hand, carboxylate concentrations, as found in the rhizosphere soil of many other plant species, are far below the reported threshold concentrations for efficient P mobilization (Jones, 1998). Attempts to assess the contribution of carboxylate exudation to nutrient mobilization are further complicated by a lack of knowledge of the complex interactions and processes between organic acids and the soil, together with the soil microbial community, and methodological shortcomings (Jones et al., 2003).

High stability of complexation between carboxylates and metals is a prerequisite for efficient nutrient mobilization or detoxification. However, complex stability depends on many factors, such as (1) type and structure of the particular carboxylate, (2) the presence of other organic chelators, (3) sorption characteristics, pH, and buffering capacity of the soil, (4) root-induced changes of pH and redox potential in the rhizosphere as well as (5) soil microbial activity (Jones, 1998). The half-life of carboxylates in bulk soils is given as between 2-3 h, depending on the soil type or temperature, whereby decomposition is even 2 to 3-fold faster in rhizosphere soil (Jones, 1998; Ryan et al., 2001). Similar half-life times were given by Neumann and Römheld (2001) for different plant species and sampling conditions in soil experiments. However, biodegradation of carboxylates appears also to be highly dependent on the amount and type of sorption to soil particles, with Al and Fe hydroxides providing the greatest protective effect (Jones, 1998). The high temporal and spatial variability of soil conditions, including also small-scale spatial variations of soil microsites or in the rhizosphere along a single root (Marschner, 1995), together with variations in microbial activity, makes it very difficult to predict the effects of root exudates on nutrient mobilization in soils.

Perspectives to improve P nutrition in crop plants

Fertilization management and cropping systems

A range of alternative management strategies has been proposed with the aim to cope with the restrictions and problems of limited P availability, to avoid overfertilization, and to achieve a more sustainable practice for use of P fertilizers in agricultural production systems. Apart from a better adjustment of P applications to plant demands, an increase in P efficiency can be gained by fertilizer placement strategies in close proximity to roots or even seeds. Compared with broadcast application, more economic placement of high local P concentrations can minimize adsorption effects, it stimulates root growth, and provides a P start supply which can improve early growth and can promote the development of P acquisition mechanisms (Lægreid et al., 1999; Bagayoko et al., 2000). Organic P sources and chemically unprocessed rock phosphates without a high energy input for fertilizer production are the only P fertilizers allowed in organic farming. However, successful application of these slow-release fertilizers requires management strategies to ensure sufficient plant supply of soluble P during the culture period. Root-induced pH changes, mediating P solubilization, can be promoted by the form of applied N fertilizers triggering acidification or alkalinization of the rhizosphere (Marschner, 1998). Also intercropping and rotation systems using plant species with a high potential for P acquisition (Horst and Waschkies, 1987, Alvey et al., 2002) can contribute to a reduced input of P fertilizers. Approaches for recycling of P fertilizers during waste water management are currently under investigation. Inoculation of plant roots with P solubilizing or plant growth promoting rhizosphere microorganisms (PGPR) and mycorrhizal fungi, so called "biofertilizers", have been demonstrated to improve the P-nutritional status of crop plants (Atkinson et al., 2002; Estaún et al., 2002). However, a wide gap of knowledge exists, concerning the conditions for a successful application with reproducible responses and the related functional mechanisms. The right choice of the P source will be site-specific and depends on edaphic, climatic and economic factors (Sinaj et al., 2001). However, the complexity of the root-rhizosphere-soil system prevents general rules and recommendations to be drawn from these approaches.

Selection and breeding of P-efficient plants

Another possibility is to breed crops with enhanced yield ability in low P soils (Lynch, 2003) or to genetically engineering plants for a higher P efficiency. Although intraspecific genotypic variation in expressing plant adaptation to P deficiency has been frequently reported (Römer et al., 1995; Neumann and Römheld, 2000), approaches for selection or breeding of P-efficient genotypes have not been followed widely yet. One of the few examples of practical significance is breeding of wheat cultivars with increased efficiency in P acquisition in Northern China reported by Li and Li (2000), although the related mechanisms are not clear.

Several reports exist where a higher organic acid exudation rate was accompanied by higher activities of enzymes producing these carboxylates, such as phosphoenolpyruvate-carboxylase (PEP-C), malate dehydrogenase (MDH) or citrate synthase (CS) (Johnson et al., 1994; 1996a+b; Keerthisinghe et al., 1998; Neumann et al., 1999; Neumann and Römheld, 1999; Watt and Evans, 1999a; Uhde-Stone et al., 2003a).

Transgenic approaches with overexpression of these enzymes, however, showed unconvincing and often contradicting results. Koyama et al. (1999) described a higher CS activity and higher citrate exudation from protoplast-derived callus from carrot with transformation of cells using *Arabidopsis thaliana* mitochondrial CS. The same group (Koyama et al., 2000) also described the overexpression of mitochondrial CS from *Daucus carota* in *Arabidopsis thaliana* with a higher CS activity and increased citrate excretion from the roots. Anoop et al. (2003) used yeast knock-out mutants of citrate synthase (CS), aconitase (ACO) and isocitrate dehydrogenase (ICDH) to test Al toxicity resistance and found that only double knock-out yeast mutants of CS (two of the three possible) showed significant reduction in their citrate content. Δaco1 and Δicdh12 yeast knock-out mutants accumulated higher levels of citrate (cellular and extracellular) and showed improved Al resistance. Overexpression of the *Pseudomonas aeruginosa* citrate synthase (CS) gene was reported to increase citrate concentration, exudation, and Al tolerance in tobacco (de al Fuente et al., 1997), or showed a better growth and reproduction on a P-deficient, alkaline soil (López-Bucio et al., 2000). However, Delhaize et al. (2001) found that the same transgenic tobacco lines, and different lines producing up to 100 times more CS protein, did neither show higher citrate concentrations nor higher citrate exudation. Therefore Delhaize et al. (2003) concluded that CS activity does not limit the accumulation of internal citrate and suggest that the transport of citrate across the plasma membrane is a more likely site for regulation of citrate efflux from tobacco roots. Furthermore, the antisense inhibition of the cytosolic NADP-ICDH in tobacco did not change plant metabolism (Kruse et al., 1998).

An improved P acquisition was gained when the phytase gene from *Aspergillus niger* was transferred into *Arabidopsis thaliana* and the gene product was secreted as extracellular enzyme to hydrolyse P from externally applied phytase in agar medium (Richardson et al., 2001). However, this approach was yet unsuccessful under soil conditions and is probably limited by the very low solubility of phytates in soils (Neumann and Römheld, 2001).

Apart from the highly artificial growth conditions and tissue preparations frequently used for such experiments, a general flaw of these approaches is that the physiological background of the reactions manipulated in such a crude way is far from understood. Plant metabolism is a complex and highly regulated system where the change of one component leads to adjustments in a whole network of reactions. Increases in enzyme activity can only increase product concentrations when the reaction increased is limiting. Increasing the amount of enzyme by genetic engineering may have little effect on organic acid synthesis if the enzyme is already present in excess, or if its activity is regulated by the concentration of reaction products, by phosphorylation (Ryan et al., 2001) or otherwise.

Even less is known about the influence of additional stress factors simultaneously occurring in many P-limited soils on the expression of plant adaptations to P deficiency. These stress factors can comprise: Ca and Mg deficiency, Al toxicity, Mn toxicity, bicarbonate stress, micronutrient deficiencies, alterations in redox conditions, light intensities, or drought or heat stress, and might give unexpected results. For example, Nian et al. (2003) and Ligaba et al. (2004) found higher Al toxicity-induced malate and citrate exudation for soybean and rape, respectively, when the plants were well supplied with P. In contrast, P-deficient plants showed no carboxylate exudation under Al stress. Bicarbonate stress in rice plants led to very high citrate exudation rates similar to those observed in white lupin cluster roots under P deficiency (Hajiboland, 2000), and comparable values were found for lactate accumulation in maize roots under oxygen deprivation (Xia and Roberts, 1994).

Genetically engineering plant metabolic parameters without knowing even the basics of physiological correlations between external parameters and regulation seems not to be a very promising approach to improve P efficiency in crop plants. The following investigation was therefore aimed to increase knowledge of the metabolic characteristics leading to citrate accumulation and exudation under P deficiency in cluster roots of white lupin.

Lupinus albus – a model plant to study chemical P acquisition in plants

White lupin is the most thoroughly investigated plant species forming cluster roots. Together with *Lupinus consentinii* it is the only cluster-rooted plant species of agricultural importance. It is easy and fast to cultivate as an annual plant and produces clusters within 4 to 5 weeks after germination when cultivated without or with low levels of external P supply. White lupin is often used as a model plant to study adaptations for chemical P mobilization such as rhizosphere acidification, release of organic metal chelators, high activities of root secretory acid phosphatase, and expression of high affinity P uptake (Neumann et al., 2000).

Cluster roots of white lupin release high amounts of carboxylates, mainly citrate and malate, in variable ratios, depending on the developmental stage of individual root clusters. These stages can be visually differentiated by the length of the rootlets and the colour of the clusters (Neumann et al., 1999; Kamh et al., 1999) and comprise: (1) still growing, white coloured young clusters (2) light brown, mature clusters fully developed without growth activity of the lateral rootlets, and (3) senescent clusters with extensive browning. The brown colour probably originates from oxidized phenolic compounds produced and exuded during cluster root development. The different stages are separated from each other by sections of roots without clusters. The production of a new generation of clusters might be brought about by a regulatory cascade involving phytohormones such as auxins and cytokinins, where P is remobilized in older tissues of the root and the shoot and is re-invested into a new generation of leaves and cluster roots (Watt and Evans, 1999a; Neumann et al., 1999; Lamont, 2003).

Investigating different developmental stages of cluster roots and not only responses of the whole root system revealed that malate and citrate tissue concentrations in the clusters can change within one day to the other while the clusters age (Johnson et al., 1996b; Keerthisinghe et al., 1998; Neumann et al., 1999; Watt and Evans, 1999a; Kamh et al., 1999). While malate concentrations and exudation rates are highest in young clusters and decrease during cluster root development, citrate concentrations are highest in mature and senescent clusters, but exudation only peaks in mature ones. This was a strong hint to a regulated citrate release. In addition, the finding that anion channel inhibitors can decrease citrate exudation rates led to the conclusion that citrate exudation takes place via an anion channel in white lupin (Neumann et al., 1999).

Carboxylate exudation in cluster roots exhibits a diurnal rhythm. Watt and Evans (1999b) described exudation only during the day, and Kamh et al. (1999) reported the highest citrate exudation rate directly at the beginning of the light period, and an exponential decline in exudation

rate during the light period. It would be interesting to investigate how internal carboxylate concentrations, supply of carbohydrates from the shoot, and enzyme activities of carboxylate metabolism fit into this pattern.

Intense P deficiency-induced carboxylate exudation from plant roots was frequently correlated with high accumulation of carboxylates in the root tissue (Neumann and Römheld, 2001; Peñaloza et al., 2002). Several investigations considered citrate metabolism under P deficiency. It was found that a substantial proportion of the C exuded by P-deficient lupin is derived from nonphotosynthetic C fixation in roots (Johnson et al., 1996a). On the other hand, citrate accumulation might not exclusively be due to a higher production, but also be promoted by a lower degradation. A decreased aconitase activity in white lupin roots under P deficiency was a first hint into this direction (Neumann et al., 1999; Neumann and Römheld, 1999).

In combination with an increased P mobilization in the cluster roots, P uptake rates are enhanced compared to non-cluster or non-deficient roots (Lamont, 1982; Keerthisinghe et al., 1998). Especially on the basis of root surface area, P uptake rates were found to be more than twice in -P cluster roots compared to -P non-clusters or +P control roots (Neumann et al., 1999, 2000). Higher P uptake rates might be due to higher expression (Liu et al., 2001) and/or a higher density (Neumann et al., 1999, 2000) of P transporters, and the induction of a high-affinity P uptake system under P deficiency was described (Schachtman et al., 1998).

Although a lot is already known about the metabolic pathways leading to citrate accumulation, the regulation of the key reactions is still unclear. Furthermore, almost nothing is known about the factors which trigger and regulate citrate exudation and how the plants react to a combination of environmental changes under P deficiency.

Therefore the aim of this work was to

1. characterize metabolic key reactions involved in P deficiency-induced citrate accumulation in cluster roots during their life cycle.
2. characterize the mechanisms for citrate export from cluster roots.
3. characterize the impact of environmental factors on root exudation and rhizosphere processes in cluster roots using the example of elevated atmospheric CO_2 concentrations, changing the supply of assimilates to the roots.

General methods

Plant cultivation and harvest

White lupin seedlings (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzucht, 76437 Rastatt, Germany) were desinfected with 30 % H_2O_2 for 15 min, rinsed with tap water, soaked in 10 m*M* $CaSO_4$ for 4 h and pre-germinated in wet filter paper containing 2.5 m*M* $CaSO_4$ for 4 d in the dark at 25°C. After emerging of the seedlings, the plants were illuminated in a growth chamber for another two days before they were transferred to nutrient solution. For this, 10 seedlings were cultivated in a 2.5-L aerated pot containing 2 m*M* $Ca(NO_3)_2$; 0.7 m*M* K_2SO_4; 0.1 m*M* KCl; 0.5 m*M* $MgSO_4$; 30 μM Fe-EDTA; 10 μM H_3BO_3; 0.5 μM $MnSO_4$; 0.5 μM $ZnSO_4$; 0.2 μM $CuSO_4$; 0.01 μM $(NH_4)_6Mo_7O_{24}$, with addition of 2.5 m*M* $CaSO_4$ per pot in solid form to prevent Ca deficiency due to high transpiration rates of the plants. For +P control plants 250 μM KH_2PO_4 were added. P-deficient plants were cultivated without any P in the nutrient solution. Growth chamber conditions were adjusted to a 16/8 h day/night cycle at a light intensity of 150 μmol m^{-2} s^{-1} and a constant temperature of 25°C with a relative humidity of 60 %.

Harvest of the four to five weeks old plants was done 3 to 4 h after beginning of the day cycle to prevent the influence of possible diurnal rhythms. The roots were rinsed twice in 1 m*M* $CaSO_2$ solution, cut, the different root segments wrapped in aluminum foil, shock-frozen in liquid N_2, and stored at – 80ºC until further use.

The root segments were differentiated according to their physiological age with the parameters of position along the lateral root, rootlet length and colour of the rootlet. Probably due to phenol exudation, followed by oxidation, cluster roots get darker when they age. In P-deficient plants four different segments were distinguished:

-P a: the apical root zone of a lateral root up to 1 cm behind the root tip

-P y: young cluster root with still growing rootlets, white colour

-P m: mature cluster rootlets; the youngest fully grown cluster with light brown to reddish colour

-P s: senescent cluster rootlets, darker brown, the next older cluster rootlets referring to the –P m ones; the very rootlet tips being even darker than the rest of the rootlet, but still without signs of decay

+P: as +P control, apical root zones of P-sufficient plants up to 1 cm behind the root tips were harvested.

Determination of carboxylates by HPLC

Root tissue concentrations

Root tissue concentrations of carboxylates after partial root incubation with respiration inhibitors (azide and salicylhydroxamic acid (SHAM)), H_2O_2, hydroxycitrate, Na_2WO_4 or monofluoroacetate (MFA) were determined via RP-HPLC.

For this, root segments collected as described above after the incubation were ground with a mortar and a pestle in 5 % (v/v) H_3PO_4 [50 mg root FW mL^{-1}]. Due to the low pH value of the extraction solution, resulting in degradation of membranes and enzymes, extraction was done without previously grinding the tissue in liquid N_2. After centrifugation of the homogenate (for 10 min at 10,000 *g*) the supernatant was diluted 10-fold with the HPLC eluent and used for HPLC injection.

Root exudate collection

For the localized collection of root exudates from nutrient solution-grown white lupin, small pieces of wet thick filter paper (Machery and Nagel), previously washed with methanol and ddH_2O, were placed on the root segments of the plants spread on plastic plates and covered with moistened filter paper to prevent the plants from drying out. After 3 h, the filter papers used for the sampling were removed and the corresponding root segments were cut, frozen in liquid N_2, and stored at –80°C. Extraction of the filter papers was done with HPLC buffer (see below), with 150 μL cm^{-2} of filter or 50 μL per filter rondelle. After centrifugation and transfer of the supernatant into a new vial the paper-free exudates were used for HPLC injection.

To test how seedlings react to aluminium or aconitase inhibitor stress, one week old white lupin and maize seedling root tips were incubated over night with 20 μM $AlCl_3$ or 10 m*M* MFA in Eppendorf vials and immediately afterwards root exudates were collected from the incubated root zones for 2 h in 250 μL ddH_2O in Eppendorf vials. The exudates were centrifuged and the supernatant used for organic acid analysis.

Conditions for HPLC

Separation of the organic acids were isocratically performed on a reversed-phase C-18 column (GROM-SIL 120 ODS-5 ST, particle size 5 μm; length 250 mm, ID 4.6 mm), with a guard column (length 20 mm, ID 4.6 mm; GROM, Herrenberg, Germany) with the same column material. A sample volume of 20 μL were injected into the flow of the eluent (18 m*M* KH_2PO_4, pH 2.25, 35°C, with a constant flow rate of 0.5 mL min^{-1}), and detected photometrically at 215 nm with a UV detector. Identification and quantification of the organic acids were done by comparing the retention times and peak areas with those of known standards.

Statistics

For statistical analysis of the data, the program Sigma Stat® 2.03 (Jandel Scientific) was used. Differences between the root segments were tested with a one-way ANOVA and Tukey test ($p < 0.05$) and significant differences were indicated by different letters. Differences between two treatments were tested with a Student's *t*-test.

Chapter 1: Phosphorus deficiency-induced alterations of organic acid metabolism during cluster root development

Introduction

In *Lupinus albus*, citrate accumulates during cluster root development under conditions of P deficiency, associated with declining levels of malate in the cluster root tissue. After reaching a threshold concentration of 20-30 μmol citrate g^{-1} root FW in mature root clusters (Neumann et al., 2000; Peñaloza et al., 2002), a transient pulse of intense citrate exudation, associated with rhizosphere acidification, occurs over a time period of 2-3 days. The transient pattern of carboxyate exudation is comparable for lupin plants grown in hydroponics and under soil conditions (Neumann et al., 1999; Kamh et al., 1999), and has been similarly described also for other cluster-rooted plant species (Dinkelaker et al., 1989; Shane et al., 2004). These findings suggest a causal relationship between the accumulation of extraordinarily high tissue concentrations of citrate and the transient burst of citrate release into the rhizosphere. Therefore the question arises which metabolic processes are responsible for the shift from malate to citrate accumulation in the tissue of mature root clusters and the sudden release of citrate as one of the most efficient carboxyates mobilizing sparingly soluble P forms in soils. Increased biosynthesis and reduced turnover of citrate have been discussed as possible metabolic processes responsible for citrate accumulation in cluster roots (Neumann et al., 1999, 2000; Neumann and Martinoia, 2002).

Phosphorus deficiency-induced biosynthesis of carboxylates

On the anabolic side of carboxylate metabolism, induction of several glycolytic bypass reactions have been described, circumventing P-depending metabolic reactions under conditions of P deficiency (Theodorou and Plaxton, 1993, 1995; Plaxton, 1998). As an example, fructokinase, phosphoglucomutase, and sucrose synthase transcript levels and enzyme activities were increased in young and mature cluster roots of white lupin (Massonneau et al., 2001). Uhde-

Stone et al. (2003b) found increased expression of ESTs with homology to a glyoxysomal malate synthase, PP_i-dependent phosphofructokinase, phospho*enol*pyruvate carboxylase (PEP-C) and malate dehydrogenase (MDH), together with other enzymes of the glycolytic pathway in cluster roots of P-deficient white lupin.

Higher activities of PEP-C and MDH were found for white lupin (Johnson et al., 1994; 1996a+b; Neumann et al., 1999; Neumann and Römheld, 1999; Keerthisinghe et al., 1998; Watt and Evans, 1999b; Uhde-Stone et al., 2003a). In other plant species, higher PEP-C activity under P-deficient conditions were described for *Brassica napus* (Hoffland et al., 1992), tomato (Pilbeam et al., 1993; Neumann and Römheld, 1999), chickpea and wheat (Neumann and Römheld, 1999) or *Catharanthus roseus* (Nagano et al., 1994). In white lupin, PEP-C mRNA (Uhde-Stone et al., 2003a) and PEP-C protein, determined by immunoblot analysis (Neumann et al., 1999; Uhde-Stone et al., 2003a), were more expressed in P-deficient cluster roots. Phospho*enol*pyruvate carboxylase catalyzes the carboxylation of phospho*enol*pyruvate (PEP) to oxaloacetate by non-photosynthetic CO_2 fixation, whereby P_i is set free. Moreover, in cluster roots of white lupin the PEP-C reaction provides up to 30 % of the carbon released as citrate into the rhizosphere under P-deficient conditions (Johnson et al., 1996a+b).

As a result, these metabolic pathways allow the operation of glycolysis under conditions of low P availability and provide additional carbon for root exudates that are released. The products of those reactions are oxaloacetate and malate, which can act as precursors for the biosynthesis of citrate (Fig. 2).

Citrate synthase (CS) activity as the metabolic step leading directly to citrate production was found to be increased under P deficiency in carrot cells (Takita et al., 1999) and in white lupin (Johnson et al., 1994), but was not found by Neumann et al. (1999) or Kihara et al. (2003a) for white lupin or by Aono et al. (2001) for *Sesbania rostrata* CS mRNA.

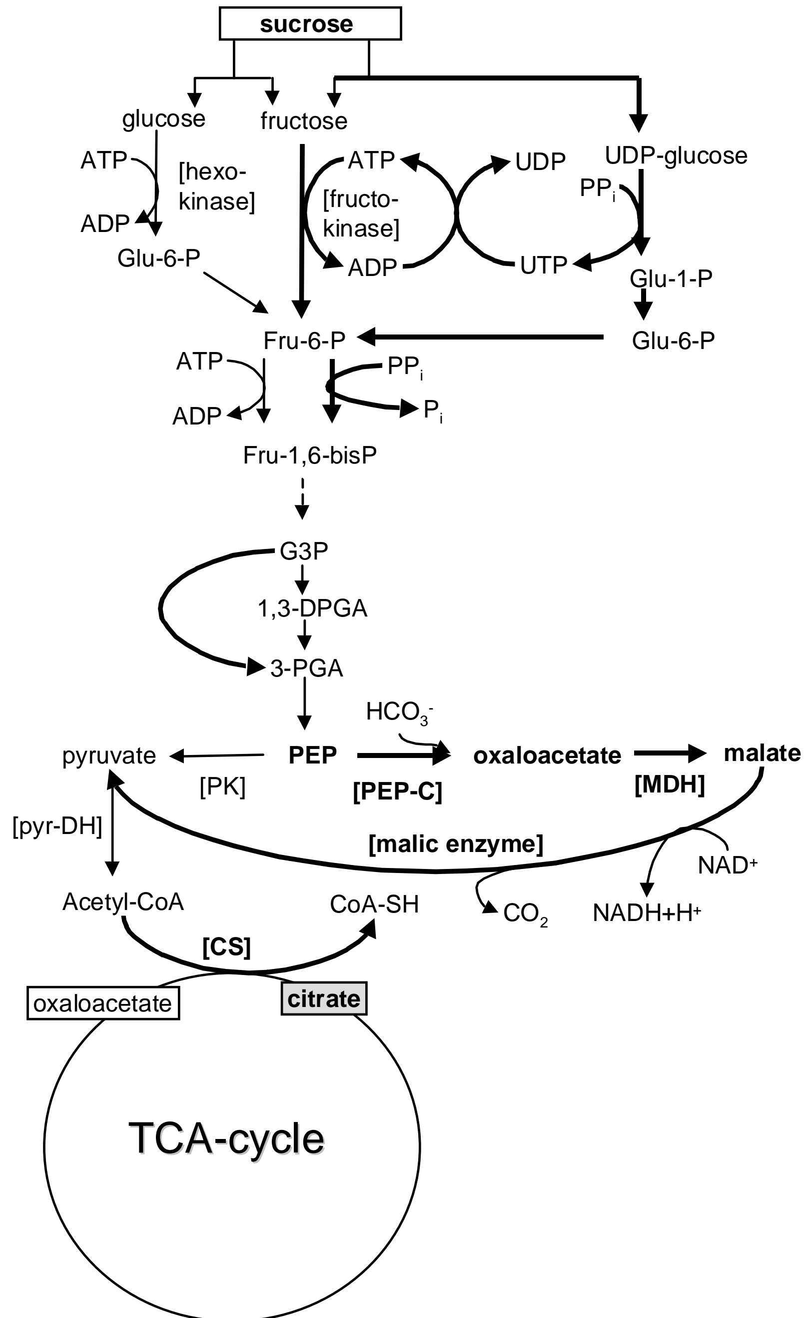


Fig. 2: Schematic representation of P deficiency-induced metabolic processes, as indicated by heavy arrows, that may circumvent P-dependent basic reactions of carboxylate metabolism, resulting in carboxylate production (adapted from Plaxton, 1998, and Heldt, 2003).

Phosphorus deficiency-induced inhibition of citrate turnover

Inhibition of aconitase

First arguments for an impaired citrate turnover as a possible cause for citrate accumulation under P-deficient conditions have been provided by Neumann et al. (1999) and Kihara et al. (2003) (Fig. 3), reporting decreased activities of aconitase for mature cluster roots in *Lupinus albus*. A similar correlation between increased accumulation of citrate and reduced activity of aconitase was found for P-deficient tomato and chickpea, but not for wheat (Neumann and Römheld, 1999). Aconitase, catalyzing the turnover of citrate to cis-aconitate and isocitrate, is rapidly inactivated by H_2O_2 (Verniquet, 1991), which can be produced at increased rates under P limitation (Parsons et al., 1999; Juszczuk et al., 2001b; Malusà et al., 2002) or under inhibited respiration (Purvis and Shewfelt, 1993; Minagawa et al., 1992) (see below).
At least two aconitase isoenzymes are present in intact plant cells: one is present in the cytosol, the other in mitochondria (Brouquisse et al., 1986, 1987). While the mitochondrial enzyme most likely participates in the tricarboxylic acid (TCA) cycle, the cytosolic enzyme might play a role in different metabolic pathways (Sadka et al., 2000). Its participation was suggested in the glyoxylate cycle in pumpkin cotyledons (Hayashi et al., 1995). Aconitase was found to be inhibited by yet unknown factors in sour lemon in contrast to sweet lime, which has been related to increased citrate accumulation (Sadka et al., 2001).

Reduced assimilation of nitrate with 2-oxoglutarate as N acceptor

Isocitrate as the product of aconitase is further metabolized to 2-oxoglutarate (2-OG) via the enzyme isocitrate-dehydrogenase (ICDH). Two forms of this enzyme exist: NAD-dependent ICDH occurs only in mitochondria, whereas the NADP-dependent form can be found in mitochondria as well as in other compartments such as peroxisomes, chloroplasts and the cytosol (Schnarrenberger and Martin, 2002).
NADP-ICDH was detected in all tissues and organs investigated in higher plants (Chen and Gadal, 1990b) and represents 90 % to 100 % of the activity detected in any plant organ, but little is known about its physiological role (Palomo et al., 1998). It has been suggested that NADP-specific ICDH represents an additional or alternative path to the TCA cycle enzyme, when large quantities of 2-oxoglutarate (2-OG) are required (Chen and Gadal, 1990a; Gálvez and Gadal, 1995), e.g. for supplying the 2-OG for amino acid biosynthesis and ammonia assimilation (Chen

et al., 1988; Gallardo et al., 1995). Chen and Gadal (1990a) proposed that in higher plants the cytosol is the major site for generation of 2-OG used in N assimilation. On the other hand, till date, the exact enzymatic origin of 2-OG for plant NH_4 assimilation is still unknown. ICDHs and aspartate aminotransferases are the two main candidates, and arguments exist for and against the role of cytosolic ICDH in NH_4 assimilation (Hodges et al., 2003). Nevertheless, a reduced uptake and assimilation of nitrate is a common feature in P-deficient plants (Rufty et al., 1990; Pilbeam et al., 1993; Buwalda and Warmenhoven, 1999; Gniazdowska et al., 1999; Neumann et al., 2000), and reduced N-assimilation may therefore also affect the turnover of citrate which is the precursor for 2-OG as a potential acceptor molecule for NH_4 assimilation (Neumann et al., 2000).

ATP-citrate lyase (ACL)

Differential display analysis of gene expression during cluster root development in *Lupinus albus* using the RFLP approach revealed high expression of ATP-citrate lyase (ACL) in young cluster roots, which declined during cluster root development (Langlade et al., 2002). The enzyme catalyzes the ATP-dependent cleavage of citrate into oxaloacetate and acetyl-CoA and may provide an anaplerotic pathway for acetyl-CoA production under P-deficient conditions where phosophо*enol*pyruvate is preferentially converted to oxaloacetate via the PEP-C reaction (Duff et al., 1989a+b; Theodorou and Plaxton, 1991; Kihara et al., 2003). Declining expression of ACL during cluster root development, possibly caused by ATP limitation, may therefore re-present an additional pathway for citrate turnover, which is repressed under severe P-deficient conditions.

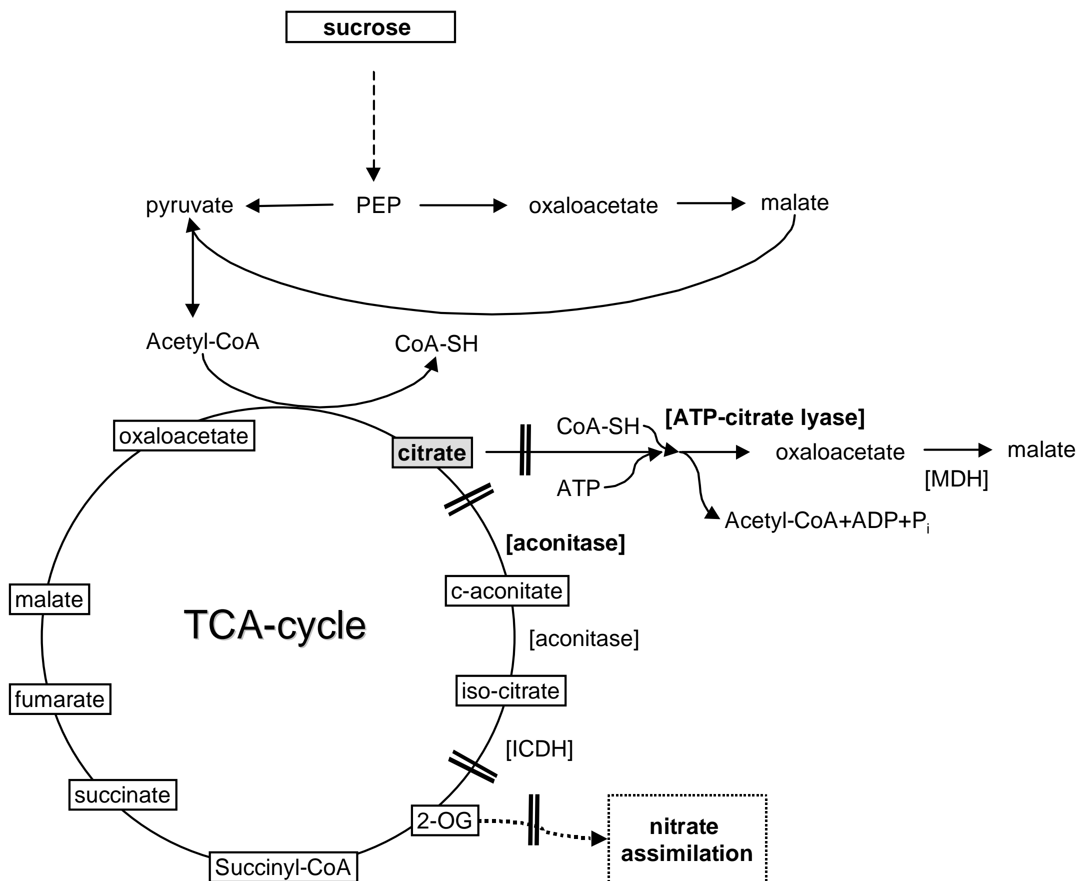


Fig. 3: Schematic representation suggesting metabolic processes being inhibited under P deficiency, as indicated by " = ", promoting a reduced turnover of citrate (adapted from Plaxton, 1998; Neumann and Martinoia, 2002; Langlade et al., 2002, and Heldt, 2003).

Reduced respiration

Lower respiration rates have also been reported for cluster roots of P-deficient white lupin (Neumann et al., 1999; Massonneau et al., 2001) and might therefore affect citrate accumulation directly by reduced consumption of citrate in the TCA cycle or indirectly by H_2O_2-induced inhibition of aconitase activity.

Respiration provides energy in form of ATP for many metabolic processes. Oxidation of C-containing compounds in glycolysis and the TCA cycle provide reduced pyridine nucleotides (NADH), which are oxidized in the mitochondrial electron transport chain (Douce, 1985;

Parsons et al., 1999). The energy which is set free by the transport of electrons along the cytochrome pathway in the mitochondrial inner membrane is used to create an electrochemical gradient, which dissipation is coupled to the production of ATP from ADP and P_i, whereby O_2 serves as terminal electron acceptor and is reduced to water. A number of factors may limit the rate of root respiration under P limitation, such as availability of P_i and adenylates as respiratory substrates, or a more general impairment of mitochondrial function by limited biosynthesis of proteins etc. (Bingham and Farrar, 1988; Williams and Farrar, 1990; Bingham and Stevenson, 1993; Wanke et al., 1998). Due to a reduced respiration, electron transport through the cytochromes would cease, and the TCA cycle would be inhibited through a shortage of oxidized pyridine nucleotides (Lee, 1979). This might be one explanation for the increased citrate concentrations observed in mature cluster roots of white lupin (Fig. 4).
The mitochondrial electron transport chain can produce significant quantities of reactive oxygen species (ROS), primarily due to the presence of the ubisemiquinone radical which can transfer a single electron to oxygen and produce superoxide (Sweetlove et al., 2002). This superoxide can be oxidized to H_2O_2, which might inhibit the aconitase enzyme.

As known so far, all plants, and some fungi and protists, additionally have an alternative respiratory pathway, brought about by a single protein, the so-called alternative oxidase (AOX). This enzyme is a cyanide-resistant terminal quinol oxidase and shunts electrons off the cyanide-sensitive cytochrome pathway at the level of ubiquinone, and reduces molecular oxygen to water in a single four-electron step without conservation of energy (Day et al., 1996). This means that this non-phosphorylating pathway is independent of P-containing respiratory substances and therfore does not contribute to a transmembrane potential (reviewed by Vanlerberghe and McIntosh, 1997). Only the phosphorylating potential from site I (NADH dehydrogenase) is retained, thus allowing some energy production (Vanlerberghe and McIntosh, 1997).
Although a clear function for the AOX pathway has yet to be established (Millenaar et al., 2001), its main effect might be to prevent an overreduction of the respiratory chain components and ROS production when there is an imbalance between carbon metabolism and electron transport (Purvis and Shewfelt, 1993; Wagner and Krab, 1995). Under this aspect, the AOX pathway could be taken as a bypass reaction under P deficiency to overcome the limiting cytochrome electron transport (Yip et al., 2001) due to a lack of ADP and P_i. In accordance with this, an increased alternative pathway capacity was observed under P-deficient conditions (Rychter and Mikulska, 1990; Hoefnagel et al., 1993a; Parsons et al., 1999; Juszczuk et al., 2001a; Yip et al., 2001).

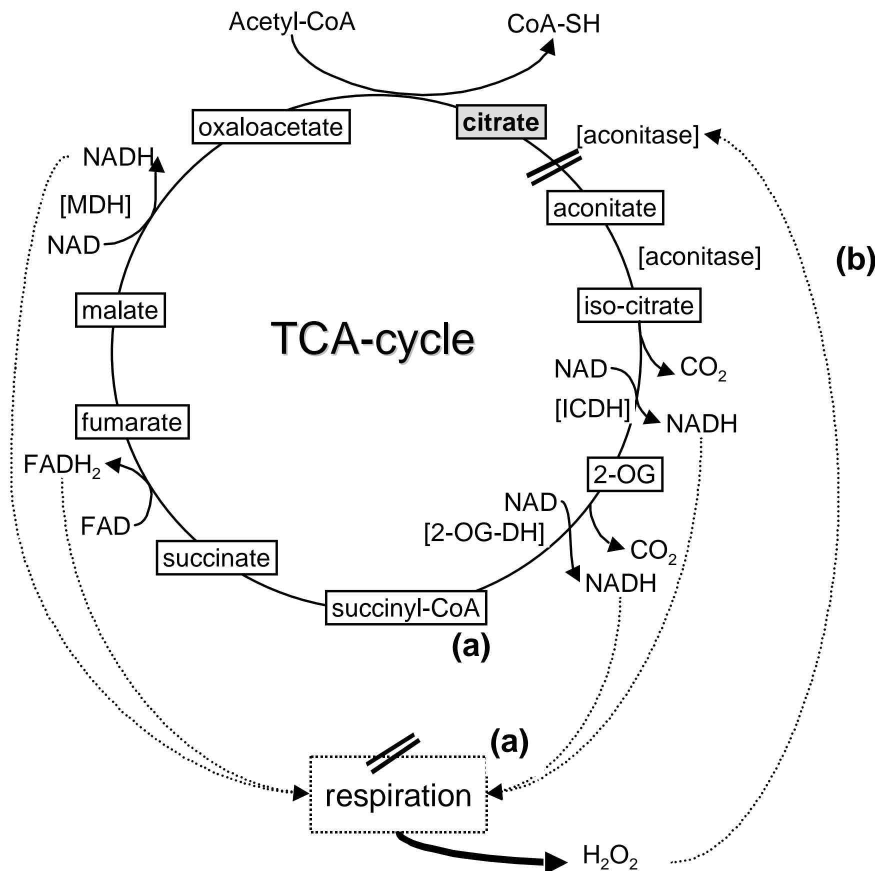


Fig. 4: Schematic representation of P deficiency-induced modifications of respiration with potential impact on citrate accumulation (a) reduced respiration leading to a feedback inhibition of the TCA cycle by overreduction of the reduction equivalents or (b) increased production of H_2O_2, caused by an impaired respiration, leading to inhibition of the aconitase enzyme (adapted from Plaxton, 1998; Neumann and Martinoia, 2002, and Heldt, 2003).

Based on the working hypothesis that citrate accumulation in cluster roots of P-deficient white lupin, preceeding the exudative burst of citrate, is a consequence of (1) increased production of citrate precursors and (2) reduced consumption of citrate, the aim of this work was to characterize the key regulatory steps in citrate metabolism which might affect citrate accumulation during cluster root development. The experimental approach comprises the characterization of possible key enzymes, regulatory factors, and metabolic sequences involved in citrate metabolism during different stages of cluster root development as related to accumulation and root exudation of citrate.

Materials and methods

Enzyme assays

Determination of *in vitro* activities of phospho*enol*pyruvate carboxylase (PEP-C) (EC 4.1.1.31) and citrate synthase (CS) (EC 4.1.3.7) enzymes was carried out by G. Neumann as described by Johnson et al. (1994; 1996a) and Neumann et al. (1999). For the determination of PEP-C activity, lactate dehydrogenase (LDH) (EC 1.1.1.27) was additionally included into the assay buffer (3 U mL^{-1}) to compensate for decarboxylation of oxaloacetate during the test. Crude enzyme extracts were prepared by homogenization of frozen tissue with mortar and pestle (200 mg FW mL^{-1} of the appropriate extraction buffer), and subsequent centrifugation at 10,000 *g* (4°C). The clear supernatant was used for the enzyme assays.

PEP-C immunodetection

Determination was carried out by G. Neumann as described by Neumann et al. (1999). For immunoblot analysis of the PEP-C protein, crude extracts of root material, prepared according to the procedure for PEP-C activity determinations (400 mg FW mL^{-1} extraction buffer), were separated by SDS-PAGE (10 % acrylamide, application of 60 μg protein per lane), and proteins were electrophoretically transferred to nitrocellulose sheets by semi-dry blotting. A polyclonal antiserum directed to the phosphorylation site of sorghum PEP-C, which exhibits cross-reaction with PEP-C of many other plant species (J. Vidal, Institut des Biotechnologie des Plantes, CNRS, Université de Paris-Sud, Paris France, pers. comm.), was used for the detection of the PEP-C protein of white lupin in a coupled assay with alkaline phosphatase staining (Sigma, Deisenhofen, Germany). Protein determinations were performed according to Bradford (1976).

Pyruvate determination

Pyruvate determination was done according to Lamprecht and Heinz (1984), and was adjusted for white lupin root material.

Pyruvate concentrations were determined from root segment material. For extraction, 5 mL of ice-cold 1 *M* perchloric acid were added per 1 g of root fresh weight and homogenized on ice

with a mortar and a pestle. The homogenate was transferred into an Eppendorf vial and a spatula tip of activated charcoal was added. The mixture was vortexed and stored on ice while the other root samples were extracted. The mixture was centrifuged at 20,000 *g* for 15 min at 4°C. The supernatant was transferred into new Eppendorf vials and neutralized with about 115 μL of 2 *M* K_2CO_3 per 500 μL extract to a pH of about 7.5. Since CO_2 is built and produces bubbles, the vial should not be filled too high to avoid the liquid to flow over. Allow the neutralized solution to stand for 15 min on ice to almost complete the neutralizing reaction. After mixing again, the tips of the vials were perforated to let the CO_2 evaporate. To remove the bubbles (which interfere with spectrophotometric determinations) the solution was sonicated for 5 min in an ultrasonication bath with ice added to keep the solution cold. After another centrifugation step for 5 min with 20,000 *g* at 4°C to pellet the precipitated material, the supernatant was used for pyruvate determination.

Measurement was performed according to the scheme given by Lamprecht and Heinz (1984) and changed for the white lupin root material:
The given incubation times were the times to wait for getting a constant absorbance reading. They were determined by time-scan (kinetic) measurements of samples.

pipette successively into a semi-microcuvette:		concentration in assay mixture
sample solution	0.50 mL	pyruvate up to 0.12 m*M*
TEA solution	0.25 mL	TEA 0.16 M, EDTA 1.8 m*M*
NADH solution	0.02 mL	NADH 0.179 m*M*
Mix thoroughly with a plastic spatula, incubate for 8 min and then read absorbance A1.		
Add H_2O to get the change of absorbance by the addition of a 10 μL volume, wait for 5 min and read absorbance A2.	0.01 mL	
Add LDH solution	0.01 mL	LDH 5.76 U mL^{-1}
Mix thoroughly, incubate for 10 min and read absorbance A3.		

ΔA = A3-(A1-A2) was used for calculation.

According to the law of Lambert-Beer, ε (340 nm NADH) = 6.3 l L x $mmol^{-1}$ x cm^{-1} was used for calculation.

Solutions used:

1. Triethanolamine/EDTA buffer (TEA, 0.5 mol L^{-1}, pH 7.6; EDTA 5 mmol L^{-1}):
   16.67 mL of a triethanolamine solution is diluted to ~ 200 mL, 0.47 g EDTA-Na_2H_2 x 2 H_2O are added and the pH adjusted with HCl to pH 7.6; the solution is then filled up to 250 mL.

2. perchloric acid solution (1 *M*):
   8.6 mL perchloric acid (70 % w/v) are diluted with water to 100 mL.
   Or: 10.03 mL of 60 % perchloric acid (w/v) are diluted with water to 100 mL.

3. potassium carbonate solution (2 mol L^{-1}):
   27.64 g K_2CO_3 are dissolved with water and made up to 100 mL.

4. reduced nicotinamide-adenine dinucleotide solution (ß-NADH, 7 mmol L^{-1}): 10 mg NADH, disodium salt, are dissolved in 2.0 mL 5 % (w/v) $NaHCO_3$ solution.

5. lactate dehydrogenase (LDH, 225 kU L^{-1}): commercially available crystalline enzyme suspensions are dissolved with cold water appropriately. Use commercially available enzyme solutions from skeletal muscle or heart.

Malic enzyme (ME) activity determination

Malic enzyme (ME) (EC 1.1.1.39) activity was determined according to Outlaw and Springer (1983) and Dittrich (1976) from root segment material. The root material was ground in liquid N_2 with a mortar and a pestle, and 500 mg of the ground material was extracted with 3 mL of an extraction cocktail containing 50 m*M* HEPES, pH 7.6, $MnCl_2$ 2 m*M*, DTT 10 m*M*, BSA 1 % (w/v), and 1 % PVP-40 with addition of 150 mg activated charcoal and 0.5 m*M* PMSF (15 μL from a 100 m*M* stock solution in 100 % ethanol) and filled into an Eppendorf vial.

For protein determination, part of the frozen ground root material was extracted separately with the same extraction cocktail, but without BSA, frozen in liquid N_2, and stored at –80°C till protein determination according to Bradford (1976), using BSA as a standard.

To set the ME protein free from the mitochondria, the tissue extract for enzyme activity measurement was ultrasonicated with a sonication rod (Bandelin Sonopuls; HF generator GM 2070) two times for 15 sec with an energy of 20-28 %, and frozen in liquid N_2 until measurement. Sonication time and energy was chosen to the values giving the highest ME activity for a certain sample.

For the photometric determination of the ME activity, the tissue extract was thawed, 15 μL Triton X-100 and 15 μL of the PMSF stock solution per 3 mL tissue extract were added, vortexed and centrifuged for 10 min at 10,000 *g* at 4°C. 12.5 µL of the supernatant was given to 0.75 mL of the assay cocktail solution containing 50 m*M* HEPES, 5 m*M* malic acid, 2 m*M* NAD, 0.2 m*M* EDTA, 0.1 m*M* CoA; 5 m*M* DTT, 5 μ*M* NADH, and 500 U L^{-1} NAD-MDH. The

production of NADH from NAD due to the ME activity was monitored photometrically by its absorption change at λ = 340 nm, measured against water. Calculations for ME activity in the tissue extract were given as $b = 9921 \times \Delta A \times \Delta t^{-1}$ [U L^{-1}].

Aconitase and NADP$^+$-Isocitrate-Dehydrogenase (NADP-ICDH) activity assay

Aconitase (EC 4.2.1.3) and NADP$^+$-Isocitrate-Dehydrogenase (NADP-ICDH) (EC 1.1.1.42) activity assays were performed in succession in the same sample. Both activities were determined photometrically in a coupled assay in which the formation of NADPH was followed at λ = 340 nm by its increase of absorbance, using aconitate (for aconitase activity) and isocitrate (for NADP-ICDH activity) as substrates, in essence according to de Vos et al. (1986). NADP-ICDH activity was monitored directly by the production of NADPH from NADP due to its catalytic activity. Aconitase activity, however, was monitored in a coupled assay, where the isocitrate produced from aconitate, due to the aconitase activity, was quantitatively transformed to 2-oxoglutarate by NADP-ICDH. NADPH, formed from NADP, actually was monitored.

Enzyme activity was measured in (a): P-deficient white lupin cluster root segments and in +P control plant root tips, and (b): in –P y and –P m cluster roots segments after localized incubation with 10 m*M* monofluoroacetate (MFA) for 8 h and successive localized collection of root exudates with filter papers. For enzyme extraction, the root segments were ground in liquid N_2 with a mortar and a pestle, and (a) 100 mg of the ground powder or (b) 50 mg of the ground powder suspended in 1 mL ice-cold extraction buffer with addition of PVPP (5 mg mL^{-1}) and PMSF (10 μL mL^{-1} of a 0.1 *M* stock solution in methanol). The suspension was stored again in liquid N_2 and each sample thawed separately directly before use to prevent degradation of the instable enzymes (Krebs and Eggleston, 1944). For activity determination, the frozen sample was thawed, centrifuged at 10,000 *g* for 5 min at 4°C, and 50 μL of the supernatant added to the assay solution.

extraction buffer for aconitase and NADP-ICDH determination

extraction buffer:
- 0.1 *M* HEPES
- 10 m*M* tricarballylic acid
- 2 m*M* DTT

pH 7.5

assay solution for aconitase and NADP-ICDH determination

assay solution: 50 μL 5 m*M* $MgSO_4$
50 μL 5 m*M* $MnSO_4$
200 μL extraction buffer
50 μL 6 m*M* NADP (4.7 mg mL^{-1} suspended in HEPES-buffer), pH 7.5

Base reaction was followed for 5 min after the addition of 50 μL of enzyme extract. The aconitase reaction was started by the addition of 50 μL of 10 m*M* c-aconitate (17.4 mg c-aconitate mL^{-1} HEPES-buffer, pH 7.5; pH further adjusted with 5 *M* NaOH to pH 7.5) and followed until the reaction was linear for at least 3 min.
To measure NADP-ICDH activity, 50 μL of a 120 m*M* dl-isocitrate solution (35 mg dl-isocitrate x 2 H_2O mL^{-1} HEPES-buffer, pH 7.5) was added and followed for another 3 min.
Calculation was done according to the law of Lambert-Beer: $E = \varepsilon \times c \times d$,
with an extinction coefficient ε NADPH (340 nm) = 6.22 [L x $mmol^{-1}$ x cm^{-1}]

Hydrogen-peroxide (H_2O_2) determination

Determination was done according to Ngo and Lenhoff (1980), Okuda et al. (1991), and Veljovic-Jovanovic et al. (2002). Root segment material was ground to a fine powder in liquid N_2 and 100 mg of the powder was extracted in 2 mL 1 *M* $HClO_4$ with addition of 5 % PVPP, referring to the extraction volume. Homogenates were frozen in liquid N_2 and stored at -80°C. For further use, the thawed homogenates were centrifuged at 12,000 *g* for 10 min at 4°C, a defined volume of supernatant was transferred into a new Eppendorf vial and neutralized with 8 *M* KOH to pH 7 in the presence of 50 μL of a 0.3 *M* Na-phosphate buffer, pH 5.6. Fine-adjustment was done with 1 *M* KOH and 1 *M* $HClO_4$. All volumes used and added were noted and considered for calculation. This new homogenate was centrifuged at 12,000 *g* for 1 min at 4°C to remove precipitated $KClO_4$. The supernatant was incubated prior to assay for 10 min with 1 U ascorbate oxidase mL^{-1} to oxidize ascorbate at room temperature.
The colour-producing reaction was initiated by addition of a 100 μL aliquot of the sample to 1 mL of reaction mixture (0.1 *M* Na-Phosphate buffer, pH 6.5; 3.3 m*M* DMAB; 0.07 m*M* MBTH, and 7 ng horseradish peroxidase (POX), type VI). The absorbance change at 590 nm was monitored photometrically at 25°C for several minutes. For each assay, the H_2O_2 concentration in the extract was quantified by reference to an internal standard (5 μL of a 0.5 m*M* H_2O_2 solution), added to the reaction mixture on completion of the absorbance change due to the sample.

