In vivo 31 P NMR Spectroscopy for the study of P Pools and their Dynamics in Arbuscular Mycorrhizal Fungi

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In vivo ^{31}P NMR Spectroscopy for the Study of P Pools and their Dynamics in Arbuscular Mycorrhizal Fungi

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Ph.D. Thesis

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Preface

The present thesis is submitted as the written part required to obtain the Ph.D. degree at Roskilde University. The work was carried out at the Plant Research Department at Risø National Laboratory under supervision of Iver Jakobsen and at the Department of Life Sciences and Chemistry at Roskilde University under supervision of Poul Erik Hansen. The work was financially supported by grants from the Danish Research Foundation.

First, I would like to thank my supervisors, Iver Jakobsen and Poul Erik Hansen, for their encouragement and numerous constructive discussions during the work. They have both been great inspirators and have always made time for me. Further, I would like to thank George Ratcliffe at the Department of Plant Sciences, University of Oxford, for letting me work in his *in vivo* NMR laboratory and for providing me with the airlift system. Anne Olsen, Anette Olsen, Anette Christensen, Anne Lise Gudmundsson and Rita Buch are thanked for skilful technical assistance and for providing me with sweets and sweet words when weekends were confiscated in order to perform experiments. Colleagues are thanked for making both Risø and Roskilde University such nice places to work, in particular thanks to Anne Marie Scharff for fruitful discussions about much more than just science and to Jock Nielsen for always having time to answer questions about biology, when my chemical mind tried to learn about fungi. Many people have read various parts of earlier versions of the thesis. I am grateful for their comments and constructive critics. Special thanks to Sally Smith for always providing inspiration and for careful proof reading most of the thesis. Last but certainly not least my family for their patience, help and encouragement all the way through. Especially my dearest husband Christian for always giving me support and love and for providing Lærke and Caroline with at least one fulltime parent.

The thesis is divided into five chapters and a reference list: Chapter 1 is a general introduction to the subject of this thesis; it contains a literature review and the main questions asked in this thesis. Chapter 2 contains an introduction to the methods used together with preliminary experimental work. More experimental work is presented in Chapters 3 and 4; Chapter 3 is reprint of a paper reproduced by kind permission from the copyright holder and Chapter 4 is prepared as a paper manuscript which will shortly be submitted for publication in Plant Physiology in a shortened form. Chapter 5 contains conclusions and perspectives.

Nanna Rasmussen, July 2002

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Summary

The main objective of the studies described in the present Ph.D. thesis was to investigate the phosphate (P) metabolism of arbuscular mycorrhizal (AM) fungi by *in vivo* ^{31}P nuclear magnetic resonance (NMR) spectroscopy.

P is an essential nutrient for all organisms. It is required in relatively large amounts and is often limiting to plant growth. The availability of P is limited by the slow rate of diffusion of inorganic orthophosphate (P_i) through the soil. As plants remove P_i from the soil solution close to the root an area surrounding the root drained for soluble P_i may be formed (the P-depletion zone). AM fungi colonize the roots of most land plants and the symbiosis between AM fungi and plants is characterized by bi-directional nutrient transport; the AM fungus receiving an indispensable supply of fixed carbon (C) in return for improved inorganic nutrient (mainly P) uptake by the host plant.

The extraradical mycelium of an AM fungus forms an extensive hyphal network and allows the plant to access P_i in the soil solution beyond the P-depletion zone. Once the association is established, the fungus takes up P_i from the soil through the extraradical mycelium in an active process like plants. However, P_i is accumulated also as polyphosphate (polyP). PolyP is translocated to the intraradical mycelium in vacuoles in a tubular streaming system. At the symbiotic interface inside the root, polyP is hydrolyzed and P_i is subsequently transferred to the plant root cells. Accordingly, polyP is considered to have an important role in the P_i translocation process. However, the amount, size and other roles of polyP present in the extraradical and intraradical mycelium is a matter of debate. Invasive methods have commonly been used to identify polyP and therefore artifacts of specimen preparation could possibly have interfered with the polyP chain length. More detailed information of P pools and polyP accumulation would benefit from non-invasive and non-destructive measurements of the dynamic incorporation of P_i into various P pools within extraradical mycelium and mycorrhizal roots. *In vivo* ^{31}P NMR spectroscopy provides an analytical method for identifying and quantifying particular metabolites in living tissue. Moreover, it allows for measuring intracellular pH, for probing the subcellular compartmentation of certain ions and for following the flux through metabolic pathways. Thus, *in vivo* ^{31}P NMR spectroscopy is a unique analytical method for the investigation of P pools and their dynamics in AM fungi.