Malondialdehyde (MDA) determination

Malondialdehyde (MDA) determination was done according to the thiobarbituric acid method of Heath and Packer (1968) and Dhindsa et al. (1981) with minor modifications to evaluate lipid peroxidation of membranes.

Many exogenic stress factors cause an increased oxidation of membrane lipids in living organisms due to activity of free radicals. As a result, malondialdehyde (MDA) is set free from these lipids. It can be detected in cell extracts via a reaction with thiobarbituric acid (TBA), producing a colour complex which can be measured at 532 nm in a spectrophotometer. The result can be taken as a parameter for the degree of membrane damage.

Extraction and determination from plant material was performed according to Dhindsa et al. (1981). Production of MDA was tested in the different root segments. Root fresh material was ground in liquid N_2 with a mortar and a pestle and 1 g of the ground material was extracted in 5 mL of a pre-cooled 0.1 % trichloro-acetic acid (TCA) with addition of 50 μL of a BHT solution (5 % butylated hydroxytoluene in 100 % ethanol) to prevent oxidative rancidity of lipids according to Juszczuk et al. (2001). Extracts were stored on ice until all the samples were extracted. The homogenate was centrifuged at 10,000 *g* for 5 min at 4°C and 0.25 mL of the supernatant mixed with 1 mL of the assay solution (0.5 % thiobarbituric acid (TBA) in 20 % TCA; has to be heated to be resolved) and then heated to 95°C for 30 min. Do not boil, since this will cause severe turbidity and oily precipitation in the sample solution.

To get rid of the bubbles forming in the solution, the solution was vortexed, sonicated for 10 min, vortexed again and centrifuged for 10 min at 10,000 *g* at room temperature. Malondialdehyde concentration was measured photometrically at 532 nm against a blank and the values at 650 nm were subtracted as turbidity correction.

To determine the MDA concentration, the law of Lambert-Beer was used with $\varepsilon = 155$ L x $mmol^{-1}$ x cm^{-1} as extinction coefficient.

To test the own procedure, a standard (TEP; 1,1,3,3-tetraethoxypropan; a fluid) can be measured: 220 μL TEP are mixed with 0.78 mL of 80 % ethanol. This mixture is diluted 1:20,000 in 0.1 % TCA. This dilution is then used in the same way as the centrifuged samples.

Histology of dehydrogenase activities

The histological staining of dehydrogenase activities has the advantage to show not only the average activity of an enzyme as determined by enzyme activity tests of tissue extracts, but also the location inside the tissue where the activity occurs. In principle, living root segments are incubated with a dehydrogenase substrate and a soluble tetrazolium dye, which serves as hydrogen acceptor, whereas the hydrogen is supplied via NADH or NADPH by the substrate due to the corresponding dehydrogenase activity oxidizing the substrate. The tetrazolium is reduced to an unsoluble bluish-purple formazan product which precipitates in the cells where the reduction takes place.

The method was performed according to Seligman and Butenberg (1951), and adapted for plant root material. Whole roots were incubated overnight in 2.5 m*M* of an aerated $CaSO_4$ solution to deplete the roots of TCA cycle substrates. For fixation, the roots were embedded in 4 % of a boiled and then cooled-down agar in a high-diameter test-tube. The solidified agar with the embedded roots were removed from the test tube and root cross sections were cut with a razor blade as thin as possible. The cross sections were layered on microscope slides and then incubated in the dark for 5 h at 35°C with a NBT solution consisting of 100 m*M* Tris-HCl, pH 7.5, nitro-blue tetrazolium (NBT; 2,2‘-di-p-nitrophenyl-5,5‘-diphenyl-3,3‘-[3,3‘-dimethoxy-4,4‘-diphenylene]-ditetra-zolium-chloride) 0.025 %, and 50 m*M* dehydrogenase substrate in the form of citrate, isocitrate, c-aconitate, malate, succinate, or fumarate. Control incubations were done with the NBT solution without dehydrogenase substrate. Colour development was documented with a digital camera with 50-fold magnification. Since quite thick cross-sections were used, enough intact cells existed so that co-factors such as NAD, NADP or FMN or FAD and others were still available in the tissues for the dehydrogenase reactions.

Respiration

Root O_2 uptake

Respiration was measured as O_2 uptake in excised root segments (200-400 mg FW) of cluster roots in different developmental stages or 1 cm apical root zones of lateral roots with a Clark-type O_2 electrode (Tri-Oxmatic EO 200; WTW© Weilheim, Germany, connected with an OXI 530 reading unit (WTW)) at a temperature of 25°C in 30 mL of an air-saturated 2 m*M* $Ca(NO_3)_2$

solution according to Neumann et al. (1999). Readings of the O_2 concentrations in the solution were taken each 30 s and O_2 depletion calculated for the O_2 uptake by the root segments on basis of time and root mass (fresh weight and dry weight). Calibration of the probe was done in air as OxiCal® fast calibration with an in-air calibration vessel PE/OXI.

Respiration inhibitors were used at final concentrations of 0.5 m*M* and 7.5 m*M* for KCN and SHAM (salicylhydroxamic acid), respectively. The optimum concentrations for inhibitor supply were estimated from titration curves according to Møller et al. (1988). As an uncoupler of oxidative phosphorylation CCCP (carbonyl cyanide-3-chlorophenyl-hydrazone) was used at concentrations from 0.2 to 20 *µM*. Each measurement was performed with separate root samples. Fresh and dry weight of the root material was recorded when the measurements were finished.

Cytochrome pathway (COX) capacity was defined as apparent respiration when the alternative pathway was inhibited by SHAM and alternative pathway (AOX) capacity as the apparent respiration with simultaneous inhibition of the cytochrome pathway by KCN. All data were corrected for residual respiration, measured by application of both, KCN and SHAM.

AOX Western Blot analysis

Extracts for non-reducing SDS-PAGE were prepared from fresh root material. Root tissue was ground in liquid N_2 using a mortar and a pestle. Five hundred mg of the ground tissue were suspended in a 1 mL volume of protein sample mixture (62.5 m*M* Tris-HCl, pH 6.8; 2 % (w/v) SDS; 10 % glycerol, 2 m*M* EDTA, and 0.002 % bromophenol blue). PMSF (final concentration 1 m*M*) was added to inhibit proteases, and samples were immediately boiled for 5 min. For separations under reducing conditions, 5 % (v/v) 2-mercaptoethanol was added.

After cooling on ice, the samples were centrifuged for 10 min at 16,000 *g* and 8ºC in a microliter centrifuge to precipitate cell debris. Proteins were separated by SDS-PAGE according to the method of Laemmli (1970) with a 6 % (w/v) polyacrylamide stacking gel and a 12 % (w/v) polyacrylamide resolving gel. The separated proteins were subsequently transferred to a nitrocellulose blotting membrane (0.2 *µ*m pore size; Sartorius, Göttingen, Germany) by semi-dry blotting (Khyse-Andersen, 1984) according to Neumann et al. (1999).

For immunoblot analysis, the blot was incubated in a 1:75 dilution of a monoclonal antibody raised against alternative oxidase (AOX) of *Sauromatum guttatum*. The AOX antibody was kindly provided by Dr. T.E. Elthon (Elthon et al., 1989). Anti-mouse IgG (whole molecule) alkaline phosphatase conjugate was used as a secondary antibody (dilution 1:17,500). Colour

development was performed with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma, Deisenhofen, Germany).

Solutions used for Western Blot analysis:

resolving gel 12 %:

7.76 mL	Rotiphorese A
3.6 mL	Rotiphorese B
5.6 mL	2 *M* Tris-HCl pH 8.8
2.8 mL	H_2O
300 µL	SDS
15 µL	TEMED
300 µL	10 % (w/v) APS

Σ 20.4 mL

stacking gel 6 %:

2 mL	Rotiphorese A
0.9 ml	Rotiphorese B
2.5 mL	0.5 *M* Tris-HCl pH 6.8
4.4 mL	H_2O
100 µL	SDS
10 µL	TEMED
100 µL	10 % (w/v) APS

Σ 10.01 mL

elektrophoresis buffer (10- fold concentrated stock solution)

15.143 g	Tris	0.25 *M*
5 g	SDS	1 % (w/v)
72.07 g	Glycin (as electrolyte)	1.92 *M*

filled up to 500 mL with H_2O (no adjustment of the pH necessary)

2-fold sample buffer:

8 mL	0.5 *M*	Tris-HCl pH 6.8
10 mL	10 %	SDS
5.8 mL	87 %	glycerine
1 mL	0.2 %	bromophenol blue

10-fold sample buffer:

25 mL	1.6 *M*	Tris-HCl pH 6.8
5 g		SDS
16.5 mL	87 %	glycerine
0.004 %	(w/v)	bromophenol blue

solution for decoloration:

450 mL	H_2O
450 mL	methanol
100 mL	glacial acetic acid

Coomassie solution:

450 mL	H_2O
450 mL	methanol
100 mL	glacial acetic acid
2 g	Coomassie blue R 250

extraction buffer:

50 m*M*	Na-phosphate buffer pH 7.2
1 % w/v	PVP
2 % v/v	mercaptoethanol
0.02 % w/v	SDS
2 % v/v	DMSO

derivatisation of the samples for the SDS-PAGE:

100 μL	crude extract
100 μL	sample buffer 2-fold
10 μL	mercaptoethanol

denaturate for 5 min at 100°C; store at –20°C till separation

Bradford-solution:

25 mg	Serva Brilliant blue G 250
12.5 mL	ethanol
25 mL	H_3PO_4 (85 % v/v)

The dye is presolved in ethanol, phosphoric acid is added and filled with bidistilled H_2O up to 250 mL. The solution is filtered, and after > 12 h filtered again.

Blotting-buffer:

kathode buffer, pH 9.4:

0.025 *M*	Tris	1.51375 g 500 mL^{-1}
0.04 *M*	6-amino-capronic acid	2.6236 g 500 mL^{-1}
20 % (v/v)	methanol	
0.02 % (w/v)	NaN_3	

anode buffer, pH 10.4:

0.3 *M*	Tris	18.165 g 500 mL^{-1}
20 % (v/v)	methanol	
0.02 % (w/v)	NaN_3	100 mg 500 mL^{-1}

TU buffer, pH 9.4:

0.025 *M*	Tris	
20 % (v/v)	methanol	
0.02 % (w/v)	NaN_3	100 mg 500 mL^{-1}

Ponceau S solution: dissolve 2 g Ponceau S in 100 mL 3 % (w/v) TCA

TBS buffer (10-fold):

0.5 *M*	Tris	15.138 g 250 mL^{-1}
1.5 *M*	NaCl	21.94 g 250 mL^{-1}
	Tween 80	1.25 mL 250 ml^{-1}
	NaN_3	50 mg 250 mL^{-1}

blocking buffer:

TBS-Puffer with 0.1 % (v/v) Tween 80® (again added 0.5 mL for 1000 mL altogether) and 0.25 % (w/v) BSA (Fraction V) = 2.5 g BSA 1000 mL^{-1}

ATP-citrate lyase (ACL) assay

The assay of ATP-citrate lyase (ACL) (EC 4.1.3.8) activity was done by N. Langlade and E. Martinoia, Université de Neuchâtel, Suisse (Kania et al., 2003). Frozen plant tissues were ground in liquid N_2 and homogenized with 3 vol. of extraction buffer (0.1 *M* HEPES-KOH, pH 7.5, 5 m*M* $MgCl_2$, 2.5 m*M* DTT, 3 m*M* Na-DEDTC (diethyldithiocarbamate), 1 m*M* EDTA, 1 m*M* benzamidine, 1 m*M* PMSF, and 3 % PVPP K30). After centrifugation (25 min, 12,000 *g*, 4°C) the supernatant was rapidly used to determine ACL activities and protein concentrations (DC

Protein Assay kit; Bio-Rad). ACL activity was determined spectrophotometrically at room temperature, using the malate dehydrogenase coupled assay. The assay mixture contained 0.2 *M* Tris, pH 8.4, 10 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 20 m*M* Na_3-citrate, 0.2 m*M* Coenzyme A, 10 m*M* ATP (omitted in blanks), 0.2 m*M* NADH, 0.4 U mL^{-1} malate dehydrogenase. Values were taken after 30 min. Blanks were performed by omitting ATP or Coenzyme A, resulting in similar values.

Inhibitor treatments

Cluster roots accumulate citrate during their development and exude it when a threshold concentration is reached. To examine which mechanism(s) may lead to the observed citrate accumulation during cluster root development, the following approach was chosen: Still young cluster roots were incubated with several metabolic inhibitors to find out if they can be forced to react like mature ones when the metabolic step, decreased naturally in mature cluster roots, is artificially inhibited in the still young ones.

A part of the root system of four to five weeks old P-deficient white lupin plants were incubated in a small jar (15 - 30 mL), containing nutrient solution and the metabolic inhibitor which reaction was to be investigated (H_2O_2; hydroxycitrate, or monofluoro-acetic acid (MFA)). This small jar was fixed to the inside of a bigger pot with a volume of 750 mL with adhesive tape. The rest of the root system was kept in the bigger pot containing nutrient solution without the inhibitor. Both solutions did not mix and were aerated independently (Fig. 5). Control plants were incubated in the same way, but only with nutrient solution without inhibitor in the small jar. After the incubation time the roots were rinsed two times in 1 m*M* $CaSO_4$ solution to remove adhering nutrient solution and inhibitor. The different root segments were cut, frozen separately in liquid N_2, and stored at –80°C till further use. In case of the MFA incubation, when the organic acid concentrations in the MFA-treated root segments showed strong effects, further incubations were performed, where root exudates were collected localized from young and mature cluster root segments after the MFA incubation. For this, the plant root system was rinsed in 1 m*M* $CaSO_4$ solution and spread on a plastic tray after the incubation, and small filter papers were put above and below the cluster root segments incubated in the MFA-containing solution to collect the root exudates. The rest of the root system was covered with wet paper to prevent drying of the roots. After the exudate collection, the filter papers were removed, frozen in liquid N_2 and stored at -80°C till analysis of organic acid contents. The root segments where the

exudates were collected from were weighted, equally frozen in liquid N_2 and stored at –80°C till analysis of aconitase- and NADP-ICDH activities.

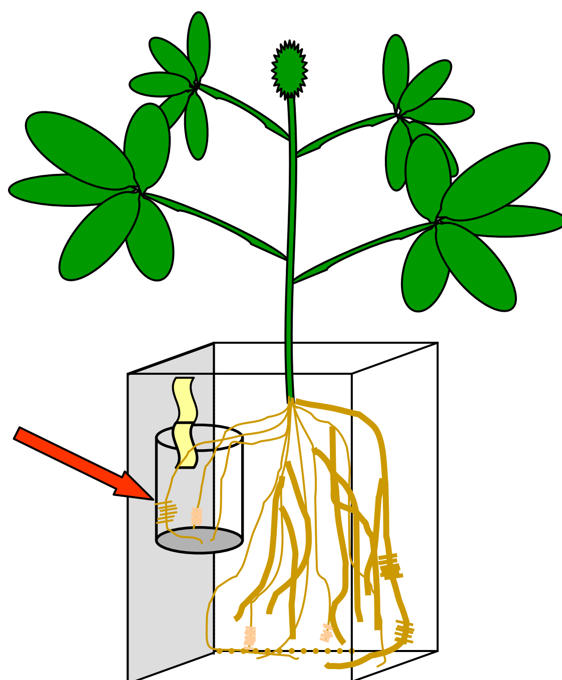


Fig. 5: Incubation of part of the root system with metabolic inhibitors in a separate jar.

Incubation of and root exudate collection from white lupin seedling root tips were done in small plastic vials. Incubation was performed in nutrient solution alone (control) or in nutrient solution with the addition of 10 m*M* MFA, 20 μ*M* Al or a combination of both, 10 m*M* MFA and 20 μ*M* Al. All solutions were titrated to a pH of 4.5 to keep Al solubilized.

For incubation, two root tips per vial were put into a 2-mL vial containing the incubation solution. The plants were covered with wet filter paper to prevent them from drying. After 12 h of incubation, the roots were shortly rinsed in 1 m*M* $CaSO_4$ solution and the root tips put into new vials containing 250 μL of distilled water for exudate collection. After a collection time of 2 h, the vials containing the water together with the exudates were frozen in liquid N_2 and stored at -80°C until analysis. The parts of the root tips reaching into the incubation solution were cut, weighted for exudate rate calculation on the base of the root fresh weight, frozen in liquid N_2 and stored at –80°C. For exudate determination, the liquid containing the exudates were thawed, centrifuged, and 20 μL injected for HPLC determination of organic acids.

Results

Phosphorus deficiency-induced biosynthesis of carboxylates

Carboxylate accumulation and metabolic activity status in different developmental stages of cluster roots

During cluster root development from apical root zones via young and mature clusters to senescent clusters, a shift from malate to citrate accumulation can be observed up to a threshold concentration, where a burst of citrate exudation occurs. In parallel, P nutritional status decreases, and P remobilization from P_i storage pools, ATP, and mainly from ribosomal RNA takes place, followed by lower protein concentrations and a lower energy status seen as lower ATP concentrations (Tab. 1).

The aim of the following investigations was to elucidate how the key reactions of citrate metabolism are influenced by these conditions and how citrate accumulation is brought about.

Table 1: Characteristics of different developmental stages of cluster roots I (Data from Neumann et al., 1999; Massonneau et al., 2001, and protein concentrations from this work). +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11.

developmental stage	root concentration		root exudation rate		root concentration			
	malate	citrate	malate	citrate	P_i	ATP	RNA	protein
	[μmol g^{-1} FW]		[μmol g^{-1} FW h^{-1}]		[μmol g^{-1} FW]	[nmol g^{-1} FW]	[mg g^{-1} FW]	
+ P						nd	0.247 ± 0.068	3.43 ± 0.42
-P a	12.09 ± 4.2	8.65 ± 0.1	0.65 ± 0.21	0.36 ± 0.14	nd	24.3 ± 4.5	0.068 ± 0.049	2.62 ± 0.41
-P y	12.69 ± 2.3	17.68 ± 5.0	0.14 ± 0.04	0.12 ± 0.05	1.82 ± 0.42	90.9 ± 8.7	0.573 ± 0.073	3.83 ± 0.57
-P m	3.59 ± 2.2	22.92 ± 3.2	0.09 ± 0.05	0.73 ± 0.26	0.42 ± 0.09	42.6 ± 8.9	0.062 ± 0.029	2.52 ± 0.45
-P s	0.67 ± 0.3	23.58 ± 2.0	0.0 ± 0.0	0.04 ± 0.03	0.23 ± 0.04	12.1 ± 3.9	0.016 ± 0.002	1.44 ± 0.35

Phospho*enol*pyruvate carboxylase (PEP-C) is the enzyme of the non-photosynthetic CO_2 fixation which branches from PEP via oxaloacetate to malate instead of from PEP to pyruvate via

pyruvate kinase. The activity of this enzyme was increased in mature clusters, together with an increase in specific activity in mature and senescent ones, and together with an increase in citrate concentrations (Tab. 2). This activity pattern paralleled enzyme amounts, detected by immunoblotting (Fig. 6), which hints to an enzyme regulation mainly by enzyme amount. However, in senescent clusters, where PEP-C enzyme amounts per root biomass and per protein were very low, specific activity was still very high and probably was caused by a posttranslational control of the enzyme. No correlation could be seen between citrate synthase (CS) activity and citrate accumulation during cluster root development. This enzyme seems not to limit citrate production in citrate metabolism.

Table 2: Characteristics of different developmental stages of cluster roots II (Data from Neumann et al., 1999; Massonneau et al., 2001, and G. Neumann, unpublished results). -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11. Significant differences between the root segments are indicated by different letters (One Way Anova, $p \leq 0.05$).

developmental stage	PEP-C specific activity [μmol NADH min^{-1} mg^{-1} protein]	PEP-C activity [μmol NADH min^{-1} g^{-1} root FW]	protein concentration [mg g^{-1} root FW]	malate concentration [μmol g^{-1} root FW]	citrate concentration [μmol g^{-1} root FW]
-P a	0.20 ± 0.03 a	1.10 ± 0.24 a	5.62 ± 0.6 a	15.06 a	10.22 a
-P y	0.16 ± 0.07 a	1.08 ± 0.51 a	6.03 ± 0.4 a	16.21 a	13.70 b
-P m	0.42 ± 0.03 b	1.48 ± 0.30 ab	3.56 ± 0.8 b	5.10 b	32.16 c
-P s	0.54 ± 0.1 b	0.88 ± 0.09 ac	1.72 ± 0.5 bc	0.00 c	28.23 c

	CS specific activity [μmol acetyl-CoA min^{-1} mg^{-1} protein]	CS activity [μmol acetyl-CoA min^{-1} g^{-1} root FW]
-P a	0.04 ± 0.01 a	0.22 ± 0.06 a
-P y	0.05 ± 0.03 a	0.30 ± 0.18 a
-P m	0.08 ± 0.02 ab	0.28 ± 0.07 ab
-P s	0.02 ± 0.01 c	0.03 ± 0.02 c

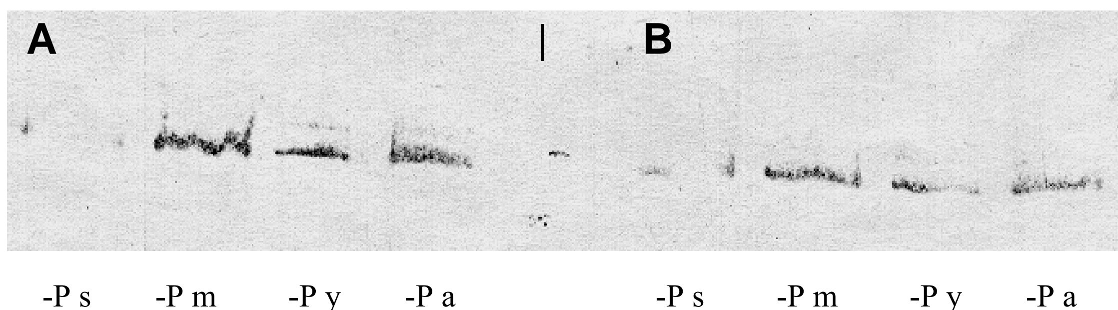


Fig. 6: Immunoblot analysis of the PEP-C protein in different white lupin root segments. **A**. with the same amount of protein per lane. **B**. with the same amount of root biomass per lane. (data from G. Neumann; method see Neumann et al., 1999). -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11.

Malic enzyme activity

Malic enzyme (ME) catalyzes the irrevesible oxidative decarboxylation of malate to pyruvate. It is the last step of the anaplerotic pathway to circumvent the ADP-demanding glycolytic step from PEP to pyruvate by pyruvate kinase. The reaction sequence follows the enzymes PEP-C, malate dehydrogenase (MDH), and ME (Plaxton, 1998). It is part of the pH-stat mechanism (Davis, 1979, Sakano, 1998). Here the NAD-dependent ME was measured. It is generally associated with mitochondria.

When malic enzyme activity was based on the root fresh weight (Fig. 7B), activities were similar in the root tips of +P control plants and P-deficient plants. In young cluster roots the activity was even higher, but decreased steeply during cluster root development. The course of activity is due to the different protein concentrations in the roots which follow the same pattern (Fig. 7C 9). Therefore, based on protein concentration (Fig 7A;C), specific ME activity was in principle the same in all root segments.

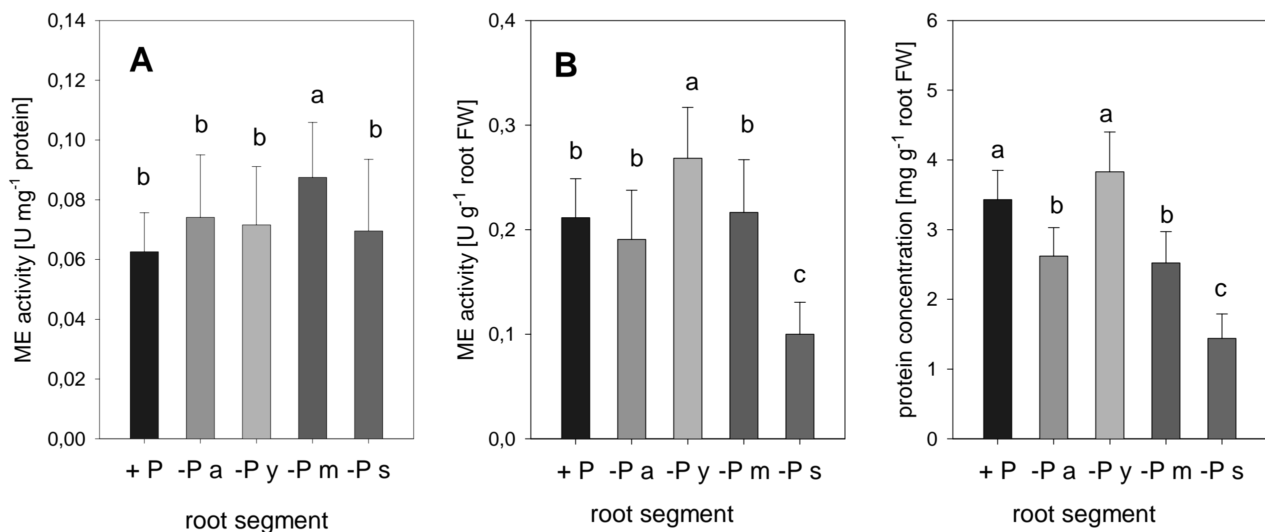


Fig. 7: Malic enzyme (ME) activities and protein concentrations in different white lupin root segments. **A:** ME activity on the basis of the protein concentration (specific ME activity). **B:** ME activity on the basis of the root fresh weight. **C:** protein concentrations. Average ± SD (n = 12 – 16) from four different harvests, each with 3-4 replicates. +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11. Significant differences between the root segments are indicated by different letters (One Way Anova, $p \leq 0.05$).

Pyruvate concentrations

Pyruvate is a key regulatory metabolite in plant glycolysis and an important branch point in metabolism (Vanlerberghe and McIntosh, 1997), linking glycolysis and respiration via the TCA cycle. It is a precursor for several amino acids, fatty acid biosynthesis and anaerobic fermentation (Juszczuk and Rychter, 2002). Since an accumulation of pyruvate in P-deficient bean plants were described as a result of its increased synthesis and decreased utilization (Juszczuk and Rychter, 2002), pyruvate was determined in white lupin root segments to investigate if an imbalance between the "normal" and anaplerotic pathways might occur, which in turn could influence organic acid metabolism.

There were no significant differences in pyruvate concentrations between lateral root tips of P-sufficient and P-deficient plants and young cluster roots (Fig. 8). However, pyruvate concentrations declined while the clusters aged, which was associated with a corresponding decrease in malic enzyme activity based on root biomass.

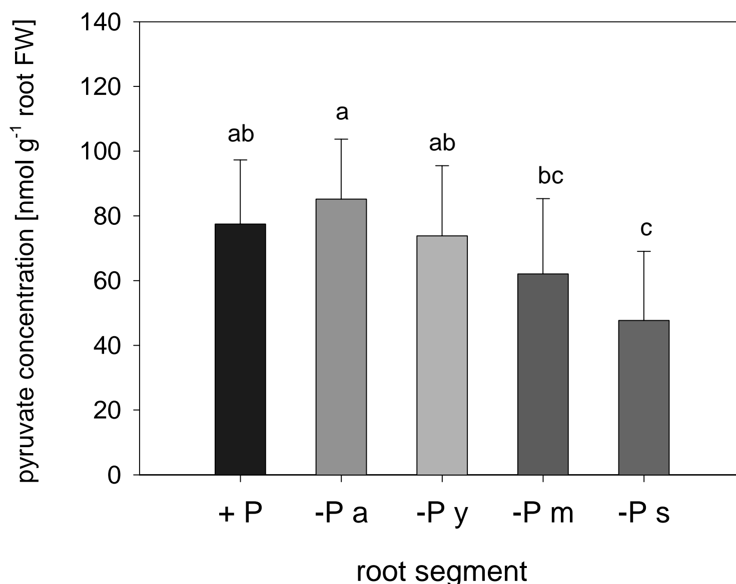


Fig. 8: Pyruvate tissue concentrations in different white lupin root segments. Data are the average ± SD (n = 14 – 21) from four different harvests, each with 3-6 resplicates. +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11. Significant differences between the root segments are indicated by different letters (One Way Anova, $p \leq 0.05$).

Phosphorus deficiency-induced inhibition of citrate turnover

Inhibition of aconitase and NADP-dependent isocitrate-dehydrogenase (NADP-ICDH)

Aconitase and NADP-dependent isocitrate-dehydrogenase (NADP-ICDH) are the enzymes converting citrate to isocitrate via cis-aconitate (aconitase) and in the next step isocitrate to 2-oxoglutarate (2-OG) (NADP-ICDH) in the TCA cycle.

In all the root segments examined, NADP-ICDH activity was always more than twice compared with the aconitase activity (Fig. 9). The pattern of activities in the different root segments were very similar to that of ME activity: On the basis of root fresh weight, the highest activities of aconitase and NADP-ICDH were found in control root tips, with similar activities in young cluster roots. Lower activities of both enzymes occured in root tips of P-deficient plants. During cluster root maturation from young to senescent ones, aconitase activities and ICDH activities declined significantly, which is parallel to the increasing citrate and decreasing malate concentrations in the respective root segments. This might explain the observed change in carboxylate concentrations. Calculated on the basis of protein concentration, specific aconitase and ICDH-activities were the same in all segments investigated with the exception of mature cluster roots, where the activities were higher. Therefore the relative enzyme activities did not change contrary to the activities per root biomass.

Whether the higher specific enzyme activities in mature root clusters were due to a higher relative aconitase and ICDH protein content or a higher enzyme activity status could only be answered by immunoblot analysis.

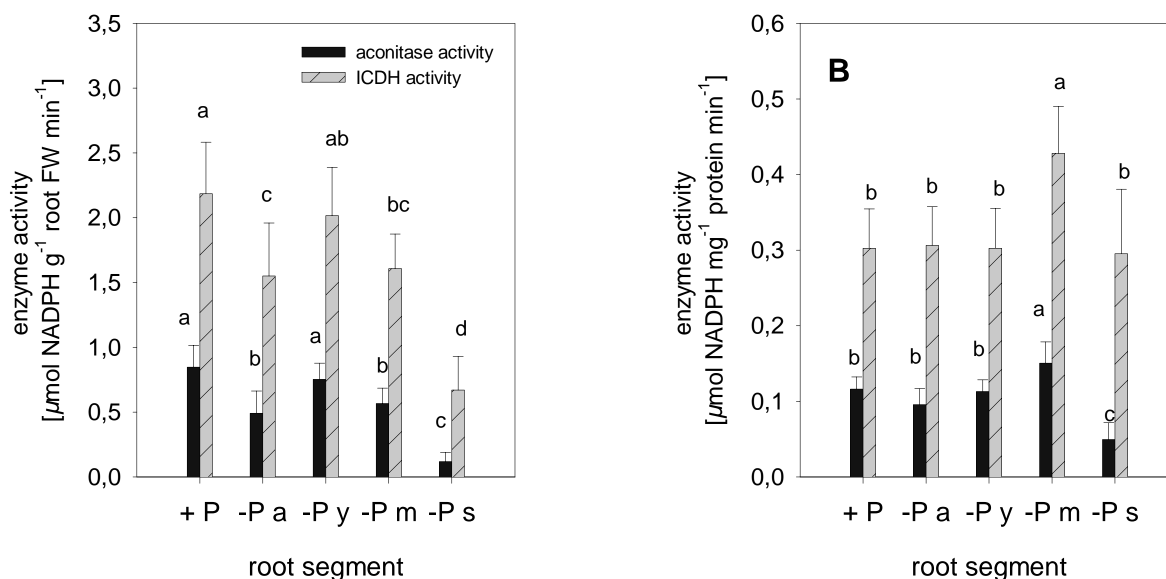


Fig. 9: Aconitase and NADP-dependent ICDH activity in different white lupin root segments.
A: activity per g root fresh weight. **B:** activity per mg protein. Average ± SD (n = 9 – 11) from three different harvests, each with 3-4 replicates. +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11. Significant differences between the root segments are indicated by different letters (One Way Anova, $p \leq 0.05$).

H_2O_2 – a natural inhibitor of aconitase in P-deficient white lupin ?

Results suggest that citrate accumulation in mature cluster roots might be caused by a reduced aconitase activity. Since it is known that H_2O_2 is a natural inhibitor of the aconitase enzyme (Verniquet et al., 1991), its relevance for a reduced aconitase activity in white lupin cluster roots and the observed citrate accumulation was tested.

Peroxide (H_2O_2) concentrations

In all the root segments investigated peroxide concentrations were essentially the same (Fig. 10), which means that aconitase inhibition is not caused by peroxide. Another explanation for the unchanged peroxide concentrations might be an oxidation of this very unstable compound before its analysis was performed. On the other hand, H_2O_2 distribution inside a tissue or inside a single cell might be very inhomogeneous and therefore a high concentration inside a certain cell compartment might not have been realized when its concentration was determined for whole root

segments. An indirect hint to oxidative stress by peroxide is malondialdehyde which is a product that results from lipid peroxidation.

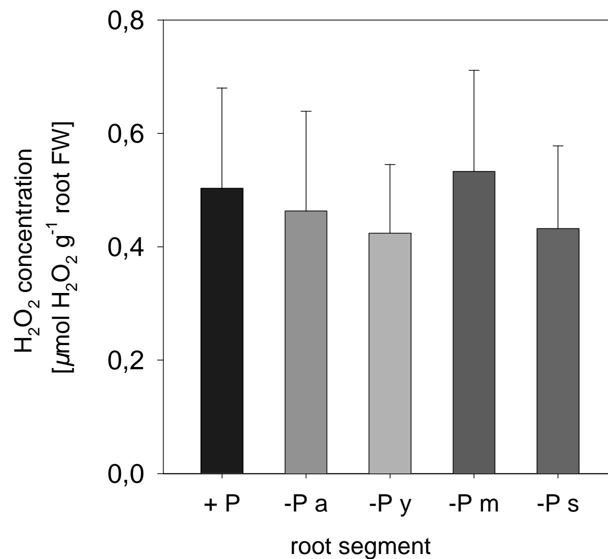


Fig. 10: Peroxide concentrations in different white lupin root segments. n = 14- 21.
One Way Anova gave no significant differences for $P \leq 0.05$. +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11.

Malondialdehyde (MDA) concentrations

Malondialdehyde (MDA) concentrations were only slightly increased in root tips and in young cluster roots of P-deficient plants compared with P-sufficient root tips (Fig. 11). In mature cluster root segments MDA concentrations reached similar values as in the root tips of P-sufficient plants, and in senescent cluster roots MDA concentrations even decreased. This indirectly supports the findings that peroxide production is not increased under P-deficient conditions in root segments of white lupin and therefore should not be responsible for the reduced aconitase activity observed.

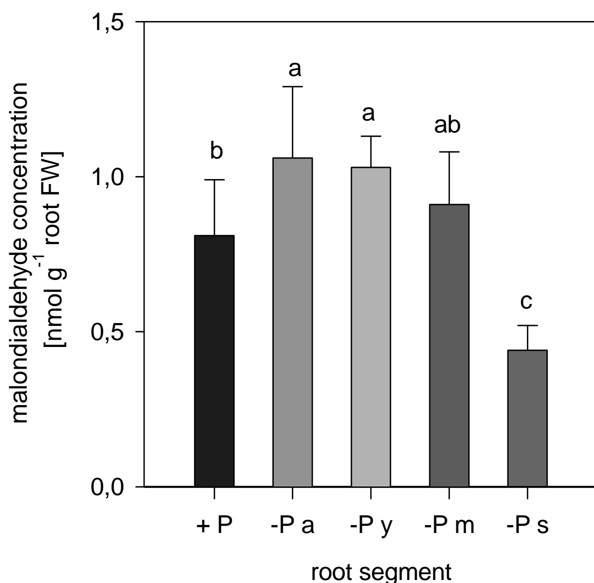


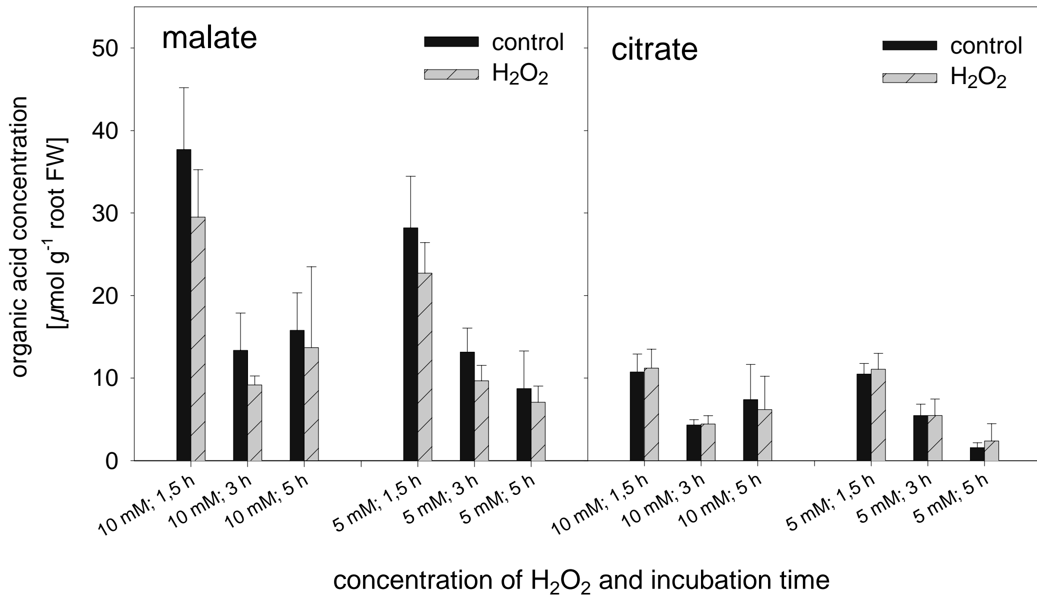
Fig. 11: Malondialdehyde (MDA) concentrations in different white lupin root segments. n = 10- 14. +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11. Significant differences between the root segments are indicated by different letters (One Way Anova, $p \leq 0.05$).

Effects of the external application of aconitase inhibitors on accumulation and root exudation of citrate

Inhibition of the enzyme aconitase by peroxide (H_2O_2)

Aconitase activities were found to be decreased in mature and especially in senescent cluster roots (Fig. 9). Although H_2O_2 tissue concentrations were not increased in mature or senescent cluster roots (Fig. 10), it was tested whether the external application of the aconitase inhibitor H_2O_2 in higher concentrations than found in the root tissue by an *in vitro* test might be able to inhibit aconitase in ageing cluster roots. Therefore H_2O_2 was applied to young cluster roots and it was tested if these clusters could be forced to react like mature cluster roots and increase their citrate concentrations and decrease their aconitase activities.

Citrate/malate tissue concentration ratios as a more sensitive parameter for a change of citrate and malate tissue concentrations were only slightly, but significantly increased at shorter incubation times of 1.5 h and 3 h at both incubation concentrations tested (5 m*M* and 10 m*M*) (Fig. 11). This increase was due to a slight, but significant decrease in malate tissue concentrations, but not due to any increase in citrate tissue concentration and might therefore not influence aconitase activity.



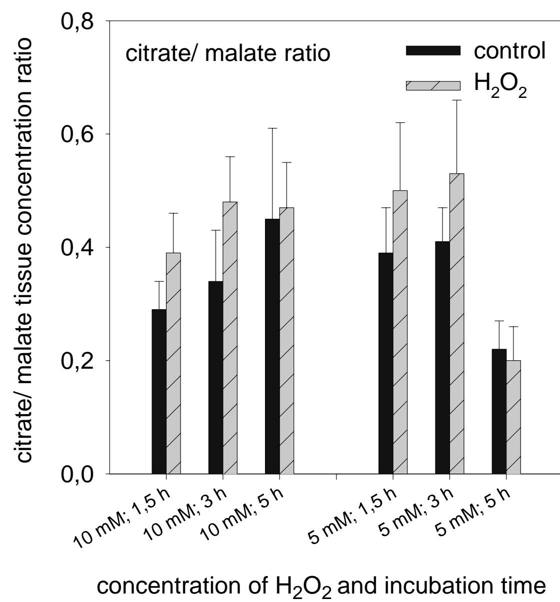


Fig. 12: Malate (left) and citrate (right) concentrations and citrate/malate tissue concentration ratios (down) in young cluster root segments after incubation with the aconitase inhibitor H_2O_2, applied at the concentrations of 10 m*M* and 5 m*M* for 1.5 h, 3 h and 5 h.

Inhibition of the enzyme aconitase by monofluoroacetate (MFA) and its influence on carboxylate accumulation in young cluster roots

Since aconitase inhibition by peroxide did not increase citrate tissue concentrations, monofluoroacetate (MFA) was tried as another known aconitase inhibitor.
MFA is a metabolic inhibitor which is incorporated into fluoroacetyl-CoA, which then reacts with oxaloacetate to form fluorocitrate. Fluorocitrate inhibits the enzyme aconitase, thus preventing the conversion of citrate to isocitrate in the TCA cycle (Quastel, 1963 and references therein; Lauble et al., 1996).

Inhibition of the citrate-metabolizing enzyme aconitase by external application of monofluoro-acetate strongly influenced citrate metabolism in young and mature cluster roots (Fig. 13). Citrate concentrations in young cluster roots incubated with 10 m*M* MFA for 8 h were twice as high as in control roots, whereas malate concentrations were reduced to half of those of control roots. Citrate/malate ratio therefore increased four-fold due to the MFA incubation. Even mature cluster roots showed a further shift towards lower malate concentrations and higher citrate concentrations when treated with MFA. The citrate/malate ratio therefore increased two-fold even in mature clusters.

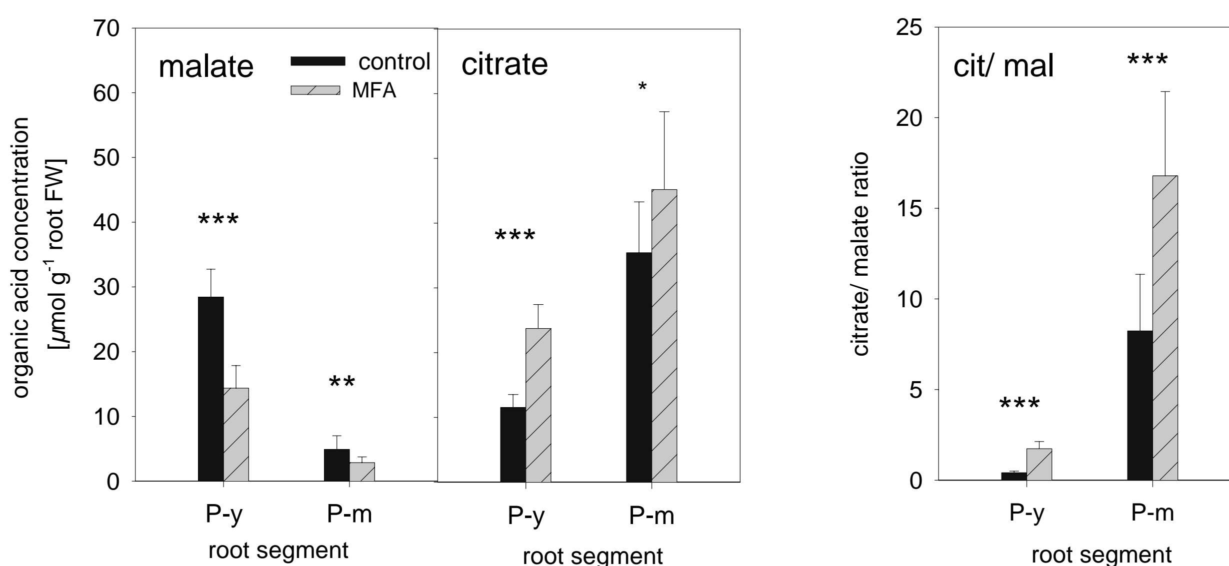


Fig. 13: Malate (left) and citrate (middle) tissue concentrations and citrate/malate tissue concentration ratios (right) in young and mature cluster root segments after incubation with 10 m*M* the aconitase inhibitor monofluoroacetate (MFA) for 8 h. -P y: young cluster roots; -P m: mature cluster roots; see also p. 11. *, **, and *** are significant at the 0.05, 0.01 and 0.001 probability levels, respectively (t-test).

Inhibition of the enzyme aconitase by monofluoroacetate (MFA) and its influence on carboxylate exudation from young cluster roots

High internal citrate concentrations are thought to be a prerequisite for high citrate exudation rates. Artificial inhibition of citrate turnover by blocking aconitase activity with MFA forced still young cluster roots to react like already mature ones and accumulate citrate. This leads to high internal citrate concentrations. Therefore it was tested if young cluster roots could also be forced to release high amounts of citrate.

Localized root exudate collection with filter papers after incubating parts of the root system with MFA (Fig. 14) revealed a four-fold increase in citrate exudation rates in young cluster roots compared to control root segments and only half of the malate exudation rates. In mature cluster roots citrate exudation rates could even be increased further, and malate exudation rates decreased significantly compared with mature control root segments. Calculated on the citrate/malate exudation ratio, MFA led to a 8.5-fold increase of the exudation rate in young and a 3.3-fold increase in mature cluster roots. Exudation rates in MFA-treated young cluster roots were even higher than in non-treated mature cluster roots. Therefore a clear relationship between a reduced aconitase activity, citrate accumulation, and citrate exudation exists.

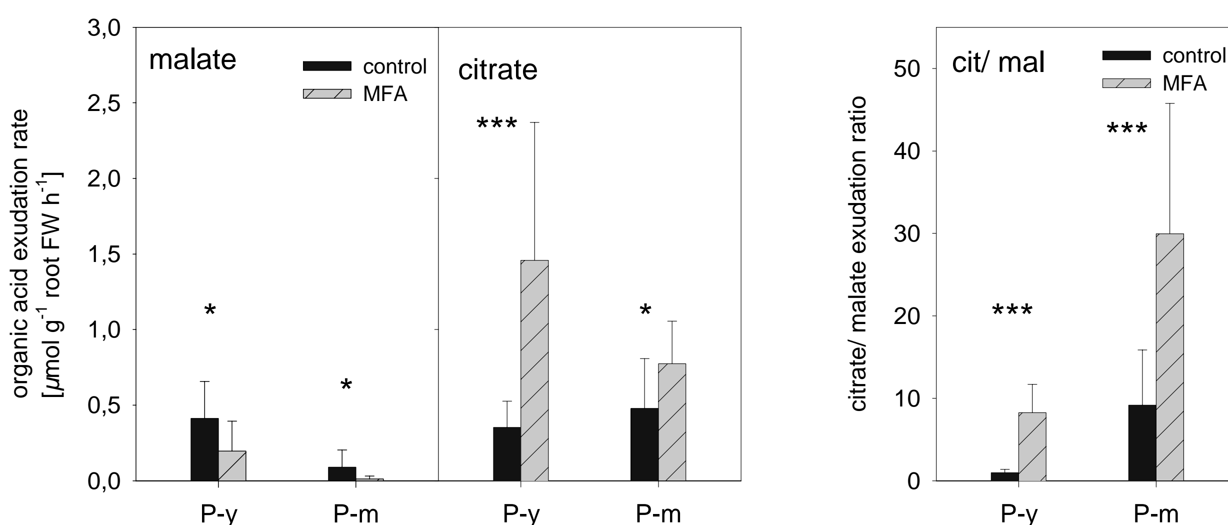


Fig. 14: Malate (left) and citrate (middle) exudation rates and citrate/malate exudation ratios (right) in young (-P y) and mature (-P m) cluster root segments after incubation with 10 m*M* of the aconitase inhibitor monofluoroacetate (MFA) for 8 h. *, **, and *** are significant at the 0.05, 0.01 and 0.001 probability levels, respectively (t-test).

Inhibition of the enzyme aconitase by monofluoroacetate (MFA) and its influence on carboxylate exudation from P-sufficient seedling root tips

Since young cluster roots can be forced to accumulate and exude citrate similar to mature cluster roots, it was investigated how seedling root tips react to aconitase inhibition. Another known factor that can trigger organic acid exudation from roots is aluminium. Several Al-tolerant plants species or plant varieties release organic acids from their root tips under Al-stress. The organic acids are thought to complex Al in the apoplast and therefore protect the root tips from Al-toxicity.

Root exudates collected from MFA, Al- or MFA +Al-incubated root tips were analyzed for several TCA organic acids and for lactate (Fig 15). Root exudates from control root tips did not contain organic acids in detectable concentrations. Root tips incubated with MFA exuded high amounts of malate and citrate, and, to lower amounts, fumarate, c-aconitate, t-aconitate and shikimate. When the root tips were incubated with Al, only citrate and lactate and a small amount of shikimate were released. The combined treatment with MFA and Al resulted in the same exudation pattern as with MFA alone, with the exception of lactate, which was not exuded in the combined treatment.

The MFA treatment initiated malate and citrate exudation rates in seedling root tips similar to those found in non-treated young cluster roots (0.25 and 0.41 μmol malate g^{-1} root FW h^{-1} in MFA-treated root tips and in non-treated young cluster roots, respectively, and 0.23 and 0.35 μmol citrate g^{-1} root FW h^{-1}, in MFA-treated root tips and in non-treated young cluster roots, respectively). Nevertheless, values are not directly comparable because incubation and collection procedures were different.

These exudation rates show that even seedling root tips can be forced to react like older tissue when aconitase activity is inhibited, which again shows a clear relationship between aconitase activity, citrate accumulation, and citrate exudation.

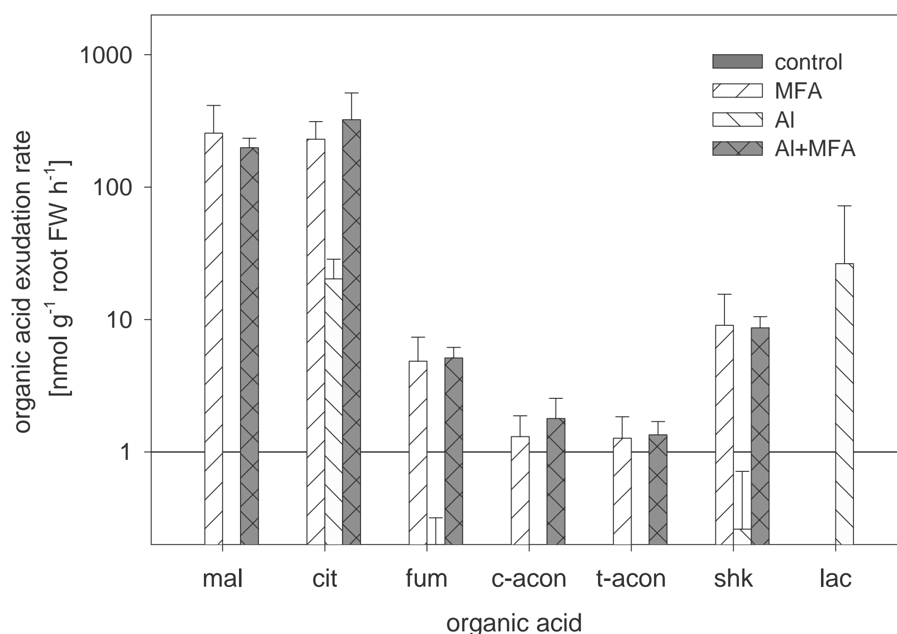


Fig. 15 Carboxylate exudation rates in seedling root tips after incubation with 10 m*M* of the aconitase inhibitor monofluoroacetate (MFA), 20 *µM* Al, and a combination of both (Al+MFA) for 12 h, followed by a localized root exudate collection for 2 h. mal: malate; cit: citrate; fum: fumarate; c-acon: cis-aconitate; t-acon: trans-aconitate; shk: shikimate; lac: lactate. Y-axis: common log-scale.

Inhibition of the enzyme aconitase by monofluoroacetate (MFA) and its influence on aconitase and NADP-ICDH activities

The root segments used for root exudate collection after the partial incubation of the root system with 10 m*M* MFA for 8 h (Fig. 15) were harvested and analyzed for aconitase and NADP-ICDH activities (Fig. 16).

Incubation with 10 m*M* of the aconitase inhibitor MFA for 8 h did not change aconitase and NADP-ICDH activities in young or in mature cluster root segments compared with the non-treated control root segments. This is in contrast to the change in the malate and citrate exudation pattern (Fig. 15) observed under the same treatment, where, in young cluster roots, malate exudation rates decreased and citrate exudation rates increased significantly. As an explanation, the results might be caused by the way the measurement was done. The MFA product (-)-erythro-2-fluorocitrate, the isomer inhibiting the enzyme, might have been removed by the dilution of the solutions the enzyme was dissolved in for enzyme activity measurements.

However, aconitase inhibition was documented to be either competitive or noncompetitive, whereby the competition was reversible, but only a 10^6-fold excess of isocitrate over the enzyme-inhibitor complex did bring enzyme activity slowly back (Lauble et al., 1996, and references therein). Therefore it is not completely conclusive that the MFA-product was really removed from the enzyme by the dilution by the activity measurement solutions. Another explanation would be a fast recovery of the aconitase enzyme or a new production of the enzyme protein after the MFA solution was removed for exudate collection.

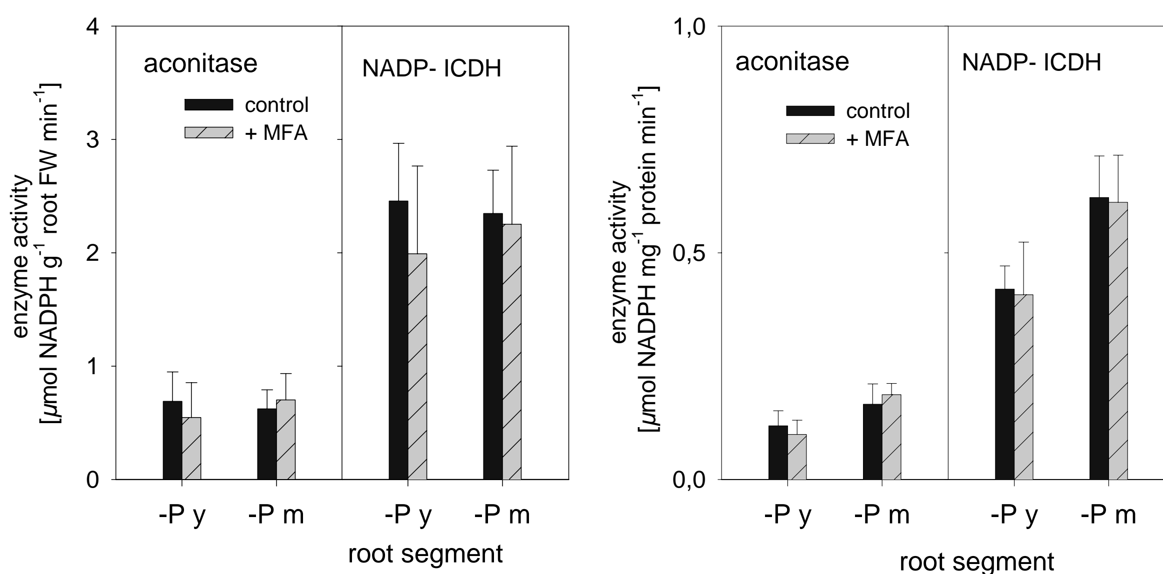


Fig. 16: Aconitase and NADP-dependent ICDH activity in young (-P y) and mature (-P m) white lupin cluster root segments after incubation with 10 m*M* MFA for 8 h.
Left group: aconitase and NADP-ICDH activity per g root fresh weight. right group: aconitase and NADP-dependent ICDH activity per mg protein. Average ± SD (n = 10 – 11) from three different harvests, each with 3-4 replicates. No significant differences between the control and the MFA-treated samples (t-test, $p \leq 0.05$).

Summarizing, the citrate accumulation and exudation in young cluster roots and citrate exudation even from P-sufficient seedling root tips, increased to levels normally found only in mature ones, by blocking aconitase activity with an artificial inhibitor, is a strong argument for a crucial role of the aconitase enzyme in citrate accumulation and exudation also in mature cluster roots.

In situ activity of dehydrogenases (DH) involved in citrate turnover

By histological staining the localization of dehydrogenase (DH) activities in a root cross-section can be seen optically. Thereby a soluble tetrazolium salt is reduced by the DH activity and by use of an organic acid as a reducing substrate. The resulting insoluble reduced tetrazolium dye precipitates and stays as a red-purple stain in the tissue where the reduction occurred.

Citrate, malate, succinate, isocitrate and cis-aconitate served as substrates for several dehydrogenases active in cross sections of the different white lupin root segments. Since the staining reactions occur in living tissue, and since most of the dehydrogenases which catalyze the oxidation of the substrates belong to the TCA cycle, substrates cannot be unequivocally related to definite dehydrogenases. Citrate and isocitrate will be oxidized by ICDH, then by ketoglutarate-DH, malate by malate-DH or by malic enzyme, and succinate by succinate-DH and via fumarate and malate by malate-DH or by the malic enzyme. However, when a staining reaction occurred, the substrate supplied must have been metabolized by its corresponding specific dehydrogenase as a first step. It just cannot be ruled out that following reactions contributed to the total staining that could be seen.

No real differences were observed in the staining intensities between the different substrates when the same root segments were compared (Fig. 17). The highest staining intensities occurred in root segments of +P control roots as well as in root tips and young cluster roots of P-deficient plants. In mature cluster root segments DH activities were much lower and were almost not visible in senescent cluster roots.

Since the cross sections were done with a razor blade by hand and were therefore not very thin, a dissolution down to single cells was not possible. But is was possible to observe different activities in different parts of the root cross sections. In cross sections of +P control root tips, P-deficient root tips and root axes of young cluster roots the highest DH activities could be observed in the area of the central cylinder, and there mainly in the pericycle.

In young cluster root laterals the highest DH activities could be found in the apical rootlet zones of just emerging clusters and in the distal parts of longer young cluster roots (Fig. 17). Interestingly, the area of the very root tips showed no colouring. In mature cluster roots, the root axis showed a similar staining distribution, though with much less intensity. Cluster root laterals showed almost no colouring. In senescent cluster roots even the central cylinder was almost not stained at all. The cortex showed no dehydrogenase activity in any of the root segments, not even in the epidermis.

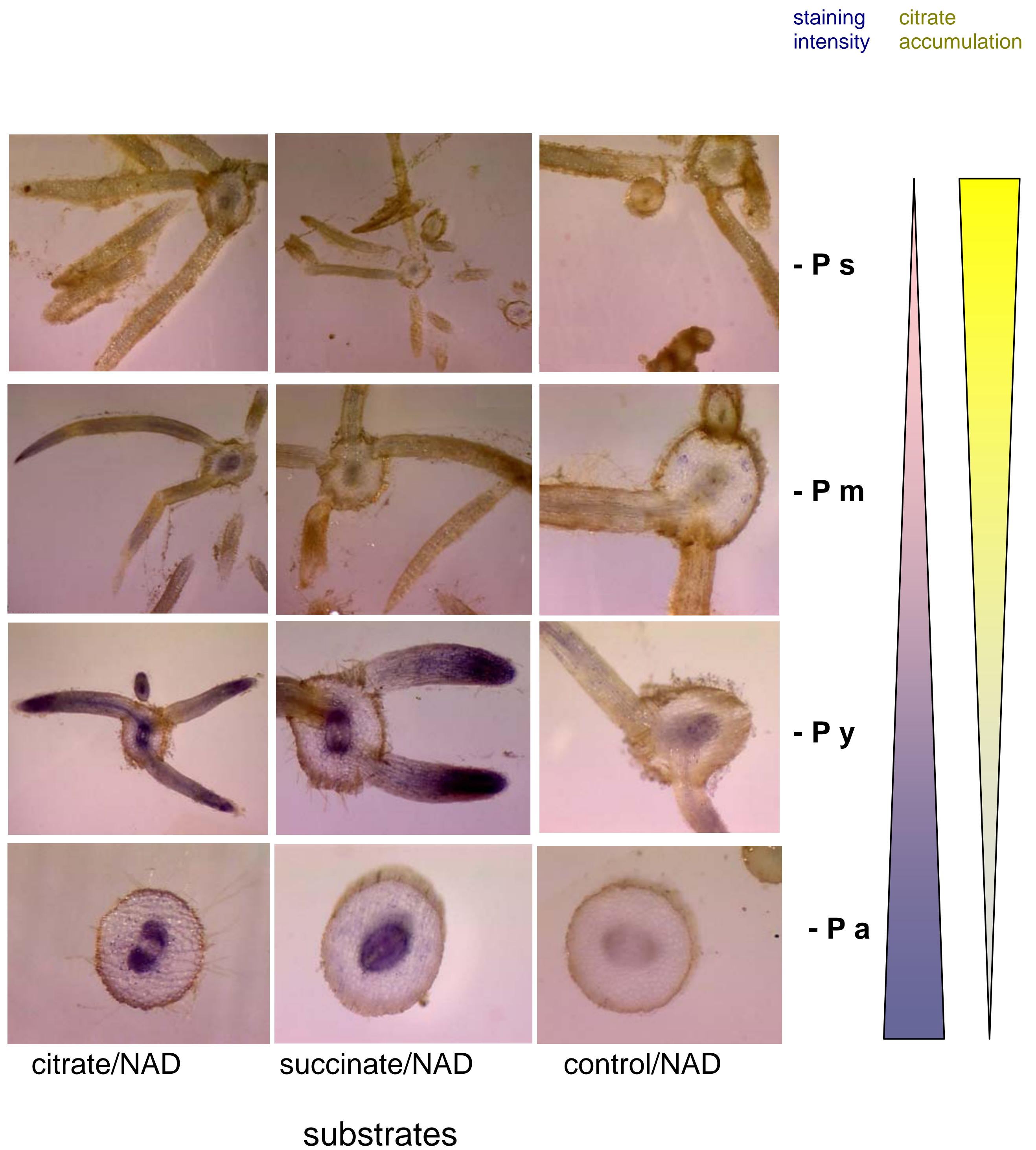


Fig. 17: Histological formazan staining of dehydrogenase activities in different root zones of P-deficient white lupin with citrate and succinate as substrates. -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11.

The decreasing DH activities during cluster root development, determined by the *in situ* staining, are in accordance with the diminishing activities of the aconitase and NADP-ICDH enzymes determined *in vitro*. They all together might contribute to the citrate accumulation observed in mature cluster roots.

Reduced respiration

Respiration provides energy in form of ATP for many metabolic processes by the cytochrome dependent respiratory chain.

In respiration, by the cytochrome-dependent respiratory chain, the energy of NADH, derived from the TCA cycle, is used to produce ATP from ADP and P_i. However, all plants, as known so far, additionally have an alternative respiratory pathway, the so-called alternative oxidase (AOX). It reduces molecular oxygen to water in a single four-electron step without conservation of energy, that is, independent of ADP or P_i (Day et al., 1996). The AOX pathway is generally assumed to maintain electron flow when the cytochrome pathway is blocked to prevent overreduction of the cytochrome chain and therefore to prevent production of reactive oxygen species.

In white lupin cluster roots the increasing P deficiency might reduce cytochrome-dependent respiration by a lack of substrate for ATP production, which might lead to a kind of feedback inhibition back to the TCA cycle by an overreduction of redox equivalents, leaving citrate unmetabolized and acccumulating.

Respiration rates and cytochrome and alternative oxidase (AOX) capacities

To determine if the origin of a root tissue and the age of a cluster root has an influence on root respiration and the distribution between the cytochrome-dependent and alternative oxidase-dependent respiration capacity, O_2 depletion was measured in the different root segments with the use of respiration inhibitors.

Total respiration was essentially the same in root tips of P-sufficient and P-deficient plants and young cluster root segments of P-deficient plants. In ageing cluster roots total respiration decreased from young via mature to senescent cluster roots (Fig. 18). AOX capacity reached only 10 % of total respiration in the root tips of P-sufficient and P-deficient plants, whereas in all

the cluster root segments this percentage was three to four times higher. The percentage of cytochrome-dependent respiration capacity compared to total respiration in all the root segments did not show any significant trend. Therefore the observed decrease in total respiration rate, being concomitant with the decreasing cytochrome-dependent respiration capacity, means that the AOX-dependent respiration capacity was not able to compensate for the decreasing cytochrome-dependent respiration.

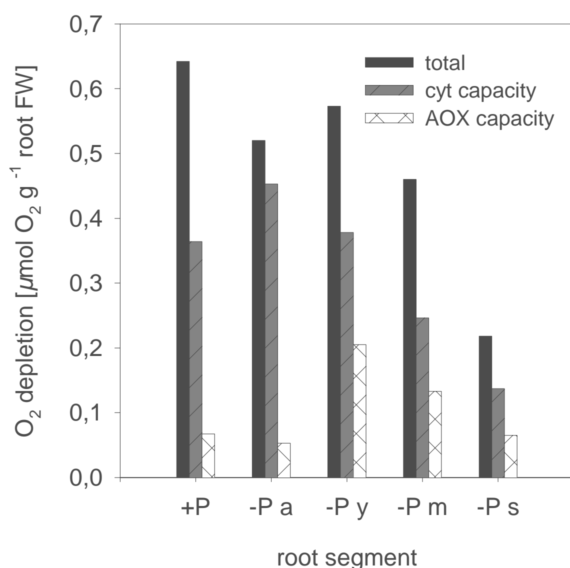


Fig. 18: Respiration, measured as O_2 depletion, in different white lupin root segments. Total: respiration without addition of inhibitors; cyt capacity: cytochrome-dependent respiration capacity, defined as the amount of KCN-sensitive O_2 uptake in the presence of 7.5 m*M* salicylhydroxamic acid (SHAM); AOX capacity: alternative oxidase-dependent respiration capacity defined as the amount of SHAM-sensitive O_2 uptake in the presence of 0.5 m*M* KCN. All values were corrected for residual respiration, measured with the addition of both, KCN and SHAM. +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11. The results are from several independent experiments on different plant subcultures (n = 3-14).

Western Blot analysis

To examine the connection between the AOX capacities and protein concentrations, immunochemical determination of the AOX protein via Western Blot analysis was performed (Fig. 19). The monoclonal antibody raised against the *Sauromatum guttatum*-AOX also reacted with white lupin AOX. Young and mature roots and +P and –P root tips were investigated. The main immunoreactive band occurred at 32 kDa. In the younger root segments (+P and –P root tips) additional multiple immunoreactive bands could be seen, mainly with molecular weights lower than 32 kDa. They are probably degradation products of the AOX protein. In the +P and -P

root tips an additional band at approximately twice the molecular weight of the main band with low intensity could be detected. Altogether, when the intensities of all the immunoreactive bands of a sample are taken together, it seems that AOX protein concentration was highest in –P root tips and young cluster roots compared to +P control roots, and decreased significantly during cluster root development. The AOX protein concentrations therefore roughly paralleled AOX respiration capacity measured as O_2 uptake and therefore the amount of the AOX protein seems to limit AOX capacity.

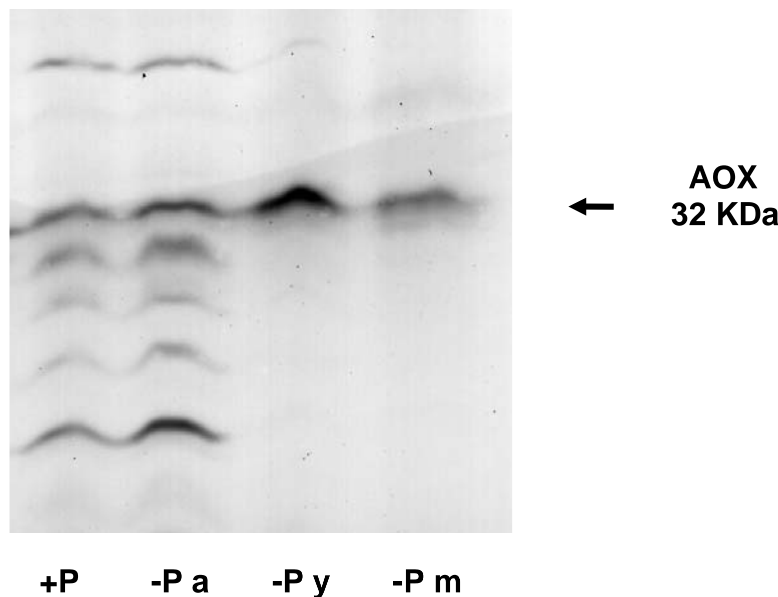


Fig. 19: Western Blot analysis of the alternative oxidase protein in different white lupin root segments. +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P a: 10 mm apical root zone of lateral roots of P-deficient plants; -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11.

Uncoupling of respiration

Energy from reduced pyridine nucleotides is used for ATP production in the respiratory chain. For this the substrates ADP and P_i are needed. Their availability might be limiting, especially under P-deficient conditions. This may then lead to an overpolarization of the inner mitochondrial membrane and a lowered electron transport rate in the respiration chain. O_2 as the terminal electron acceptor may be consumed to a lower rate, resulting in a reduced respiration rate. If respiration is inhibited by a lack of ADP and P_i for ATP production, uncoupling of the electron transport from ATP production should increase the respiration rate.

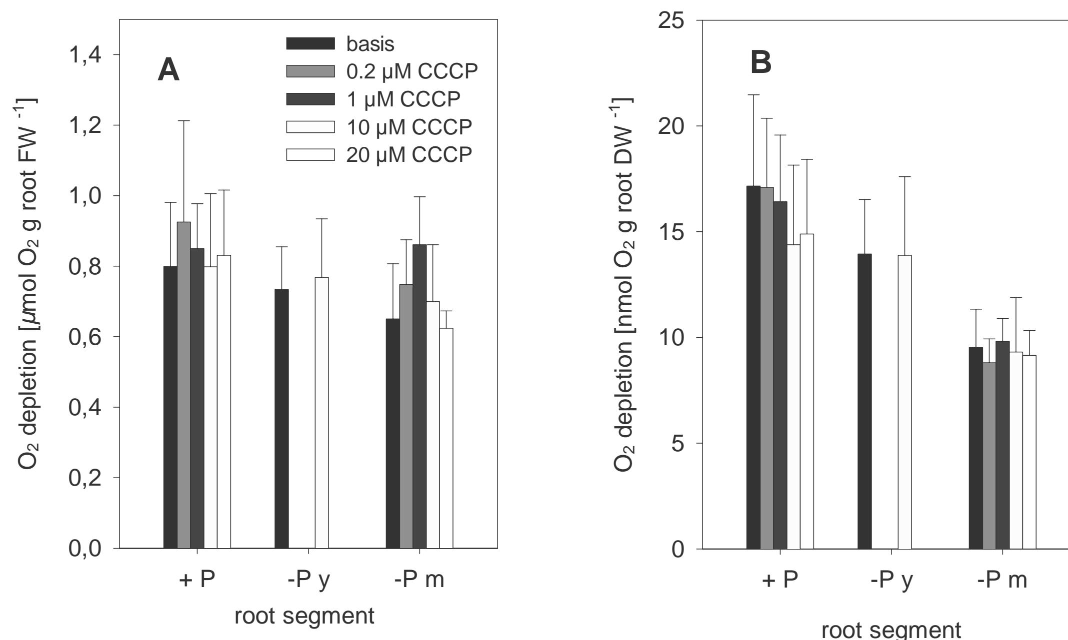


Fig. 20: The effect of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) with the concentrations of 0; 0.2; 1; 10, and 20 *µM* on respiration of different white lupin root segments. +P: 10 mm apical root zone of lateral roots of P-sufficient plants; -P y: young cluster roots; -P m: mature cluster roots; see also p. 11. A: per g root fresh weight. B: per mg root dry weight. The results are from several independent experiments on different subcultures of plants; n = 3-24).

Uncoupling the respiration of white lupin root segments with the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) did not change respiration rates in P-sufficient root tips or in young or mature cluster root segments of P-deficient plants, although a wide range of CCCP concentrations (0.2; 1; 10, and 20 *µM* CCCP) were applied (Fig. 20). This means that the respiration-limiting step, especially in the P-deficient mature and senescent cluster roots, is not oxidative phosphorylation.

Oxidative damage of the respiratory chain ?

In respiration, the formation of reactive oxygen species often increases under P-limiting conditions, when the electrochemical gradient produced by accumulation of protons outside the mitochondrial matrix and reduction of the electron transport chain is not dissipated by ATP production due to a lack of ADP and P_i. To test if this is also true for the root segments of P-deficient plants, their peroxide concentrations were determined. However, peroxide concentrations as well as malondialdehyde concentrations as a marker for oxidative damage were essentially the same for all the root segments investigated (Fig. 10 and 11). As for the

reduced activity of the aconitase enzyme, oxidative damage is rather not the reason for the reduced respiration rates observed.

Partial root incubation with respiration inhibitors

To test if citrate accumulation goes back to a block in respiration, respiration inhibitors were applied to a part of the root system, such as azide to inhibit cytochrome-dependent respiration and salicylhydroxamic acid (SHAM) to inhibit alternative oxidase-dependent respiration (Fig. 21). An artificial block of the respiratory pathway might leave the NADH, normally metabolized to produce ATP in the respiratory chain, unused, giving a feedback substrate inhibition to the TCA cycle where NADH is built. If TCA cycle enzymes are inhibited, TCA cycle substrates such as citrate stay unused and should accumulate. This was to be tested by application of the respiratory inhibitors.

Azide and SHAM had both no effect on citrate tissue concentrations in young and in mature cluster roots at both incubation times (4 h and 8 h) investigated (Fig. 21). Malate tissue concentrations in young cluster roots were lower when azide or SHAM were applied, but significant changes occured only after 8 h of incubation. No changes could be detected in mature cluster roots, concerning malate concentrations. The increase in the citrate/malate tissue concentration ratio as a more sensitive parameter for changes in both, malate and citrate concentration (Tab. 3), was therefore mainly due to lower malate concentrations and not to increased citrate concentrations as a reaction to respiration inhibitor application. It can be concluded that the reduced respiration observed during cluster root development is rather not responsible for the citrate accumulation in the respective cluster root segments.

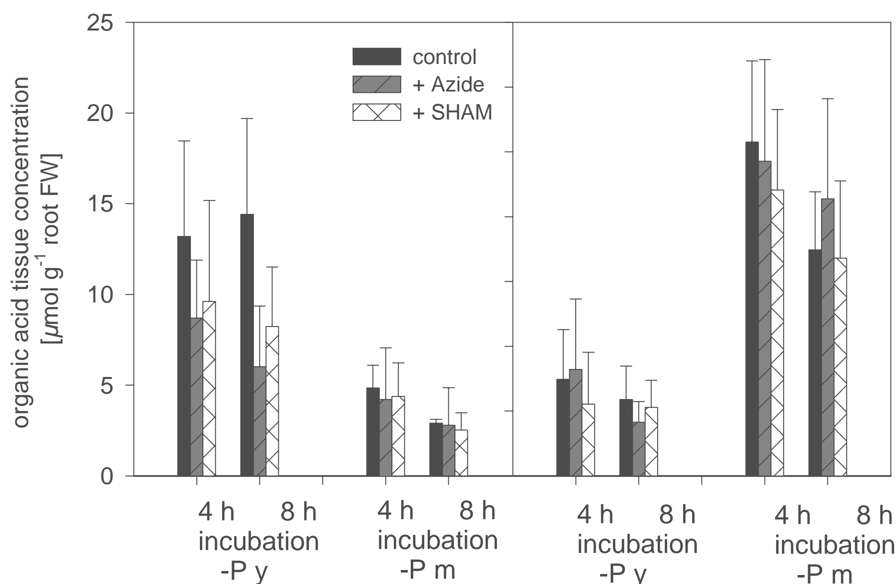


Fig. 21: The effect of partial root incubation with the respiration inhibitors azide (1 m*M*) and SHAM (7.5 m*M*) for 4 h and 8 h on malate (left) and citrate (right) concentrations in young (-P y) and mature (-P m) cluster roots of white lupin.The results are from several independent experiments on different subcultures of plants; n = 12-19 for –P y and n=8-9 for –P m with 4 h incubation and n=2 for –P m with 8 h incubation).

Table 3: The effect of partial root incubation with the respiration inhibitors azide (1 m*M*) and SHAM (7.5 m*M*) for 4 h and 8 h on citrate/malate tissue concentration ratios in young (-P y) cluster roots of white lupin.The results are from several independent experiments on different subcultures of plants. Significant differences between the root segments are indicated by different letters (One Way Anova, $p \leq 0.05$).

inhibitor treatment	citrate / malate ratio	
duration	4 h	8 h
control	0.51 a	0.39 a
SHAM	0.57 ab	0.68 b
azide	0.92 b	0.83 b

Reduced assimilation of nitrate into 2-oxoglutarate as N acceptor ?

It is generally observed that NO_3 uptake in P-deficient plants is reduced, whereas the cause is not conclusively found yet. 2-oxo-glutarate as a product of citrate degradation is the C-skeleton needed for incorporation of ammonium for amino acid synthesis. A lower nitrate reduction rate

by nitrate reductase and a lower incorporation rate of ammonium might lead to an accumulation of 2-oxoglutarate and as a feedback inhibition to an increase in citrate concentration. Therefore tungstate was applied as a nitrate reductase inhibitor and the malate and citrate concentrations in the different root segments were determined.

At all Na_2WO_4 concentrations tested (300 μM, 600 μM, and 1000 μM), citrate concentrations in young cluster roots were only slightly increased, and malate concentrations only slightly decreased at the two lower Na_2WO_4 concentrations applied, without any change at the highest Na_2WO_4 concentration (Fig. 22). The citrate/malate ratio as a more sensitive parameter for a change in malate as well as in citrate concentration increased by around 50 % at 600 and 1000 μM Na_2WO_4, when the nitrate reductase inhibitor was applied. However, citrate concentrations were much lower than usually reached in mature cluster roots. A reduced use of 2-oxoglutarate for N incorporation seems rather not to be an important factor to cause the high citrate concentrations found in mature cluster roots.

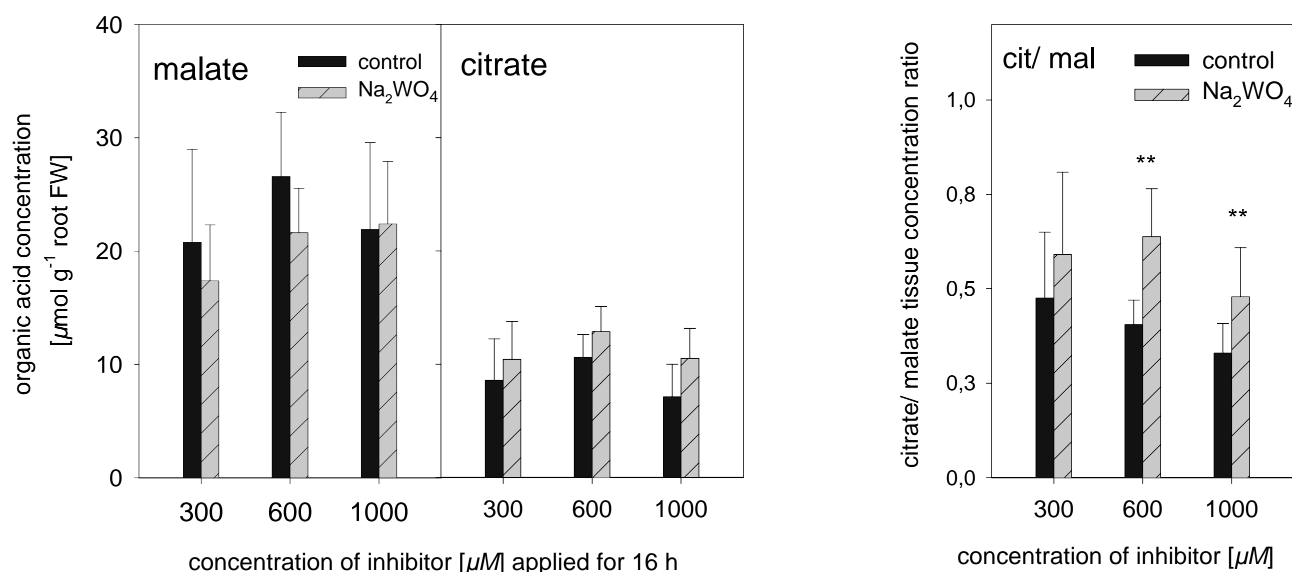


Fig. 22: Malate (left) and citrate (middle) concentrations and citrate/malate tissue concentration ratios (right) in young cluster root segments after incubation with the nitrate reductase inhibitor Na_2WO_4, applied at concentrations of 300 μM, 600 μM and 1000 μM for 16 h. **: significant at the 0.01 probability level (t-test).

ATP-citrate lyase (ACL) activity

The possible involvement of the enzyme ATP-citrate lyase (ACL) in citrate accumulation during cluster root development was investigated in cooperation with N. Langlade and E. Martinoia (Université de Neuchâtel, Suisse). The enzyme ACL cleaves citrate to acetyl-CoA and oxaloacetate by use of the energy of ATP (Fig. 23) and therefore is a possible regulation point in citrate metabolism. The enzyme's activity and expression declined with maturation of the cluster roots, parallel to the declining malate concentrations measured in the corresponding clusters (Fig. 24). Malate could be produced from the ACL product oxaloacetate via malate dehydrogenase. The increasing citrate concentrations found in mature and senescent clusters might be caused by a reduced turnover by the decreasing activity of the ACL in connection with a reduced ACL expression.

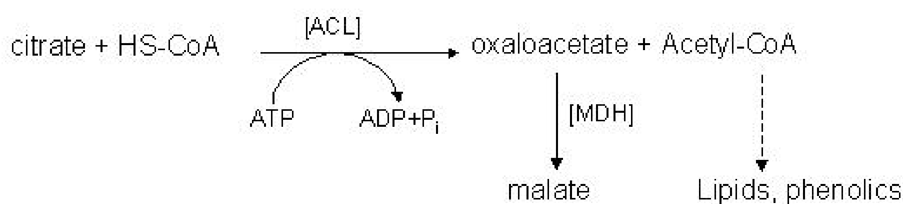
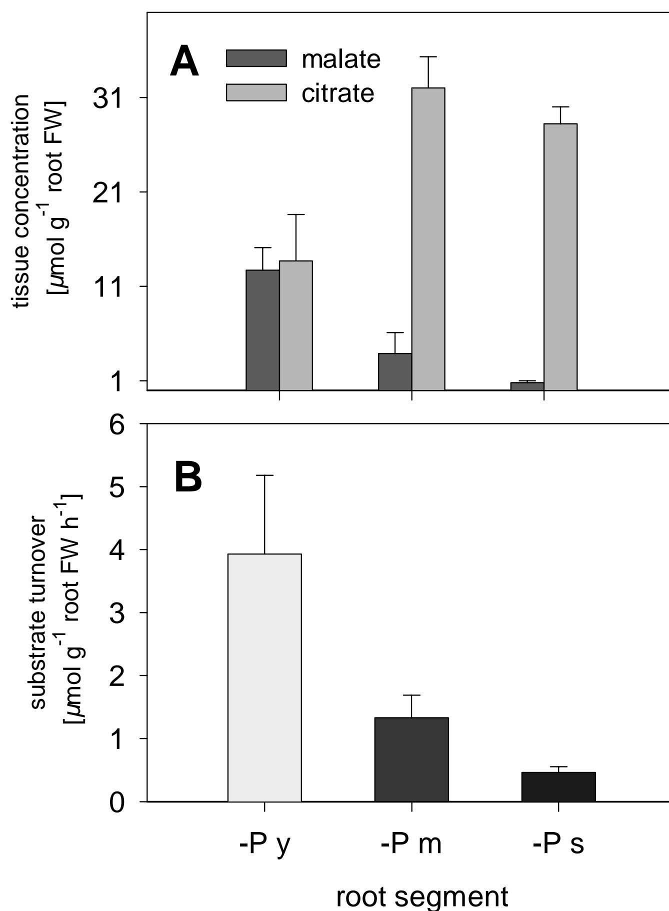


Fig. 23: reaction scheme of the enzyme ATP-citrate lyase



C ACL expression

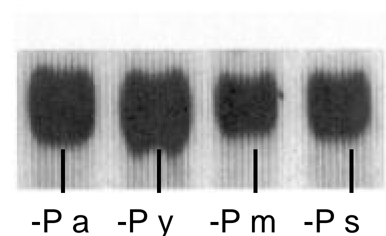


Fig. 24: **A**: malate and citrate root segment tissue concentrations. **B**: *in vitro* activities of ATP-citrate lyase in different white lupin root segments; **C**: transcript levels of ACL. -P y: young cluster roots; -P m: mature cluster roots; -P s: senescent cluster roots; see also p. 11.
(data from N. Langlade and E. Martinoia; Université de Neuchâtel, Suisse)

Inhibition of the citrate-cleaving enzyme ATP-citrate lyase (ACL)

Citrate degradation was found to be facilitated by ATP-citrate lyase, with decreasing enzyme activities when the cluster roots grew older (Langlade et al., 2002). Therefore a partial root incubation was tried with the ATP-citrate lyase inhibitor hydroxycitrate at concentrations of 5 m*M* for 12 h and of 100 m*M* for 8 h. However, at both hydroxycitrate concentrations, malate and citrate tissue concentrations in young cluster roots and therefore the citrate/malate ratios did not show any differences (Fig. 25).

Besides the obvious explanation that hydroxycitrate had no influence on the ACL and on the organic acid concentrations in the root tissue, hydroxycitrate might not have been taken up into the root cells. Citrate does not diffuse across the plasma membrane to a higher extent, and exudation is probably regulated via anion channels. Therefore, vice versa, hydroxycitrate, being chemically very similar to citrate, might be excluded from uptake by the plasma membrane, especially because it has to be taken up against an electrical gradient.

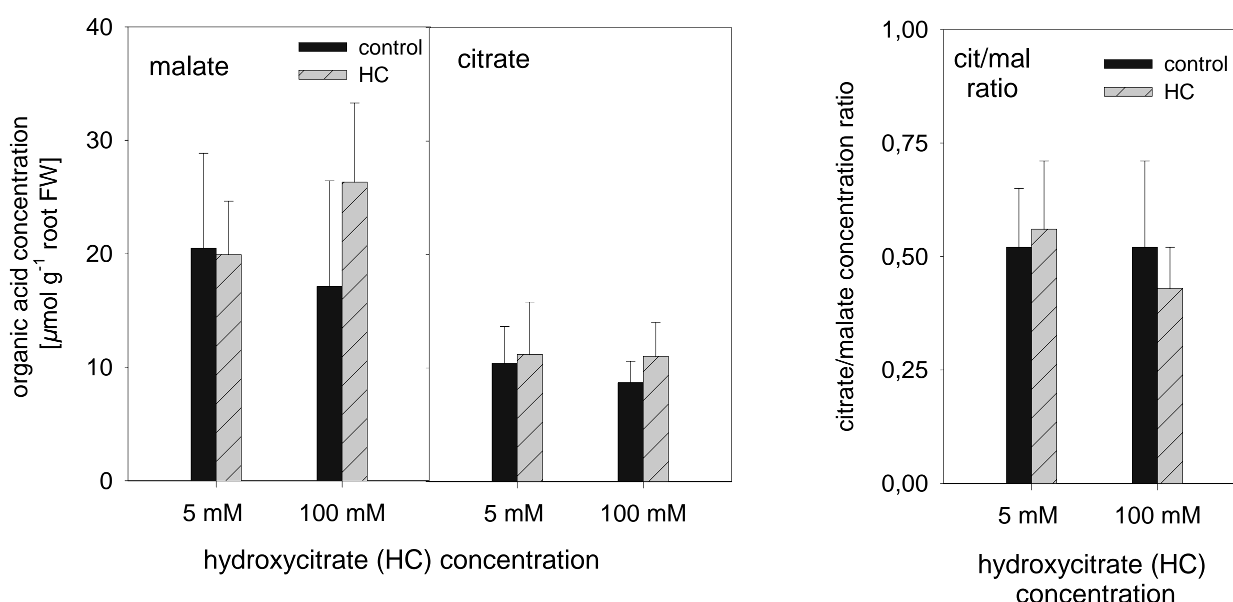


Fig. 25: Malate (left) and citrate (middle) concentrations and citrate/malate tissue concentration ratios (right) in young cluster root segments after incubation with the ATP-citrate lyase inhibitor hydroxycitrate (HC), applied at the concentrations of 5 m*M* for 12 h and of 100 m*M* for 8 h.

Discussion

Increased biosynthesis of carboxylates under P-deficient conditions

High levels of citrate accumulation in mature cluster roots of P-deficient white lupin, followed by a pulse of citrate exudation, might be due to a variety of metabolic processes. It has been reported that enzymatic pathways, leading to citrate production, are stimulated under P deficiency, such as the activity of PEP-C and MDH, or the citrate synthase (Johnson et al., 1994; 1996a+b; Neumann et al., 1999; Neumann and Römheld, 1999; Keerthisinghe et al., 1998; Watt and Evans, 1999a; Uhde-Stone et al., 2003a).

Phospho*enol*pyruvate carboxylase (PEP-C) activity

In vitro activity of phospho*enol*pyruvate-carboxylase (PEP-C) (EC 4.1.1.31) per root biomass as well as the specific activity was found to be increased in developing cluster roots (Table 1), paralleled by a higher amount of the immunodetectable PEP-C protein on fresh weight and on protein base (Fig. 6). Interestingly, high specific PEP-C activities in mature and particularly in senescent clusters are associated with a declining abundance of the PEP-C protein (Tab. 2). This may indicate a posttranslational regulation of the enzyme. Mechanisms of posttranslational regulation of PEP-C comprise both, positive (sugar-P) and negative (malate) allosteric control (Lepiniec et al., 1994) and protein phosphorylation (Gilbert et al., 1998).
Maximum PEP-C activity one day before the onset of citrate efflux and two days before its peak, followed by a 75 % decrease of PEP-C activity over a period of 3 days, as reported by Watt and Evans (1999b), fits to the observation that maximum PEP-C activity precedes citrate efflux and suggests a contribution to increased accumulation of citrate in the cluster root tissue prior to exudation (Neumann et al., 1999). Accordingly, $^{14}C-CO_2$ labelling studies revealed that a substantial proportion of the C exuded by P-deficient lupin is derived from nonphotosynthetic C fixation in roots (Johnson et al., 1996a), probably to replenish the carbon loss by citrate exudation.
Increased PEP-C activity may also reflect a P_i-releasing, anapleurotic reaction, induced under P-deficient conditions, to circumvent the ATP-dependent pyruvate kinase (PK) reaction, in consequence leading to an enhanced malic acid production. Kihara et al. (2003) described a higher PEP-C activity at the expense of the PK activity in mature cluster roots of white lupin. Additionally, PEP-C is part of the pH stat mechanism (Sakano, 1998; see also Chapter 2). A

lower uptake of nitrate under P deficiency (Neumann et al., 2000) leads to an excess uptake of cations (a higher cation/anion uptake ratio) particularly expressed in dicotyledonous plants with a high inherent demand for Ca, which was also described for white lupin (Dinkelaker et al., 1989; Pilbeam et al., 1993; Neumann et al., 2000; Sas et al., 2001). In consequence, a higher electrical potential due to the surplus of cations taken up into the cytosol causes a depolarization of the plasma membrane (PM), activating the PM H^+-ATPase to preserve the electrical gradient across the plasma membrane. However, the protons released across the PM for electrical reasons alkalize the cytosol, which activates PEP-C together with the (anaplerotic) glycolytic pathway leading to net production of protons.

Citrate synthase (CS) activity

Citrate synthase (CS) (EC 4.1.3.7) activity as the metabolic step converting oxaloacetate and acetyl-CoA to citrate did not show a direct relation to citrate accumulation during cluster root development (Table 2), as similarly reported by Neumann et al. (1999) and Kihara et al. (2003) in a similar way for cluster roots of white lupin, or by Aono et al. (2001) for *Sesbania rostrata* CS mRNA. However, CS activity was described to be increased in P-deficient carrot cells (Takita et al., 1999) but also in white lupin (Johnson et al., 1994), although differences between developmental stages of cluster roots were not considered in this study. These findings suggest that the activity of citrate synthase is probably no limiting step in citrate production even in mature cluster roots. Accordingly, so far, overexpression of bacterial CS genes in tobacco under P deficiency or Al-stress (Delhaize et al., 2001; Betekong, 2004) did not show citrate accumulation, whereas de la Fuente et al. (1997) and López-Bucio et al. (2000) reported higher citrate accumulation and exudation in tobacco overexpressing bacterial CS from *Pseudomonas aeruginosa*.

Pyruvate concentration and malic enzyme activity

Pyruvate is a key metabolite that links glycolysis with mitochondrial respiration via the TCA cycle, and it is a precursor for various amino acids, fatty acid biosynthesis, anaerobic fermentation, and in the end, is a precursor for citrate. Even a function as an antioxidant was proposed (Juszczuk and Rychter, 2002).

The trend for a continuous decline of pyruvate concentrations observed in developing cluster roots (Fig. 8) might be explained by the increased PEP-C and MDH activities which promote anapleurotic oxaloacetate and malate production while circumventing direct pyruvate production.

In a subsequent reaction leading to an anaplerotic pyruvate production, malic enzyme (ME) converts malate to pyruvate, whereby NAD^+ or $NADP^+$ is reduced to NADH or NADPH and CO_2 is released. NAD^+-malic enzyme was found to be associated with mitochondria and shows an absolute specificity for NAD (Dittrich, 1976). The decrease observed in ME (EC 1.1.1.39) activity (Fig. 7) parallels the decreasing pyruvate concentrations in the root segments. However, when ME activity was calculated on protein basis, the activity did not decrease during cluster root development and was even slightly higher in mature clusters. Therefore declining ME activity in ageing cluster roots is probably due to lower protein concentrations in these tissues (Fig. 7). Accordingly, high intramitochondrial ME activity was reported in young and nutrient-sufficient plants (Millar et al., 1998).

In tobacco suspension cells the concentrations of pyruvate-derived amino acids declined under P-deficiency. This was explained by a limited availability of pyruvate, due to adenylate control of the enzyme pyruvate kinase (Parsons et al., 1999). Contrary to this, pyruvate accumulation in (severely) P-deficient bean plants was observed (Juszczuk and Rychter, 2002). These authors explained this by an increased synthesis via PEP-C and PEP phosphatase, and a decreased utilization due to an imbalance between the activity of the cytochrome pathway (reduced respiration) and the oxidation of organic acids, promoting the accumulation of pyruvate.

PEP-phosphatase activity increased in mature cluster roots of white lupin compared to root tips of +P and –P-plants (Kihara et al., 2003), as also found for *Brassica nigra* (Duff et al., 1989a+b) or *Selenastrum minutum* suspension cells (Theodorou et al., 1991). Thus pyruvate production via PEP-phosphatase-mediated dephosphorylation of PEP might prevent pyruvate concentrations to decrease more severely than observed, and could keep pyruvate concentrations high enough to provide substrate for citrate production.

Reduced turnover of citrate under P-deficient conditions

Although the metabolic changes on the anabolic side of citrate metabolism such as an increased PEP-C activity, or increased MDH and PEP-phosphatase activities, contribute to production of

malate, oxaloacetate and pyruvate as citrate precursors, this would still not explain the highly selective accumulation of citrate observed in mature cluster roots under P deficiency. Additionally, other mechanisms must exist to determine citrate accumulation. A first hint to such a mechanism was found by a decreased aconitase activity in mature cluster roots of white lupin (Neumann et al., 1999; Neumann and Römheld, 1999), which might explain citrate accumulation by a reduced citrate turnover in response to P limitation.

Reduced aconitase activity

Conversion of citrate to isocitrate with the intermediate product cis-aconitate, mediated by the activity of aconitase, is the first step of citrate degradation. While the mitochondrial aconitase enzyme most likely participates in the TCA cycle, the cytosolic enzyme might play a role in different metabolic pathways (Sadka et al., 2000a).

It is known that inactivation of aconitase could cause an accumulation of citrate or other metabolic intermediates (Gardner and Fridovich, 1991), although mitochondrial aconitase is not normally considered a rate-limiting component of the TCA cycle (Chen et al., 1997). Investigation of aconitase in P-deficient white lupin root segments revealed decreasing activities during cluster root development (Fig. 9). Accordingly, citrate accumulation, followed by compartmentation into vacuoles and concomitant proton influx into the vacuoles was found to be caused by a reduction in aconitase activity in sour lemon (Sadka et al., 2000a) and hints to aconitase as a key metabolic step in citrate degradation.

By the first view, the relatively low decrease in aconitase activities seem not to be very convincing to explain the very high citrate accumulation observed in mature and senescent cluster roots of *Lupinus albus*. However, the optimal conditions for enzyme activities used in *in vitro* tests might not always mirror the conditions under which the enzymes react *in vivo* (Watt and Evans, 1999; Cots et al., 2002). Variability in enzyme activity that might exist between neighbouring root tissues is also not considered, and therefore direct correlation of changes in metabolite pools with *in vitro* enzyme activity data is difficult. Moreover, apart from aconitase activity, a range of additional metabolic reactions involved in citrate turnover may be affected by P limitation and were subject of further investigations.