The plant chosen for the work was cucumber, in the majority of the work grown in symbiosis with the AM fungus *Glomus intraradices* in a compartmented growth system. Other species of AM fungi used included *Scutellospora calospora*, *G. mosseae* and *Gigaspora rosea*. The cucumber plants were grown in a central mesh-bag, which prevents root penetration but allow free passage of AM fungal hyphae. The extraradical mycelium grew into sand surrounding the mesh-bag and could be collected from the sand, while root material could be collected from the mesh-bag. A circulation system was constructed for oxygenating the excised hyphae or roots while in the NMR tube. Both the efficiency of P_i uptake and the turn-over of P metabolites by excised hyphae were investigated in order to clarify the

metabolic status of excised fungus. Furthermore, an attempt was made to measure phosphatase activity in the extraradical mycelium and mycorrhizal roots using the enzyme-labeled-fluorescence (ELF) method in order to localize aspects of P metabolism. Alkaline phosphatase activity was observed in all species of AM fungi used, which indicated metabolically active fungi.

In this study, polyP of a short chain length was seen in actively metabolizing extraradical AM fungal hyphae for the first time by the use of *in vivo* ^{31}P NMR spectroscopy. Furthermore, a time-course ^{31}P NMR investigation of the formation of P pools in differently P-treated AM hyphae and mycorrhizal roots was performed. It was demonstrated that P_i taken up by extraradical mycelium accumulated firstly into polyP and subsequently into vacuolar P_i within the extraradical mycelium. Furthermore, a time lag was observed before any P metabolites appeared in mycorrhizal roots. The amount of polyP in extraradical mycelium was considerably higher than vacuolar P_i and synthesis of polyP was therefore suggested to be important for effective P_i uptake in AM fungi. The polyP was located in vacuoles and the measured average chain length was short, supporting a role for polyP in translocation of P_i from soil to host root by AM fungi. Cytoplasmic P_i in the extraradical mycelium could not be detected by *in vivo* ^{31}P NMR possibly because of a small cytoplasmic volume or low concentration of cytoplasmic P_i.

The average polyP chain length was further characterized by the use of extraction procedures and colorimetric measurements. Combining the results obtained from these methods and NMR revealed small amounts of long-chain and granular polyP in the extraradical mycelium when supplied with high P amounts. Moreover, possible interfungal variation in P pools, polyP content and polyP average chain length was investigated for the purpose of understanding the diversity in the ability of different species of AM fungi to supply P to the host plant. The results of this preliminary investigation suggested that there are differences between species of AM fungi in P pools sizes within extraradical mycelium and also in effectiveness of translocating the P to the root.

Dansk resume

Formålet med studierne beskrevet i denne ph.d afhandling var at undersøge fosfatstofskiftet hos arbuskulære mykorrhizasvampe (AM) ved anvendelse af *in vivo* ^{31}P NMR spektroskopi.

Fosfat er et livsvigtigt næringsstof for alle planter. Det er nødvendigt i relativt store mængder og derfor ofte begrænsende for planters vækst. Tilgængeligheden af fosfat er begrænset af den langsomme diffusion af uorganisk orthofosfat gennem jorden. Når planter optager fosfat fra jordvandet tæt på roden dannes hurtigt et område omkring roden som er udtømt for opløselig fosfat (P-udtømningszonen). AM svampe koloniserer rødderne af de fleste landplanter, og symbiosen mellem AM svampe og planter er karakteriseret ved en transport af næringsstoffer i begge retninger; AM svampen optager nødvendige kulstofforbindelser fra planten, som til gengæld modtager uorganiske næringsstoffer (især fosfat) fra svampen.