Reduced activity of dehydrogenases

NADP-dependent isocitrate dehydrogenase (NADP-ICDH) activity

Isocitrate dehydrogenase is the second step in metabolizing citrate by oxidizing isocitrate to 2-oxoglutarate (2-OG). Thereby adenosine nucleotides are reduced and CO_2 is released. The NAD-dependent enzyme is associated with the mitochondria as part of the TCA cycle, while the NADP-ICDH exists as different isoenzymes linked to cytosolic, chloroplastic, peroxisomal and mitochondrial compartments. The cytosolic NADP-ICDH isoform seems to be the predominant isoenzyme in higher plants, as the major part of the activity detected in leaves and roots is associated with this isoenzyme (Gallardo et al., 1995). Hence this was the enzyme examined in white lupin cluster roots.

NADP-ICDH activities paralleled aconitase acitivies in all the different root segments investigated, although on a two- to threefold higher level (Fig. 9). On the other hand, the NAD-specific ICDH generally seems to make up less than 10 % of all ICDH activity (reviewed by Palomo et al., 1998). It has been suggested that NADP-specific ICDH (EC 1.1.1.42) represents an additional or alternative path to the the NAD-dependent ICDH (EC 1.1.1.41) TCA cycle enzyme, and supplies the 2-OG for amino acid biosynthesis and ammonia assimilation (Chen and Gadal, 1990a). The antisense inhibition of NADP-ICDH in transgenic tomato plants left amino acid concentrations and respiration unchanged. Only the levels of isocitrate and citrate increased. The authors assumed that potato can cope with a severe reduction in cytosolic NADP-ICDH activity without major shifts in growth and metabolism (Kruse et al., 1998). Gallardo et al. (1995) found a reduced NAD-ICDH, and an increased NADP-ICDH activity in ripening tomato fruits and associated this with a higher demand of 2-OG for glutamate production. In mature cluster roots of white lupin cytosolic ICDH transcripts were lower and mitochondrial ICDH transcripts similar compared with +P control root tips (Kihara et al., 2003). Specific activities of aconitase and NADP-ICDH were the same in all the root segments investigated, with the exception of mature cluster roots, where specific activity was even slightly higher. Since total protein concentrations decreased during cluster root development, the decreasing aconitase and NADP-ICDH activities in the root segments might be due to lower enzyme concentrations in the roots. In a similar way, the decline in aconitase abundance under Fe deficiency was speculated to be due to an overall functional impairment of mitochondria (Chen et al., 1997).

In contrast to the results presented here, Kihara et al. (2003) found a higher aconitase activity in –P lupin root tips compared to the +P controls, but also a 30 % lower activity in mature clusters than in the controls, both, on the base of protein and root biomass. Similarly, they found slightly

lower NADP-ICDH activities in –P lupin root tips and only half the activity in mature cluster roots compared to the +P control root tips, again in both, on a protein and root fresh weight base. Their Western Blot analysis showed that the ICDH protein content paralleled activity, probably being regulated at the transcript level. The differences in both investigations might be due to the different experimental conditions and another plant variety. The similar activity pattern found independent of a protein or root fresh weight base would mean that protein concentrations were the same in all the segments investigated, which contrasts to the results gained by Neumann et al. (1999), Massonneau et al. (2001) and in this work.
De Vos et al. (1986) showed that citrate and malate accumulation in Fe-deficient bean roots was not due to lower specific aconitase activities, as was supposed, because aconitase contains a 4Fe-4S-cluster in its active site (DeKock et al., 1960; Bacon et al., 1961; Venkat Raju et al., 1972). They suggested a hormonal unbalance, caused by Fe deficiency and leading to proton extrusion, which was followed by citrate and malate production as a pH-stat regulation (Landsberg, 1981).
To decide whether citrate accumulation is definitely due to aconitase and ICDH impairment, further investigations are needed. The results so far suggest that a general decrease in protein concentration may limit the enzymes' activities, and other factors such as ROS or IRP regulation (see below) might play an additional role.

In-situ staining of dehydrogenase activities

In situ activity staining of dehydrogenase (DH) using tetrazolium salts and a selection of suitable carboxylates as substrates (citrate, aconitate, isocitrate, succinate, malate) made it possible to visualize the areas of DH activities in cross sections of different root segments which may be involved in citrate turnover.
Staining intensities were similar in cross sections of the same root segment irrespective of the substrate supplied. This can be explained by co-ordinated reactions of the various dehydrogenases present in the tissues, where the product of one reaction is the substrate for another one.
The highest DH activities could be found in growing areas such as the tips of lateral roots of P-sufficient or P-deficient plants or the tips of young cluster rootlets. The area of the very root tip, however, seemed not to show any DH activity. Activity generally was associated with dividing cells such as the pericycle in the root cross sections, showing the highest metabolic turnover in these growing parts of the tissue, and lower activities in the cortex region of the root cross section. In a similar way, Uhde-Stone et al. (2003a) found PEP-C expression localized primarily

in the cortex and in the meristem. For *in situ* hybridization of malate-DH these authors could show that expression occurred throughout the just emerging cluster rootlet and in the cortex, but not in the apices of mature clusters, and no detectable expression at tip and base of the clusters. They interpreted this as a hint that oxaloacetate in part might be directed to citrate rather than to malate, which is in accordance with the reports that malate exudation shifts towards citrate exudation during cluster root development (Neumann et al., 1999).

The decreasing dehydrogenase activities found during cluster root development are in accordance with the decreasing enzyme activities measured in the different root segments such as aconitase, NADP-ICDH or malic enzyme *in vitro*. This general decrease in activity for different enzymes probably has its common cause in the reduced protein and RNA concentrations in the respective root segments.

Lower staining intensities due to bigger, more vacuolated cells in older tissues, which dilute the areas where enzyme reactions and therefore staining can occur seem rather not to be the case. In mature and even more so in senescent cluster roots small cells with a low percentage of vacuoles do also exist around the distal parts of the cluster rootlets. But there no dehydrogenase activities could be detected. This finding fits to the observation that cluster rootlet growth is determined, although the tissue itself is still young compared to the age of the whole plant. The cells around the root tips keep small and do not elongate, and the small, former meristematic cells just stop their cell division activity, parallel to a decrease in dehydrogenase activity. It might be speculated that a causal relationship exists between the reduction of meristematic activity and the reduction of enzyme activities.

Reduced respiration

During cluster root development, root respiration (Fig. 18) is severely reduced parallel to declining concentrations of intracellular soluble P_i, total RNA, and ATP (Massonneau et al., 2001), suggesting P limitation of respiration, whereas apical root zones of P-deficient plants, compared to P-sufficient ones, show only small changes in total respiration. A reduced respiratory activity may thereby also affect activities of dehydrogenases.

To investigate partitioning of the electron flow between the cytochrome and the AOX pathways, specific inhibitors for both pathways were used: KCN or azide for the cytochrome and hydroxamic acids such as salicylhydroxamic acid (SHAM) for the AOX pathway, allowing a rough estimation of cytochrome and AOX respiration capacities. Both, the capacities of the cytochrome and of the alternative pathway declined during cluster root development, suggesting

an impairment of both pathways under the conditions of increasing P limitation during ageing of cluster roots. However, interpretation of results obtained with use of respiration inhibitors are complicated by several problems: (1) They show a lack of specificity and problems with penetration into tissues (Vanlerberghe and McIntosh, 1997). (2) Their usefulness depends on the assumption that AOX cannot compete with the cytochrome pathway for electrons and is active only when the cytochrome pathway is saturated, which is generally not the case (Day et al., 1996; Vanlerberghe and McIntosh, 1997). The only method at present for quantitative measurements of AOX pathway activity would be the use of oxygen isotope fractionation (Robinson et al., 1995). Cytochrome c oxidase and AOX differentially fractionate ^{18}O when reducing oxygen to water, and this fractionation can be accurately measured with a mass spectrometer. This allows calculation of the partitioning of electron flow between the two pathways in the absence of added inhibitors.

Decreasing respiration rates as described for cluster roots of P-deficient white lupin were also reported for other plant species with a suboptimal nutrient supply (Hoefnagel et al., 1993b), or P deficiency (Theodorou et al., 1991). In contrast, P deficiency did not affect total respiration in bean roots (Rychter and Mikulska, 1990) or tobacco suspension cultures (Parsons et al., 1999). Both authors attributed this to a higher relative contribution of the alternative oxidase (AOX) pathway. Although a clear function for the AOX pathway has yet to be established (Millenaar et al., 2001), an increase in AOX activity was found under several stress conditions under which the cytochrome pathway may limit carbon flux such as nutrient deprivation, chilling temperatures or salt stress (Vanlerberghe et al., 1997). Under P deficiency, the AOX could bypass the ADP- and P_i-demanding ATP production and circumvent the dependence on the transmembrane potential of the cytochrome pathway.

A higher AOX capacity was also found for cluster roots of white lupin compared to +P control root tips and root tips of P-deficient plants, irrespective of the age of the cluster (Fig. 18). However, this increase was not high enough to compensate for the loss of cytochrome pathway capacity and continuously declined during cluster root development. Therefore total respiration decreased.

To elucidate whether AOX protein concentrations were responsible for the comparatively low upregulation of alternative respiration, immunochemical determination of the AOX protein via Western blot analysis was performed (Fig. 19). The AOX antibody binds to a highly conserved region of the protein (Finnegan et al., 1999), therefore the signal obtained on immunoblots is probably a reflection of the AOX concentration (Millenaar et al., 2001).

The main immunoreactive band in white lupin root segments occured at 32 kDa, which is in accordance with the molecular weight given for the AOX protein, which is between 32 and 39

kDa as reported for other plant species (McIntosh, 1994). The highest amount of AOX protein was detectable in young clusters, whereas the amount was very low in mature clusters, reflecting the corresponding decline in respiratory activity. Interestingly, Shane et al. (2004) presented similar results for changes in AOX protein during the developmental time course in cluster roots of *Hakea prostrata*. In white lupin, multiple immunoreactive bands occurred in immunoblots in +P and –P root tips. They had a lower molecular weight than the MW of the main band, and probably represent degrading products of the AOX protein, cleaved by proteases. Interestingly, they occurred only in the youngest root segments. In these root segments also an additional band at approximately twice the molecular weight of the main band with low intensity could be detected. It could be speculated that this band represents the less active AOX dimer, which was described as the precursor of the more active monomeric protein, covalently bound with a disulfid bond at the Cys-126 residues. Part of the protein might have been activated during cluster root development by reduction of the AOX dimer. This could be in connection with the high citrate concentrations found in mature and senescent cluster roots. Vanlerberghe and McIntosh (1997) described an increase of AOX activity mediated by citrate, which was probably due to the production of NADPH during citrate oxidation. They concluded that NADPH seemed to be required for AOX reduction via a thioredoxin or glutathion system. Declining levels of AOX protein during cluster root development suggest a regulation at the transcriptional level, determined by the concentration of the AOX protein. Since total protein concentration decreases during cluster root development, AOX protein concentration per root biomass decreases even more. Therefore generally lower protein concentrations in older cluster roots cannot be the only reason for the lower AOX protein concentrations. Other factors such as overproportionally lower rates of transcription or lower mRNA or AOX protein stability could also play a role. Additionally, AOX actual flux was suggested to be subject to fine metabolic control (Vanlerberghe and McIntosh, 1997). Under P deficiency and in the highly specialized clusters, metabolism might differ to a large extent compared to other plant species without nutrient deficiencies.

Reduced nitrate assimilation

Nitrate uptake and N assimilation decreases under P deficiency (Lee, 1982; Schjorring, 1986; Rufty et al., 1990; 1993; Buwalda and Warmenhoven, 1999; Neumann et al., 1999) and nitrate accumulates in the roots (Gniazdowska and Rychter, 2000). 2-oxoglutarate is the C-skeleton used for N assimilation and might accumulate under P deficiency, leading to a feedback inhibition of citrate turnover. However, the causes for lower nitrate uptake under P-deficient

conditions are still not clear. Several, sometimes contradicting, explanations were given: (1) a lower ATP availability due to the P deficiency lowers the adenylate energy charge and energy-driven pro-cesses such as nitrate uptake (Rufty et al., 1993) and limits the synthesis of the membrane transport system for NO_3, which was indicated in kinetic experiments with P-stressed barley (Rufty et al., 1991). However, ATP availability seems to be high enough to increase H^+-ATPase activity in mature cluster roots to support acidification of the rhizosphere and the export of H^+ as counter-ions for the citrate released (Kania et al., 2001). On the other hand, under P limitation ATP might be used only for crucial metabolic steps, or the need for N assimilates might be reduced, leading to a lower nitrate reduction; (2) a lower root hydraulic conductivity due to a lower fluidity of the plasma membrane (Radin and Matthew, 1989; Carvajal et al., 1996a) might cause lower nitrate transport from root to shoot and might result in an increased nitrate accumulation in the roots (Rufty et al., 1993; Gniazdowska et al., 1999), whereby nitrate uptake might be decreased by feedback inhibition (Gniazdowska et al., 1999); (3) a reduced nitrate reductase (NR) activity (Pilbeam et al., 1993; Gniazdowska and Rychter, 2000), although enough reducing power in form of NADH and NADPH were available (Gniazdowska et al., 1999). Nitrate reductase activity may also be regulated by reactive oxygen species, causing a reversible inactivation of the enzyme (Solomonson and Barber, 1990).

Contrary to the hypothesis that the use of 2-OG in N assimilation is decreased and therefore 2-OG accumulates due to a lower nitrate reduction by inhibition of NR, Vanlerberghe et al. (1990) described a large increase in the glutamine (Gln) to glutamate (Glu) ratio in a green alga during P limitation. They explained it as an indication that there was ample reduced N available to the Gln synthetase-Glu synthase (GS/GOGAT) cycle and that it was the availability of C in form of 2-OG to the cycle that limited N assimilation.

In conclusion, several approaches could explain a reduced nitrate assimilation, and it might depend on many metabolic conditions which of the ways actually cause a reduced nitrate assimilation. On the other hand, tungstate has a lot of different effects on metabolism and might increase citrate/malate ratio in another way than in inhibiting nitrate reductase, especially since it only worked slightly after a long incubation time and higher concentrations applied.

Reduced ATP-citrate lyase activity

Searching for genes with differential expression in young and mature cluster roots, Langlade et al. (2002) identified an ATP-dependent citrate lyase (ACL). This enzyme cleaves citrate by use of HS-CoA and ATP to produce oxaloacetate and acetyl-CoA and releases ADP and P_i. It

showed its highest activity in young cluster roots. Activity successively declined during cluster root maturation down to less than 20 % in senescent clusters (Fig. 24; Langlade et al., 2002; Kania et al., 2003). Specific activity, on the base of root segment protein concentration, showed the same pattern of decline. The change from malate to citrate accumulation during cluster root development is paralleled by the reduction in ACL activity, and a good correlation was found between the malate/citrate ratio in root exudates and ACL activity. This indicates that ACL may play a key role as a metabolic switch between malate and citrate accumulation during cluster root development under P deficiency (Langlade et al., 2002).

The function of the enzyme has been linked with the formation of acetyl-CoA in seedlings as a precursor for the biosynthesis of lipids and terpenoids (Ratledge et al., 1997). Fatty acid production is localized in plastids or in the cytosol, depending on the plant species. However, *de novo* synthesis of acetyl-CoA from pyruvate, decarboxylated via the pyruvate-DH, probably takes place mainly in mitochondria. Pyruvate dehydrogenase activity is insufficient in nongreen plastids to account for the observed rates of fatty acid biosynthesis (Lenmark and Gardeström, 1994). This results in the situation that acetyl-CoA is produced anew in mitochondria, but is needed in the cytosol or in plastids for lipid biosynthesis, and cannot be transported across subcellular membranes. Therefore it is thought that citrate can act as an acetyl-CoA transport substitute. The citrate-malate shuttle system (Watson and Lowenstein, 1970) provides convincing evidence that citrate generated in the mitochondria can be exported into the cytosol, and thus would be able to enter the plastids, where the citrate then could be cleaved by ACL to give acetyl-CoA in the compartment needed for fatty acid synthesis. The oxaloacetate produced would be converted to malate via the malate dehydrogenase and transported back to the mitochondria, replenishing the TCA cycle (Rangasamy and Ratledge, 2000b). The correlation of the highest ACL activities with the elongation zone of roots suggests that one role of ACL may indeed be linked to lipid biosynthesis (Langlade et al., 2002).

ACL might have an additional function in plants: PEP metabolism is shifted towards the production of oxaloacatate and malate by the activities of PEP-C and MDH at the expense of pyruvate production via PK as an anapleurotic reaction under P-deficiency (see above). However, via the PEP phosphatase and the PEP-C, MDH and the malic enzyme reactions, two metabolic pathways still exist to produce the pyruvate needed for the *de novo* synthesis of citrate. Since the metabolic block leading to citrate accumulation under P-deficient conditions might be at the side of citrate degradation, ACL then might have the task to prevent citrate accumulation. This functions at less severe P-deficiency in young cluster roots, where ACL activity is upregulated and prevents citrate accumulation by cleaving it. Oxaloacetate thereby is reduced to malate, which was observed to accumulate in young cluster roots. Only in later stages of cluster

root development, ACL activity and ATP availability decrease, leading to the citrate accumulation observed. In this point of view, under less severe P deficiency, ACL in its second function reacts as a kind of backup system to prevent citrate accumulation, which ceases to function under more severe P deficiency. Langlade et al. (2002) found two gene products for the enzyme, whereby the expression pattern of both paralleled activity, meaning that the enzyme probably is regulated at the transcriptional level. This is in accordance with the decreasing protein and RNA concentrations found in ageing cluster roots, and the generally decreasing dehydrogenase activities found in cross sections of different root segments by activity staining. Decreasing specific activities of ACL during cluster root development hints to an overproportional decrease in activity in addition to the decrease due to the generally lower protein concentrations.

An additional way of citrate degradation exists. The glyoxysomal pathway is a 5-enzyme short cut of the TCA cycle, and in addition to its established function in the postgerminative growth of oilseeds, it has also been detected as a salvage pathway for structural lipids in various plant tissues under natural or induced senescence conditions (Gut and Matile, 1988; De Bellis and Nishimura, 1991; Chen et al., 2000; Cots et al., 2002). Interestingly, in white lupin citrate accumulation occurs in senescing cluster roots, and an EST of malate synthase as part of the glyoxysomal pathway showed higher expression in ageing cluster roots (Uhde-Stone et al., 2003b). Therefore it might be worth to investigate if the glyoxylate cycle prevents an even higher citrate accumulation as already observed and has an anapleurotic function similar to ACL, but takes effect later in cluster root development. ACL, which is more active in younger clusters, might bring about fatty acid production, and the glyoxylate cycle, which is more active in older clusters, might salvage lipids in these ageing tissues.

Factors determining reduced citrate turnover

The present study revealed downregulation of various metabolic sequences potentially involved in citrate turnover during cluster root development in P-deficient white lupin. Possible reactions comprise a reduced aconitase activity, lower dehydrogenase activities, a lower respiration, a lower use of 2-oxoglutarate for N assimilation, and reduced ATP-citrate lyase activity. However, regulatory factors determining the P deficiency-induced inhibition of these metabolic pathways and their relative contribution to citrate accumulation are still unknown and were in the scope of more detailed investigations.

Reduced activity of aconitase

Inhibition of aconitase by H_2O_2 production ?

Plant mitochondrial aconitase is rapidly inactivated by H_2O_2 (Verniquet et al., 1991). The enzyme is very susceptible to reactive oxygen species such as the superoxide radical or H_2O_2 due to its 4Fe-4S-cluster in its active site, where the Fe_α can be oxidized and lost from the enzyme (Hausladen and Fridovich, 1996). Increased H_2O_2 concentrations could be expected in plants under P-deficiency (Parsons et al., 1999; Juszczuk et al., 2001b; Malusà et al., 2002), but H_2O_2 determination in different cluster root segments showed that concentrations rather declined when P deficiency became more severe during cluster root development (Fig. 10). Arguments for and against an aconitase inhibition by H_2O_2 exist. The concentrations of 0.4 – 0.5 μmol H_2O_2 g^{-1} root FW in cluster roots of P deficient white lupin (Fig. 10) are comparable to those found in nutrient-sufficient winter wheat seedling leaves (0.5 – 0.8 μmol H_2O_2 g^{-1} leaf FW (Okuda et al., 1991) or for bean, spinach and mungbean leaves (~0.35-0.65 μmol H_2O_2 g^{-1} leaf FW) (Patterson et al., 1984). This makes an aconitase inhibition due to high H_2O_2 concentrations rather improbable. Additionally, citrate protects aconitase from H_2O_2-derived inactivation (Verniquet et al., 1991). However, the lowest aconitase activities were found in the root segments with the highest citrate concentrations. Perhaps aconitase impairment might have been even more severe without the protection by the high citrate concentrations in mature and senescent cluster roots.

Verniquet et al. (1991) found inhibition of aconitase activity in isolated potato tuber mitochondria at concentrations of less than 100 μM H_2O_2. Very low concentrations of H_2O_2 such as 25 μM inhibited over 70 % of tobacco aconitase activity (Navarre et al., 2000). Estimating the H_2O_2 concentration in the cluster roots as 500 to 800 μM when a root density of 1g mL^{-1} is assumed, then the measured H_2O_2 concentrations are far above the H_2O_2 inhibition level for aconitase activity. To maintain aconitase activity, H_2O_2 then must be intercepted very effectively, or the enzyme aconitase must continuously be produced anew. Since protein concentrations and mRNA levels decrease during ageing of cluster roots, new enzyme production could be inhibited under more severe P deficiency and therefore aconitase activity might decrease due to a lower replacement of the oxidized enzyme. Interestingly, the H_2O_2 treatment of P-sufficient tobacco suspension cells caused a dramatic, but short-term increase in citrate (Vanlerberghe and McIntosh, 1996), whereby the authors could not understand the transient nature of this increase. Perhaps H_2O_2 was degraded quite fast (half-lives for H_2O_2 were given within minutes for cells, and up to 1 h when applied to protoplasts (Neill et al., 2002)), and the plants were able to produce new aconitase till citrate concentrations were measured.

To explain the unaltered H_2O_2 concentrations measured under P deficiency, H_2O_2 as a highly reactive substance might have reacted with other substrates before its determination was completed, especially at higher concentrations. On the other hand, its distribution inside a tissue or inside a single cell might be very inhomogeneous and therefore a high concentration inside a certain cell compartment might not have been realized when its concentration was determined for whole root segments.

To simulate such a supposed, local high peroxide concentration, H_2O_2 at higher concentrations than found in the root tissue in *in vitro* measurements were applied to a part of the root system. It was to be tested if young cluster roots could be forced to react like mature clusters in increasing their citrate concentration and decreasing their aconitase activity.

Citrate/malate tissue concentration ratios as a more sensitive parameter for a change of citrate and malate tissue concentrations were only slightly, but significantly increased at shorter incubation times of 1.5 h and 3 h at both incubation concentrations tested (5 m*M* and 10 m*M* H_2O_2) (Fig. 11). This increase was due to a slight, but significant decrease in malate tissue concentrations, but not due to any increase in citrate tissue concentrations. Peroxide might therefore not influence aconitase acitivity. However, in cannot be completely ruled out that the high peroxide concentrations applied impaired plant metabolism independent of any aconitase reaction. Peroxide is known to be a strong oxidative agent which reacts quite unspecific. A metabolic disturbance on a more general level therefore might also have prevented any citrate accumulation.

Nitric oxide (NO) as an aconitase inhibitor ?

On the other hand, aconitase activity might be inhibited by other factors than H_2O_2. Nitric oxide (NO), in addition to its free radical behaviour, was recognized as a signal molecule, implicated for a variety of plant processes, including leaf expansion (Leshem, 1996), root growth (Gouvea et al., 1997), or for the full activation of plant disease resistance and programmed cell death (Delledonne et al., 1998). Tobacco aconitases, like their mammalian counterparts, are inhibited by NO (Navarre et al., 2000). Aconitase is more vulnerable than other Fe-S- or heme-containing enzymes to this inhibition (Castro et al., 1994). In animals, NO reversibly inactivates aconitases by promoting the loss of the 4Fe-4S cluster (Drapier, 1997). Since cluster root development is associated with rapid appearance of senescence symptoms, which could be interpreted as programmed cell death, inhibition of aconitase could be due to NO production. Nitric oxide

synthase activity, producing NO from arginine, was described for white lupin roots and nodules (Cueto et al., 1996).

An increase in citrate concentration in Fe-deficient sunflower was assumed to be due to an inhibition in citrate breakdown (Venkat Raju et al., 1972). Additionally, under Fe-deficient conditions white lupin produces cluster roots like under P deficiency, although with differences in morphology and physiology, and with an even greater citrate release from -Fe than from -P plants (Hagström et al., 2001). A link between citrate metabolism in Fe-deficient and in P-deficient plants in connection with IRP (iron-regulated proteins) and NO regulation might exist.

Substrate limitation of respiration ?

Cytochrome pathway capacity decreased parallel to total respiration, suggesting that the cytochrome pathway determines total respiration, whereby the increase in AOX capacity that did take place was not high enough to compensate for the loss of the cytochrome capacity (Fig. 18).

Especially under P-deficient conditions, cytochrome respiration might be under adenylate control (Vanlerberghe and McIntosh, 1992): A low availability of ADP and P_i inhibits ATP synthesis, which is brought about by dissipating the electrochemical gradient, and is established by electron transport via the cytochrome pathway. Additionally, a lower respiration rate would mean a lower turnover of reduced nucleotides, which may lead to an inhibition of the TCA cycle by a feedback mechanism and an accumulation of citrate. Uncoupling of the electron flow from ATP production would increase O_2 consumption, as was found for *Catharanthus* suspension cultures under P deficiency (Hoefnagel et al., 1993a), or in tobacco suspension cells under P-deficient and P-sufficient conditions (Parsons et al., 1999). To test if root respiration in white lupin is under adenylate control (a lack of ADP limits respiration), respiration was measured by use of the uncoupler CCCP (Fig. 20). Although a whole range of concentrations were tested, no increase in respiration could be observed in root tips of P-sufficient plants nor in cluster roots. Therefore even under the severe P-deficient conditions in senescent cluster roots, respiration was not limited by deficiency of ADP or P_i. Similarly, in roots of bean plants, the application of an uncoupler increased O_2 consumption only in P-sufficient plants, but not under P limitation (Rychter and Mikulska, 1990). The authors attributed this to a partial inhibition of the cytochrome pathway. External supply of glucose in addition to the uncoupler again increased respiration in bean only in P-sufficient roots, and failed to stimulate respiration under P deficiency. This indicates that factors other than substrates (ADP, P_i) and metabolic demand for ATP limit the respiration rate under P-deficient conditions (Wanke et al., 1998).

P deficiency-induced oxidative damage ?

Increased production of reactive oxygen species (ROS) under P deficiency and oxidative damage of membranes and respiratory chain components by the activity of reactive oxygen species (ROS) such as the superoxide radical ($O2^{\bullet -}$) are reported (Parsons et al., 1999). The superoxide radical then may be rapidly converted to peroxide (H_2O_2) by mitochondrial superoxide dismutase (SOD). The mitochondrial electron transport chain is a major source of ROS generation in eucaryotic cells, including plant cells (Purvis et al., 1995; Parsons et al., 1999), where the main production site is at complex III with a one-electron transfer to molecular oxygen to generate the superoxide anion (Boveris et al., 1976; Parsons et al., 1999; Sweetlove et al., 2002).
To elucidate whether oxidative stress could be responsible for the reduced respiration found in ageing cluster roots of white lupin, H_2O_2 concentrations were determined in the different root segments (Fig. 10). Surprisingly, H_2O_2 concentrations rather declined when P deficiency became more severe during cluster root development and therefore could not be accounted for the reduced respiration rates observed. Since H_2O_2 as a very aggressive molecule might have already reacted with other parts of the cell before its determination was performed, or ROS other than H_2O_2 might cause the oxidation of respiratory components, malondialdehyde (MDA), which is formed by lipid peroxidation due to oxidative stress (Halliwell and Gutteridge, 1989), was determined as well. However, MDA concentrations were also not increased during cluster root development, and so far there is no indication for oxidative damage by enhanced production of ROS in cluster roots. This leaves a general impairment of the respiratory components or their insufficient production due to lower protein concentrations to explain the lower respiration rates.
On the other hand, there is nothing known in plants about mechanisms by which cytochrome capacity is regulated (Vanlerberghe et al., 2002). It was speculated that cytochrome pathway capacity in plant mitochondria is controlled by a phosphorylation cascade modulated by redox signals. This idea is especially interesting in the view that P deficiency might influence phosphorylation and can be associated with redox processes.

Pyruvate acts allosterically on the reduced AOX to stimulate its activity in isolated mitochondria (Millar et al., 1993), and there is genetic evidence that pyruvate will accumulate if there is an imbalance between respiratory carbon metabolism and electron transport (Vanlerberghe et al., 1997). In this way, no such imbalance seems to be the case in white lupin cluster roots, and the lower pyruvate concentrations in ageing cluster roots are rather not responsible for the decreasing AOX capacity measured. On the other hand, so far it was not possible to directly assess the influence of pyruvate on AOX activity *in vivo* (Millenaar et al., 2001). Pyruvate

probably does not play a role *in vivo* at all: The pyuvate concentrations needed for AOX activation are very low and normal pyruvate concentrations are far above this threshold value (Millenaar et al., 1998).

The most established hypothesis for AOX function is that this protein reduces the generation of reactive oxygen species (ROS) in mitochondria. It presumably prevents overreduction of respiratory chain components such as ubiquinone (Day and Wiskich, 1995; Vanlerberghe and McIntosh, 1997; Maxwell et al., 1999; Parsons et al., 1999). Thereby it shunts electrons off the cytochrome pathway directly from ubiquinone to molecular oxygen, and is independent of oxidative phosphorylation. Accordingly, it was found that external H_2O_2 application to plant or fungal cells can increase AOX expression (Vanlerberghe and McIntosh, 1996). In consequence, it was hypothesized that ROS acts as a signal to increase AOX expression (Vanlerberghe et al., 2002). Therefore it is tempting to suggest that the low increase in AOX capacity in cluster roots of white lupin, compared to P-deficient bean or tobacco plants, can be attributed to a low increase in H_2O_2 concentrations, giving a signal too small for enough AOX production to compensate for the loss of cytochrome capacity.

Citrate treatment of cells or inhibited internal citrate turnover can induce the synthesis of AOX (Vanlerberghe and McIntosh, 1996; Pastore et al., 2001). However, this was not the case in white lupin cluster roots. Quite contrary, the higher the citrate concentrations in cluster roots, the lower the AOX activity. In accordance with this finding, an inhibition of the alternative pathway was described by Lambers et al. (1997) when massive amounts of citric acid were produced. These authors also concluded that the increased carbon costs associated with the release of organic acids, when P is in short supply, are partly balanced by lower carbon costs associated with respiration via the alternative path.

It was suggested that a plant cell maintains signal pathway(s) able to actively regulate cytochrome pathway capacity, and that the fate of such cells (survival or programmed cell death) is dependent upon the level of AOX (Vanlerberghe et al., 2002). Interestingly, it was suggested (Neumann, pers. comm.) that cluster root structures might be a kind of “throw away” structures “designed” to be abandoned after a short life-span. This could mean a programmed cell death as defined by Vanlerberghe et al. (2002).

In addition, decreases in respiratory rate often occur as plant tissues age (Azcon-Bieto et al., 1993; McDonnell and Farrar, 1993; Atkin and Cummins, 1994). The decline in cytochrome pathway activity with age was supposed to reflect a decline in the demand for ATP associated with the slower growth rates in ageing tissue (Amthor, 1989; Millar et al., 1998). In white lupin clusters two factors might combine: the P deficiency which limits ATP availability, and the

aging of the cluster roots. Both together may lead to a more severely limited respiration with a reduction of both, cytochrome and alternative pathway capacity.
More generally, the low P_i, mRNA, and protein levels might prevent the production of urgently needed proteins such as the aconitase or nitrate reductase proteins, or proteins necessary for membrane integrity and intactness. Interestingly, it was not possible to isolate intact mitochondria from mature and senescent cluster roots (Watt, Dissertation) nor was it possible to isolate protoplasts from mature clusters which were tight enough for patch clamp studies (Zhang et al., 2004; Yan (pers. comm.); this work). Such a general impairment of enzyme production and therefore also of membrane components might explain the fact that so far no single cause for the citrate accumulation observed under P-deficient conditions could be found.

Citrate accumulation as related to artificial inhibition of selected metabolic sequences

As an additional approach to identify metabolic sequences of citrate turnover in P deficiency-induced accumulation of citrate in cluster roots of white lupin, the effects of inhibitors for the respective metabolic pathways on citrate accumulation were investigated. To minimize the problem of systemic side effects, inhibitors were applied locally to selected root zones only for short time periods.

Influence of an inhibited respiration by azide or SHAM on citrate tissue concentrations

Reduced respiration seems to have only a small feedback influence on carboxylate concentrations in cluster roots. When respiration in young cluster roots was inhibited by partial root incubation with respiration inhibitors, citrate/malate tissue concentration ratio increased (Fig. 21). The increase was higher when the cytochrome compared to the AOX pathway was inhibited, which supports the view that the AOX pathway does not play such an important role under cytochrome-limiting conditions as it does in other plant species. The increase in the citrate/malate ratio was mainly due to lower malate concentrations and not to increased citrate concentrations. This suggests that the reduced respiration rates found in cluster roots under P deficiency are not primarily responsible for the increase in citrate concentrations found in mature cluster roots.

Inhibition of the aconitase enzyme by external application of MFA

Monofluoroacetate (MFA) is a metabolic inhibitor that acts by being incorporated into fluoroacetyl CoA, which then reacts with oxaloacetate to form fluorocitrate. Fluorocitrate inhibits the enzyme aconitase, thus preventing the conversion of citrate to isocitrate in the TCA cycle (Quastel, 1963, and references therein; Lauble et al., 1996).

Inhibition of the citrate-metabolizing enzyme aconitase by external application of monofluoroacetate strongly influenced organic acid metabolism in young and mature cluster roots (Fig. 13). The strong increase in citrate concentrations and strong decrease in malate concentrations in the young cluster roots means a shift in their carboxylate pattern normally found only in more mature clusters. Even mature cluster roots showed a further shift towards lower malate concentrations and higher citrate concentrations when treated with MFA. Aconitase therefore seems to be a key enzyme which determines organic acid metabolism while a cluster root develops. Aconitase activity might therefore be responsible for the transition of malate accumulation towards citrate accumulation while the cluster roots reach their mature state.

It was suggested that citrate exudation is triggered when a treshold value of citrate concentration is reached (Neumann et al., 2000; Peñaloza et al., 2002). This treshold seems to be reached in young, MFA-treated clusters, because they released high amounts of citrate (Fig. 14). The citrate exudation rates of these MFA-treated young clusters were even higher than in non-treated mature clusters. Since citrate exudation is supposed to also be triggered by cytosolic acidification (Kania et al., 2003), it would be interesting to investigate changes in cytosolic pH under MFA treatment.

Even white lupin seedlings, not suffering from any nutrient deficiency, showed malate and citrate exudation from their root tips when aconitase activity was blocked by MFA (Fig. 15). Accordingly, citrate accumulation was observed in MFA-treated tobacco suspension cells (Vanlerberghe and McIntosh, 1996). Maize seedling roots reacted to MFA incubation with malate and shikimate exudation (data not shown). Therefore it can be assumed that aconitase generally is a key metabolic step, limiting normal citrate degradation when its activity is impaired. The exudation of malate and shikimate in maize might be due to alternative metabolic steps to transform citrate.

Summarizing, artificial reduction in aconitase activity can increase citrate concentrations and citrate exudation rates in root segments normally not accumulating citrate and not exuding citrate. This leaves the aconitase enzyme as a key metabolic step that determines citrate accumulation in mature cluster roots under P deficiency. Additionally, this finding supports the

concept of a citrate threshold concentration which has to be reached before citrate is exuded. Furthermore, anion channels responsible for high citrate exudation rates already exist under P-sufficient conditions or are inducible in these roots within hours when citrate concentrations increase.

When aconitase activity was measured in young and mature cluster roots after incubation with MFA, no change in activity could be observed (Fig. 16). This is in contrast to the increase in citrate concentration and exudation rates which occurred after the same incubation. However, the root segments used for determination of aconitase activity were harvested after exudate collection for 2 h, which was done without further application of MFA. According to the conjecture that the loss of aconitase activity might be due to a lower replacement of enzyme, aconitase perhaps was newly produced in these still growing tissues after removal of the inhibitor and re-gained the activity it had before MFA was applied. On the other hand, the MFA product (-)-erythro-2-fluorocitrate, the inhibitory isomer of the enzyme, might have been removed by dilution by the solutions the enzyme was dissolved in for enzyme activity measurements. However, aconitase inhibition was documented to be reversible only by a 10^6-fold excess of isocitrate over the enzyme-inhibitor complex (Lauble et al., 1996, and references therein). Therefore it is not completely conclusive that the MFA-product was really removed from the enzyme by dilution from the activity measurement solutions.

Inhibition of nitrate reductase (NR) by tungstate

The nitrate reductase inhibitor tungstate (Na_2WO_4) was applied to young cluster roots to examine if a loss of nitrate reductase activity influences carboxylate concentrations in cluster roots due to a possible lower nitrogen incorporation into 2-oxoglutarate (2-OG), which would lead to a reduced consumption of citrate as precursor for 2-OG. Citrate concentrations increased slightly in response to tungstate treatments at moderate concentrations, while malate concentrations were slightly reduced, resulting in a corresponding increase of the citrate/malate ratio (Fig. 22). However, the observed changes in carboxylate concentrations were not comparable with the alterations of malate and citrate levels characteristic for maturation of cluster roots. Therefore nitrate reduction as the first step of inorganic N incorporation into C skeletons via nitrate reduction and the GS/GOGAT pathway seems to have a small, but not a crucial influence on the balance of TCA cycle metabolites, at least not enough to explain the high citrate accumulation observed in mature cluster roots. On the other hand, tungstate, a molybdate analogue, inhibits

formation of the active nitrate reductase enzyme *in vivo* by preventing incorporation of molybdenum (Wray and Filner, 1970; Deng et al., 1989). Therefore the NR enzyme is still synthesized, but the nitrate-reducing activity is defective. As treatment of plants with tungstate inhibits formation of new active nitrate reductase, the decrease in NR activity reflects the actual rate of NR degradation (Lillo et al., 2003). The low effect of tungstate application on carboxylate concentrations in the cluster roots of white lupin might be in accordance with this. The nitrate reductase was already produced before the tungstate treatment started. Therefore the influence of a P deficiency-induced nitrate reductase activity on organic acid metabolism might be much higher than estimated from this tungstate application experiment.

Inhibition of the ATP-citrate lyase (ACL) by external application of hydroxycitrate

The attempt to inhibit the ATP-citrate lyase (ACL) enzyme by application of different concentrations and different durations of incubation with hydroxycitrate did not show any effect on citrate or malate concentrations in the young cluster roots (Fig. 25). Two possibilities exist: (1) an inhibition of the ACL does not influence internal concentrations of citrate or malate at all, or the activity of the ACL is not an important or limiting step in organic acid metabolism under P deficiency. (2) The inhibitor hydroxycitrate, being chemically very similar to the organic acid citrate (a hydroxyl-group at the C2 instead of a –H) might not be taken up into the root cells in sufficient amounts to exert inhibitory effects on ACL.

Conclusions and outlook

Citrate accumulation in mature cluster roots is probably not only be caused by an increased production by a higher activity of enyzmes such as glycolytic bypass reactions, and the activities of the PEP-C, Malate-DH and citrate synthase enzymes. Many hints exist that citrate also accumulates due to a reduced degradation. In consideration can be a reduced aconitase activity, decreased activities of dehydrogenases in the TCA cycle, reduced nitrate assimilation with its lower use of organic acid carbon skeletons, or a lower respiration, and a lower degradation by ATP-citrate lyase. Higher enzyme activities on the anabolic side cannot completely explain the high citrate accumulation observed.

Although the artificial inhibition of respiration and nitrate reductase did slightly increase the citrate/malate ratio in young cluster roots, and therefore seem to influence citrate and malate

metabolism, the highest effect was seen when aconitase was inhibited by MFA. Citrate concentrations in still young clusters as well as citrate exudation rates were increased to amounts usually only found in mature ones, and even P-sufficient seedling root tips could be forced to exude citrate. This are strong hints for a crucial role of aconitase in citrate accumulation and exudation in white lupin cluster roots. A lower aconitase activity was also identified as the cause for citrate accumulation in sour lemon during fruit growth compared to sweet lime (Sadka et al., 2001; 2002). Citrate exudation could even be triggered by MFA in P-sufficient seedling root tips, which shows a clear relationship between citrate accumulation and citrate exudation. In yeast, aconitase or ICDH gene-deficient mutants showed higher citrate accumulation (Anoop et al., 2003). Transgenic lines of plants or yeast, missing or overexpressing single or multiple genes influencing citrate production or degradation, might help to elucidate the importance of each single enzyme in citrate metabolism.

Nitric oxide (NO) has numerous physiological effects in plants (Bethke et al., 2004) and might play a crucial role in plant metabolism also under P deficiency, as a stimulated NO flux was found under P limitation (Stöhr and Ullrich, 2002). It presumably inhibits aconitase activity by oxidizing its catalytic Fe-S cluster, or via a connection with IRP proteins (Navarre et al., 2000). It decreases the respiratory cytochrome c oxidase activity (Millar and Day, 1996), and Zottini et al. (2002) have shown that NO induces an increase in AOX expression in plant cell cultures. It would be interesting to investigate the influence of NO also as a regulatory agent on metabolism of white lupin under P deficiency.

Chapter 2: Mechanisms of citrate export in cluster roots

Introduction

Cluster roots of P-deficient white lupin plants show specialized metabolic adaptations to the lack of phosphorus. An increase in carboxylate production and tissue concentration, followed by exudation, especially of citrate in mature clusters, was often described (e.g. Dinkelaker et al., 1989; Johnson et al., 1996a+b; Keerthisinghe et al., 1998; Gilbert et al., 1999; Neumann and Römheld, 1999; Neumann et al., 1999, 2000). Much less is known about the carboxylate transport itself. Due to the pH stat mechanism (Sakano, 1998) increased production of carboxylates also produces protons. This provokes metabolic disturbances since the cytosolic pH has to be kept constant at a pH of 7.3 – 7.5 (Sanders and Bethke, 2000), and high concentrations of carboxylates might complex cations such as Ca^{2+}, which as a second messenger has to be kept at defined low concentrations in the cytosol (Marschner, 1995). White lupin is able to cope with this situation by exuding the citrate from mature clusters across the plasma membrane, probably when its internal concentration reaches a threshold value of 20-30 µmol g^{-1} root FW (Neumann et al., 2000; Peñaloza et al., 2002). However, in senescent clusters the citrate concentrations are still very high, but almost no exudation occurs, which is not due to an artifact by microbial degradation of released citrate (Neumann et al., 1999). Contrary to this, malate concentrations and exudation rates decrease in parallel during cluster root development. Citrate exudation therefore cannot be explained by passive diffusion across leaky membranes impaired by P deficiency. Thus a regulated release mechanism was suggested (Neumann et al., 1999). This view was further supported by the finding that anion channel inhibitors such as ethacrynic acid or anthracene-9-carboxylic acid (A-9-C) inhibited citrate exudation, and citrate exudation via an anion channel was postulated (Neumann et al., 1999).

Based on inhibitor studies, a similar transport mechanism has been postulated for Al-induced release of malate in wheat (Ryan et al., 1995; 1997), where malate exudation also was reduced when the anion channel inhibitors niflumate or A-9-C were applied. A direct proof for the involvement of anion channels in Al-induced exudation of malate in wheat and of citrate in maize has been recently demonstrated by patch-clamp studies (Kollmeier et al., 2001; Piñeros and Kochian, 2001; Zhang et al., 2001).

Ion transport across membranes via channels is brought about by the effect of the electrochemical potential gradient. The plasma membrane enzyme H^+-ATPase uses the energy of ATP to transport protons from the cytoplasm out of the cell against their diffusional direction, which establishes an electrical gradient due to the accumulation of positive charges in the apoplasm, leaving more negative charges in the cytosol. Concomitantly a chemical gradient is established for the higher proton concentration outside the cell (lower apoplastic pH) and a lower proton concentration in the cytosol (higher cytosolic pH). Both gradients, taken together, are called the electrochemical gradient or proton motive force, which is described mathematically by the Nernst equation. Potentials of about –120 mV and pH differences of two pH units are routinely observed across plasma membranes (Palmgren, 1998), and even values of –200 to –300 mV are common in plant cells (Sanders and Bethke, 2000). The energy stored in the electrochemical gradient can then be used for ion transport across channels or transporters, energetically downhill, and is called secondary active transport.

Generally, transport activated by the plant H^+-ATPase is involved in many physiological functions, e.g. mineral nutrition in the root, metabolic translocation, regulation of cytoplasmic pH, and cell turgor-related functions, such as organ movement and cellular growth (reviewed by Arango et al., 2003). More specifically, the intracellular pH is mainly regulated by the PM H^+-ATPase and by malate production or degradation (Palmgren, 1998) by way of the pH stat mechanism (Sakano, 1998). The PM H^+-ATPase, however, is not the only factor which determines the size of the membrane potential and contributes to H^+ extrusion. Metabolic processes, e.g. CO_2 and lactate production, and changing permeability of the membrane to anions and cations may be equally important factors (Palmgren, 1998).

It is known that certain cell types have much higher concentrations of H^+-ATPase than others. In general, cell types with abundant H^+-ATPase are specialized for intense active transport and accumulate solutes from their surroundings. For example, H^+-ATPase is localized in root hairs and root epidermal cells and is important for nutrient uptake and active loading of solutes into the xylem (Palmgren, 2001).

H^+-ATPases were identified in plants, fungi, protozoa and Archaebacteria (Palmgren, 1998). Many H^+-ATPase isoforms were described, e.g. 12 H^+-ATPase genes in Arabidopsis, 9 in *Nicotiana plumbaginifolia* (Palmgren, 2001) and 10 in rice (Arango et al., 2003). Plasma membrane H^+-ATPases are believed to have ten transmembrane segments (Palmgren, 2001), consisting of a single catalytic subunit of about 100 kDa. How many subunits exist altogether in the native state is uncertain, but a cluster of 3 x 2 subunits, arranged as a hexameric structure, was proposed (Boutry, pers. comm.). A number of quantitative differences in catalytic and regulatory properties between isoforms exist (Palmgren, 2001).

Citrate exudation from mature clusters across the plasma membrane via the postulated anion channel is probably also a secondary active transport. In the neutral pH of the cytosol citrate exists to about 93 % as citrate $^{3-}$ (Marin et al., 1981) and therefore has to be transported as an anion. In addition to the electrical gradient supporting the efflux of citrate from the cytosol, a concentration gradient also exists since the citrate concentration is higher in the cytosol than in the outer medium. Therefore citrate exudation across an anion channel would even rather be a facilitated diffusion.

In comparison to the bulk soil with a pH of 7.8 (Dinkelaker et al., 1989), rhizosphere acidification to a pH of 4.5 as reached in mature clusters of P-deficient white lupin, means that the H^+-ATPase has to establish a 500-fold higher proton concentration in the apoplast compared to the cytosol.

The following hypothesis can therefore be stated: Under P deficiency citrate accumulates in white lupin cluster roots. The physiological changes brought about by citrate accumulation leads to the opening of a putative anion channel in mature clusters when a certain threshold value of citrate concentration is reached, which results in a burst of citrate exudation. To maintain the electrical gradient across the plasma membrane, and for charge balance, protons are concomitantly released by an activated H^+-ATPase.

The aim of this work was to characterize the physiological changes of the PM H^+-ATPase in cluster roots of white lupin under P-deficient conditions in relation to citrate transport across the plasma membrane via the putative anion channel. Another question was how this putative channel is characterized and how it is regulated. Until this study was finished, no direct evidence for such an anion channel was given.

Two methodological approaches were chosen:

1. Hydrolytic and proton transport activity of the H^+-ATPase was to be determined in highly purified inside-out plasma membrane (PM) vesicles in a membrane-physiological approach. In inside-out plasma membrane vesicles the physiologically inner membrane side of the living cell is on the outer side of the vesicle and therefore accessible for externally supplied substrates such as ATP or citrate. Vesicles isolated from roots of P-deficient and P-sufficient control plants were to be investigated with respect to the supposed increase in proton transport necessary to enable the high acidification observed in mature clusters. Activity of

the H^+-ATPase was also to be measured with respect to the pH and electrical balance in the cytosol when a high amount of citrate transport out of the cell dissipates the electrochemical gradient. Isolated vesicles offer several advantages over intact cells in studying transport mechanisms: the vesicles are devoid of cytoplasmic constituents of the intact cell, and their metabolic activities are limited to those enzymes associated with the membrane itself. The energy source for transport of a particular solute can be determined by studying which substrate stimulates solute accumulation (Sze, 1985). Another question was the relation between membrane energetization, H^+-ATPase, and citrate transport across the plasma membrane. This work with isolated PM vesicles was done in co-operation with Stefano Cesco and Roberto Pinton from the University of Udine, Italy.

2. Protoplasts were to be isolated for patch-clamping studies to characterize the putative citrate channel in the plasma membrane of cluster roots of white lupin.

Materials and methods

Vesicle isolation and characterization

Plasma membrane (PM) vesicles were isolated from root systems with cluster roots of P-deficient plants (-P) and from roots of +P control plants in a small scale procedure according to Giannini et al. (1988), using a sucrose gradient centrifugation. The aim was to investigate PM transport properties important for citrate exudation of mature cluster roots under P deficiency.

For this, 14 g of freshly harvested root material was cut and rinsed in a solution of ice-cold non-aerated 0.1 m*M* $CaSO_4$ solution. After removing the water on a filter paper and weighing, the roots were ground thoroughly in a mortar with a pestle with 56 mL of the homogenization (MO) solution (Tab. 7), 1 m*M* PMSF, 20 μg Chymostatin mL^{-1} and PVPP (0.5 g g^{-1} root FW). All the steps were done on ice, all centrifugations at 4°C. The breis was filtered through six layers of cheese cloth into a beaker. The filtrate was pipetted into 2 mL Eppendorf vials and centrifuged at 17,130 *g* for 6 min to precipitate cell debris, and the supernatant was transferred into new 2 mL vials. After another centrifugation at 17,130 *g* for 25 min the tips of the vials were cut, the supernatant was poured away and a total of 1 mL of the resuspension solution (Tab. 6), containing 20 μg of freshly added Chymostatin solution, was distributed evenly into all the vials. The pellets were resuspended in the solution. Care has to be taken that no air gets into the solution to avoid oxidation of the membranes. The resuspended solution was transferred into a total of four vials and MO solution was added to fill each vial to a volume of 2 mL, then the solutions were mixed and centrifuged for another 25 min at 17,130 *g*. After removal of the supernatant the pellets were resuspended in a total of 800 μL of MO solution, giving the microsomal fraction.

To get a pure plasma membrane-derived fraction, a sucrose-gradient separation of the membranes was performed: 540 μL of the 38 %-sucrose GS solution (Tab. 8) was filled into 2-mL vials. The surface of the solution has to be smooth. 1260 μL of the 25 %-sucrose gradient GS solution was layered on top with help of an insulin syringe with a very thin needle. To get a smooth interlayer, the tip of the needle was put into the 38 %-sucrose gradient and pulled up above the surface. A thread of the 38 % solution evolved, along which the 25% solution was carefully filled in to avoid any turbulence in the interlayer between the two solutions. 200 μL of the vesicle suspension was layered on top of each vial containing the gradient solution using the same procedure, and centrifuged for 60 min at 20,820 *g* with slow accelaration and deacceleration to avoid mixing of the gradients. After centrifugation, the interlayers were drawn off with an insulin syringe, whereby care had to be taken that the pellet on the inner side of the

vial and the suspension forming the interlayer were both carefully removed. The suspension of the four gradients were transferred into six new vials and MO solution was added until all the vials were filled. After mixing thoroughly, the suspensions were centrifuged for another 60 min at 20,820 *g*, the supernatant removed and an amount of MR solution (Tab. 6) added until a certain protein concentration was gained (here: 1 mL of MR solution). This suspension gave the highly purified vesicles.

Table 4: Homogenization stock solution (MO) for vesicle isolation (for preparation of 1000 mL, stocks of 50 or 75 mL each are stored at -20°C).

sucrose	85.575 g	250 m*M*
glycerol	126 g	10 % v/v
glycerol-1-phosphate	2.16 g	10 m*M*
$MgSO_4$ x $7H_2O$	0.4929 g	2 m*M*
EGTA	0.761 g	2 m*M*
EDTA-acid	1.1688 g	4 m*M*
BTP	7.057 g	25 m*M*

titrate to pH 7.6 with solid MES

Table 5: GS stock solution: for preparation of 0.5 L, stocks of 25 mL each are stored at -20°C.

EGTA	0.38 g	2 m*M*
$MgSO_4$ x 7 H_2O	0.24 g	2 m*M*
BTP	0.7 g	1.24 m*M*
glycerol-1-phosphate	1.08 g	10 m*M*
EDTA-acid	0.292 g	2 m*M*

titrate to pH 7.4 with solid MES

Table 6: MR stock solution (resuspension medium) (for preparation of 50 mLwith stocks of 1 mLeach, stored at -20°C)

BTP	0.0282 g	2 m*M*
glycerol	12.6 g	20 % v/v
EGTA	0.03805 g	2 m*M*
EDTA	0.0292 g	2 m*M*

titrate to pH 7.0 with MES

Table 7: Homogenization solution:

stock solution stored at –20°C (per 50 mL)

choline iodide	2.85 g	
ATP	0.0551 g	2 m*M*
DTT	0.0154 g	2 m*M*
PMSF	830 μL	1 m*M*
chymostatin	50 μL	20 μg/mL
ascorbic acid	0.044 g	5 m*M*
PVPP	0.5g g^{-1} root FW	directly into the mortar

BSA was not used to permit the measurement of the protein concentration. The choline iodide, ATP, DTT and ascorbic acid (can be stored in the fridge overnight) were weighted into a beaker. The MO stock solution was added just before the beginning of the vesicle isolation, as well as the PMSF and Chymostatin stock solutions.

Table 8: Gradient Solution (GS) Stock solution (per 25 mL)

choline iodide	1.425 g
ATP	0.0275 g
DTT	0.0077 g
ascorbic acid	0.022 g
PMSF	415 μL
chymostatin	25 μL
25 und 38 % (w/w) sucrose	

Protein concentration of the vesicle solution was determined after a modified method of Bradford (1976), with 150 μL of standard (up to 7.5 μg BSA) or sample, 50 μL NaOH (20 m*M*), and 2.8 mL of Bradford solution per vial. Protein concentration was measured photometrically at 595 nm against a blank after 15 min of colour reaction time.

To characterize the vesicles according to their subcellular origin, and therewith the purity of the vesicle suspension, the activities of the several ATPases, derived from the different subcellular membrane fractions, were determined essentially according to Gallagher and Leonard (1982). For this, 100 μL of vesicle suspension, containing 0.5 μg protein, were incubated for 30 min at 38ºC in a waterbath in 500 μL of a reaction medium containing buffer, ATPase substrates, SDS to break up the vesicles to reach the ATPase activities from the outside-out and inside-out vesicles, and specific inhibitors (Table 9): vanadate for plasma membrane ATPase, azide for mitochondrial ATPase, nitrate for vacuolar ATPase and molybdate for other phosphatases releasing P_i. As P-standards, 50 μL K_3PO_4 (0.5 m*M*) + 50 μL H_2O and 100 μL K_3PO_4 (0.5 m*M*) were used instead of the vesicle suspension. Blanks were done with 100 μL MR solution.

Stock solutions used for the ATPase assays:

- acridine orange (AO) 1 m*M*
- Brij 35 0.72 %
- BTP 0.45 *M*
- $CaSO_4$ 10 m*M*
- citric acid-BTP 150 m*M*, pH 6.5 (pH adjusted with solid BTP)
- EDTA-MES 200 m*M*, pH 6.5
- HCl 1.23 *N* (12.108 g 37 % HCl filled up to 100 g)
- KCl 1 *M*

- K_3PO_4 0.5 *M*
- malic acid-BTP 150 m*M*, pH 6.5 (pH adjusted with solid BTP)
- MES 0.9 *M*
- MES-BTP 150 m*M*, pH 5.5, 6.0, 6.2, 6.3, 6.4, 6.5, 6.6, 6.9, 7.0, 7.1, 7.3, 7.5, 8.0
- Mg-ATP 150 m*M* ($MgSO_4$ x 7 H_20 0.0739 g + ATP 0.1653 g, dissolved in 2 mL H_2O, prepared directly before use and stored on ice).
- Na_2MoO_4 70 m*M*
- NaN_3 70 m*M*
- PMSF 60 m*M*, dissolved in 2-propanol
- SDS 10 % (w/v) in H_2O
- succinic acid-BTP 150 m*M*, pH 6.5 (pH adjusted with solid BTP)
- sucrose 1 *M*
- vanadate 10 m*M* in 20 m*M* NaOH (for 100 mL: 0.09095g V_2O_5 in 1 mL of 2 *N* NaOH + ca. 50 mL H_20; stir overnight, then fill up to 100 mL).

Table 9: Different inhibitor treatments to determine the activities of the different ATPases in the vesicle suspension derived from different subcellular membrane fractions.

substance	inhibitor combinations								
	KNO_3	KNO_3+ V_2O_5	KNO_3+ NaN_3	KNO_3+ $NaMoO_4$	KCl		KNO_3	KNO_3 +NaN_3	KCl
	---------- Vol. [μL] ----------								
buffer pH 6.5	200	200	200	200	200	Buffer pH 8.0:	200	200	200
KNO_3	60	60	60	60	-		60	60	-
V_2O_5	-	24	-	-	-		-	-	-
NaN_3	-	-	10	-	-		-	10	-
$NaMoO_4$	-	-	-	5	-		-	-	-
KCl	-	-	-	-	60		-	-	60
Mg-ATP	20	20	20	20	20		20	20	20
Brij	8. 3	8. 3	8. 3	8. 3	8. 3		8. 3	8. 3	8. 3
H_2O	211.7	187.7	201.7	206.7	211.7		211.7	201.7	211.7
vesicle suspension	100	100	100	100	100		100	100	100
total volume [μL]	600	600	600	600	600		600	600	600

The ATPase reactions were stopped by addition of 1 mL of solution 1 (Tab. 10), then the vials were stored on ice again. 10 min after storing on ice, solution 2 (Tab. 11) (1.5 mL) was added on ice and the rack was put into the water bath at 38ºC for another 10 min, then to room temperature and the reaction of the P_i, which was set free by the activity of the ATPases, with the

colour reactants was determined photometrically at 705 nm after 10 min of colour reaction according to Forbush (1983).

Table 10: Solution 1 to stop the ATPase reaction

ascorbic acid	3 % (w/v)	6 g dissolved in 40 mL of H_2O
HCl	0.6 *N*	100 mL of 1.23 *N* HCl
ammonium molybdate	0.5 % (w/v)	10 mL of stock solution (70 m*M*)
SDS	3 % (w/v)	60 mL of a 10 % (w/v) stock solution

Solution A has to be stored in the dark and has to be used within 30 min after preparation.

Table 11: Solution 2 for the colour reaction with P_i.

tri-sodium-citrate	6 g
Na –arsenate x $7H_2O$	6 g
H_2O	300 mL
glacial acetic acid	6 mL

For the vanadate-sensitive plasma membrane ATPase assay alone, the reaction medium of Tab. 12 was used. Vanadate-sensitive and vanadate-insensitive ATPase activity was differentiated by adding 24 μL vanadate solution instead of H_2O.

Table 12: Vanadate-sensitive and -insensitive plasma membrane ATPase reaction medium.

substance	concentration	volume of the stock solutions used [μL] - vanadate	+ vanadate
BTP-MES	50 m*M* pH 6.5	200	200
Mg-ATP	5 m*M*	20	20
Na_2MoO_4	0.6 m*M*	5	5
KNO_3	100 m*M*	60	60
NaN_3	1.5 m*M*	10	10
Brij	0.01 %	8.3	8.3
V_2O_5	0.4 m*M*	-	24
H_2O		196.7	172.7
vesicle suspension (0.5 μg protein)		100	100

To determine the pH dependence of the vanadate-sensitive ATPase from the vesicles derived from the plants grown with or without P supply, the vanadate-sensitive ATPase assay was performed with MES-BTP buffer solutions with the pH values of 5, 5.5, 6.0, 6.2, 6.4, 6.5, 6.7, 6.9, 7.0, 7.3, 7.5, and 8.0 according to Tab. 13. Vanadate-sensitive and vanadate-insensitive ATPase activity was differentiated by adding 24 μL vanadate solution instead of H_2O.

Table 13: pH-dependent vanadate-sensitive and –insensitive plasma membrane ATPase reaction medium.

substance	concentration	volume of the stock solutions used [μL] - vanadate	+ vanadate
BTP-MES	50 m*M* pH 5.0 – 8.0	200	200
Mg-ATP	5 m*M*	20	20
Na_2MoO_4	0.6 m*M*	5	5
KNO_3	100 m*M*	60	60
NaN_3	1.5 m*M*	10	10
Brij	0.01 %	8.3	8.3
V_2O_5	0.4 m*M*	-	24
H_2O		196.7	172.7
vesicle suspension (0.5 μg protein)		100	100

Proton pumping activity of inside-out vesicles and therefore proton accumulation into membrane vesicles was assayed by the absorbance change of acridine orange (AO) as a pH-sensitive dye taken up by the vesicles and accumulating inside the vesicles, dependent of the acidification inside the vesicles due to the proton transport by the PM H^+-ATPase activity. For this, stock solutions of 343 μL MES-BTP buffer, pH 6.5; 10 μL AO solution; 100 μL KNO_3; 33 μL Mg-ATP; 250 μL sucrose, and vesicle suspension with 50 μg protein and a volume of H_2O filling the assay solution up to 1000 μL were mixed and the decrease in absorption of acridine orange was followed photometrically at 492 nm. To test the amount of vanadate-sensitive ATPase activity, the assay was done with addition of 40 μL V_2O_5. The influence of organic acids on the proton pumping activity was determined by adding 13 μL of 150 m*M* citrate- malate- or succinate-BTP, pH 6.5, to the assay solution. Addition of 10 m*M* EDTA-BTP chelated the Mg^{2+} ions necessary for H^+-ATPase activity, which resulted in the dissipation of the pH gradient brought about by the activity of the H^+-ATPase.

Citrate transport across vesicle membranes

Citrate transport across the vesicle membrane was determined by use of ^{14}C-citrate, whereby isolated plasma membrane vesicles were incubated for 60 min with an incubation solution at room temperature (Tab. 14). The vesicles were separated from the solution by filtration across cellulose nitrate filters (pore size 0.45 μm, 25 mm diameter), whereby the protein-containing vesicles stick to the membranes. The filters were cleaned with two-times rinsing with 1.5 mL washing solution (Tab. 15) each. The filters containing the vesicles were given into szintillation

vials, dissolved in 2 mL ethylacetate and radiation was measured after addition of 2 mL szintillation liquid. To determine unspecific binding of vesicles, the vesicle suspension was boiled to denaturate proteins before adding the labelled citrate. Alternatively 0.01 % Brij ®35 (0.72 % with 3.5 µL) was added to the vesicle suspension to open the vesicles and preventing citrate accumulation inside the vesicles. Unspecific binding of citrate to the filters was determined by incubating the filters in 10 m*M* unlabelled citric acid before the experiments were performed.

Table 14: Incubation solution for ^{14}C-Citric acid transport determination

stock solution	volume used	final concentration
MES-BTP 150 m*M* pH 6.5	84 µL	50 m*M*
sucrose solution 1 *M*	37.5 µL	150 m*M*
BSA 0.05 mg mL^{-1}	5 µL	1 µg mL^{-1}
Mg-ATP 150 m*M*	8.4 µL	5 m*M*
^{14}C citric acid 0.401 µmol mL^{-1}	16 µL if diluted 1/8	3.2 µ*M* – 0.1 µCi
vesicle suspension	80-100 µL	30 µg protein
H_2O	To fill up to 250 µL	

Table 15: Washing solution (à 100 mL)

stock solution	volume used	final concentration
MES-BTP 150 m*M* pH 6.5	33.3 mL	50 m*M*
sucrose 1 *M*	15 mL	150 m*M*
BSA 0.05 mg mL^{-1}	2 mL	1µg mL^{-1}
H_2O	To fill up to 100 mL	

Acidification of the cytosol

The investigation was done by N. Langlade and E. Martinoia, Université de Neuchâtel, Suisse (Kania et al., 2003). Different parts of lupin roots were collected as described by Massonneau et al. (2001). Four to ten root segments were rinsed briefly in distilled water and incubated during 1 h at 22°C in Eppendorf reaction tubes containing 1 mL of 20 m*M* propionate, pH 4.0, 20 m*M* MES-KOH at pH 4.0, or 20 m*M* HEPES-KOH at pH 7.0. Citrate and malate concentrations were determined in these root washings using the citric acid test kit and L-malic acid test kit (Boehringer, Mannheim, Germany). The presence of applied chemicals has been verified not to influence the determination method.

Isolation of protoplasts from mature cluster roots and root tips

The protocol for protoplast isolation was developed for white lupin cluster roots in part as a combination of methods given by Lin (1980), Xu et al. (1981), Power and Chapman (1985), Schachtman et al. (1991), Blackhall et al. (1994), Sevón et al. (1995), Spangenberg and Potrykus (1995), Gleddie (1995), and Steinecke and Schreier (1995).

Mature cluster rootlets from 4 to 6 weeks old plants were separated from their root axis with a razor blade (that is, only the lateral "brushes“ were used), given shortly on a filter paper to remove adhering water and cut into small pieces with a razor blade in a petri dish containing the enzyme solution (100 mg root fresh material mL^{-1} solution) (Tab. 16). To infiltrate the enzyme solution into the intercellular space, a slight vacuum was applied. The infiltrated roots were incubated in the petri dish at 30°C in the dark at 50 rpm in an incubator. Because the phenols exuded from the roots tend to inactivate the enzymes, the enzyme solution was changed after two hours of incubation.
Protoplasts were isolated after a total of 5 hours of incubation. The whole volume was separated into aliquots of 0.5 mL, being transferred separately to other petri dishes with a cut pipette tip and squeezed very carefully with a small glass vial to release the protoplasts from the cell walls. This suspension was filtered through a 300 μm plastic mesh into test tubes. The mesh was rinsed with a five-fold volume of flotation solution and scratched carefully with the pipette tip on the mesh to release more protoplasts. The contents of each test tube were carefully mixed and 1 mL of washing solution layered on top of each test tube, followed by centrifugation for 10 min at 175 *g*. The upper solution was collected directly above the interlayer with a pasteur pipette with a broad opening. The content of one test tube was divided to two Eppendorf reaction tubes and filled up with washing solution. After mixing and centrifugating for 6 min at 175 *g*, the supernatant was removed and the pellet carefully resuspended in fresh washing solution. This step was repeated once or twice again. Finally the supernatant was removed after the last centrifugation and the pellet resuspended in the remaining supernatant (approx. 70–100 μL). Subsamples of this suspension were used to determine protoplast density in a Fuchs-Rosenthal counting chamber. The vitality of the protoplasts were tested with a 0.1 % (w/v) Phenosafranin solution (phenosafranin dissolved in washing solution) and added 1:1 (v/v) to the suspended protoplasts. Intact protoplasts are able to keep the red phenosafranin out of their cells and therefore are not coloured red.

Table 16: Solutions used for protoplast isolation

Enzyme solution	Cellulase "Onozuka" RS	2 % (w/v)	
	Cellulase YC	1 %	
	Macerozyme R-10	1 %	
	Pectolyase Y-23	0.1 %	
	sucrose	0.4 *M* (= 13.7 %)	results in 610 mOsm for the complete enzyme solution
	KNO_3	1 m*M*	
	$CaCl_2$ x 2 H_2O	1 m*M*	
	$MgSO_4$ x 7 H_2O	1 m*M*	
	KI	0.964 μM	
	$CuSO_4$ x 5 H_2O	0.1 μM	
	MES	5 m*M*	
	BSA	0.5 %	
	PVP	2 %	
	DTT	1 m*M*	
	Na-ascorbate	10 m*M*	
	pH	adjusted to 5.5	

Flotation solution	sucrose	0.4 *M* (= 13.7 %)	results in 610 mOsm for the complete enzyme solution
	KNO_3	1 m*M*	
	$CaCl_2$ x 2 H_2O	10 m*M*	
	$MgSO_4$ x 7 H_2O	1 m*M*	
	KI	0.964 μM	
	$CuSO_4$ x 5 H_2O	0.1 μM	
	MES	5 m*M*	
	Ficoll	12 % (w/v)	
	PH	adjusted to 5.5	

Washing solution	sorbitol	0.5 *M*	results in 610 mOsm for the complete enzyme solution
	KNO_3	1 m*M*	
	$CaCl_2$ x 2 H_2O	10 m*M*	
	$MgSO_4$ x 7 H_2O	1 m*M*	
	KI	0.964 μM	
	$CuSO_4$ x 5 H_2O	0.1 μM	
	MES	5 m*M*	
	Ficoll	12 % (w/v)	
	pH	adjusted to 5.5	

Isolation of root hair protoplasts from seedlings and cluster roots

Root hair protoplasts from seedlings and cluster roots were isolated using a protocol modified from Cocking (1985) and Gassman and Schroeder (1994) with adjustments for white lupin.
Cluster root segments of white lupin or seedling root tip segments (up to several cm from the apex) were placed into enzyme solution (Tab. 17) in a water bath shaker at 30°C and 100 rpm for

about 30 min. After the enzyme treatment the root segments were washed twice with washing solution for 5 min at 4°C on a rotary shaker at 100 rpm and then were put into a small volume (approx. 200 – 400 μL) of isolation solution on a microscope slide for about 30 min. The lower osmolality of the isolation solution led to osmotic expansion and hatching of root hair protoplasts. After the 30 min the root segments were shaken gently to free the protoplasts and pulled out of the isolation solution. The protoplast density then could be counted in a Fuchs-Rosenthal chamber and the vitality determined with the phenosafranin method (see above).
Osmolality was calculated from the reduction of the freezing point in the solution measured with a KNAUER-Osmometer with a NaCl solution as a standard.

Table 17: Solutions used protoplast isolation from root hairs

Enzyme solution	Cellulase "Onozuka" RS	1 % (w/v)	
	Cellulysin	1 %	
	Macerozyme R-10	1 %	
	Pectolyase Y-23	0.02 %	
	sucrose	0.68 *M* (= 12.4 %)	results in 860 mOsm
	KCl	10 m*M*	of the complete solution
	$CaCl_2$ x 2 H_2O	1 m*M*	
	$MgCl_2$ x 2 H_2O	2 m*M*	
	MES	10 m*M*	
	BSA	0.1 %	
	Na-ascorbate	10 m*M*	
	pH	adjusted to 5.5	
Washing solution	sucrose	0.75 *M* (= 13.7 %)	results in 860 mOsm
	KCl	10 m*M*	of the complete solution
	$CaCl_2$ x 2 H_2O	1 m*M*	
	$MgCl_2$ x 2 H_2O	2 m*M*	
	MES	5 m*M*	
	ascorbic acid	10 m*M*	
	pH	adjusted to 5.5	
Isolating solution	sucrose	0.15 – 0.333 *M*	results in 200-400 mOsm
	KCl	10 m*M*	of the complete solution
	$CaCl_2$ x 2 H_2O	1 m*M*	
	$MgCl_2$	2 m*M*	
	MES	10 m*M*	
	pH	adjusted to 5.5	

Results

Purification of plasma membrane vesicles

Since various ATPases have been localized in membranes of different subcellular compartments, high purification of plasma membrane (PM) fractions is a prerequisite for investigations of PM H^+-ATPases. Highly purified plasma membrane vesicles were isolated from root systems with cluster roots of P-deficient plants (-P) and from roots of +P control plants in a small scale procedure using a sucrose gradient centrifugation.

The purity of the vesicle preparations was tested by determining the activity of marker enzymes with use of phosphatase inhibitors and specific inhibitors for the several H^+-ATPases originating from different plant membranes. For this, the ortho-phosphate (P_i) set free by the phosphatase and ATPase activities were measured (Table 18). The high vanadate-sensitive ATPase activities of around 90 % of vesicles derived from –P and +P plants showed that the vesicle preparations were enriched with PM-derived membranes. Only minor impurities of vacoular membranes (nitrate-sensitive vacuolar ATPases), mitochondrial membranes (azide-sensitive mitochondrial ATPases) or free phosphatases (molybdate-sensitive) were detected. Therefore this method was viable to isolate vesicles which could be used to further investigate the phosphorylating and proton transport activities of the PM H^+-ATPase. The latency, which gives the percentage of tight vesicles, was high enough to allow proton transport studies. The latency is defined as the percentage of the difference between the total phosphatase activity of vesicles and phosphatase activity of tight vesicles to the total phosphatase activity of the vesicles. The total phosphatase activity of vesicles is determined when the vesicles are opened by the addition of a detergent, so that the ATP-cleaving areas of the ATPases on both, the inside and the outside of the vesicle, can react. The activity of tight vesicles is determined without the addition of a detergent, where only the ATP-cleaving areas of the ATPases on the outer side of the vesicle can react.

Table 18: Purity of vesicle preparations characterized by inhibition of marker enzymes (ATPases).

	ATPase or phosphatase activity [μmol P_i mg^{-1} protein h^{-1}]			
inhibitor	assay pH	marker ATPase	+ P	- P
vanadate	6.5	plasma membrane	64.9	83.4
molybdate	6.5	phosphatase	17.3	43.1
azide	8.0	mitochondria	0.9	2.7
nitrate	8.0	vacuole	14.7	5.6
latency [%]			62	42

Characterization of the plasma membrane H^+-ATPase in roots of white lupin as related to the P-nutritional status

Alterations of plasma membrane H^+-ATPase activity during cluster root development

When vanadate-sensitive PM H^+-ATPase was measured at different stages of cluster root development, the highest activity per cluster was found in mature clusters (Fig. 28 A), which is in accordance with the highest acidification and the highest rate of citrate exudation found in these root segments (Fig. 26). A lower pH of the nutrient solution as the cultivation medium of the plants is paralleled by an increase in citrate exudation into the nutrient solution (Fig. 27). Accordingly, immunoblotting of the PM H^+-ATPase protein (Fig. 33) showed a 97 kDa H^+-ATPase band with a 69 % higher intensity when the vesicles were derived from cluster roots of P-deficient plants compared with roots from P-sufficient control plants. This increase in PM H^+-ATPase protein amount parallels H^+-ATPase hydrolytic and proton transport activity (Fig. 29+32).

On the basis of cluster root fresh weight, activity was highest in young clusters, with decreasing activities towards older stages (Fig. 28 B), whereas on the basis of protein concentration H^+-ATPase was the same in young and mature clusters and activity decreased in senescent ones (Fig. 28 C).

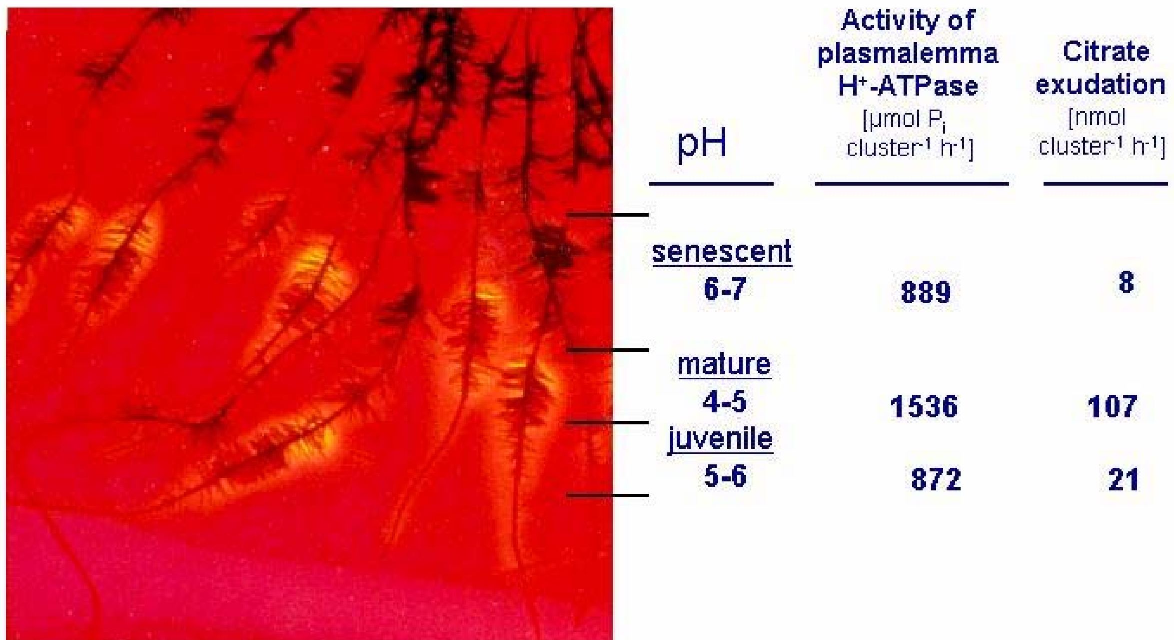


Fig. 26: Spatial variation of pH, plasma membrane H^+-ATPase activity, and exudation of citrate along cluster roots of P-deficient white lupin.

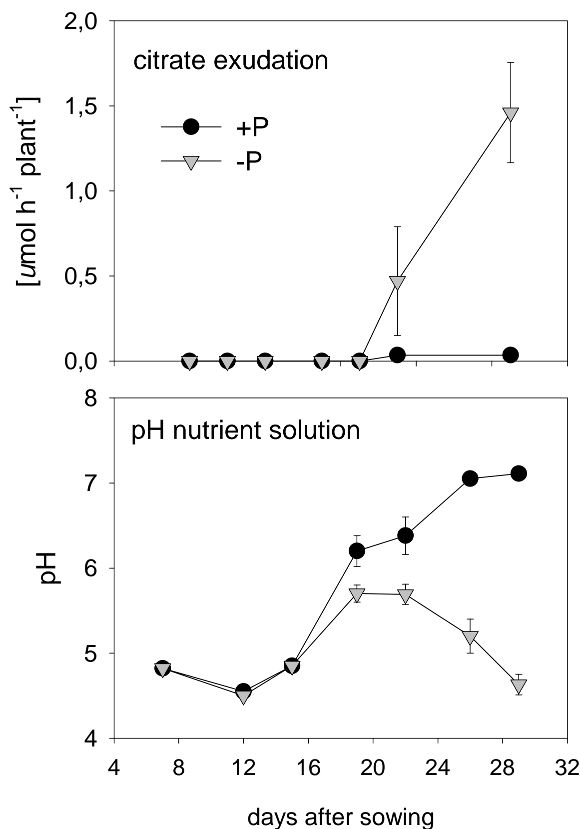


Fig. 27: Development of citrate exudation rate per plant (above) and pH in the nutrient solution (below) in +P control plants and P-deficient plants during plant growth (from: Neumann et al., 1999)

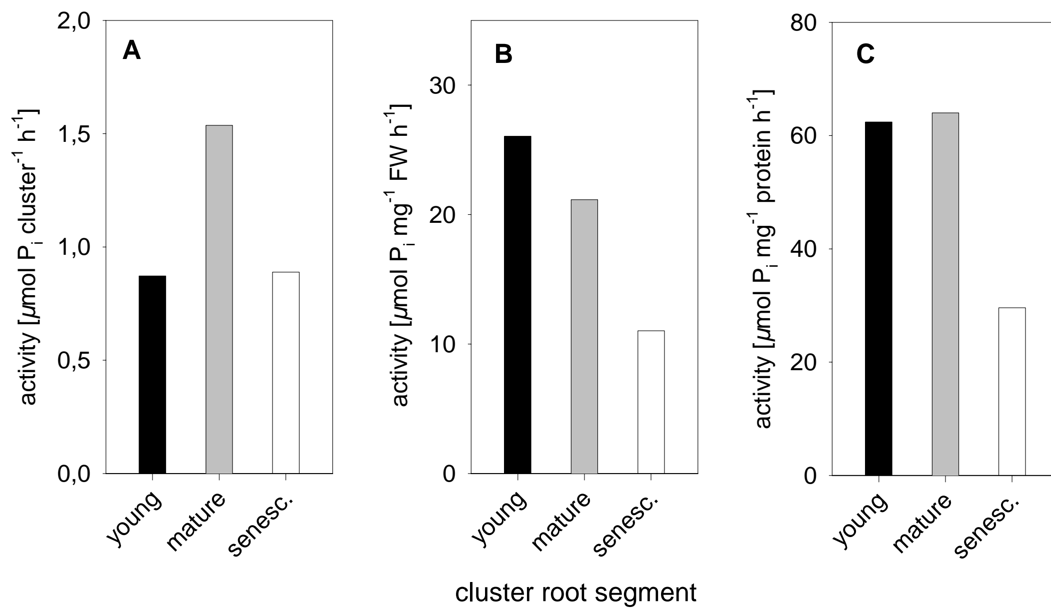


Fig. 28: Vanadate-sensitive PM H^+-ATPase hydrolytic activity measured as phosphate release by ATP cleavage in PM vesicles derived from different segments of cluster roots of P-deficient plants. **A**: H^+-ATPase activity per cluster; **B**: per cluster fresh weight; **C**: per protein.

ATP hydrolysis

Plasma membrane H^+-ATPase activity, measured as phosphate release by ATP hydrolysis in PM vesicles (Fig. 29), was the same when the vesicles were derived from two weeks old + P and –P plants. After three to five weeks of plant growth H^+-ATPase activity was on average 60 % higher in vesicles derived from -P plants compared to +P plants, demonstrating that the plants increased their H^+-ATPase activity as a reaction to P deficiency after consumption of the P seed reserves.

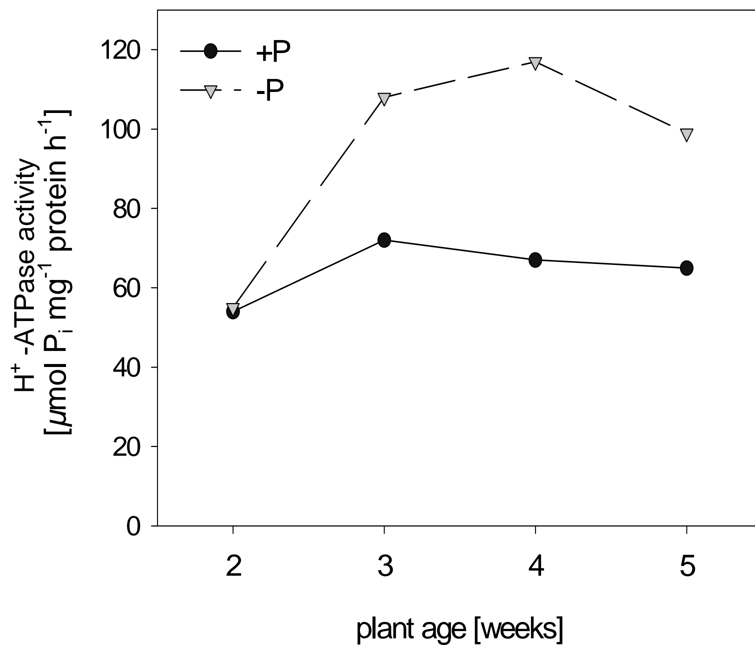


Fig. 29: Plasma membrane H^+-ATPase hydrolytic activity measured as phosphate release by ATP cleavage in PM vesicles derived from roots of two to five weeks old P-sufficient (+P) or P-deficient (-P) plants.

pH optimum

Hydrolytic activity of the H^+-ATPase, measured as phosphate release by ATP cleavage in PM vesicles (Fig. 30), was determined at different pH values in the assay solution to elucidate how the PM H^+-ATPase activity depends on the surrounding pH. Activity approximated a Gauss distribution with the highest H^+-ATPase activity at a pH of 6.35 in vesicles from -P plants and a pH of 6.50 from +P control plants, calculated from a 5th order linear regression. This demonstrates that under P deficiency the pH optimum of the PM H^+-ATPase is shifted by 0.15 pH units to more acidic conditions.

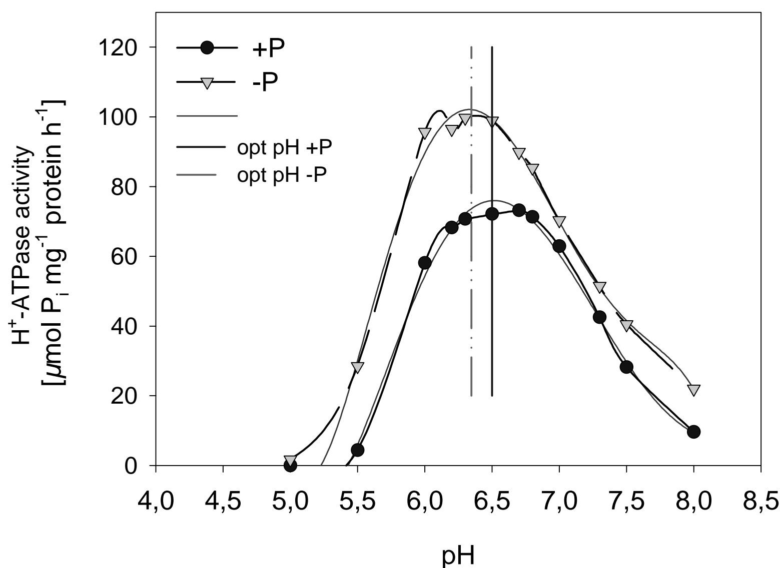


Fig. 30: PM H^+-ATPase hydrolytic activity measured as phosphate release by ATP cleavage in PM vesicles derived from roots of five weeks old P-sufficient (+P) or P-deficient (-P) plants at different pH values in the assay solution. Maximum activity for +P at pH 6.50; for -P at pH 6.35.

Plasma membrane H^+-ATPase activity as affected by carboxylates

To determine whether citrate or malate in the cytosol might influence H^+-ATPase activity, citrate and malate were applied to the H^+-ATPase assay solution at concentrations of up to 10 and 15 m*M* for citrate and malate, respectively. The different concentrations for citrate and malate were chosen to apply the same amount of carboxylic groups for both, the citrate and the malate solution.

Independent of the kind of carboxylate and the concentration supplied, H^+-ATPase activity was always higher in vesicles derived from plants grown under P-deficient conditions. Increasing malate concentrations had no influence on the H^+-ATPase activity in the -P and the +P vesicles (Fig. 31). However, low citrate concentrations of < 0.1 m*M* increased H^+-ATPase activity, but when citrate concentrations were higher than a threshold value of approximately 4 m*M*, H^+-ATPase activity decreased, independent of the P status of the plants the vesicles were derived from. This means that citrate interferes with H^+-ATPase activity. A low amount of citrate activates H^+-ATPase, whereas a higher amount of citrate inhibits the H^+-ATPase and decreases its activity.

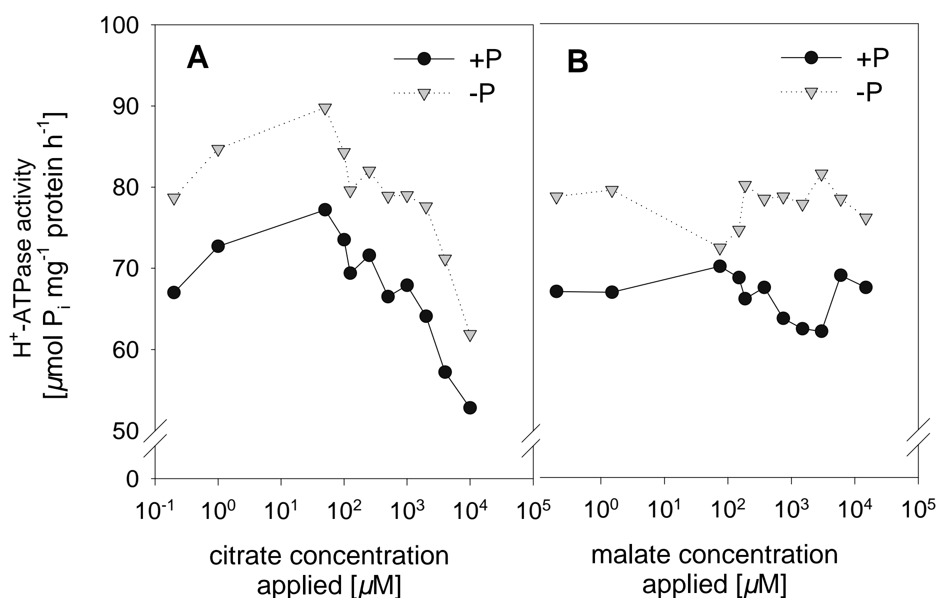


Fig. 31: Plasma membrane H^+-ATPase hydrolytic activity measured as phosphate release by ATP cleavage in PM vesicles derived from -P and +P control plant roots dependent on citrate (A) and malate (B) concentrations in the assay solution.

Proton transport across membranes

Proton transport activity of the H^+-ATPase in vesicles derived from P-deficient plants was monitored by the use of acridine orange as a dye that changes its absorbance dependent on the pH and acridine orange accumulation inside vesicles at a lower pH (Fig. 32). Proton pumping activity was largely insensitive to nitrate, but was almost completely inhibited by vanadate, which indicates the presence of tightly sealed inside-out plasma membrane vesicles. Absorption quenching was reversed by the addition of 10 m*M* EDTA as a chelator of Mg^{2+}. Addition of 7.5 m*M* malate increased proton pumping activity, whereas 5 m*M* citrate and therefore the same concentration of carboxylic groups inhibited this activity. This is in accordance with the altogether unchanged hydrolytic activity of the H^+-ATPase at higher malate concentrations and the lower activity at higher citrate concentrations (Fig. 31).

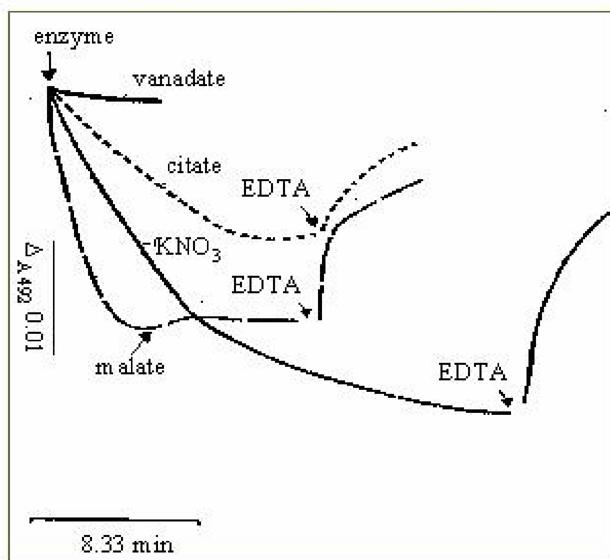


Fig. 32: Effect of NO_3 (100 m*M*), vanadate (0.1 m*M*), malate (7.5 m*M*) and citrate (5 m*M*) on the pH gradient formation in PM vesicles, determined by the absorbance change of acridine orange at λ = 492 nm. Vesicles were isolated from P-deficient roots. Acidification was stopped by the addition of 10 m*M* EDTA-BTP.

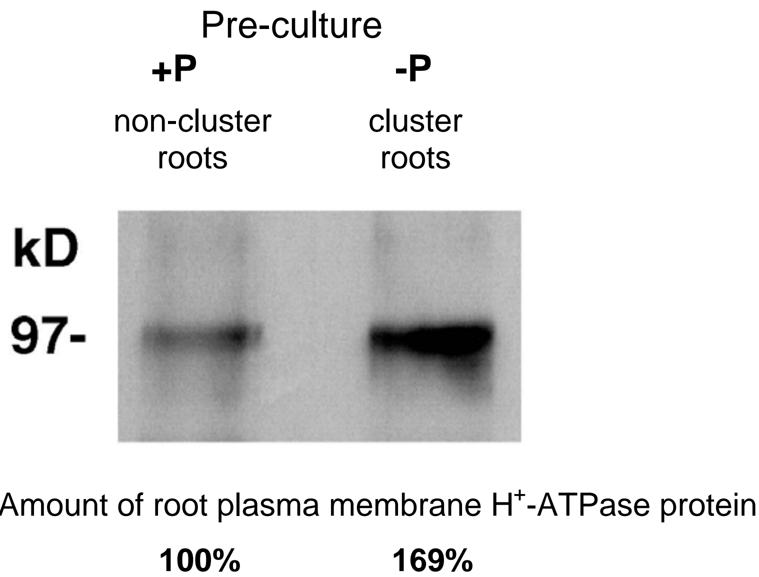


Fig. 33: Immunodetection of the H^+-ATPase protein by Western blotting of PM vesicles isolated from roots of +P control plants or of P-deficient (-P) cluster-containing plants (data from S. Cesco and R. Pinton).

^{14}C-citrate transport into inside-out vesicles

The only direct determination of citrate transport across vesicles can be done by use of radioactively labelled citrate. When ^{14}C-citrate was applied to the vesicle suspension, the amount of citrate transported across the plasma membrane was around twice in vesicles derived from -P plants compared to the vesicles derived from +P control plants when the test solution contained Mg-ATP for membrane energetization. This means that citrate transport is accelerated under P-deficient conditions, additionally to a basal rate of citrate transport already under P-sufficient conditions.

Estimating the rate of proton accumulation inside the vesicles from the initial slope of absorption quenching of the acridine orange, citrate itself increased proton pumping activity in vesicles derived from P-deficient plants, compared to +P control plants, even without addition of Mg-ATP to enable H^+-ATPase activity (Tab. 19). When Mg-ATP was added to the solution to support H^+-ATPase activity, proton transport was further increased slightly in +P control vesicles and to a high amount in –P vesicles compared to a solution not containing Mg-ATP.

Table 19: Uptake of ^{14}C labelled citrate and H^+ pumping activity by addition of citrate alone or citrate + Mg-ATP for energetization in PM vesicles derived from P-deficient (-P) and +P control plants.

	+ P	- P
uptake of ^{14}C-citrate [pmol h^{-1} mg^{-1} protein]	34.2	76.7
H^+ pumping activity [Δ_{A492} min^{-1} mg^{-1} protein]:		
2 m*M* citrate	3.6×10^{-3}	6.0×10^{-3}
2 m*M* citrate + Mg-ATP	4.4×10^{-3}	16.0×10^{-3}

Carboxylate exudation and intracellular pH

The pulse of citrate exudation in mature cluster roots after a threshold level of citrate concentration is reached suggests a controlled citrate exudation mechanism. Reduced citrate exudation when anion channel inhibitors were applied support this idea. The release of carboxylates via anion channels under Al-stress were recently proven by patch-clamp studies in wheat and in maize (Kollmeier et al., 2001; Piñeros and Kochian, 2001; Zhang et al., 2001). However, so far nothing is known about factors which might trigger the opening of the channels. The following investigations were done by Langlade and Martinoia, Université de Neuchâtel, Suisse. To assess whether modifications in the intracellular pH may be involved in the induction of carboxylate exudation in roots of P-deficient white lupin, weak organic acids such as propionic acid, acetic acid and methyl trichloroacetic acid (methyl-TCA), which are not metabolized by plants, were applied to the root medium at pH 4.0. According to the ion-trap principle, the protonated acids easily cross the plasma membrane and are deprotonated at the neutral pH of the cytosol, which decreases cytosolic pH.

During an incubation period of 1 h with propionic acid, release of citrate and malate dramatically increased, especially in +P control plants and young, P-deficient root tissue such as apical root zones and young clusters, normally not releasing high amounts of carboxylates (Fig. 34). Similar results were obtained with acetic acid or methyl-TCA (data not shown). This high exudation rates, triggered by cytosolic acidification, were not an effect of low external pH values, since a low external pH value alone (MES-buffer at a pH of 4.0) did not change carboxylate exudation rates in the root segments which showed high carboxylate release rates when propionic acid was

applied. In contrast, there were no consistent effects in mature and senescent clusters. This may be explained by a low cytosolic pH in these root segments already before propionate application, induced by the high levels of intracellular citrate accumulation.

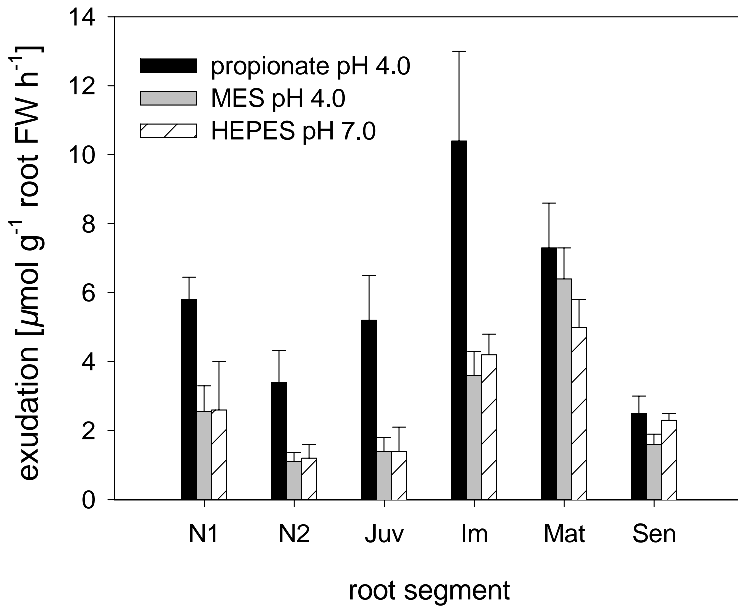


Fig. 34: Citrate and malate concentrations in different root segments of white lupin as affected by buffering the external pH and application of propionate. N1: 10 mm apical root zone of first order laterals in P-sufficient plants; N2: 10 mm apical root zones of first-order laterals in P-deficient plants; Juv: juvenile root clusters; Imm: immature root clusters completely developed but secretory activity not yet expressed; Mat: mature root clusters; Sen: senescent root clusters (data from Langlade and Martinoia).

Protoplast isolation

Protoplast isolation from mature cluster root laterals

Protoplasts were to be isolated for patch-clamp studies from mature cluster roots of P-deficient lupin plants to investigate the putative carboxylate channel responsible for the citrate exudation pulse observed in mature clusters when a threshold value of citrate tissue concentration is reached. The aim was to establish a protoplast purification protocol to gain protoplasts viable for tight sealing in patch-clamp studies.

Yield of protoplasts derived from mature cluster root laterals were generally very low and varied much between different isolations. The highest yield reached was approx. 100,000 protoplasts g^{-1} root biomass of laterals, but mostly it was much lower, meaning that the protoplasts were very fragile. Staining with phenosafranin (Fig. 35 A+B) revealed a percentage of 70 % of living protoplasts directly after isolation and ~ 50 % several hours later. Incubation in enzyme solution for more than 8 h decreased protoplast yield and viability sharply. Incubation for more than 12 h resulted in no protoplasts at all.

Mannitol or sorbitol as osmoticum in the enzyme solution led to much lower yields compared to sucrose. Most protoplasts appeared to be desintegrated at the same osmolality of mannitol or sorbitol compared with sucrose.

Oxidizing phenols might have been one cause for protoplast destruction due to membrane damage, since the enzyme solution and the root tissue got darker in the course of incubation although high amounts of antioxidants and protecting agents were used. Even the incubating solution was changed after two hours to remove phenolic compounds.

The root tissue, put under the microscope directly from the enzyme solution, was optically completely unchanged compared with non-incubated tissue. The tissue could be macerated only mechanically with pressure and rubbing in the petri dish with the bottom of a glass vial, meaning that the cell wall bonds between cells and the cell wall structure itself could not be dissolved, but only loosened, making it available for mechanical breaking. Using a salt solution for washing the protoplasts gave a much higher protoplast yield. Since these high salt concentrations might be disadvantageous at clamping, this procedure was not followed further.

Patch-clamping of mature cluster root protoplasts isolated by the described procedure with adjustments was tried by Yan et al. (pers. comm.), but the protoplasts broke before measurements could be performed.

To find out whether mature cluster root protoplasts were just too old for isolation, or if the isolation procedure had a crucial flaw, or if protoplast isolation from white lupin tissue is generally not possible, the same isolation procedure was applied to cotyledons, which are often used for protoplast isolation. Here the yield was orders of magnitude higher (Fig. 35 C), being in the range of counts given in literature, showing that the problem rather was on the side of the protoplasts isolated from mature cluster roots, which probably are physiologically too old for isolation.

Protoplast isolation from root tips

As an alternative, protoplast isolation was performed with root tips of P-deficient plants, which gave higher protoplast yields than mature cluster roots. However, the calyptra, the root cap covering the root tip itself, could not be removed completely from the root tip before isolation. Therefore the origin of the protoplasts could not be determined without doubt, although protoplasts derived from the calyptra seemed to contain less inner structure together with a thinner plasma membrane layer than those from the root tip itself. The already loosely connected cells of the calyptra seemed to be isolated much easier than the root cap cells themselves.

Protoplast isolation from root hairs

There are still contradicting results whether root hair cells themselves are able to release carboxylates. To answer this question, protoplasts derived from root hairs were also to be patch-clamped. Root hairs in white lupin exist along seedling roots, beginning about one cm behind the root tip, and ending some cm more basal. Root hairs also grow on cluster roots, beginning on young cluster roots, and building dense mats of long root hairs in mature and senescent clusters.
The isolation procedure was done according to Cocking (1985) and Gassmann and Schroeder (1994) with modifications for white lupin. When the root segments were put into the hypo-osmotic isolating solution on the microscope slide, the swelling of root hair protoplasts emerging from the tips of root hair cells could be observed directly (Fig. 35 D-G). The root hair tip is the area where the cell walls get thin and breakeable enough due to the digestive enzyme activity so that the expanding protoplasts can break through. Root hair protoplasts could be produced by this procedure only from young root hairs from seedling root tips; the shorter the root hairs, the

higher the percentage of root hairs that released protoplasts. No protoplasts could be released from root hairs from any cluster roots, not even from very young ones.

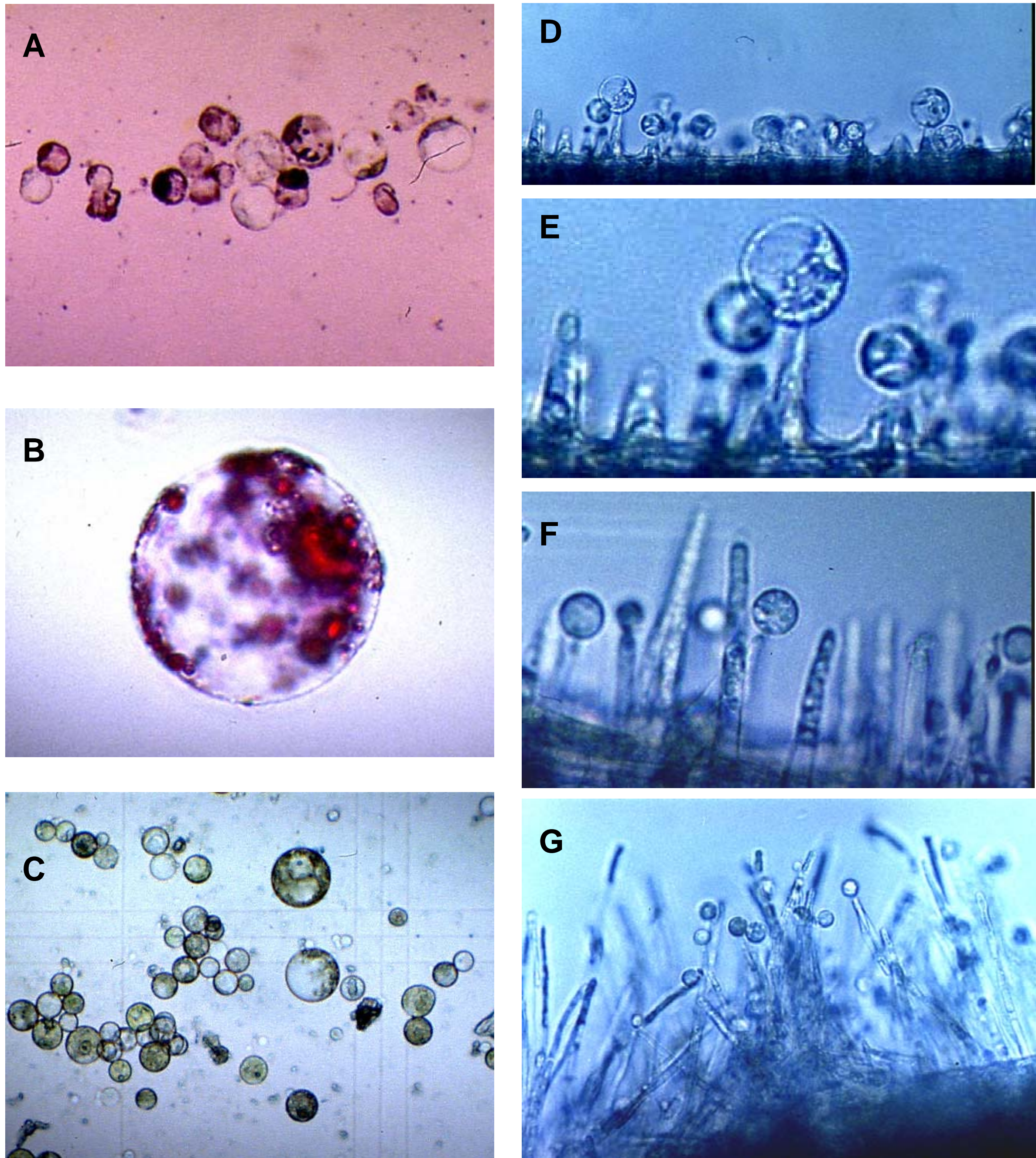


Fig. 35: Protoplasts isolated from: A+B: mature cluster root laterals. Red colouration inside the protoplasts means low viability. C: from cotyledons. D-G: root hair protoplasts isolated according to the methods of Cocking (1985) and Gassmann and Schroeder (1994).

Discussion

Plasma membrane H^+-ATPase characterization

The concomitant release of citrate and protons (Neumann et al., 1999) (Fig. 26) from a specific root zone (Marschner et al., 1987; Dinkelaker et al., 1989; Neumann et al., 1999), later called “mature“ cluster roots of P-deficient white lupin plants, hints to a common regulation of citrate exudation and H^+-ATPase activity. Therefore H^+-ATPase activity was to be tested in relation to P status, citrate accumulation and exudation in different root zones of white lupin plants. For this, plasma membrane (PM)-derived vesicles from P-deficient, cluster-containing roots of white lupin and from roots of +P control plants were isolated in a small-scale procedure using a sucrose gradient for further purification.

Characterization of the root H^+-ATPase as related to the P-nutritional status

Hydrolytic activity of the PM H^+-ATPase, measured as ortho-phosphate (P_i) release by ATP cleavage, was the same in vesicles derived from two weeks old plants independent of the P supply during growth. This means that the plants did not suffer from an internal P deficiency high enough to impair the activity of the PM H^+-ATPase. The plants probably still had a sufficient amount of P available from the seed reserves. After three to five weeks of growth, however, hydrolytic activity of the H^+-ATPase increased in vesicles derived from P-deficient plants, whereas vesicles derived from +P control plants did not show changes in activity (Fig. 29). This is in accordance with the acidification of the root’s surrounding, observed as a lower pH in the nutrient solution of plants grown under P deficiency, together with high citrate exudation rates from the root system (Fig. 27). Stoichiometric calculations between the amounts of carboxylates (citrate + malate) and protons, released by P-deficient white lupin roots, and different exudation curves over time from both groups of substances, revealed that the exudation of carboxylates is not the only cause for acidification (Sas et al., 2001). Phosphate deficiency results in a higher cation/anion uptake ratio (Dinkelaker et al., 1989; Sas et al., 2001) due to a lower nitrate uptake (Neumann et al., 1999). For reasons of charge balance more protons are exuded by the H^+-ATPase when more cations than anions are taken up.

Already under non-limiting conditions the H^+-ATPase is expected to consume a significant part of the available ATP in highly metabolizing cells (Arango et al., 2003). A higher ATP turnover

for hydrolysis by PM H^+-ATPase in spite of a lower ATP concentration status under P-deficient conditions (Massonneau et al., 2001) means that an even higher percentage of ATP must have been used for H^+-ATPase activity than under non-limiting conditions. This high priority of the use of ATP for H^+-ATPase activity under P-deficient conditions stresses the importance of this metabolic step.

Western blot analysis, using a polyclonal antibody against the maize PM H^+-ATPase, revealed a higher protein amount of a 97 kDa immunostained band in samples derived from vesicles isolated from P-deficient roots compared to vesicles from +P control plants, which hints to a transcriptional regulation. The molecular weight found is in accordance with the monomeric PM H^+-ATPase with a molecular weight of around 100 kDa on average found in many plants (reviewed by Palmgren, 2001). The fact that the enzyme is encoded, in various species, by a multigene family hints at the possibility of regulation at the transcriptional level (Arango et al., 2003). However, it is not possible to test whether an antibody can discriminate between different isoforms (Williams and Gregory, 2004). H^+-ATPase genes might be activated by various abiotic and biotic environmental factors and the amount of H^+-ATPase might be increased under conditions requiring greater transport activity (Arango et al., 2003). The density of the H^+-ATPase protein also changes with the developmental stage of the plant tissue, and is influenced by sink-source relations (Williams and Gregory, 2004), since sucrose is taken up in a sucrose/H^+ symport (Giaquinta, 1977; Bush, 1989). Cluster roots show a fast transformation from a sink to a source organ during cluster root development, which might contribute to the lower H^+-ATPase protein found in older cluster roots.

Plasma membrane H^+-ATPase activity in different cluster root segments

The amount of H^+-ATPase hydrolytic activity in different developmental stages of cluster roots was dependent on the basis of the measurements. H^+-ATPase activity per cluster root segment was highest in mature clusters (Fig. 28), which was to be expected due to the strong acidification brought about by this root segment. Here also the highest citrate exudation rates were found (Fig. 26). This effect of the highest activities might at least in part be due to the highest surface area in this root segment. Mature clusters have fully developed cluster rootlets with a long and dense mat of root hairs. Young clusters have a much lower mass than mature and senescent ones, shorter rootlets and no or shorter root hairs. On the basis of fresh weight, H^+-ATPase activity decreased while the cluster segments grew older. The young clusters are quite small compared to the mature or senescent clusters because they consist of still growing tissue with short laterals,

but they already have a high H^+-ATPase activity. Perhaps the high percentage of the still growing root tips of the young cluster laterals reacted like root tips of non-cluster roots and therefore acidified. Hydrolytic activity per amount of protein decreased only in senescent cluster roots.

According to the acidification of the rhizosphere of the different cluster root segments a lower activity of the young clusters compared to the mature ones was to be expected. The relatively high activity of the young clusters compared to the mature ones might also have been caused by classification of the clusters, where the young ones might have already been in transition to being mature ones. However, the low H^+-ATPase activity in senescent cluster roots in all calculations hints to a general P limitation of the H^+-ATPase as also found for other metabolic processes in these old root zones (Chapter 1).

Plasma membrane H^+-ATPase activity depending on the external pH

An adaptation of the H^+-ATPase to a lower apoplastic pH created by a higher H^+-ATPase activity of P-deficient plants was observed in form of a shift of the optimal pH value by 0.15 pH-units to more acidic conditions in the reaction medium for the maximum hydrolytic activity of the isolated vesicles derived from P-deficient plant roots compared to +P control roots. The general pH optimum of a proton pump with less than a pH of 7 is already more acidic than the normal pH value of the cytoplasm (Mimura, 2001). This finding is in accordance with those of Yan et al. (2002), who also reported a more acidic pH optimum in active cluster roots. A lower pH optimum could be seen as an adaptation to a lower cytosolic pH. The accumulation of carboxylates under P deficiency by a higher synthesis and reduced degradation (see Chapter 1), causing a higher proton production and lower proton incorporation due to the pH stat mechanism (Sakano, 1998), results in lower cytosolic pH values.

Differential expression of H^+-ATPase isoforms seems to be common in plants (Baxter et al., 2003; Arango, 2003), and might also explain the lower pH optimum of the lupin H^+-ATPase in mature cluster roots (Yan et al., 2002). A number of quantitative differences in catalytic and regulatory properties between isoforms were described (Palmgren, 2001). Even five subfamilies were separated, which differed functionally in their regulatory properties (Dambly and Boutry, 2001; Arango et al., 2003). It was found that different H^+-ATPase isoforms with distinct kinetics (Luo et al., 1999) might operate within the same cell (Oufattole et al., 2000). The overlapping expression found e.g. in *Nicotiana plumbaginifolia* (Moriau et al., 1999) suggests caution in interpreting enzyme kinetics for H^+-ATPases analyzed at the organ level, since the data reflect

contributions from several isoforms. Different isoforms with different K_m and different sensitivities to orthovanadate were described and it was mentioned that such findings reveal the futility of using crude plant homogenates as the starting material for biochemical analyses of enzymes that occur in multiple isoforms (Sanders and Bethke, 2000).

Plasma membrane H^+-ATPase activity as affected by carboxylates

Mature cluster roots are characterized by very high internal citrate concentrations of 20-25 µmol g^{-1} root fr. wt. (Neumann et al., 1999), which is seen as a prerequisite for the pulse of citrate exudation observed in these root segments (Neumann et al., 2000; Peñaloza et al., 2002), together with a strong acidification of the mature cluster root rhizosphere. High carboxylate concentrations may have detrimental effects on plant cell physiology. Citrate as a chelator can complex Ca^{2+} and Mg^{2+} ions, therefore disturb Ca- and Mg homeostasis, and might decrease cytosolic pH and impair the cytosolic pH stat mechanism (Neumann et al., 2000). To avoid this, cytosolic citrate concentrations usually are kept below 5 m*M* (Jones, 1998). Therefore the influence of carboxylates at different concentrations on the activity of the H^+-ATPase was to be tested.

In the present work, higher citrate concentrations of more than 4 m*M* inhibited H^+-ATPase hydrolytic activity in purified vesicles, whereas malate at the same concentration of carboxylic groups did not have any significant influence, independent of the P status of the plants the vesicles were isolated from (Fig. 31). This hints to a citrate-specific inhibitory effect on H^+-ATPase activity, independent of the acidic nature of citrate. An artifact due to Mg^{2+} complexation by the citrate and therefore a lack of available Mg-ATP as substrate for the ATPase can be ruled out, since an even ten times higher Mg concentration did not give any differences in activity (data not shown).
When H^+-ATPase proton transport across the vesicle membrane, derived from P-deficient plant roots, was monitored from the initial slope of absorbance quenching of acridine orange (AO), 5 m*M* citrate again showed an inhibitory effect on proton transport compared to the proton transport determined with the addition of NO_3^- alone as a counter-ion for proton transport (Fig. 32). However, malate at the same carboxylic group concentration even increased proton transport. The low impurities of membranes of other origin than the plasma membrane was documented with the almost complete proton transport inhibition by addition of vanadate. This

means that both, the hydrolytic and proton transport activity of the plasma membrane H^+ -ATPase, was impaired by higher citrate concentrations.

Protein pumping activity was also monitored from the initial slope of AO absorbance quenching in vesicles derived from P-deficient plants and from +P control plants when only 2 m*M* citrate were applied (Tab. 19). Even without energetization of the plasma membrane and inactivity of the H^+-ATPase due to a lack of its substrate Mg-ATP, citrate transport occurred. This might be explained by a pH difference between the outer medium (pH 6.5) and the inside of the vesicles (pH 7.0). A passive proton flux took place due to the addition of NO_3^- to the outer medium and its easy influx into the vesicle lumen, supporting proton transport. Without addition of ATP, proton transport activity was higher in vesicles derived from P-deficient plants compared to vesicles from +P control plants, which hints to differences in membrane transport or membrane permeability to NO_3^- and protons independent of any activity of the H^+-ATPase. When ATP was added and H^+-ATPase was able to be active, proton transport in +P control vesicles stayed the same, whereas proton transport in -P vesicles was increased 2.5-fold. Therefore the main proton transport across the plasma membrane under P-deficient conditions is brought about by the activity of the P deficiency-induced H^+-ATPase.

Unfortunately, a direct correlation between the hydrolytic and the proton transport activity cannot be calculated, since the monitoring of acridine absorbance quenching is rather not quantitative. The AO signal is not linearly related to changes in the rate of H^+ pumping (Venema and Palmgren, 1995). Even the mechanism by which AO reports pH gradients is not yet fully understood (Palmgren, 1991), and it is suggested that the observed absorbance changes during the H^+-ATPase assay are simply due to accumulation of free protonated dye in the intravesicular lumen and subsequent dimerization, dependent on the concentration inside. What is measured as absorbance decrease at 495 nm is thus the disappearance of AO monomers (Palmgren, 1991, 1998).

From the results of an increased H^+-ATPase activity at moderate citrate concentrations and a decreased activity at high citrate concentrations it might be concluded that high citrate exudation rates serve as a means to prevent detrimental effects of high citrate concentrations on plant metabolism and H^+-ATPase activity *in vivo*. So far it is not known how this inhibitory effect of high citrate concentrations on H^+-ATPase activity is brought about. Phenolics were described to inhibit H^+-ATPase (Erdei et al., 1994). Furthermore, it would be interesting to investigate if the ratio of ATP hydrolysis and proton transport is sustained. A 1:1 stoichiometric relationship between ATP hydrolysis and proton transport is supported by measurements (Palmgren, 2001) and was suggested to be reasonable for the ATPase under conditions where it generates maximal

pH gradients and membrane potentials (Venema and Palmgren, 1995). But these authors have also demonstrated a change in coupling ratio of an ion pump induced by glucose under *in vivo* conditions. They interpreted this as pointing to a physiological role for uncoupling as a mechanism for regulating pump activity, although kinetic controls must operate to avoid undesired H^+ leakage or futile consumption of ATP. The phenomenon of variable transport coupling ratio may be of biological relevance (Palmgren, 1998). In *Beta vulgaris*, for example, an indication was found that osmotic regulation of H^+-ATPase in the plasma membrane is achieved via modulation of the coupling between H^+ transport and ATP hydrolysis, and that such regulation involves 14-3-3 proteins (Babakov et al., 2000).

The enzyme H^+-ATPase is known to be tightly regulated. The C-terminal end of the PM H^+-ATPase serves a role as an autoinhibitory regulatory domain, whereby 14-3-3 proteins will bind if a serine or threonine is phosphorylated in the binding site of the target. This binding activates the enzyme (reviewed e.g. by Palmgren, 1998; 2001). 14-3-3 proteins are present as multigene families in most organisms, are dimeric, and bring about signal-induced changes in the target by conformational changes that mediate their biological effects. Apart from the H^+-ATPase, over 100 proteins have been found to interact with 14-3-3 proteins, such as various protein kinases, receptor proteins, enzymes such as nitrate reductase or proteins involved in transcriptional control of gene expression (reviewed e.g. by Yaffe, 2002; Ferl, 2004). It would be interesting to investigate the effect of citrate on the H^+-ATPase in respect of this control mechanism.

Apart from the regulatory mechanism itself, citrate exudation might prevent a reduced H^+-ATPase activity caused by citrate accumulation in the cytosol. This citrate exudation across the plasma membrane might be an alternative reaction due to an impaired vacuolar citrate compartimentation as a result of P deficiency. The energy-consuming process of citrate accumulation inside the vacuoles might already be impaired when plasma membrane transport is still possible, or the vacuoles might be too leaky to allow the high citrate accumulation necessary to prevent cytosolic accumulation.

Citrate transport across the plasma membrane

A direct way to determine citrate transport across membranes is by incubating tight vesicles derived from P-deficient roots and from +P control roots with radioactively labelled ^{14}C-citrate. Vesicles derived from P-deficient lupin roots showed a 2.2-fold increase in citrate transport in comparison to the +P control when Mg-ATP was supplied to support H^+-ATPase activity, together with a three-fold increase in vesicle acidification (Tab. 19). This hints to an induced

citrate transport across the plasma membrane of P-deficient cluster roots in connection with a higher activity of the PM H^+-ATPase. However, citrate transport already seems to occur in P-sufficient plant roots, although on a lower level. Higher citrate transport rates might have occurred if only mature cluster roots were used for the investigation instead of the whole root system. The whole root system was used to get enough plant material to make the experiments possible.

Protoplast isolation from mature cluster root laterals

To characterize citrate transport across the putative citrate channel in the plasma membrane of mature clusters of P-deficient white lupin plants, patch-clamp studies were to be performed with protoplasts isolated from mature cluster roots.

By a combination of several protoplast isolation protocols (Lin, 1980; Xu et al., 1981; Power and Chapman, 1985; Schachtman et al., 1991; Blackhall et al., 1994; Sevón et al., 1995; Spangenberg and Potrykus, 1995; Gleddie, 1995, and Steinecke and Schreier, 1995), protoplast preparations were gained after 5 h of cell wall digestion to up to 100,000 protoplasts g^{-1} root biomass with a vitality of 70 %. Similar yields were gained by Sinha et al. (2003) from white lupin, too, although from primary roots of 14-d-old *in-vitro* seedlings grown under full-nutrient conditions. Protoplasts from such plants are much easier to isolate. However, protoplast yields from the mature cluster roots of P-deficient plants were often much lower, and vitality decreased sharply within hours. Membrane intactness was very critical due to oxidative damage. The brown colour of the incubation medium was probably due to phenols and their oxidation. Even the change of the incubation medium after half of the digestion time and addition of high amounts of antioxidants and phenol-binding substances did not prevent protoplast damage. Only relatively short digestion times did bring protoplast yield at all. Incubation in enzyme solution for more than 8 h decreased protoplast yield and viability sharply. However, protoplast yield to amounts usually described in literature could be gained when white lupin cotyledons were used. This shows that the origin of the tissue the protoplasts were to be isolated from was the problem and rather not the isolation method *per se*.

Patch-clamp studies of the protoplasts derived from mature clusters according to this protocol were not possible. The seals between the membrane and the pipette were not tight enough to make measurements possible (Yan, pers. comm.). More vital protoplasts from root hairs, isolated by a special short time isolation procedure (~ 30 min) (Cocking, 1985; Gassmann and Schroeder, 1994), could only be gained from seedling root hairs, but not from mature or even young cluster

root hairs. This is another hint that cluster root development differs strongly from the development of tissue without P deficiency, even at the same time after emergence. However, Zhang et al. (2004) were able to isolate epidermal protoplasts from mature clusters in a similar short-time digestion procedure and identified a citrate-permeable anion channel by patch-clamping. Citrate efflux was stimulated by hyperpolarization of the plasma membrane. Interestingly, they also found citrate transport mechanisms in roots of P-sufficient plants, although to a lower extent, similar to the findings in the citrate transport experiments with inside-out vesicles in the present work. Zhang et al. (2004) even described citrate transport inhibition by the anion channel antagonist anthracene-9-carboxylic acid, which is in accordance with the reports of Neumann et al. (1999) for intact plants.

Al-induced exudation of malate in wheat and of citrate in maize via anion channels has also been recently demonstrated by patch-clamp studies (Kollmeier et al., 2001; Piñeros and Kochian, 2001; Zhang et al., 2001). Additionally, a wheat gene encoding an aluminium-activated malate transporter was found and cloned (Sasaki et al., 2004).

Regulation of the citrate release mechanism in cluster roots of P-deficient white lupin

Results so far support the hypothesis that in mature cluster roots of P-deficient white lupin citrate is exuded transiently across the plasma membrane via an anion channel, together with an increased proton transport due to an increased H^+-ATPase activity to maintain the electrochemical gradient of the plasma membrane. However, the nature of the signal which triggers the citrate exudation pulse is still unknown. The high citrate concentrations of ~ 30 μmol citrate g^{-1} root biomass observed before the citrate exudation pulse occurs suggests a link between both events. This link is supported by the finding that an artificial increase in citrate concentrations already in young cluster roots or seedling roots tips by application of the aconitase inhibitor monofluoroacetate induces citrate exudation rates in these young root tissues comparable to those of mature cluster roots. Anion channels can be modulated by carboxylate anions themselves, as demonstrated for vacuolar anion channels of CAM plants (Cerana et al., 1995) and for stomatal guard cells (Hedrich and Marten, 1993). Possibly a connection exists between citrate exudation and P deficiency-induced changes in cytosolic pH.

The accumulation of high amounts of carboxylates in cluster roots of P-deficient white lupin due to a higher production rate should decrease cytosolic pH by the pH stat mechanism as described by Sakano (1998). It is not the production of carboxylates *per se* that leads to the accumulation

of protons and therefore a lower pH. The explanation is found in metabolic steps in glycolysis, where a hydrogen atom bound undissociable in a hydroxyl or aldehyde group in a molecule, or as H_2O, is dissociable after the reaction and therefore reacts as a 'pH-active' proton. Examples are the reactions of hexokinase, phosphofructokinase and glycerinaldehyde-P-dehydrogenase. Summarized, the production of carboxylates in mature cluster roots in combination with a lower respiration (Neumann et al., 1999; Massonneau et al., 2001) may acidify the cell, which might be the sources of protons which acidify the rhizosphere.

The concept of the pH stat in plants was only recently widely recognized. Interestingly, in humans, intense exercise leading to the production of lactate is generally still believed to contribute to the muscular acidosis observed. However, by a similar way as in plants, the protons are produced in glycolysis and not by the production of lactate (Robergs und Amann, 2003).

However, cytosolic pH must stay stable for metabolic reasons. High citrate concentrations in plants might interfere with cytosolic Ca- and Mg-concentrations and metabolic regulation. Therefore the concominant occurence of citrate accumulation and cytosolic acidification might serve as a signal to release citrate via anion channels.

In order to examine whether cytosolic acidification is a signal to release carboxylates, the cytosol of cells of different root segments were acidified artificially. For this, roots were incubated with a solution containing weak organic acids such as propionic acid, acetic acid and methyl trichloroacetic acids at a pH of 4.0. These substances can cross the plasma membrane easily in their protonated form due to the low external pH and deprotonate when taken up into the cytosol with its much higher pH, and therewith lowering the cytosolic pH. After one hour of incubation, malate and citrate exudation increased dramatically in root tips and young and immature cluster roots, which usually do not release high amounts of these acids (Fig. 34). Acetic and methyl trichloroacetic acid had similar effects (data not shown). It was ruled out that the exudation was triggered just by the low external pH by applying a buffer solution with MES at the pH 4.0. Here the exudation rates did not change compared to the control solution at pH 7.0. Interestingly, Zhang et al. (2004) reported the opening of a citrate channel in white lupin cluster root protoplasts by hyperpolarization of the plasma membrane.

No effect on malate and citrate release rates by cytosolic acidification was seen in mature and senescent cluster roots. There the cytosolic pH should already be low due to the citrate accumulation. Moreover, malate and citrate exudation rates were already very high in mature clusters and could not be increased further. In senescent cluster roots carboxylate exudation obviously is no longer regulated by a decreased cytosolic pH, since exudation rates stayed low, with and without artificial cytosolic acidification. The fact that root tips and young and immature clusters can release high amounts of carboxylates might be explained by channels that already

exist in the membranes of even very young tissue and are responsible for release of carboxylates. Perhaps it is the opening of the channels that determines carboxylate release, as also suggested by Delhaize et al. (2001). The short reaction time of one hour shows that production of the channels were not newly initiated in this young tissue. The channels already existed and were sensitive to a changing cytosolic pH. To confirm that the exudation was really a cytosolic pH effect, cytosolic acidification was prevented by either pre-incubating the roots for 24 h with phosphonate or resupply of phosphate to re-establish P-sufficient conditions. Here incubation with propionic acid did not trigger carboxylate exudation (data not shown).

High citrate concentrations could conceivably alter the osmotic balance and metal-ion homeostasis of the cell due to its metal-complexing features (Gardner and Fridovich, 1991). Therefore it can be expected that the concentrations of carboxylates in the cytosol must be closely controlled in order to conform to the kinetic and inhibitory requirements of the enzymes involved in cellular metabolism (Jones, 1998). Under these aspects, citrate accumulation can be seen as a metabolic disorder the plant has to cope with, either by compartmentation, export, or exudation. White lupin has the advantage that it can release the citrate accumulating to high amounts in a kind of exudation burst from the special structures of cluster roots, which, in this combination, has the positive side effect of efficient P mobilization (Neumann and Martinoia, 2002). Release of carboxylates due to P deficiency also occurs in other plant species. However, the spectrum of carboxylates released depends on the plant species (Gerke, 1995; Gerke et al., 2000a). Moreover, the release rates in non-cluster root systems are much smaller and P mobilization is much lower as seen in species producing cluster roots.

As a summary of the findings so far, a possible reaction scheme for citrate-permeable anion channels in cluster roots of P-deficient white lupin plants will be presented:

Cytosolic pH can be lowered by P deficiency or MFA-induced citrate accumulation, similar to the external application of weak organic acids (e.g. propionic acid). The P deficiency-induced biosynthesis of carboxylates via higher glycolysis results in a higher production of protons by the pH stat mechanism. Inhibited citrate turnover further supports proton accumulation (Sakano, 1998). The PM H^+-ATPase, showing a lower pH optimum under P deficiency, is stimulated by the reduced cytosolic pH, and hyperpolarizes the plasma membrane, which activates the opening of the anion channel. Citrate release depolarizes the plasma membrane, and the anion channels close again. Due to the severe P deficiency in senescent cluster roots, H^+-ATPase activity is finally limited, hyperpolarization of the plasma membrane ceases and with this citrate exudation is stopped. A similar relation between carboxylate exudation and cytosolic acidification was described for maize root tips under anaerobiosis, leading to accumulation and exudation of

lactate (Xia and Roberts, 1994). The induction of PM H^+-ATPase activity and a hyperpolarized plasma membrane is also discussed for the Al-induced malate exudation from apical root zones of Al-tolerant wheat varieties (Ahn et al., 2004). To test this hypothesis, it would be necessary to investigate the subcellular distribution of citrate concentrations, pH values, and membrane potentials.

Conclusions

The results presented here give hints to a H^+-ATPase-coupled citrate transport via an anion channel, which is preferentially expressed in citrate-releasing mature cluster roots. Since unphysiologically high citrate concentrations inhibit the H^+-ATPase, a tight regulation between citrate concentrations and H^+-ATPase activity can be postulated.

Citrate exudation, induced by MFA or weak organic acids, shows that exudation is not specific for citrate itself. Other carboxylates such as malate or fumarate are also exuded to a higher amount. In mature clusters citrate accumulation and therefore citrate exudation predominates over all other carboxylates. Induced carboxylate exudation from young cluster roots or even P-sufficient seedling root tips confirms the observation that citrate channels were already found in +P control plants (Zhang et al., 2004).

Carboxylate channels which exist already in seedling root tips explain why in investigations of differential gene expression no hints to citrate channels in cluster roots of P-deficient plants were found. Recently, near-isogenic wheat lines differing in Al tolerance at a single locus (*Alt1*), showed differences in malate exudation under Al stress. This means that the *Alt1* codes for an anion channel or for channel-regulating properties (Ahn et al., 2004). If the *Alt1* codes for a carboxylate channel, its sequence information might be helpful to identify and characterize carboxylate channels also in cluster roots of white lupin on a molecular level. More precise information about the regulation of the H^+-ATPase on the several levels possible (transcriptional, translational and post-translational, such as the regulation by phosphorylation and 14-3-3 proteins, or regulation via phytohormones) and its relation with carboxylates needs further investigation.

Chapter 3: Role of modifications of atmospheric CO_2 concentrations on root exudation and rhizosphere processes in cluster roots

Introduction

Root-induced P mobilization by cluster roots of P-deficient white lupin so far was mostly investigated under P deficiency under otherwise optimal growth conditions. Additional stress factors such as heat, drought, Al-toxicity, pH values of the soil, or nitrogen or micronutrient deficiencies were not considered. However, under natural growth conditions additional stress factors are often combined with P deficiency and modify the plant's reaction to P deficiency. Additionally, plant-physiological investigations are mostly done in nutrient solution.
The aim of the present study was to investigate elevated atmospheric CO_2 concentrations as one example of modified growth conditions on mechanisms of P mobilization in P-deficient white lupin.

Atmospheric CO_2 concentrations have increased from 280 μmol mol^{-1} in the pre-industrial age (mid 18th century) to up to 373 μmol mol^{-1} in the year 2002 (Keeling and Whorf, 2003), mainly due to combustion of fossil fuels, and deforestation (IPCC, 2001). In the last 40 years, CO_2 concentrations increased by 17 %. When the data so far are extrapolated, CO_2 concentrations will double till the end of this century compared with the pre-industrial value (IPCC, 2001).
Independent of a higher global temperature, this increase in CO_2 concentration will influence plant growth and the interaction of plants with nutrients and soils. Most experiments conducted with elevated CO_2 concentrations showed a higher biomass gain when the plants were growing at sufficient nutrient supply (Hodge and Millard, 1998). However, no increase in biomass occurred when nutrients were limiting, as described for Sitka spruce (Murray et al., 2000), for tropical tree species (Winter et al., 2001), for soybean (Sa and Israel, 1998), wheat (McKee and Woodward, 1994), clover (Duchein et al., 1993), cork oak (Maroco et al., 2002), pine (Conroy et al., 1988, 1990b), *Poa alpina* (Baxter et al., 1997), grassland communities (Stöcklin et al., 1998; Stöcklin and Körner, 1999), or lucerne, faba bean, perennial ryegrass, wheat, maize, poplar and tomato (Goudriaan and de Ruiter, 1983). However, some species show an increase in biomass even under limiting nutrient supply, e.g. *Quercus alba* (Norby et al., 1986), N_2-fixing tree species (Norby, 1987; Norby and O'Neill, 1989) or *Eucalyptus* (Conroy et al., 1992).

Similarly, the data of effects of CO_2 concentrations on root/shoot (R/S) ratios and therefore the allocation of carbohydrates between shoot and root are confusing and ambiguous. The compilation of the published data, however, suggests that, on average, R/S changes little at elevated CO_2 concentrations (Norby, 1994; Rogers, H. et al., 1996), although differences between plant groups were described. A higher root/shoot ratio was documented for herbaceous plants, a decrease for trees, and no change for cereals (Farrar and Williams, 1991). It was suggested that the sensitivity of resource allocation is due to factors other than CO_2 concentration, e.g. nutrient supply (Salsman et al., 1999), or due to the plant species investigated, and dependent on whether a plant is annual or perennial (Yoder et al., 2000). Often it is not clear if the increased root/shoot ratio is an effect of an elevated CO_2 concentration or indirectly induced by nutrient deficiencies.

The higher biomass production observed at elevated CO_2 concentrations is deducible to a higher assimilation rate (Eamus, 2000). Photosynthetic CO_2 fixation is supported by higher CO_2 concentrations since RUBISCO is not CO_2-saturated at ambient CO_2 concentrations, and photorespiration is reduced due to the higher CO_2 to O_2 ratio at elevated CO_2 concentrations (Sage et al., 1989; Betsche, 1994; Eamus, 2000).

In a survey of 60 experiments, growth at elevated CO_2 concentrations increased photosynthesis by 58 %. The frequently observed acclimation of photosynthesis to elevated CO_2, which is characterized by higher carbohydrate concentrations, lower concentrations of soluble proteins and RUBISCO, and an inhibition of photosynthetic capacity, cannot completely compensate for the stimulation of the assimilation rate by high CO_2 concentrations (Drake et al., 1997). However, a restriced rooting volume and therefore an artificially restricted sink strength might be responsible for some of the acclimation effects described (Arp, 1991; Barrett and Gifford, 1995), if not for most of them (Eamus, 2000). However, a downregulation of photosynthesis was also described when the rooting volume was not restricted (Socias et al., 1993; Drake et al., 1996; Bernacchi et al., 2003).

The phenomenon of the ‘locally missing carbon’, in which leaf photosynthetic rate is much higher than the biomass gain, was hypothesized to be explainable by root turnover, root respiration, or exudation (Cheng and Johnson, 1998). In a mixed grass experiment at elevated CO_2 concentration, total root rhizosphere deposition was increased by 56 % and root biomass by less than 25 % (Hungate et al., 1997). An increased allocation of ^{14}C-labelled photosynthate (Hodge and Millard, 1998) and increased carbohydrate concentrations (Norby, 1994) have been found in roots at elevated CO_2. This increased input of C to roots could stimulate higher rates of exudation of soluble organic compounds (Norby, 1994), and increase nutrient availability.

However, no general trend for exudation rates at elevated CO_2 concentrations were found, and exudation rates depend on plant species and experimental conditions. In some experiments higher root exudation rates were found (Cheng and Johnson, 1998), and a 60 % increase in soluble C at elevated CO_2 concentration was explained by a higher root exudation (Cheng et al., 1993). Sometimes the increase was only marginal (Norby et al., 1987), sometimes exudation rates were unchanged (Uselman et al., 2000), and even lower exudation rates were described as for *Lolium perenne* (Hodge et al., 1998). The nature of the exudates, however, may be more important for ecosystem function than their overall quantity (Cardon, 1996), which e.g. may be viable for carboxylates which mobilize sparingly soluble phosphates, or plant-derived phosphatases. An important aspect is the question whether elevated CO_2 concentrations increase the concentrations of nutrient-mobilizing root exudates in the rhizosphere or whether the effect of higher exudation rates are the result of a bigger root system with unchanged exudation rates per root mass or root length, and therefore unchanged rhizosphere concentrations.

The question arises how plants with very specialized strategies as reaction to nutrient deficiencies can cope with an elevated CO_2 concentration. White lupin was chosen as a model plant because under P deficiency it produces cluster roots with an exceptionally high ability for P mobilization by exuding organic metal chelators (carboxylates, phenolics), protons and phosphatases. The question was how a plant with such a specialized adaptation to P-deficient conditions reacts to elevated CO_2 concentrations with respect to formation and function of cluster roots, expression of chemical changes in the rhizosphere and P acquisition from sparingly soluble P sources.

Experiments were performed in a collaboration with the Institute of Soil Science and Land Evaluation, Section Soil Biology (A. Rothe, J. Wasaki, and E. Kandeler) aiming to characterize related modifications of rhizosphere-microbial community structures.

Materials and methods

Plant cultivation and harvest

White lupin seedlings (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzucht, 76437 Rastatt, Germany) were desinfected with 30 % H_2O_2 for 15 min, rinsed with tap water, soaked in 10 m*M* $CaSO_4$ for 4 h and pre-germinated in wet filter paper containing 2.5 m*M* $CaSO_4$ for 4 d in the dark at 25°C. After emerging of the seedlings, the plants were illuminated in a growth chamber for another two days before they were transferred to nutrient solution or rhizoboxes.
Growth chamber conditions were adjusted to a 16/8 h day/night cycle with a light intensity of 300 μmol m^{-2} s^{-1} and a constant temperature of 25°C at day and 20°C at night with a relative humidity of 60 %. When the plants were transplanted either into nutrient solution or into rhizoboxes, they were further cultivated in two different growth chambers with either 400 μmol mol^{-1} (ambient) or 800 μmol mol^{-1} (elevated) atmospheric CO_2 concentrations but otherwise the same growth conditions as described above.

For experiments in nutrient solution, 10 seedlings were cultivated in a 2.5-L aerated pot containing 2 m*M* $Ca(NO_3)_2$; 0.7 m*M* K_2SO_4; 0.1 m*M* KCl; 0.5 m*M* $MgSO_4$; 30 μM Fe-EDTA; 10 μM H_3BO_3; 0.5 μM $MnSO_4$; 0.5 μM $ZnSO_4$; 0.2 μM $CuSO_4$; 0.01 μM $(NH_4)_6Mo_7O_{24}$, with addition of 2.5 m*M* $CaSO_4$ per pot in solid form to prevent Ca deficiency due to high transpiration rates of the plants. For +P control plants 250 μM KH_2PO_4 were added. P-deficient plants were cultivated without any additional P source in the nutrient solution.

For experiments in rhizoboxes, two seedlings each were transplanted into a rhizobox. The rhizoboxes were prepared as follows: Air-dried soil (C-horizon of a luvisol, a calcareous sub-soil; Wippenhausen, Weihenstephan, Germany; $CaCO_3$-content 21.5 %; pH 7.5; P_2O_5 content (CAL): < 10 mg kg^{-1} soil; K_2O content < 40 mg kg^{-1} soil, and a low-P loamy soil) were sieved to a grain size of 2 mm. A mixture of 90 % of the loess soil and 10 % of the loamy soil were mixed, 250 g of the dry mass of the mixture were fertilized with 100 mg N kg^{-1} soil as $Ca(NO_3)_2$; 150 mg K kg^{-1} as K_2SO_4; 50 mg Mg kg^{-1} as $MgSO_4$ and 20 μmol Fe kg^{-1} as Fe-EDTA. For –P treatments no P was added, whereas +P controls were additionally fertilized with 80 mg P kg^{-1} as $Ca(H_2PO_4)_2$. The soil mixture was watered to 8 % water content and filled into one rhizobox. For this, a wet fleece was layered on the back wall of the rhizobox and the fertilized soil was filled in and distributed evenly. The seedlings were planted into the rhizobox, a plastic foil layered on the

soil and the cover plate tightened. The foil between the soil and the cover plate prevents the roots to stick to the cover plate. 30 mL of water were added into the holes in the back of each rhizobox. With this, soil water content was adjusted to 20 %. The rhizoboxes were weighted and watered each day to this weight.

Root exudate collection and determination of carboxylates

Localized root exudate collection with use of filter papers, determination of the numbers of cluster roots and harvest of the plants grown in nutrient solution was performed at 21, 24, 27, 30, 33, and 36 days after sowing, each time starting 3 h after beginning of the day cycle to prevent the influence of possible diurnal rhythms. The high number of six harvests was used to cover a dense time course of the metabolic and growth changes induced by the two CO_2 concentrations. The original idea to collect root exudates and to harvest plants each at the same physiological age under both CO_2 concentrations turned out to be not viable. Plant habitus and morphology changed within hours and in different fastness at the different CO_2 concentrations. It became apparent that it needs direct comparison between plants to determine the same physiological age. However, in the present study, a time span of several days occurred between the same physiological age.

Localized root exudate collection from plants in rhizoboxes with use of filter papers and harvest of the respective cluster roots was performed at the days 15, 20, 27, and 35 after sowing, and the whole plants were finally harvested at day 35.

Carboxylate determination was performed as described in “General methods”.

Plants cultivated in nutrient solution

Determination of ortho-phosphate (P_i) in root tissue and shoot tips

Inorganic ortho-phosphate (P_i) was determined in the cluster roots and in shoot tips according to the method of Bollons and Barraclough (1997) to reveal the availability of soluble, “metabolic” P_i, easily to be used by the plants, and being under metabolic turnover. The frozen plant material was ground in liquid N_2 with a mortar and a pestle, 50 mg of the ground material was extracted with 1 mL of an ice-cold 2 % acetic acid solution and stored on ice until all the samples were

extracted. After centrifugation at 10,000 *g* for 10 min at 4°C, the supernatant was used for P_i determination according to the molybdate method of Murphy and Riley (1962). For this, 1 mL of the supernatant or 400 µL of the supernatant + 600 µL of 2 % acetic acid solution was added to 1 mL of the mixed reagent (Table 20), the optical density was measured at 720 nm in a spectrophotometer after 20 min of colour development and absorptions were compared with that of known P_i standards (up to 50 *µM* P_i).

Table 20: Preparation of the mixed reagent for P_i determination

sulphuric acid 5 *N*	dilute 70 mL of concentrated sulphuric acid to 500 mL
ascorbic acid (0.1 *M*)	dissolve 1.32 g of ascorbic acid in 75 mL of water; prepare fresh each day.
potassium antimonyl tartrate (1 mg Sb mL^{-1})	dissolve 0.2743 g of potassium antimonyl tartrate in distilled water and dilute to 100 mL
mixed reagent	mix thoroughly 125 mL of 5 *N* sulphuric acid and 37.5 mL of ammonium molybdate; add 75 mL of ascorbic acid solution and 12.5 mL of potassium antimonyl tartrate solution; this reagent should be prepared as required as it is not stable for more than 24 h

Plants cultivated in rhizoboxes

Enzymatic citrate determination

Citrate determination was done with an enzymatic test kit (r-biopharm, Darmstadt, Germany - Boehringer Mannheim, Cat. No. 139 076) adjusted for white lupin root material.

Citrate concentrations were determined from root segment material. For extraction, 5 mL of ice-cold 5 % H_3PO_4 were added per 1 g root fresh weight and homogenized on ice with a mortar and a pestle. The homogenate was transferred into an Eppendorf vial and a spatula tip of activated charcoal was added. The mixture was vortexed and stored on ice while the other root samples were extracted. The mixture was centrifuged at 20,000 *g* for 15 min at 4°C. The supernatant was transferred into a new Eppendorf vial and neutralized with 5 *M* KOH to a neutral pH. The volumes of the supernatant and of the KOH added were noted. The neutralized solution was centrifuged again and the new supernatant was used for citrate determination according to the test protocol supplied with the test kit.

Determination of total phosphate in root and shoot tissue

Root and shoot dry material was analyzed for its total P concentration according to Gericke and Kurmies (1952). For this, 200 mg of the dry material was filled into a porcelain dish and ashed at 500°C for 4 h in a muffle furnace. After cooling down, several drops of deionized water and then HNO_3 1:3 (v/v) were added, dried on a heating plate and again ashed at 500°C for another two hours. After cooling down, the samples were two times heated with 2 mL of HNO_3 1:3 (v/v) till dryness to precipitate SiO_2. The ash was then dissolved in 2 mL HCl 1:3 in the dish and transferred into a 20 mL flask, whereby the dish was washed with ~ 10 mL of hot water which was also filled into the flask. The solution was boiled for at least 2 min in the flask on a heating plate with addition of one boiling stone to transmute the meta- and pyrophosphates produced by the ashing and heating to dryness back to orthophosphate. Only orthophosphate reacts with the molybdate-vanadate solution to a yellow colour complex. After cooling down, the solution was filled up to 20 mL with H_2O and filtered with a blue-band filter. For spectrophotometric analysis, 1 mL of the filtered solution was added to 1.5 mL of the molybdate-vanadate reagent (Table 22) and 2.5 mL of HCl 1:30 to a total volume of 5 mL. The optical density was measured at 436 nm in a spectrophotometer after 20 hours of colour development and absorptions were compared with that of known P_i standards (up to 15 mg phosphate-P L^{-1}).

Table 21: Preparation of acids and molybdate-vanadate solution to determine phosphate-P.

1:3 HNO_3	dilute 1 part of 65 % HNO_3 with 2 parts of deionized water
1:3 HCl	dilute 1 part of 37 % HCl with 2 parts of deionized water
molybdate-vanadate solution	a) 1:3 HNO_3
	b) ammonium vanadate solution 0.25 % (w/v)
	c) ammonium molybdate solution 5 % (w/v)
	mix the solutions a – c in the relation of 1:1:1

Determination of acid and alkaline phosphatase in rhizosphere soil

Acid and alkaline phosphatase activity was determined by hydrolysis of the artificial substrate methylumbelliferyl (MUB)-phosphate yielding the fluorescent product methylumbelliferone. One to five g of soil were suspended in 100 mL of sterilized water and sonicated for 2 min with an energy of 50 J s^{-1}. 50 μL of the soil suspension were added to 50 μL of a 0.1 *M* MES buffer solution, pH 6.1 (for acid phosphatase) or 50 μL of a 0.1 *M* Trizma buffer solution, pH 7.6 (for alkaline phosphatase) and 100 μL of substrate (10 m*M* MUB-phosphate in the corresponding buffer) into a microplate. The plates were incubated at 30°C and fluorescence was registered with a microplate reader (Company Town State©) after 0, 30, 60, 120, and 180 min of incubation with an excitation wavelength of 360 nm and fluorescence reading at an emission wavelength of 460 nm. Standards were prepared in the same buffers and 50 % methanol with concentrations up to 1 m*M*.

Results

Plants grown in nutrient solution

Plant growth and development

The influence of an elevated atmospheric CO_2 concentration on plant growth and physiological parameters in P-deficient white lupin grown in hydroponic culture was measured each third day from day 21 to day 36 after sowing.

Plant development was generally accelerated at elevated CO_2 concentrations. No clear effects on shoot growth but earlier P deficiency-induced expression of senescence symptoms in older leaves could be seen. Root growth was clearly stimulated at elevated CO_2 (Fig. 36+37).

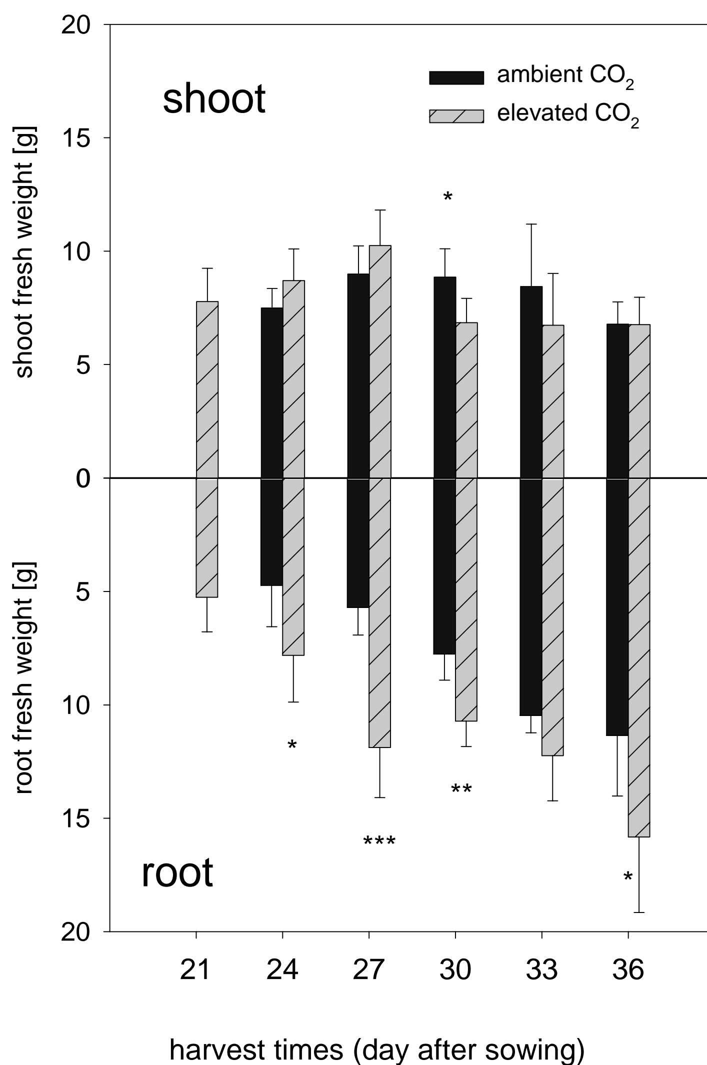


Fig. 36: Shoot and root fresh weight of P-deficient white lupin plants under ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing). *, **, and *** are significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

Fig. 37: Above: P-deficient white lupin plants cultivated at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations 26 days after sowing. Below: Shoots and roots of P-deficient white lupin plants cultivated at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations 26 days after sowing.

Root/shoot ratio increased during plant growth from day 24 to day 36 at ambient CO_2 concentrations while P deficiency became more severe (Fig. 38). At the elevated CO_2 concentration root/shoot ratio was even higher at each single harvest from day 24 to day 36, compared to the values at ambient CO_2 concentration, with an average increase by 58 %. Part of this increase in root/shoot ratio was due to earlier wilting and abscission of leaves from day 30 to 36, but can be predominantly attributed to stimulation of root growth.

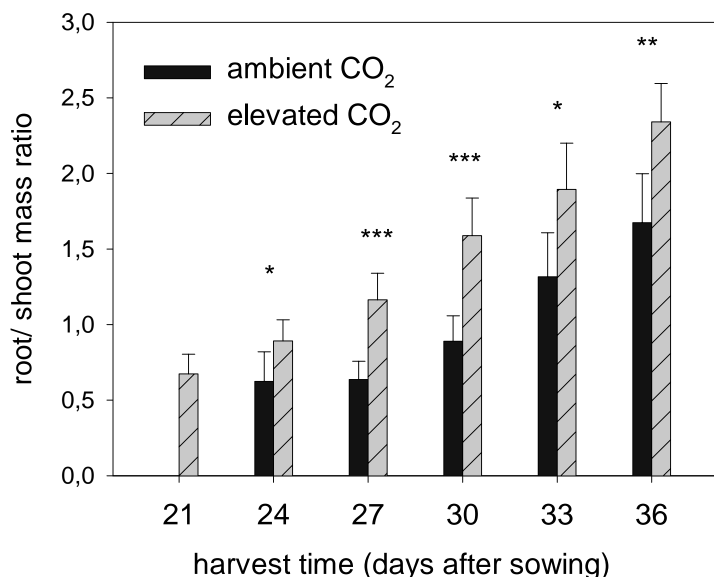


Fig. 38: Root/shoot mass ratio of P-deficient white lupin plants under ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing).
*, **, and *** are significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

Cluster root development

Cluster roots emerged earlier at elevated CO_2 concentrations (Fig. 39), leading to higher numbers of cluster roots between 21 and 30 days after sowing in plants grown at elevated CO_2. Thereafter, there was no difference between CO_2 treatments.

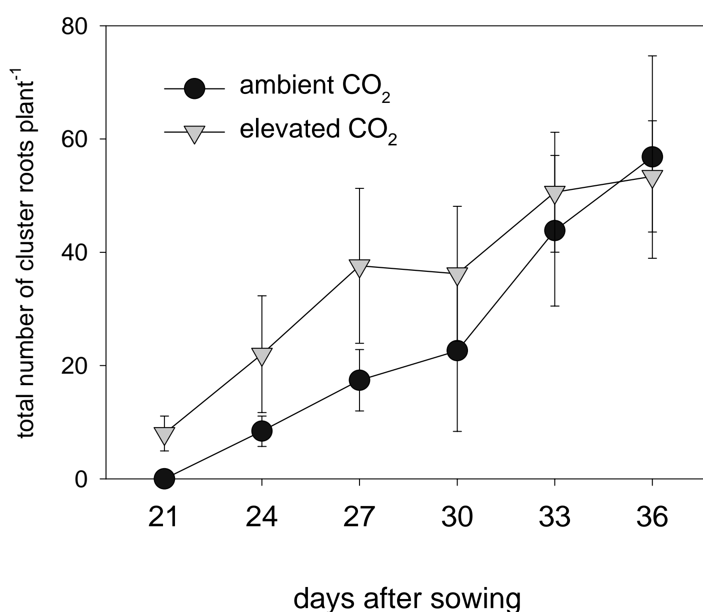


Fig. 39: Total number of cluster roots per plant of P-deficient white lupin plants at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing).

No significant differences between CO_2 treatments were observed for the proportion of cluster roots relative to the whole root system (Fig. 40), demonstrating that increased formation of

cluster roots at the elevated CO_2 concentration was a consequence of a general stimulation of root growth and not of an overproportional induction of cluster root formation.

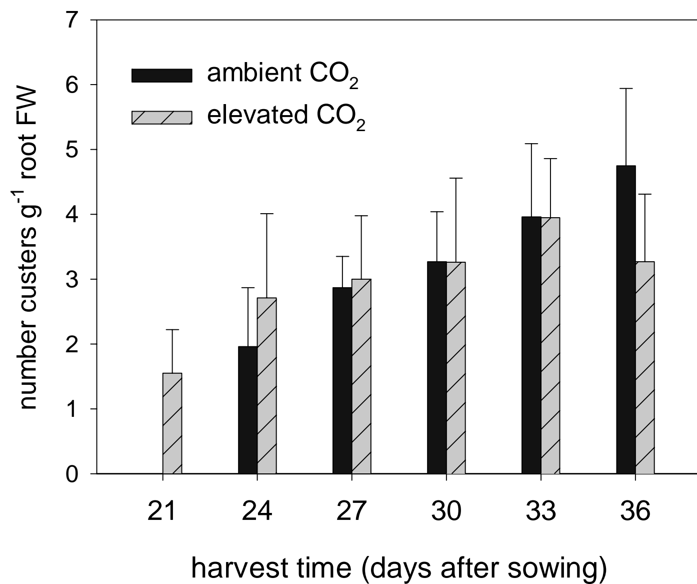


Fig. 40: total number of cluster roots per root fresh biomass of P-deficient white lupin plants at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing).

Accelerated cluster root development at elevated CO_2 concentrations was also reflected by differences in the proportion of cluster roots in the different developmental stages (Fig. 41). According to the total number of cluster roots between 21 and 30 days after sowing the proportion of young, mature and senescent clusters was always higher at the elevated CO_2 concentration, but thereafter the differences disappeared.

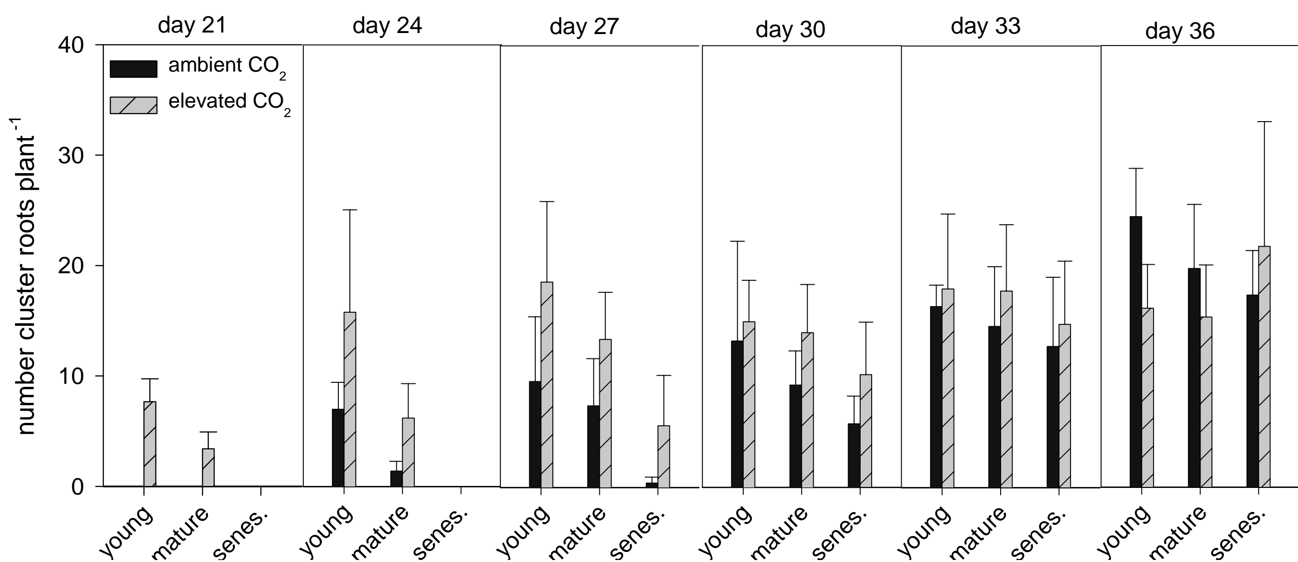


Fig. 41: Distribution of cluster roots of different developmental stages (young, mature, and senescent) at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing).

Cluster root function

According to earlier observations (Chapter 1), citrate and malate were the dominant carboxylates in root exudates collected from individual root clusters of white lupin. Exudation of malate declined with increasing age of the clusters while a peak of citrate exudation was observed in mature clusters. Based on root biomass, there were no significant differences in root exudation of carboxylates between CO_2 concentrations. However, carboxylate exudation rates showed a high variability. This is typical for exudate collections from individual clusters. The classification into three developmental stages according to morphological characteristics (see "General methods", p. 11), cannot account for gradual changes in cluster root activity within and between the different stages of development.

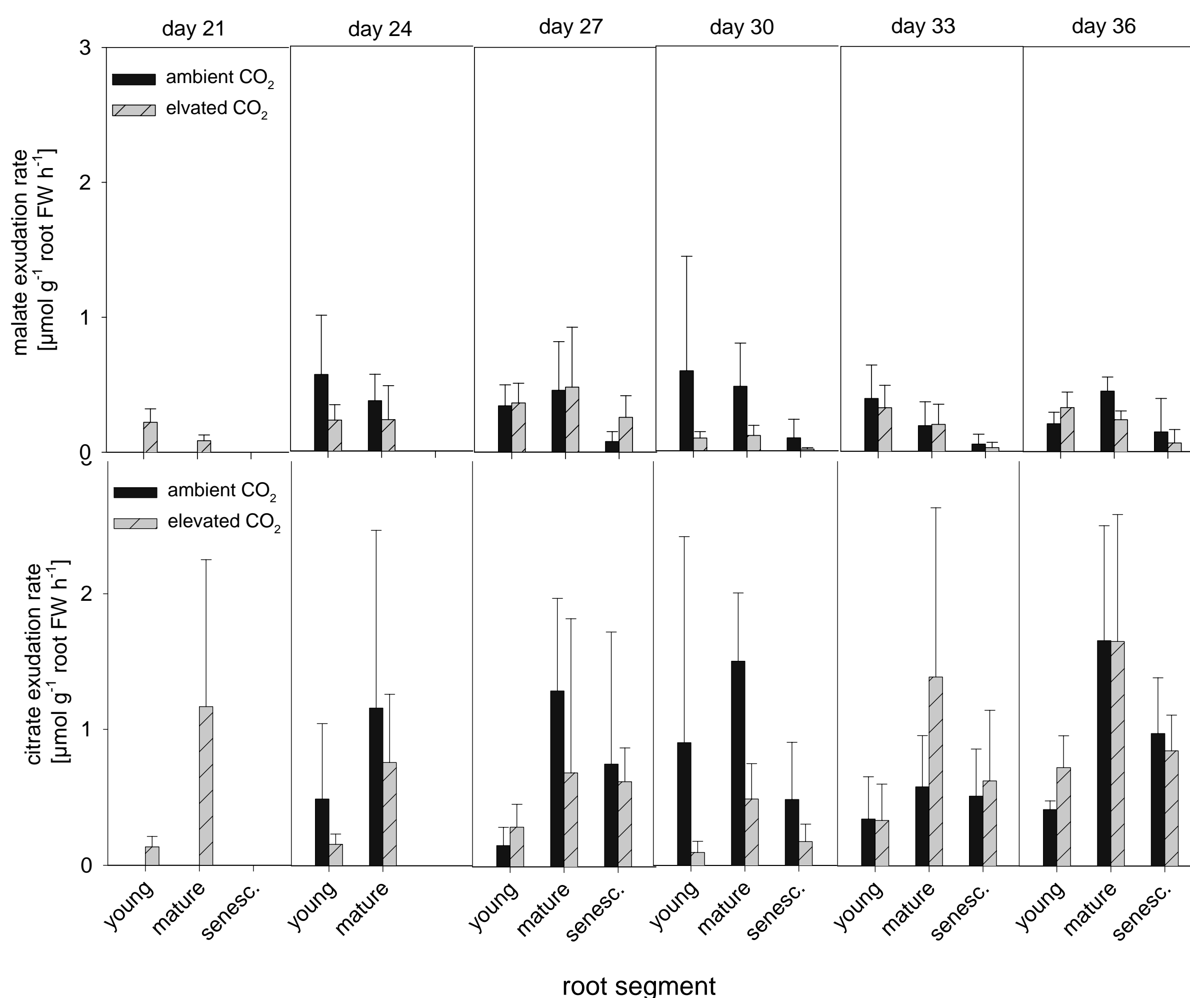


Fig. 42: Malate (upper row) and citrate exudation rates (lower row) in different white lupin cluster root segments (young, mature, and senescent, see also p. 11) at six different days after sowing at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations.

Accumulation and exudation of citrate in mature cluster roots seems to be related to a declining metabolic availability of P_i (Chapter 1). Since elevated CO_2 concentrations accelerated plant development and expression of P deficiency symptoms in white lupin, the effects of elevated CO_2 concentrations on P_i availability was determined in different developmental stages of cluster roots. According to earlier findings (Tab. 1), P_i concentrations strongly declined with increasing age of the root clusters but there were no clear differences between CO_2 concentrations (Fig. 43). This is in accordance with the observation that citrate exudation from individual root clusters was also not affected by elevated CO_2 concentrations.

During the whole growth period, high P_i concentrations were maintained in young, growing tissues with a high P_i demand, such as young cluster roots and apical buds of the shoot (Fig. 44), suggesting a high capacity for P_i retranslocation from older tissues.

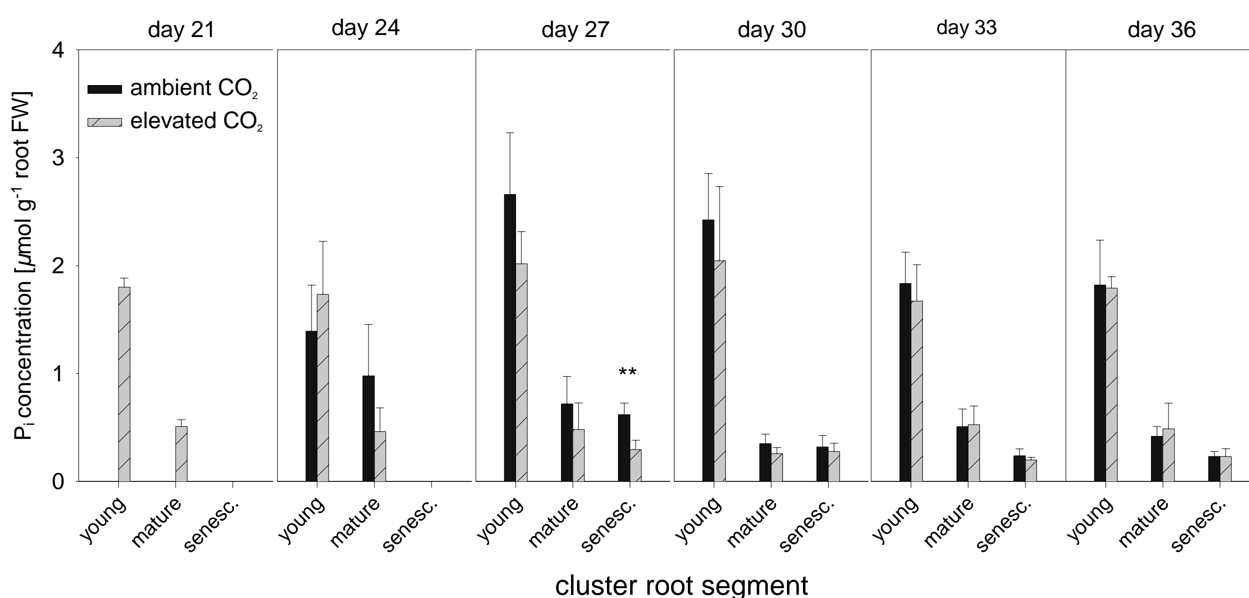


Fig. 43: P_i concentrations per root biomass in different cluster root segments (young, mature, and senescent, see also p. 11) at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing). **: significant at the 0.01 probability level.

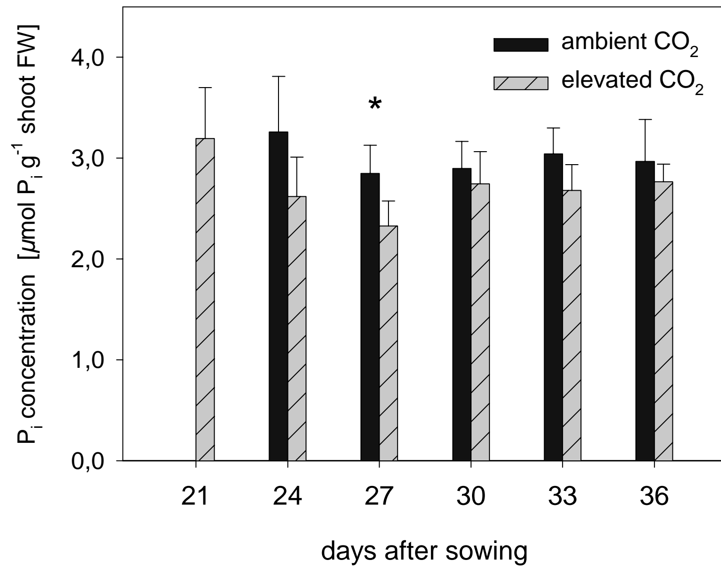


Fig. 44: P_i concentrations per shoot biomass in the shoot tip at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations at six different days of harvest (days after sowing). *: significant at the 0.05 probability level.

Plants grown in rhizoboxes

To investigate P deficiency responses of *Lupinus albus* in soil culture as affected by elevated atmospheric CO_2 concentrations, plants were grown in rhizoboxes in a calcareous Loess subsoil containing sparingly soluble Ca-phosphates as dominant P fraction, with and without additional P fertilization at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) CO_2 concentrations.
Plant growth and cluster root function and development was monitored over a period of 35 days with sequential exudate collections after 15, 20, 27, and 35 days after sowing. At each harvest date cluster roots in different developmental stages appearing at the soil surface of the rhizoboxes were selected for collection of root exudates and rhizosphere soil solution by use of chromatography paper (Dinkelaker et al., 1997; Engels et al., 2000). Rhizosphere soil was collected to determine phosphatase activities, and the respective clusters with adhering rhizosphere soil were excised for microbial diversity studies and rhizosphere phosphatase activity determinations in a cooperation project with the Institute of Soil Science and Land Evaluation, Section Soil Biology (A. Rothe, J. Wasaki, and E. Kandeler).

Plant growth and development

Comparing the 35 days old plants between the two P supplies, there were no significant differences in root and shoot biomass production at both CO_2 concentrations. However, at the elevated CO_2 concentration a slight but significant increase in shoot biomass was detectable at both P supplies (Fig. 45).

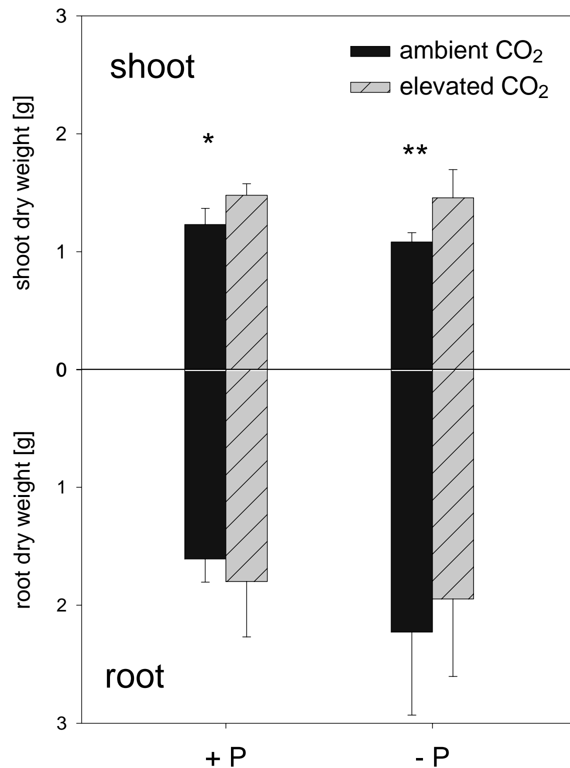


Fig. 45: Shoot and root dry weight of white lupin plants grown in rhizoboxes at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentration with sufficient (+P) and without (-P) external P supply, 35 days after sowing. *; **: significant at the 0.05 and 0.01 probability level, respectively (t-test) (data from J. Wasaki and A. Rothe).

Cluster root development

Cluster root formation was observed in the +P and the -P treatments. However, the total number of cluster roots for each harvest was significantly higher in -P treatments compared to P-sufficient control plants, independent of the CO_2 concentration (Tab. 22). In both P treatments, elevated atmospheric CO_2 concentrations tended to increase the number of cluster roots and accelerated cluster root development (higher proportion of older clusters) during 27 days after sowing for +P control plants and during 20 days after in the P-deficient plants (Fig. 46). Similar to the results obtained in hydroponic culture, the differences disappeared in later stages of plant development.

Tab. 22: Number of cluster roots of white lupin plants removed for further investigations at different harvest times (DAS: days after sowing). Plants were grown in rhizoboxes at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations grown with sufficient (+P) and without (-P) external P supply (data from J. Wasaki and A. Rothe).

harvest [DAS]	+P		-P	
	400 µmol mol^{-1} CO_2	800 µmol mol^{-1} CO_2	400 µmol mol^{-1} CO_2	800 µmol mol^{-1} CO_2
15	9	12	15	24
20	9	20	22	24
27	8	15	23	23
35	10	9	26	19
total	**36**	**56**	**86**	**90**
mean	**9.0**	**14**	**21.5**	**22.5**
SD	**0.8**	**4.7**	**4.7**	**2.4**

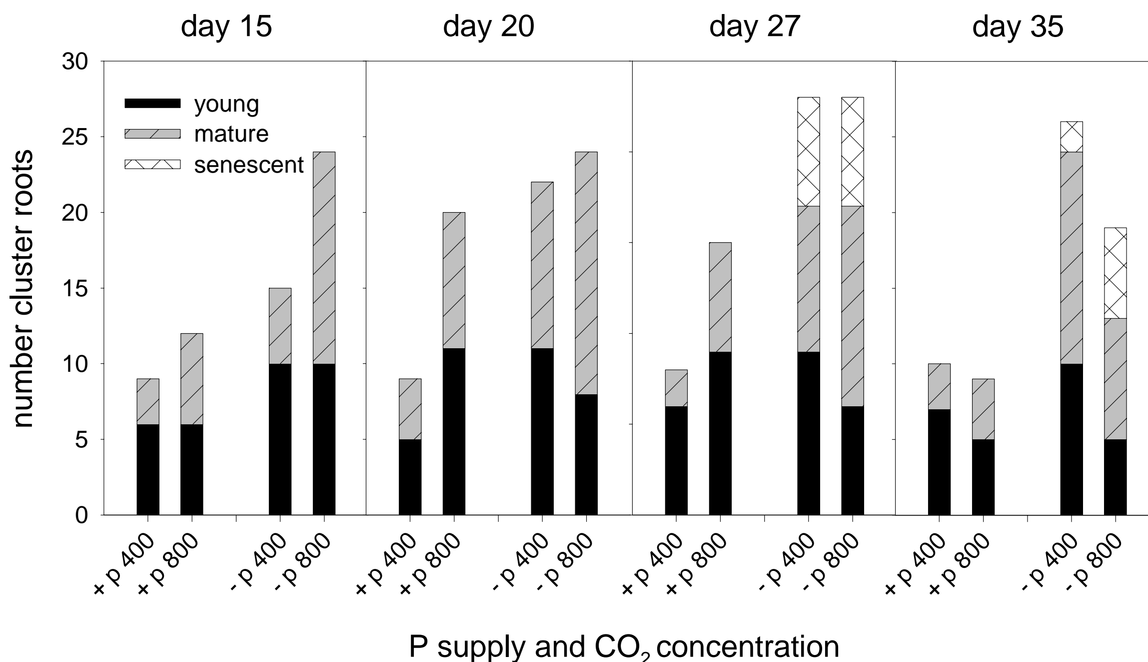


Fig. 46: Distribution of cluster roots at different developmental stages (four harvest dates) of white lupin plants grown in rhizoboxes, at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations grown with sufficient (+P) and without (-P) external P supply (data from J. Wasaki and A. Rothe).

Cluster root function

Citrate exudation

Rhizosphere soil solution containing root exudates was collected from different root zones of white lupin grown in rhizoboxes by use of filter papers with a high soaking capacity. Short-term collection (2 h) was performed to minimize microbial degradation of carboxylates and to recover a high proportion of root exudates (Neumann and Römheld, 2000). Citrate as the major carboxylate released under P-deficient conditions was analyzed by an enzymatic test.

Citrate was detected in all samples, but particularly high amounts were found in samples obtained from cluster roots and especially from mature and senescent ones (Fig. 47). Phosphorus deficiency significantly increased citrate exudation in mature and senescent clusters with a trend for increased exudation at elevated CO_2 concentrations although the differences compared with plants grown at ambient CO_2 were not significant.

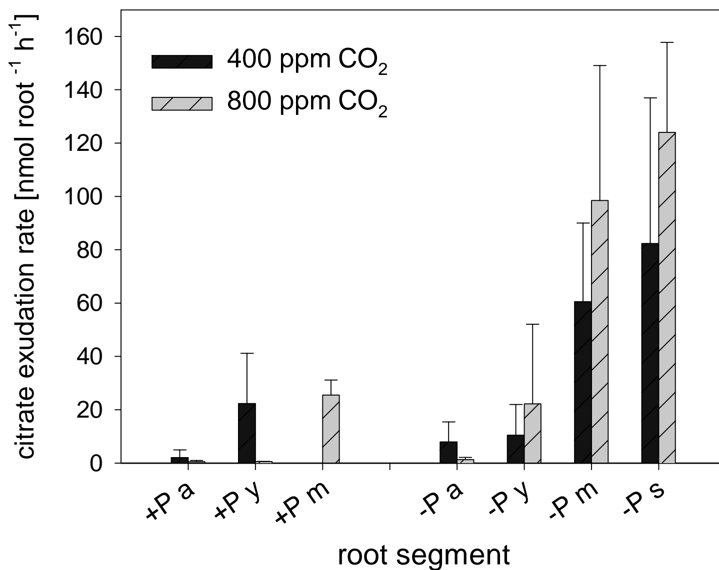


Fig. 47: Citrate exudation from root segments of P-sufficient (+P) and P-deficient (-P) plants (a: 10 mm apical root zone of lateral roots; y: young cluster roots; m: mature cluster roots; s: senescent cluster roots; see also p. 11) 35 days after sowing at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations (data from G. Neumann).

Phosphatase activity

Acid and alkaline phosphatases are enzymes released from P-deficient plant roots (acid phosphatases) and microorganisms (acid and alkaline phosphatases) and are involved in mineralization of organic P forms in soils. Root secretory acid phosphatases, detected in *Lupinus albus* and many other plant species under P-deficient conditions may contribute to some extent to acquisition and retrieval of organic P in the rhizosphere (Neumann and Römheld, 2000).

There was a very similar activity distribution pattern of acid and alkaline phosphatases in the rhizosphere soil obtained from different root segments of white lupin in rhizobox cultures, although activity of alkaline phosphatase was generally lower than that of acid phosphatase (Fig. 48). Phosphorus deficiency increased phosphatase activities in all root segments, but particularly in senescent cluster roots. There was a trend for increased phosphatase activities at elevated CO_2 concentrations although differences were in most cases not significant.

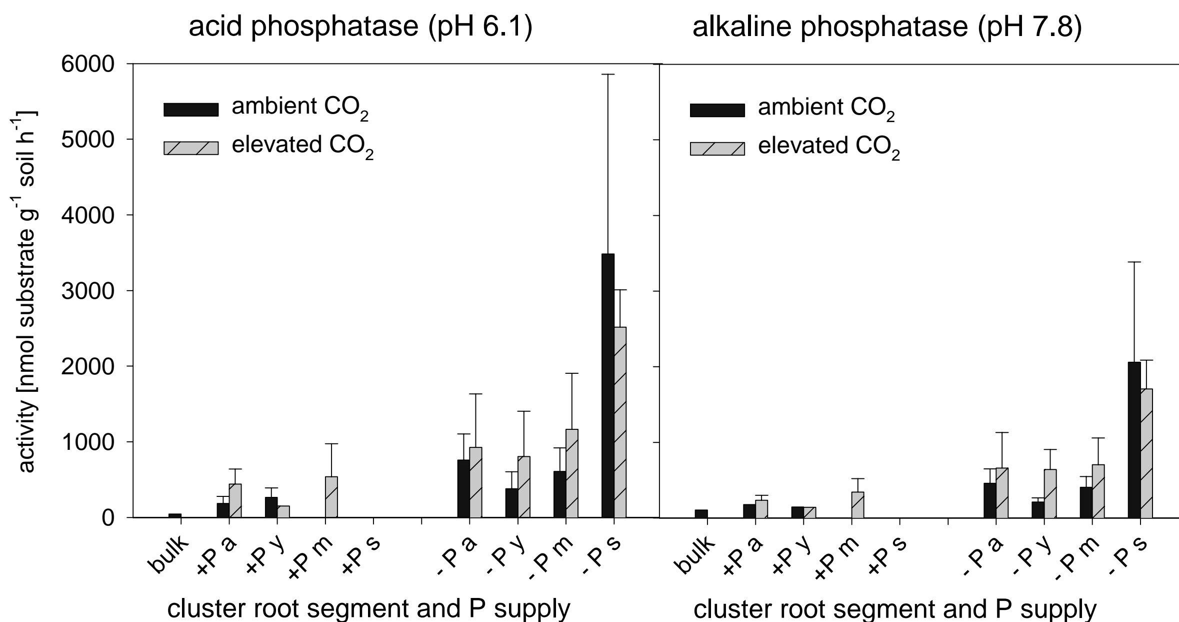


Fig. 48: Rhizosphere acid and alkaline phosphatase activity [nmol substrate turnover h^{-1} g^{-1} rhizosphere soil] of white lupin plants grown in rhizoboxes at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations grown with sufficient (+P) and without (-P) external P supply (a: 10 mm apical root zone of lateral roots; y: young cluster roots; m: mature cluster roots; s: senescent cluster roots; see also p. 11) 35 days after sowing (data from J. Wasaki and A. Rothe).

Phosphate concentrations and contents

Root and shoot total P concentrations were lower when the plants were grown without P addition to the soil (Fig. 49). However, there were no differences in root or shoot P concentrations between CO_2 concentrations. Comparison of shoot P concentrations revealed that in all treatments the plants suffered from P deficiency although this was more severely expressed in the -P treatments.

Total P content declined only in the shoot tissue of P-deficient plants but there were no differences in P root content between P treatments. CO_2 concentrations had no effect on P contents in white lupin grown with or without additional P supply. Independent of the CO_2 concentration, P contents were higher in the roots than in the shoots under sufficient P supply and under P-deficient conditions.

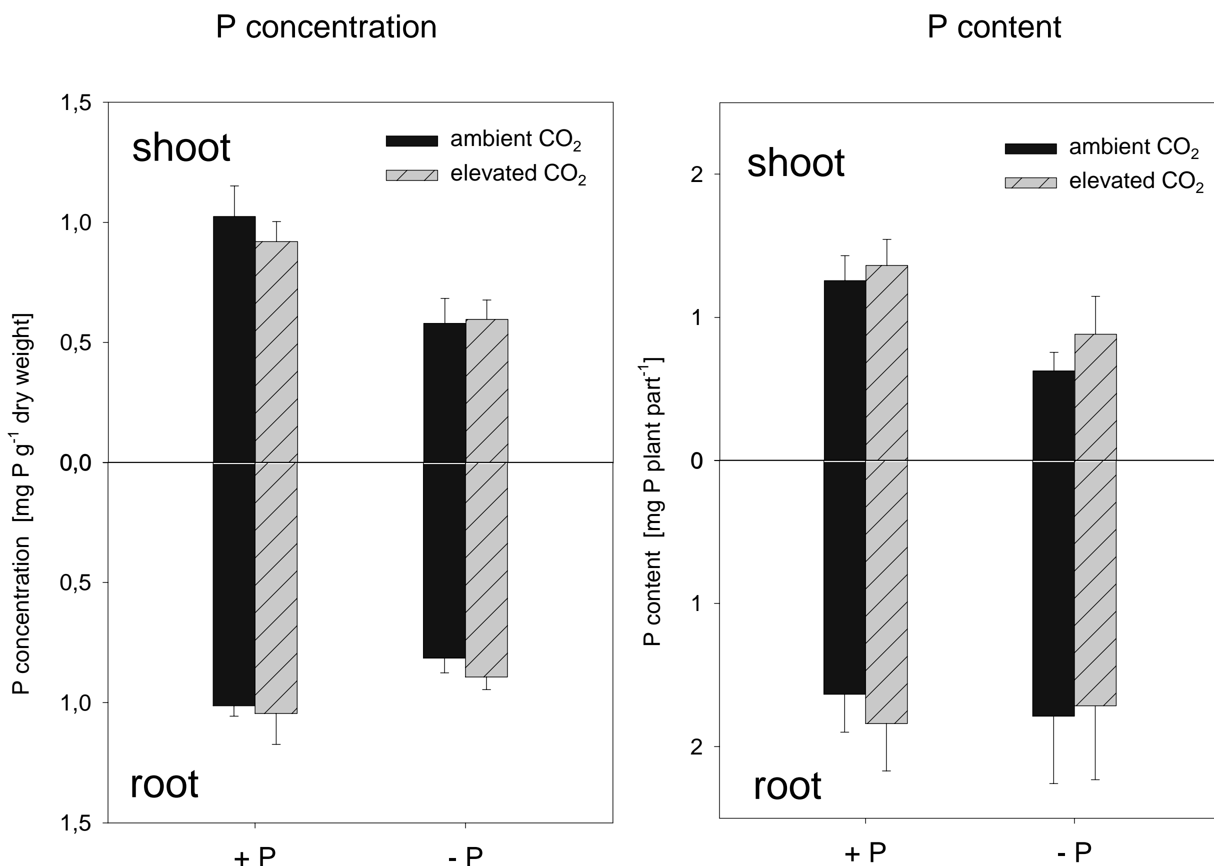


Fig. 49: Shoot and root total P concentration per dry weight (left) and P root and shoot contents (right) of white lupin plants grown in rhizoboxes at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations grown with sufficient (+P) and without (-P) external P supply 35 days after sowing. No significant differences (t-test) (data from J. Wasaki and A. Rothe).

Discussion

The aim of this investigation was to elucidate white lupin's metabolic prerequisites for internal P efficiency and P-mobilizing strategies in combination with an additional factor, in this case an elevated CO_2 concentration. Root exudation rates in white lupin plants at elevated CO_2 concentrations were determined previously, following the exudation pattern of one generation of clusters over their life time (Watt and Evans, 1999b), or by exudate collection over the whole root system in quite young plants (Campbell and Sage, 2002). Here the exudation rates were determined from different cluster root segments over a time span of two weeks. Another question was if lupins are able to mobilize more P from sparingly soluble P sources in soil at elevated CO_2 concentrations.

Plant growth and development

Independent of the cultivation method, plant development was accelerated at elevated CO_2 concentrations, and P deficiency and senescence symptoms such as yellowing, wilting, and abscission of leaves could be seen much earlier, especially in the plants grown in nutrient solution. At elevated CO_2 concentrations premature leaf senescence and more pronounced P deficiency symptoms were also found in strawberry (Chen and Lenz, 1997). Even in nutrient-sufficient wheat, premature senescence contributed to photosynthetic decline (Sicher and Bunce, 1998), and in cotton leaves, yellowing and photosynthetic decline was described at elevated CO_2 concentrations (Chang, 1975; Betsche, 1994). These findings were interpreted by Betsche as a 'perturbation' at the metabolic level.

In white lupin cultivated in nutrient solution, shoot growth was rather unaffected by the CO_2 concentration (Fig. 36). However, leaf senescence occurred much earlier and stronger and might have opposed any possible growth effects. Root growth was much faster at the elevated CO_2 concentration.

CO_2-induced maturation due to induced P_i limitation should be aggravated under already limiting P supply. P-limiting conditions would deteriorate metabolism in chloroplasts even more as already described for nutrient-sufficient conditions (Betsche, 1994), which might contribute to the yellowing, wilting, and abscission of leaves.

Quite contrary, shoot growth was slightly higher at elevated CO_2 concentrations in plants in rhizobox culture, but root growth was unchanged. The harvest of a higher amount of cluster roots from plants at elevated CO_2 (Fig. 45) might have reduced root growth. More generally,

especially the root-related data at both CO_2 concentrations have to be considered with care. Due to three intermediate harvests of cluster roots the system was already changed before the plants were harvested as a whole. Additionally, the calcareous soil with its high pH value, high bicarbonate content and low micronutrient availability, which was used for plant cultivation, might have reduced root growth. It is known that *Lupinus albus* prefers more acidic soils (Peiter et al., 2000; 2001).

However, the same root biomass at both CO_2 concentrations might also be no artifact as a result of the experimental conditions. Plants that do not react with an increase in biomass even at high P supply when the CO_2 concentration is elevated tend to accumulate starch, as described for cotton (Rogers et al., 1993). This might be due to the 'metabolic perturbation' already described (Betsche, 1994). Interestingly, white lupin also accumulates starch in the leaves under P deficiency at elevated CO_2 concentrations (Campbell and Sage, 2002) to amounts similar to those described in cotton. Therefore a similar 'metabolic perturbation' might be assumed. Additionally, the greater CO_2-induced increase in non-structural carbohydrate in cotton was explained by more limited sink in cotton as a determinant plant than in other plants which are indeterminate (Rogers et al., 1993). Perhaps the sink strength in lupin roots is also limited due to the determinate growth of cluster roots, although the high amount of exudates might be seen as an additional sink.

Contrasting results were described for root/shoot ratios at elevated CO_2 concentrations, ranging from a greater root/shoot ratio (Hocking and Meyer, 1991; Rogers, H. et al., 1992; McKee and Woodward, 1994), via no change (Larigauderie et al., 1994; Hodge and Millard, 1998) to a lower root/shoot ratio (e.g. Salsman et al., 1999). In white lupin, root/shoot ratio increased during plant growth at ambient CO_2 while P deficiency became more severe (Fig. 38). At the elevated CO_2 concentration this ratio was increased even further at each harvest. Interestingly, the increase in the root/shoot ratio in the plants grown at elevated CO_2 was much faster at the first harvests compared to the plants grown at ambient CO_2. This might also be explainable by a faster growth and therefore a faster growth into P deficiency at elevated CO_2 concentrations. This increase was mainly due to a faster root growth, but was further supported by the earlier wilting and abscission of leaves.

In contrast, Campbell and Sage (2002) found no increase in the root/shoot ratio in 22 days old lupin plants at elevated CO_2 concentrations under P deficiency, and only a slight increase in the root/shoot ratio compared to P-sufficient plants when both were grown at ambient CO_2 concentration. Perhaps the low amounts of P supplied to the P-deficient plants in their experiment already prevented severe P deficiency, enough to suppress a greater change in root/shoot ratio.

A higher root/shoot ratio needs a higher C partitioning into the root to allow the necessary increase in root biomass compared to the shoot biomass.

Cluster root development

A higher amount of cluster roots at elevated CO_2 concentrations at the earlier harvests found in nutrient solution and in rhizoboxes (Fig. 39+46) was also described by Campbell and Sage (2002) in earlier stages of plant growth. Accelerated cluster root development at elevated CO_2 concentrations was also reflected by differences in the proportion of cluster roots in the different developmental stages. According to the total number of cluster roots between 21 and 30 days after sowing the proportion of young, mature and senescent clusters was always higher at the elevated CO_2 concentration and thereafter the differences disappeared, cluster root production ceased and the amount of clusters eventually became the same at both CO_2 concentrations. This is probably due to a lower photosynthesis at this already severe P deficiency (Fredeen et al., 1990; Barrett and Gifford, 1995). Therefore less carbohydrates are available for the production of new clusters, a state which is reached earlier at elevated CO_2 concentrations.
However, the capacity for an increase in the amount of cluster roots at higher CO_2 concentrations seems to be limited. Comparing the increase in the number of cluster roots from a CO_2 concentration of 200 μmol mol^{-1} to ambient and from ambient to 740 μmol mol^{-1} CO_2, Campbell and Sage (2002) found indications that cluster root allocation might already meet its limits at the current atmospheric CO_2 concentration. Similar results for plant growth were found for several annual weedy herbaceous species (Bunce, 2001).
No significant differences between CO_2 treatments were observed for the proportion of cluster roots relative to the whole root system (Fig. 40), demonstrating that increased formation of cluster roots at 800 μmol mol^{-1} CO_2 was a consequence of a general stimulation of root growth, due to more lateral root initiation (Skene, 2000) and not of an overproportional induction of cluster root formation.

Function of cluster roots

Independent of the cultivation method, root exudation per cluster or per cluster root weight was unchanged at elevated CO_2 concentrations (Fig. 42+47). In nutrient solution the carboxylate exudation pattern in different stages of cluster root development with a citrate exudation peak in

mature clusters and decreasing malate exudation rates during growth (Neumann et al., 1999; 2000) was also confirmed at elevated CO_2 concentrations. A transient carboxylate exudation was also described for *Lupinus albus* (Kamh et al., 1999) and for *Hakea undulata* (Dinkelaker et al., 1997) when cultivated in soil. However, in the present study citrate exudation rates in mature and senescent clusters were essentially the same from plants grown in rhizoboxes. This might be explained by the problem to differentiate mature from senescent clusters in soil. Additionally, it is known that root life-span is increased when the plants grow in soil, probably due to P mobilization and P uptake, resulting in a better P status than in plants grown in nutrient solution without any P supply. In contrast to plants grown in nutrient solution with optimal P supply, plants grown in soil often have a slight P deficiency even when they are supplied with P. In white lupin, even +P control plants produced cluster roots and exuded citrate, although to a much lower amount. The P added to the soil often binds to the soil matrix, and since it is transported to the plant only by diffusion, its availability is lower than in nutrient solution where all the P is easily accessible. This was seen in the +P control plants after 35 days of growth, which also suffered from slight P deficiency (~ 1 mg P g^{-1} plant dry weight is already well below the value of around 2 mg P g^{-1} dry weight given for well-supplied plants (Marschner, 1995)). However, P concentrations in +P control plants were still higher than in -P plants, but might explain cluster root production and citrate exudation.

The higher carbohydrate transport from the shoot into the root, as seen in the higher root/shoot ratio at elevated CO_2 concentrations, did not result in a higher citrate release from individual clusters. Similar results were described by Watt and Evans (1999b) who documented the exudation rates of one generation of cluster roots during their life cycle and found that elevated CO_2 concentrations did not change the rate of citrate efflux per unit of length of cluster root, although it did shorten the period over which this efflux occurred.

The same exudation rates of citrate and malate on the basis of root dry weight were also found when collected over the whole root system of 22 days old P-deficient lupin plants (Campbell and Sage, 2002). Taken together, the exudation rates of the cluster roots themselves do not react to elevated CO_2 concentrations. But considering that, at least in younger plants in the fourth week of growth, when the amount of cluster roots which do release citrate is higher at elevated CO_2 concentrations, then the exudation rate per plant should be higher. Exactly this was found by Campbell and Sage (2002) for citrate in 22 days old whole root systems of P-deficient plants at elevated CO_2 concentrations. In consequence, white lupin probably enhances its P-mobilizing ability not by higher concentrations of root exudates in the rhizosphere, but mainly by more sites per plant where intensive mobilization of P occurs. Similarly, a higher cumulative release of C in *Plantago* (Hodge and Millard, 1998) was due to a larger root system, which was also described

for both, crop and pasture species (Hocking and Barrett, 2003). It was generally suggested that rhizodeposition does scale linearly with root mass (Darrah, 1996). On the other hand, a higher citrate production on a unit root dry weight base under P deficiency at elevated CO_2 in *Eucalyptus* was cited by Cardon (1996), and a higher production and exudation of citrate was described in an Australian pasture grass and other plant species (Gifford et al., 1996).

In white lupin under P deficiency, the increased C distribution into the roots at elevated CO_2 concentrations are not transformed into higher cluster root exudation rates. This might be explained by a reduced citrate turnover as cause for citrate accumulation rather than an increased citrate production. Citrate degradation is independent of the C supply and therefore also independent of the CO_2 concentration. Quite contrary, phytosiderophore production under Fe-deficiency in barley (and other gramineous species) root tips depend on photosynthetic activity (Erenoğlu et al., 1996). Therefore phytosiderophore exudation increased at elevated CO_2 concentrations (unpublished results).

Activity of phosphatases

Acid and alkaline phosphatase activities in the rhizosphere of *Lupinus albus* continually increased during cluster root development (Fig. 48), parallel to an increasing P deficiency, and were especially high in senescent clusters. However, atmospheric CO_2 concentrations did not significantly increase phosphatase activities.

The increased activity of acid phosphatase, an enzyme to hydrolyse the ester bonds of organic P compounds, is known to be an adaptation of plants to low internal P concentrations (Dracup et al., 1984; Lefebvre et al., 1990; Hunter and Leung, 2000; Miller et al., 2001; Gaume et al., 2001). The strict correlation between phosphatase activities and P concentrations in the roots might explain the highest phosphatase activities in the senescent clusters, which was also found in nutrient solution (Neumann et al., 1999; Wasaki et al., 1997, 2003). Accumulation of the enzyme might also explain that the highest activities were found in sensecent clusters, especially since the white lupin-derived enzyme is known to be very stable for several days even in soil.

Acid phosphatases mainly originate from roots, and only to a low amount from microorganisms, whereas alkaline phosphatases mainly originate from microorganisms (Marschner, 1995). The acid phosphatase secreted from white lupin roots has a broad pH optimum and an even higher pH stability (Tadano et al., 1993). For this reason the alkaline phosphatase activities measured in the present study might actually originate from white lupin-derived acid phosphatases. This would explain the almost identical distribution pattern of alkaline phosphatase activities in

comparison to the acid phosphatase activities. The "alkaline phosphatase" activities measured therefore probably were also the acid phosphatase activities, but measured at a less optimal pH and therefore with lower activities.

Essentially unaltered phosphatase activities at ambient and elevated CO_2 concentrations might be caused by the unaltered P concentrations in the respective cluster roots at the different CO_2 concentrations. Internal phosphate concentrations in the end determine acid phosphatase secretion.

However, the properties of white lupin phosphatases might be an exception in plants, because a higher acid phosphatase activity at elevated CO_2 concentrations was described for wheat (Barrett et al., 1998), an Australian pasture grass (Gifford et al., 1996), for *Bromus madritensis* (Dhillion et al., 1996), or for *Eriophorum* in tussock tundra in Alaska (Moorhead and Linkins, 1997), although not for pine, where acid phosphatase activity even decreased (DeLucia et al., 1997). It was suggested that in case of a generally higher phosphatase activity as a general response to elevated atmospheric CO_2 concentrations under P limiting soil conditions, a higher P acquisition from the organic P pool in the field could be assumed for the future (Barrett et al., 1998). Especially increased exudation of both, phosphatases and organic acids, was assumed to be an important driver of the ecosystem long-term response to elevated CO_2 concentrations in P-limited ecosystems (Canadell et al., 1996).

P acquisition and P nutritional status of the plants

Citrate accumulation, followed by a pulse of citrate exudation in mature cluster roots under P deficiency, seems to be related with the internal P status of the corresponding cluster (Chapter 1). To investigate this relation to CO_2 supply, ortho-phosphate concentrations were determined in different cluster root segments at several harvests in plants cultivated in nutrient solution. Ortho-phosphate was chosen as parameter since P_i concentrations give a better view of the physiological status of P deficiency. It is the P_i that is physiologically active.

As already found for citrate concentrations and exudation rates, P_i concentrations were also not changed by different CO_2 concentrations in the corresponding cluster root segments (Fig. 43). Therefore it can be stated that at elevated CO_2 concentrations plants develop faster and show P deficiency earlier, leading to earlier production and faster development of cluster roots. However, the sensecence program of individual cluster roots, which in consequence determines P concentration, P remobilization and citrate accumulation, is quantitatively unchanged.

Interestingly, even after 36 days of growth, P_i concentrations in young cluster roots still had similar high P_i levels as two weeks before. This means that the young root tissue had a very high P demand which was met even as the other tissues were already severely P-deficient. Since the plants did not get any external P and had to use the P originally derived from the seeds, P must have very efficiently been resupplied within the plant. A similar efficient retranslocation of P could be observed in the shoot tips, where P concentration also was kept at the same high levels for two weeks, and independent of the atmospheric CO_2 concentration. Correspondingly, it was suggested that cytosolic P concentrations are conserved at levels sufficient to maintain C supply at rates required by growing tissues under conditions of chronic low P supply (Gifford et al., 2000).

Lupinus albus grown in rhizoboxes did not grow better when supplied with soluble P, which documents the high P efficiency of this plant species. A general inhibition of root growth by the soil used (calcareous subsoil with a high pH) is rather improbable because biomass production was similar between plants grown in rhizoboxes and those grown in nutrient solution.
Furthermore, total P uptake was in the same order of magnitude between +P control plants (1.1 mg P $plant^{-1}$) and -P plants (0.6 mg P $plant^{-1}$), when the seed reserves of 1.8 mg P $plant^{-1}$ were also considered. For the +P plants the P taken up was 10 % of the P added to the soil, which is a very high percentage. The P taken up by the P-deficient plants is the total amount of the plant-available P (P_{CAL} = 1.25 mg per rhizobox), which means that most of the P taken up under P deficiency was mobilized by the roots from the sparingly soluble Ca-P fraction (82.5 mg P per rhizobox). P mobilization from organic P sources was rather low, because 90 % of the soil was an anorganic subsoil. Accordingly, an intense growth and activity of cluster roots could be observed under P deficiency. However, even in the +P control plants shoot P concentrations were in the range of P deficiency, although P concentrations were slightly higher than in the -P plants. Root P concentrations however were the same, which means a P retention in the roots under P deficiency. This might be explained with the high P demand for cluster root production and P-mobilizing activity.
A compensatory adjustment to nutrient deficiency at elevated CO_2 concentrations, when the availablity of nutrients is limited, could be an increased phosphate use efficiency (PUE), meaning that per unit phosphate more biomass can be achieved, or, in other words, the minimum P tissue concentration can be lowered further at elevated CO_2. However, this was not the case in white lupin grown in rhizoboxes with the same total P concentrations at both atmospheric CO_2 concentrations. Interestingly, nutrient solution-grown white lupin plants did show an increased PUE. The total P content in the plants were the same at both CO_2 concentrations since the P

solely came from the seed P reserves. Even under severe P deficiency total plant biomass was higher at elevated CO_2 concentrations due to a higher root biomass, which results in a higher plant biomass per unit phosphate.

Generally, there are few consistent responses emerging between species or life forms concerning the effects of an elevated atmospheric CO_2 on tissue P concentration, giving examples for and against effects (Gifford et al., 2000). However, it seems that nitrogen use efficiency (NUE) could be increased at elevated CO_2 concentrations (Woodin et al., 1992; Reeves et al., 1994; Murray et al., 2000), probably due to a lower RUBISCO concentration (Stitt, 1991; Tissue et al., 1993; Drake et al., 1997). The same carboxylation rate with a lower RUBISCO concentration is possible since a higher intercellular CO_2 concentration increases the carboxylation rate of RUBISCO (Sage et al., 1989). Phosphorus use efficiency (PUE) rather is not altered (Norby et al., 1986; Reeves et al., 1994; Temperton et al., 2003), although a higher PUE was also described (Woodin et al., 1992; Conroy et al., 1992; Bassirirad et al., 1996; Roberntz and Linder, 1999). Even differences in PUE exist between roots and shoots (Tremblay et al., 1988).

No CO_2 effect on P nutrient status could be observed in white lupin plants cultivated in rhizoboxes. However, this was rather not to be expected in this experiment. A higher cluster root production as it occurred under moderate P deficiency at earlier stages of plant growth might have resulted in a higher P mobilization and a better P nutritional status of the plants at elevated CO_2 concentrations. But the cluster roots had to be harvested for microbial diversity measurements, and were therefore not available for further P mobilization. In a culture system with an unlimited soil volume and undisturbed cluster root development a CO_2 effect might occur, leading to a higher amount of active cluster roots per plant, a higher P mobilization and a higher biomass, especially since elevated CO_2 is supposed to lead to increased mineralization rates as a direct result of increased root activity (Zak et al., 1993). Additionally, an increased growth rate at elevated CO_2 concentrations is thought to be sustainable only with a concomitant increase in availability and/or acquisition of growth-limiting nutrients (Bassirirad et al., 2001).

Another parameter might have influenced plant growth and P efficiency at the different CO_2 concentrations. Plants grown in small pots exhibit many of the responses found in plants acclimated to high CO_2 concentrations (Arp, 1991), such as higher carbohydrate concentrations and inhibition of photosynthetic capacity. In the end, photosynthetic rates of plants grown at elevated CO_2 concentrations are lower than the rates of plants grown at ambient CO_2 at the same C_i (intercellular CO_2 concentration) (Sage et al., 1989; Stitt, 1991; Xu et al., 1994). A reduced sink strength of the roots by a limited rooting volume by small pots which induces a source-sink imbalance and therefore a feedback inhibition of photosynthesis and a lower biomass gain

especially in the roots might be responsible for the acclimation effect (Thomas and Strain, 1991). The rhizobox volume for growth of white lupin was about 300 cm^3 and therefore far below the pot size where pot effects were described (Arp, 1991). To prevent root restriction the plants were grown only for a short time and therefore did not need a big soil volume. However, the rooting volume was further restricted by the construction of the rhizoboxes, where the roots are forced to grow along a plexiglass plate preventing part of the root system from growing into the soil volume. Therefore acclimation due to a restricted root growth by the size and construction of the rhizoboxes is possible and might explain that root growth, in contrast to shoot growth, especially in the P-deficient plants, was not increased at elevated CO_2 concentrations. On the other hand, root restriction might have been alleviated by the intermediate harvests of cluster roots.

Root morphology is crucial for the plant's reaction to P limitation. Watt and Evans (2003) reported similar data for biomass and P content when they compared plant growth and P acquisition between white lupin and soybean. They found that white lupin acquires its P from clusters by extraction of P from sparingly soluble P sources, especially under a limited supply of soluble P. The root system is characterized by a low capacity to use soluble P, which is expressed in a similar biomass between plants grown in soil with and without additional soluble P, as also found in this study. The small soil volume exploited by white lupin clusters contains only a small percentage of the soluble P available over the whole soil volume, but the clusters can mobilize the fixed P in this small part of soil efficiently, especially with the higher amount of clusters under P-limiting conditions. Contrary to this, soybean as an example for another root morphology is able to produce a longer and finer root system and acquires soluble P more readily, and reaches more of the soluble P when it is added to the soil. This is expressed in a higher biomass and higher P concentrations in shoot and root. Similarly, an increased P acquisition efficiency in bean was not related to chemical modification of the rhizosphere, but to root architecture and morphology (Lynch, 2003).
Plants like white lupin, specialized in mobilizing sparingly soluble P by cluster roots, might be able to cope with an elevated CO_2 concentration at low-P sites without a loss of biomass, although with no increase in growth when P supply is high.

In conclusion, the question remains if the reactions of P-deficient white lupin to elevated CO_2 concentrations are due to an aggravated P deficiency, brought about by a faster development and therefore a faster development of P-deficient metabolic conditions, or if an elevated CO_2 concentration changes the plant’s metabolism *per se*. Most of the effects described so far can be explained by a faster development, and are also seen in plants under P deficiency at ambient CO_2

at a later stage, such as a higher root/shoot ratio, lower P_i concentrations at the transition from slight to severe P deficiency, faster leaf wilting, and abscision and its explanation by an induced P deficiency, higher P contents, and the same exudation rates from the cluster roots on a mass or length basis. A more rapid maturation of plants exposed to elevated CO_2 concentrations were also described for bean (Porter and Grodzinski, 1989), rice and wheat (Conroy et al., 1994), or native annual plant species of North America (Omer and Horvath, 1983). On the other hand, it cannot be ruled out that the faster development of P-deficient conditions causes changes that do not occur when the transition rate is slower. Many parameters were not examined at all, such as the influence of phytohormones like auxins or ethylene, which are involved in cluster root formation (Gilbert et al., 2000; Skene and James, 2000) or P concentrations within different plant tissues or even its distribution within a cell.

Microbial diversity

An analysis of the structural and microbial diversity of rhizosphere microorganisms of white lupin was performed in a co-operation with the Institute of Soil Science and Land Evaluation, Section Soil Biology (A. Rothe, J. Wasaki, and E. Kandeler). The functional characterization of the rhizosphere microorganisms by use of marker enzymes of the C, N, and P cycle and the analysis of the structural diversity of bacterial populations via DGGE analysis of the 16 S rDNA showed an influence of the CO_2 concentrations especially by the different developmental stages of the cluster roots. This might be explainable by differences in exudation activities. Similar results were also gained in preliminary experiments by Marschner et al. (2002). Since the exudation activities of the cluster roots are not influenced by atmospheric CO_2 concentrations, no significant effects of different CO_2 concentrations were seen on microbial diversity in the white lupin rhizosphere.

General conclusions and outlook

Chemical mobilization of sparingly available soil P forms by plant roots requires intense expression of root-induced chemical changes in the rhizosphere, comprising alterations of rhizosphere pH, release of metal chelating compounds, and secretion of phosphohydrolases. Cluster rooted plant species, such as members of the Proteaceae, Casuarinaceae, and several leguminous plants, including *Lupinus albus*, are adapted to habitats of extremely low soil fertility. These plant species are among the few proven examples for an efficient chemical P mobilization in soils (Jones, 1998; Neumann and Römheld, 2000) and can therefore serve as model systems to study regulatory aspects of the related mechanisms (Neumann et al., 1999; Neumann and Martinoia, 2002). A detailed understanding of these mechanisms is a prerequisite for attempts to improve efficiency for P acquisition in crop plants by strategies for management of rhizosphere chemistry using approaches of fertilization management, breeding, or biotechnology.

In cluster-rooted plant species, the efficient expression of chemical P mobilization in the rhizosphere seems to be determined by two factors, comprising (1) a morphological and (2) a physiological component. The morphological component includes adaptive responses to P deficiency leading to formation of closely-spaced clusters of lateral rootlets with limited growth, densely covered with root hairs. These root structures increase the secretory surface area involved in release of P-mobilizing root exudates, leading to a concentration effect in the small soil volume around the root clusters. Since cluster root formation can occupy up to 60 % of the total root system, this strategy provides an efficient step by step extraction of small soil compartments during plant development.

The physiological component comprises the preferential expression of metabolic alterations, leading to selective accumulation and intense secretion of P-mobilizing root exudates in cluster roots. The elucidation of regulatory mechanisms which determine the related physiological adaptations was the scope of the present study.

Citrate is among the most efficient metal-chelating carboxylates which can mediate P_i desorption and solubilization from Fe-, Al- and Ca-phosphates. The present work presents further evidence that selective accumulation of high amounts of citrate prior to a transient burst of intense citrate exudation during cluster root development in P-deficient white lupin is probably a consequence of two general mechanisms: (1) a higher production rate, and (2) a decline in citrate turnover. The pulse of citrate exudation from mature root clusters during a period of 1-3 days is mediated

by activation of a citrate transport mechanism linked with increased expression and activity of the plasma membrane H^+-ATPase, which leads to a concomitant extrusion of protons. The involvement of an anion channel, postulated on base of the data from inhibitor experiments (Neumann et al., 1999) has been recently confirmed by patch-clamp studies (Zhang et al., 2004).
A higher production rate of citrate precursors is brought about by an activation of P_i-independent glycolytic bypass reactions and the induction of non-photosynthetic CO_2 fixation via PEP-Carboxylase, with possible adaptive functions to P limitation such as: (1) more economic P_i utilization at the metabolic level under P-deficient conditions (Plaxton, 1998); (2) stabilization of the cytosolic pH in response to P deficiency-induced excess cation uptake (Dinkelaker et al., 1989; Sakano, 1998; Sas et al., 2001), and anaplerotic carbon supply to balance C-losses associated with increased root exudation under P stress (Johnson et al., 1996a). Due to the PEP-C-mediated non-photosynthetic CO_2 fixation, these adaptive responses are unequivocally linked with enhanced biosynthesis of carboxylates, such as oxaloacetate and malate as precursors for citrate production. However, the selective accumulation of citrate during cluster root development seems to be rather determined by reduced turnover of citrate as a consequence of limited P availability for various metabolic sequences involved in citrate degradation. Declining concentrations of soluble P_i, ATP, and ribosomal RNA during cluster root development are associated with a reduction of respiration, and a reduced activity of enzymes involved in citrate turnover in the TCA cycle. In this context, the inhibition of aconitase may play a crucial role in mediating citrate accumulation, since artificial inhibition of aconitase by local application of monofluoro-acetic acid was able to induce citrate accumulation as also citrate exudation, at a rate comparable with mature cluster roots even in root tissues such as young cluster roots and roots of P-sufficient seedlings, usually inactive in root exudation of citrate. Although the present study demonstrates down-regulation of various additional enzymes involved in citrate catabolism in the TCA cycle, it is not clarified yet whether the generally lower respiration rate observed in mature and senescent cluster roots leads to a feedback inhibition of citrate turnover in the TCA cycle, or whether there is a specific inhibition of aconitase activity. Increased production of H_2O_2 in the P-deficient tissue, which could act as a potent inhibitor of aconitase (Verniquet et al., 1991), was not detectable during cluster root development, and accordingly there was no indication for increased lipid peroxidation, frequently associated with accumulation of H_2O_2. Another factor with high potential for aconitase inhibition could be an increased production of NO. Nitric oxide was found to impair the aconitase enzyme (Navarre et al., 2000). This aspect requires further investigation. Interestingly, the lower respiration rate seems rather be related to a general impairment of the respiratory apparatus (e.g. limitation of protein biosynthesis reflected in declining protein concentrations during cluster root development) and not to a limited

availability of ADP and P_i as respiratory substrates. This is indicated by the absence of any increase in respiration after uncoupling oxidative phosphorylation by use of CCCP. Other potentially P-limited metabolic pathways involved in citrate turnover may comprise citrate cleavage to acetyl-CoA and oxaloacetate via ATP-citrate lyase and P deficiency-induced inhibition of nitrate assimilation. Although downregulation of enzymes involved in these pathways such as ATP-citrate lyase and NADP-isocitrate dehydrogenase has been demonstrated during cluster root development, *in vivo* inhibitor studies failed to induce a significant citrate accumulation and the rate of contribution to citrate accumulation remains to be elucidated.

The continuous decline in metabolic P_i availability during cluster root development, which limits reactions involved in citrate catabolism, probably reflects P_i retranslocation from mature and senescent cluster roots to the young, emerging clusters, characterized by a high proportion of meristematic, growing tissue with a high demand for metabolic energy. Therefore, mature cluster roots are exposed to more severe P limitation, associated with the respective modifications in citrate catabolism, while P supply to the growing tissues in young cluster roots is still maintained at a sufficient level.

From an evolutionary point of view, this suggests that the adaptive responses of the carboxylate metabolism to P limitation in cluster roots of *Lupinus albus* are based on common reactions to P deficiency with a role in internal P utilization and widespread distribution in higher plants (Neumann and Römheld, 2000), such as (1) induction of P_i-dependent metabolic bypass reactions (e.g. PEP-C induction) and (2) P retranslocation from older tissues to young, actively growing organs. A similar example for an adaptive response based on a set of common sequences in plant metabolism is the synthesis of Fe-mobilizing phytosiderophores in graminaceous plant species from nicotianamine with ubiquitous distribution in higher plants (Neumann and Römheld, 2000).

Based on calculations of carboxylate turnover and a limited correlation between root carboxylate concentrations, enzyme activities, and root exudation, Watt and Evans (1999b) suggested that citrate exudation may be rather controlled by the export mechanism than by citrate accumulation in the cluster root tissue. However, aconitase inhibitor experiments in the present study demonstrate a close relationship between citrate accumulation and subsequent exudation. Citrate accumulation to a threshold concentration of 20-30 μmol g $^{-1}$ root fresh weight prior to the pulse of exudation may indicate a role of cytosolic citrate accumulation in the regulation of the export mechanism. Differential interactions of malate and citrate have been demonstrated for the activity of the plasma membrane (PM) H^+-ATPase, which is involved in citrate export. The fact that external application of low-molecular weight organic acids with potential to decrease the

cytosolic pH can induce citrate exudation (Kania et al., 2003) suggest a contribution of cytosolic acidification, which has shown to be associated with high accumulation of carboxylates in the root tissue (Xia and Roberts, 1994). Also changes in membrane potential seem to be involved in the regulation of citrate export, since a recently identified citrate-permeable anion channel in cluster roots of *Lupinus albus* (Zhang et al., 2004) is activated by membrane hyperpolarization. A current working hypothesis is based on the assumption that citrate accumulation in cluster roots induces cytosolic acidification, which leads to activation of proton extrusion by the PM H^+-ATPase. Thereafter, the resulting hyperpolarization of the plasma membrane may activate the anion channel, mediating citrate export. Future research activities are necessary to confirm the postulated changes in cytosolic pH and cytosolic citrate concentrations during cluster root development by use of NMR techniques in combination with membrane-physiological studies to evaluate the potential effects on citrate export. Homology analysis with the recently identified gene potentially encoding for the Al-regulated malate channel in wheat roots (Ahn et al., 2004) may enable also a molecular genetic characterization of the citrate channel in *Lupinus albus*.

Compared with apical root zones in lateral roots, P deficiency-induced metabolic alterations in cluster roots are associated with an increase in root exudation by a factor 3 – 30 calculated on a base of root biomass or root length (Neumann et al., 1999; 2000). However, also the high density of lateral rootlets (and root hairs) in cluster roots contribute to accumulation of root exudates in the rhizosphere by providing an increased root surface area with secretory activity. Assuming an average length of 5 mm per rootlet and a density of 50 rootlets per cm of the lateral root axis for cluster roots in *Lupinus albus* (Dinkelaker et al., 1995), carboxylate exudation may increase at least by a factor of 25 compared with normal lateral roots. Even higher values may be expected due to the presence of root hairs, and in cluster roots of Proteaceae, reaching rootlet densities of up to 1000 per cm. Moreover, mature cluster roots with the highest secretory activity exhibit no more growth activity (Watt and Evans, 1999b), and root exudates can be released over an extended period of time (2-3 days) into the same volume of rhizosphere soil. In contrast, normal lateral roots are characterized by root growth rates of up to 1.5–2.5 cm per day, resulting in an average residence time of approximately 5 h in a given soil volume for the apical root zone which usually exhibits the highest rates of root exudation (Jones et al., 1996; Neumann and Römheld, 2000). The prolonged secretory activity of cluster roots in the same soil compartment may therefore additionally increase the accumulation of root exudates in the rhizosphere by a factor of approximately 10-15 compared with normal lateral roots. These model calculations demonstrate that, compared with metabolic alterations, characteristics of cluster root morphology and development are at least of equal importance for a significant rhizosphere accumulation of

P-mobilizing root exudates. Future attempts for biotechnological manipulation of carboxylate exudation towards improved nutrient acquisition of crop plants must therefore not only consider the complexity of regulatory processes involved in carboxylate metabolism and transport, but also aspects of root morphology. Thus, it is not surprising that up to now, simple overexpression or antisense repression strategies of single genes are frequently not reproducible (Delhaize et al., 2001).

The experiments with elevated atmospheric CO_2 concentrations demonstrate a differential influence of CO_2 on cluster root development and cluster root function. For future research activities, this underlines the importance to consider also the influence of environmental factors on expression of adaptive plant responses to P limitation.

Abstract

In many tropical and subtropical areas crop production is severely limited by a deficiency of plant-available phosphorus (P) in the soils. Therefore plant mechanisms to mobilize the sparingly soluble P fraction are of high interest. One such mechanism of P-deficient plants is the exudation of carboxylates and protons from roots. White lupin (*Lupinus albus* L.) was chosen as a model system to investigate plant metabolism under P deficiency which enable the plant to release extraordinarily high amounts of citrate and protons from its cluster roots (bottlebrush-like clusters of short rootlets of determinate growth which form along secondary lateral roots). The aim of this work was to determine the reasons for the high citrate acccumulation observed in mature cluster roots of P-deficient white lupin and to characterize the regulation of citrate release.

A threshold citrate concentration is seen as a prerequisite for the transient pulse of intense citrate exudation associated with rhizosphere acidification which occurs over a time period of 2-3 days.

Biochemical changes on the anabolic side of citrate metabolism such as increased activities of phospho*enol*pyruvate carboxylase (PEP-C) or malate dehydrogenase (MDH) cannot solely explain the very high citrate accumulation observed during cluster root development, although these reactions supply the cluster roots with citrate precursors. In addition, pyruvate concentrations decrease in developing cluster roots, probably in relation to the decreasing malic enzyme activities in the respective clusters.

Citrate accumulation might also be caused by an impaired citrate turnover. Aconitase, the enzyme catalyzing the turnover of citrate via cis-aconitate to isocitrate, showed decreasing activities during cluster root development. NADP-isocitrate dehydroganase (NADP-ICDH) activities, as the next metabolic reaction which oxidizes isocitrate to 2-oxoglutarate, paralleled aconitase activities in all the different root segments investigated, although on a two- to threefold higher level. For this, aconitase rather than NADP-ICDH activities seem to limit citrate turnover. Specific activities of aconitase and NADP-ICDH were the same in all the root segments investigated.

Aconitase is rapidly inactivated by H_2O_2, which can be produced at increased rates under P limitation. However, neither H_2O_2 concentrations nor malondialdehyde concentrations as a marker for lipid peroxidation under oxidative stress were increased in clusters with low aconitase activities. Artifical inhibition of aconitase by incubating young cluster roots with high amounts of externally applied H_2O_2 did not change citrate and malate concentrations in these root

segments. However, a strong increase in citrate concentrations and a strong decrease in malate concentrations in young cluster roots, together with high citrate exudation rates, could be observed when monofluoroacetate (MFA) as another aconitase inhibitor was applied. Inhibition of the aconitase enzyme therefore forced still young clusters to react like mature ones. This hints to aconitase as a key metabolic step in citrate turnover. High rates of carboxylate exudation were measured even from seedling root tips when incubated with MFA.

Decreasing dehydrogenase activities as found during cluster root development by *in situ* staining with formazan were independent of the substrate supplied (citrate, aconitate, isocitrate, succinate, malate). This is in accordance with the decreasing enzyme activities measured in the different root segments such as aconitase, NADP-ICDH or malic enzyme *in vitro*. A reduced nitrate reductase (NR) activity under P deficiency, resulting in a lower drainoff of 2-oxoglutarate for N assimilation, seems not to play an important role for citrate accumulation, since an artificial NR inhibition with tungstate did not significantly increase citrate concentrations in young cluster roots.

The change from malate to citrate accumulation during cluster root development is paralleled by a reduction in ATP-citrate lyase (ACL) activity, an enzyme cleaving citrate to oxaloacetate and acetyl-CoA. The good correlation between the citrate/malate ratio in root exudates and ACL activities indicates that ACL plays a key role as a metabolic switch between malate and citrate accumulation during cluster root development under P deficiency. The enzyme might prevent high citrate concentrations under less severe P deficiency, when ACL activity is not limited by ATP availability. The attempt to inhibit the ACL enzyme by application of hydroxycitrate (HC) did not show any effect on citrate or malate concentrations in the young cluster roots. However, HC was probably not taken up into the root cells and could therefore not exert any inhibitory effects.

Decreasing total respiration rates as found for developing cluster roots might affect citrate accumulation directly by reduced consumption of citrate in the TCA cycle or indirectly by H_2O_2-induced inhibition of aconitase activity. However, a reduced respiration rate did not result in higher H_2O_2 concentrations in white lupin. Cytochrome pathway capacity decreased parallel to total respiration, suggesting that the cytochrome pathway determines total respiration. An increase in alternative oxidase (AOX) capacity did take place in cluster roots, but was not high enough to compensate for the decreased cytochrome capacity. The AOX enzyme often occurs under P deficiency or under oxidative stress, probably to bypass a limiting P_i-and ADP-dependent cytochrome pathway. The amount of the AOX protein, determined by immunodetection, paralleled AOX capacity. However, the availability of P_i and adenylates was not limiting for total respiration, since uncoupling oxidative phosphorylation with CCCP did not

increase the respiration rate. The citrate/malate ratio in young clusters with high rates of respiration and low inherent levels of citrate accumulation was only slightly increased by short-term application (4 -8 h) of azide and SHAM as respiration inhibitors.

The concomitant release of citrate and protons from mature cluster roots of P-deficient white lupin plants hints to a common regulation of citrate exudation and H^+-ATPase activity in this specific root zone. Highly purified inside-out plasma membrane (PM) vesicles were isolated in a membrane-physiological approach to determine H^+-ATPase characteristics involved in citrate exudation under P deficiency.
Increased hydrolytic activity of the PM H^+-ATPase derived from P-deficient plants parallels an increase in rhizosphere acidification and citrate exudation and hints to a causal relationship. Western blot analysis revealed a higher H^+-ATPase protein amount under P deficiency. The optimum pH of the H^+-ATPase was shifted towards more acidic conditions under P-deficiency, which might be an adaptation to the supposedly decreased cytosolic pH brought about by the pH stat mechanism when carboxylates accumulate. Lower citrate concentrations (2 m*M*) stimulated PM vesicle acidification even in the absence of ATP, which was further enhanced by the addition of Mg-ATP, and particularly expressed in PM vesicles isolated from roots of P-deficient plants. Accordingly, ^{14}C-citrate was taken up at higher rates into vesicles derived from P-deficient white lupin compared with vesicles of P-sufficient control plants. Therefore citrate transport predominantly occurs in roots of P-deficient plants, and is linked with the activity of the PM H^+-ATPase to maintain the electrochemical potential gradient which is reduced by citrate export out of the cell. Citrate exudation combined with an increase in H^+-ATPase activity seems to prevent citrate accumulation up to concentrations which might exert inhibitory effects on the PM H^+-ATPase. Such an inhibition was seen by diminished intravesicular proton accumulation, detected with the pH probe acridine orange, when 5 m*M* citrate were applied to the vesicle preparation. No such inhibitory effects were observed by malate application, which hints to a citrate-specific reaction.
Lowering the cytosolic pH by external application of propionate stimulated citrate and malate exudation in non-cluster laterals and in young clusters. Therefore a causal relationship might exist between citrate accumulation and exudation by acidification of the cytosol. The threshold citrate concentration at which citrate exudation is triggered perhaps is reached when citrate accumulation leads to acidification of the cytosol. Carboxylate exudation in young cluster roots and seedling root tips hints to a putative anion channel which already exists in young tissue and might be regulated in relation with H^+-ATPase activity and cytosolic pH.

Protoplasts, isolated from mature cluster roots, did only give very low yield and were not viable for seals high enough for patch-clamp studies. This might be due to the fast senescence in the developing clusters which also seems to change membrane integrity. High yields could only be gained from seedling root tips, or cotyledons. Similarly, protoplast isolation from root hairs also was only possible from seedling root tips or non-cluster lateral root tips, but even not from just emerging root hairs of young cluster roots.

To determine the influence of a second growth factor in addition to P deficiency on citrate metabolism, white lupin was cultivated in nutrient solution and in rhizoboxes at ambient (400 μmol mol^{-1}) and at elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations.

Plant development was accelerated at elevated CO_2 concentrations, and P deficiency and senescence symptoms such as yellowing, wilting, and abscission of leaves could be seen much earlier. When cultivated in nutrient solution, shoot growth was rather unaffected by the CO_2 concentration, whereas root growth was much faster at elevated CO_2. Quite contrary, shoot growth was slightly higher at elevated CO_2 concentrations in plants in rhizobox culture, but root growth was unchanged. However, the harvest of a higher amount of cluster roots from plants at elevated CO_2 or the calcareous soil might have reduced root growth of the plants grown in rhizoboxes.

Higher root/shoot ratios under P deficiency were further increased at elevated CO_2 concentrations. The amount of clusters was higher in plants grown in nutrient solution at 800 μmol mol^{-1} CO_2 from day 21 to day 33 after sowing, but thereafter the differences disappeared. No significant differences between CO_2 treatments were observed for the proportion of cluster roots relative to the whole root system. Independent of the cultivation method, root exudation per cluster or per cluster root weight was unchanged by the elevated CO_2 concentration. The distribution of citrate and malate exudation in different cluster root segments with decreasing malate exudation and a peak of citrate exudation in mature clusters was also confirmed at 800 μmol mol^{-1} CO_2. The increased carbon distribution into the root at 800 μmol mol^{-1} CO_2, seen in a higher root/shoot ratio, was not transformed into higher exudation rates from the single cluster. Acid and alkaline phosphatase activities in the rhizosphere of *L. albus* continually increased during cluster root development independent of the CO_2 supply.

Phosphatase activities and carboxylate accumulation and exudation rates were essentially unchanged by different atmospheric CO_2 concentrations. This might be due to also unaltered P_i concentrations in the respective root segments, because internal P concentrations seem to determine these parameters. Since citrate accumulation and exudation probably depends on citrate degradation, which is not influenced by the amount of carbon supplied for anabolic

processes, elevated CO_2 concentrations do rather not change citrate concentration and exudation. Accordingly, no significant effects of different CO_2 concentrations were seen on microbial diversity in the rhizosphere of white lupin.

So far, no single cause or mechanism was found to be responsible for the high citrate concentrations measured in mature cluster roots, although citrate degradation seems to be important and aconitase probably plays a key role. A general impairment of metabolism due to decreasing concentrations of P_i, adenylates, RNA, and proteins rather seems to bring about decreasing enzyme activities and reduced respiration. Various regulatory mechanisms via phosphorylation/ dephosphorylation, phytohormones, nitric oxide, or others also have to be considered.

Zusammenfassung

In vielen tropischen und subtropischen Gebieten wird die Produktion landwirtschaftlicher Erzeugnisse durch einen Mangel an pflanzenverfügbarem Phosphat (P) limitiert. Deshalb sind Mechanismen von Pflanzen, die schwer lösliche P-Fraktionen mobilisieren, von hohem Interesse. Einer dieser Mechanismen unter P-Mangel besteht aus der Mobilisation schwer verfügbaren Phosphats durch die Wurzelexsudation von Carboxylaten und Protonen.

An Weisslupine (*Lupinus albus* L.) als Modellpflanze sollte der Stoffwechsel unter P-Mangel untersucht werden, der die Pflanze in die Lage versetzt, extrem hohe Mengen an Citrat und Protonen von ihren Clusterwurzeln (flaschenbürstenartige Bündel kurzer Seitenwurzeln mit begrenztem Längenwachstum, die an Lateralwurzeln zweiter Ordnung gebildet werden) abzugeben. Das Ziel der Arbeit war es, die Ursachen der hohen Citratakkumulation zu ermitteln, wie sie in den reifen Clusterwurzeln der Weisslupine unter P-Mangel gefunden wird. Ebenso sollte die Regulation der Citratabgabe charakterisiert werden.

Das Erreichen eines Citrat-Schwellenwertes wird als Voraussetzung für den vorübergehenden, zwei bis drei Tage andauernden Puls einer intensiven Citratabgabe gesehen, die mit einer Ansäuerung der Rhizosphäre einhergeht. Die Stoffwechselveränderungen auf der anabolen Seite des Citratstoffwechsels, wie die erhöhten Aktivitäten der Phospho*enol*pyruvat-Carboxylase oder der Malat-Dehydrogenase, können die Verschiebung von einer Malatanreicherung zu der sehr hohen Citratanreicherung, wie sie während der Clusterwurzelentwicklung beobachtet wird, nicht vollständig erklären, obwohl die Clusterwurzeln dadurch mit Vorläufersubstanzen des Citrats versorgt werden. Zusätzlich nimmt die Pyruvatkonzentration in den sich entwickelnden Clusterwurzeln ab, was wahrscheinlich in Zusammenhang mit der nachlassenden Aktivität des Malat-Enzyms in den entsprechenden Clustern zu sehen ist.

Die Citratanreicherung könnte auch durch einen gestörten Citratumsatz hervorgerufen sein. Das Enzym Aconitase, das den Umsatz von Citrat über cis-Aconitat zu Isocitrat katalysiert, zeigte in den sich entwickelnden Clusterwurzeln nachlassende Aktivität. Die Aktivität der NADP-Isocitrat-Dehydrogenase (NADP-ICDH), die als nachfolgenden Stoffwechselschritt den Umsatz des Isocitrats zu 2-Oxoglutarat katalysiert, nahm parallel zu der Aktivität der Aconitase in allen untersuchten Wurzelabschnitten ab, in den entsprechenden Wurzelabschnitten jedoch mit einer jeweils zwei-bis dreifachen höheren Aktivität. Deshalb limitiert wahrscheinlich eher die Aconitase als die NADP-ICDH den Citratumsatz. Die spezifischen Aktivitäten der Aconitase und der NADP-ICDH waren jedoch in allen untersuchten Wurzelabschnitten jeweils gleich.

Die Aconitase wird durch H_2O_2, welches bei P-Mangel mit erhöhter Rate produziert werden kann, schnell inaktiviert. Es waren jedoch weder die Konzentrationen an H_2O_2 noch die des

Malondialdehyds als Markersubstanz für Lipidperoxidation unter oxidativem Stress in den Clusterwurzeln erhöht, die geringe Aconitaseaktivität zeigten.

Eine künstliche Hemmung der Aconitase durch eine Inkubation der noch jungen Clusterwurzeln mit hohen H_2O_2 –Konzentrationen veränderte die Citrat- und Malatkonzentrationen jedoch nicht. Ein starker Anstieg der Citratkonzentration und eine starke Abnahme der Malatkonzentration, in Kombination mit hohen Citratabgaberaten, konnte jedoch in jungen Clusterwurzeln beobachtet werden, die mit dem Aconitasehemmstoff Monofluoracetat (MFA) behandelt wurden. Die Hemmung der Aconitase zwang damit die noch jungen Clusterwurzeln, wie reife Clusterwurzeln zu reagieren. Das deutet auf die Aconitaseaktivität als eine Schlüsselreaktion beim Citratumsatz hin. Hohe Abgaberaten an Carboxylaten wurden sogar in Wurzelspitzen von Keimlingen nach MFA-Inkubation beobachtet.

Nachlassende Aktivitäten von Dehydrogenasen, wie sie während der Clusterwurzelentwicklung durch *in situ*-Färbung mit Formazan festgestellt wurden, waren unabhängig vom angebotenen Substrat (Citrat, cis-Aconitat, Isocitrat, Succinat, Malat). Das stimmt mit den nachlassenden Enzymaktivitäten wie der Aconitase oder der NADP-ICDH in den verschiedenen Wurzelabschnitten überein, wie sie *in vitro* gemessen wurden. Eine nachlassende Nitratreductase (NR)- Aktivität unter P-Mangelbedingungen, die zu einem geringeren Verbrauch von 2-Oxoglutarat für die N-Assimilation führt, scheint jedoch keine wichtige Rolle für die Citratakkumulation zu spielen, da eine künstliche Hemmung der NR mit Wolframat keinen signifikanten Anstieg der Citratkonzentrationen in jungen Clusterwurzeln bewirkte.

Der Wechsel von einer Malat- zu einer Citratakkumulation während der Clusterwurzelentwicklung verläuft parallel zu einer nachlassenden Aktivität der ATP-Citrat Lyase (ACL), einem Enzym, das Citrat zu Oxalacetat und Acetyl-CoA spaltet. Die gute Korrelation zwischen dem Citrat/Malat-Verhältnis in den Wurzelexsudaten und der ACL-Aktivität weist darauf hin, dass ACL bei dem Wechsel von der Malat- zur Citratakkumulation in den sich entwickelnden Clusterwurzeln unter P-Mangel eine Schlüsselrolle spielt. Das Enzym verhindert eventuell die Anreicherung hoher Citratmengen bei mäßigem P-Mangel, solange die Aktivität der ACL noch nicht durch eine limitierte ATP-Verfügbarkeit eingeschränkt ist. Der Versuch, die ACL durch die Anwendung von Hydroxycitrat (HC) zu hemmen, zeigte keinen Einfluss auf die Citrat- und Malatkonzentrationen in den jungen Clusterwurzeln. Das HC wurde jedoch wahrscheinlich gar nicht in die Wurzelzellen aufgenommen und konnte deshalb keine hemmende Wirkung entfalten.

Nachlassende Gesamtrespirationsraten, wie sie in den sich entwickelnden Clusterwurzeln gefunden wurden, könnten eine Citratakkumulation auslösen, entweder über einen nachlassenden Verbrauch von Citrat im Citratzyklus, oder indirekt über eine H_2O_2-induzierte Hemmung der

Aconitaseaktivität. Nachlassende Gesamtrespirationsraten führten bei Weisslupine jedoch nicht zu einer erhöhten H_2O_2-Konzentration. Die Kapazität der cytochromabhängigen Atmungskette nahm parallel zur nachlassenden Gesamtrespirationsrate ab, was darauf hindeutet, dass diese die Gesamtrespirationsrate bestimmt. Eine Zunahme der Kapazität der Alternativen Oxidase (AOX) wurde zwar in den Clusterwurzeln gefunden, war aber nicht hoch genug, um den Verlust der Kapazität der cytochromabhängigen Atmungskette zu kompensieren. Der alternative Atmungsweg des Enzyms AOX tritt oft unter P-Mangel oder oxidativem Stress auf, wahrscheinlich um die gehemmte, auf P_i- und ADP angewiesene cytochromabhängige Atmungskette zu umgehen. Der durch Immunodetektion ermittelte Gehalt an AOX-Protein verlief parallel zur AOX-Kapazität. Die Verfügbarkeit von P_i oder Adenylaten limitierte die Gesamtrespiration jedoch nicht, da eine Entkopplung der oxidativen Phosphorylierung mit CCCP die Respirationsrate nicht erhöhte. Das Citrat/Malat-Verhältnis in jungen Clusterwurzeln mit hohen Respirationsraten und ursprünglich geringer Citratakkumulation erhöhte sich durch die Kurzzeit-Applikation (4-8 h) von Azid oder SHAM als Atmungsinhibitoren nur geringfügig.

Die gleichzeitige Abgabe von Citrat und Protonen von reifen Clusterwurzeln der Weisslupine unter P-Mangel weist auf eine gemeinsame Regulation der Citratabgabe und der H^+-ATPase-Aktivität hin. Hochreine Plasmamembran (PM)-Vesikel mit inside-out-Orientierung wurden in einem membranphysiologischen Ansatz isoliert, um die Eigenschaften der H^+-ATPase und ihren Zusammenhang mit der Citratexsudation unter P-Mangel zu untersuchen.
Der Anstieg der hydrolytischen Aktivität der PM H^+-ATPase von P-Mangelpflanzen verlief parallel zu einer verstärkten Ansäuerung der Rhizosphäre und der Citratabgabe, was auf eine gemeinsame Ursache hinweist. Eine Western-Blot-Analyse zeigte eine höhere Menge an H^+-ATPase-Protein in Proben, die von P-Mangelpflanzen stammten. Das pH-Optimum der H^+-ATPase war unter P-Mangelbedingungen zu einem saureren pH-Wert verschoben, was eine Anpassung an einen vermutlich verringerten cytosolischen pH-Wert sein könnte, hervorgerufen durch den pH-Stat-Mechanismus, wenn verstärkt organische Säuren unter P-Mangel gebildet werden. Geringere Citratkonzentrationen (2 m*M*) stimulierten eine Ansäuerung der PM-Vesikel sogar in Abwesenheit von ATP. Bei Zugabe von Mg-ATP verstärkte sich die Ansäuerung, dabei besonders stark in PM-Vesikeln, die von P-Mangelpflanzen isoliert worden waren. Entsprechend wurde ^{14}C-markiertes Citrat mit einer höheren Rate in die Vesikel aufgenommen, die von P-Mangelpflanzen stammten. Daraus kann geschlossen werden, dass Citrattransport hauptsächlich in den Wurzeln von P-Mangelpflanzen stattfindet und mit der Aktivität der PM H^+-ATPase gekoppelt ist, die den elektrochemischen Potentialgradienten aufrechterhält, der vom Citratexport aus der Zelle heraus verringert wird. Citratexsudation in Kombination mit einer

erhöhten H^+-ATPase-Aktivität, wie sie an isolierten PM-Vesikeln durch eine erhöhte hydrolytische und Protonentransportaktivität bei Angebot von relativ geringen Citratkonzentrationen gemessen werden konnte, scheint eine Citratakkumulation auf Konzentrationen zu verhindern, die die H^+-ATPase hemmen könnten. Solch eine Hemmung der H^+-ATPase wurde durch eine geringere intravesikuläre Protonenanreicherung, detektiert mit der pH-Sonde Acridin Orange, bei einer Applikation von 5 m*M* Citrat festgestellt. Bei der Applikation von Malat traten keine hemmenden Effekte auf, was auf eine citratspezifische Reaktion schließen lässt.
Eine Absenkung des cytosolischen pH-Werts durch die Applikation von Propionat stimulierte die Citrat- und Malatexsudation von clusterlosen Seitenwurzeln und von jungen Clustern. Der Schwellenwert der Citratkonzentration, ab dem die Citratexsudation ausgelöst wird, ist eventuell dann erreicht, wenn die Citratanreicherung zu einer Ansäuerung des Cytosols führt. Die Abgabe von Carboxylaten aus jungen Clusterwurzeln und aus Wurzelspitzen von Keimlingen weist auf einen vermuteten Anionenkanal hin, der bereits in jungem Gewebe existiert und eventuell in Zusammenhang mit der Aktivität der H^+-ATPase und dem cytosolischen pH-Wert reguliert wird.

Aus reifen Clusterwurzeln isolierte Protoplasten erreichten nur eine sehr geringe Ausbeute und waren nicht dicht genug für die angestrebten Patch-Clamp-Untersuchungen. Das mag an der schnellen Seneszenz der sich entwickelnden Cluster liegen, die ebenfalls die Membranstabilität zu verändern scheint. Hohe Ausbeuten ließen sich nur von Wurzelspitzen von Keimlingen oder von Keimblättern erreichen. In ähnlicher Weise war die Isolation von Protoplasten aus Wurzelhaaren auch nur bei Wurzelspitzen von Keimlingen oder clusterlosen Seitenwurzeln möglich, jedoch nicht einmal bei sich gerade gebildeten Wurzelhaaren junger Clusterwurzeln.

Um den Einfluss eines zweiten Wachstumsfaktors zusätzlich zum P-Mangel auf den Citratstoffwechsel zu untersuchen, wurde Weisslupine in Nährlösung und in Wurzelkästen bei 400 μmol mol^{-1} und 800 μmol mol^{-1} atmosphärischer CO_2-Konzentrationen angezogen.
Die Pflanzenentwicklung war bei einer CO_2-Konzentration von 800 μmol mol^{-1} beschleunigt, und P-Mangel-und Seneszenzsymptome wie Gelbfärbung, Welke oder Abfallen der Blätter traten viel früher auf. Bei Pflanzen in Nährlösung war das Sprosswachstum von der CO_2-Konzentration unbeeinflusst, das Wurzelwachstum jedoch bei 800 μmol mol^{-1} CO_2 viel höher. Im Gegensatz dazu war das Sprosswachstum bei Pflanzen in den Wurzelkästen leicht erhöht, aber das Wurzelwachstum unverändert. Jedoch die Ernte von größeren Mengen an Clusterwurzeln von Pflanzen, die bei 800 μmol mol^{-1} CO_2 gewachsen waren, hat wahrscheinlich das Wurzelwachstum vermindert. Der kalkhaltige Boden hat eventuell ebenfalls das

Wurzelwachstum der Pflanzen in den Wurzelkästen beeinträchtigt. Ein bereits gestiegenes Wurzel/Sprossverhältnis der unter P-Mangel gewachsenen Pflanzen war bei 800 μmol mol^{-1} CO_2 noch einmal erhöht. Die Anzahl der Cluster der Pflanzen in Nährlösung war bei 800 µmol mol^{-1} CO_2 von Tag 21 bis 33 nach Aussaat höher, danach verschwanden die Unterschiede jedoch wieder. Die Anzahl der Clusterwurzeln pro Gesamtwurzelmasse wurden von untschiedlichen CO_2-Konzentrationen nicht verändert. Unabhängig von der Anzuchtmethode veränderten 800 μmol mol^{-1} CO_2 die Wurzelexsudation pro Cluster oder pro Clustergewicht ebenfalls nicht. Die Verteilung der Citrat- und Malatexsudation in den verschiedenen Wurzelabschnitten mit sinkender Malatexsudation und einer kurzfristigen extremen Citratexsudation in reifen Clusterwurzeln trat bei 800 μmol mol^{-1} CO_2 ebenso auf wie bei 400 μmol mol^{-1} CO_2. Die erhöhte Kohlenstoff-Verlagerung in die Wurzel bei 800 μmol mol^{-1} CO_2, feststellbar durch das größere Wurzel-Spross-Verhältnis, wurde nicht in eine höhere Exsudationsrate pro Cluster umgesetzt. Die Aktivitäten der Sauren und Alkalischen Phosphatase in der Rhizosphäre von *Lupinus albus* nahmen während der Clusterwurzelentwicklung kontinuierlich zu, waren jedoch unabhängig von der CO_2-Konzentration.

Die im wesentlichen unveränderten Phosphataseaktivitäten und die unveränderte Anreicherung und Abgabe von Citrat bei 400 und 800 μmol mol^{-1} CO_2 werden wahrscheinlich durch die ebenfalls unveränderten P_i-Konzentrationen in den entsprechenden Wurzelabschnitten hervorgerufen, da die interne P-Konzentration diese Parameter zu bestimmen scheint. Da Citratanreicherung und Abgabe wahrscheinlich hauptsächlich durch die Rate des Citratabbaus geregelt wird, die unabhängig von der C-Versorgung ist, verändert eine erhöhte CO_2-Konzentration diese Parameter ebenfalls nicht. Entsprechend waren auch keine signifikanten Einflüsse des CO_2 auf die mikrobielle Diversität in der Rhizosphäre der Weisslupine festzustellen.

Nach bisherigen Erkenntnissen scheint die hohe Citratkonzentration in reifen Clusterwurzeln nicht durch einen einzelnen Faktor hervorgerufen zu werden, obwohl der Citratabbau offenbar wichtig ist und die Aconitase eventuell eine Schlüsselrolle spielt. Vielmehr scheint eine allgemeine Beeinträchtigung des Stoffwechsels durch nachlassende Konzentrationen an P_i, Adenylaten, und Protein verminderte Enzym -und Atmungsaktivitäten hervorzurufen. Verschiedene Regulationsmechanismen wie Phosphorylierung/Dephosphorylierung, der Einfluss von Phytohormonen, oder Stickstoffmonoxid, müssen ebenfalls berücksichtigt werden.

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