AM svampenes eksterne mycelium danner et udstrakt netværk af svampehyfer og tillader fosfatoptag fra jordvandet længere væk end plantens P-udtømningszone. Ifølge den aktuelle model er processen følgende: Fosfatoptagelsen i det eksterne mycelium sker ved aktivt optag fra jorden som hos planter, men fosfat inkorporeres også som polyfosfat (polyP). PolyP translokeres til det interne mycelium i et system af sammenhængende vakuoler. Ved den symbiotiske grænseflade nedbrydes polyP og fosfat overføres til planterodens celler. Som det fremgår har polyP en vigtig rolle i translokeringen af fosfat. Der er imidlertid diskussion om mængden, længden og andre roller af polyP tilstede både i det eksterne og interne mycelium. PolyP er tidligere mest blevet identificeret og studeret ved invasive metoder, hvor behandlingen kan påvirke polyP kædelængden. Det vil derfor være nyttigt med non-invasive og non-destruktive målinger af den dynamiske indbygning af fosfat til forskellige fosfatpuljer i det eksterne mycelium samt i koloniserede rødder for at opnå en mere detaljeret viden om fosfatpuljer og polyP-akkumulering. *In vivo* ^{31}P NMR spektroskopi er en analytisk metode til at identificere og kvantificere bestemte forbindelser i levende materiale. Derudover kan intracellulært pH, subcellular fordeling af bestemte ioner og strømmen gennem biosynteseveje kortlægges. Det er derfor oplagt at undersøge fosfatpuljer og deres dynamik i AM svampe ved hjælp af *in vivo* ^{31}P NMR spektroskopi.

Agurk blev valgt som værtsplante, og i de fleste eksperimenter blev agurk dyrket i symbiose med AM svampen *Glomus intraradices* i et todelt pottesystem. Desuden blev AM svampene *Scutellospora calospora*, *G. mosseae* og *Gigaspora rosea* anvendt. Agurkeplanterne voksede i en central netpose hvor udelukkende svampehyfer kunne vokse ud igennem. Det eksterne mycelium voksede ud i sand, der omringede netposen, og blev opsamlet herfra, mens rodmateriale blev opsamlet fra netposen. Under NMR målingerne forsynede et cirkulationssystem de afskårne svampehyfer og rødder med ilt. Den fysiologiske tilstand af de afskårne svampehyfer blev vurderet ved at undersøge de afskårne svampehyfers optag og omsætning af tilsat fosfat. Hos alle undersøgte AM svampe blev basisk fosfataseaktivitet i det eksterne mycelium og koloniserede rødder bestemt ved enzym-mærknings-

fluorescens (ELF), for at undersøge aspekter af fosfatstofskiftet. Basisk fosfataseaktivitet indikerer fysiologisk aktivitet.

For første gang blev kortkædet polyP identificeret med *in vivo* ^{31}P NMR spektroskopi i fysiologisk aktive eksterne AM svampehyfer. Fosfatpuljerne i AM svampehyfer og koloniserede rødder med forskellige fosfatbehandlinger blev undersøgt i et ^{31}P NMR tidsstudie. Herved blev det demonstreret at fosfatoptaget i det eksterne mycelium først akkumulerede som polyP og først derefter som orthofosfat i vakuolerne. I koloniserede rødder kunne fosfatforbindelserne først observeres senere. Den identificerede polyP i det eksterne mycelium var placeret i vakuolerne og den bestemte middelkædelængde var kort. Desuden var mængden af polyP betydeligt større end mængden af orthofosfat i vakuolerne, hvilket antyder at syntesen af polyP er vigtig for et effektivt optag af fosfat hos AM svampe. Tilsammen understøtter disse observationer at polyP har en rolle i translokeringen af fosfat fra jord til værtsplante via AM svampe. Orthofosfat kunne ikke detekteres i cytoplasma i det eksterne mycelium ved *in vivo* NMR, hvilket kan skyldes et lille cytoplasmisk volumen eller en lav koncentration af orthofosfat i cytoplasma.

Middelkædelængden af polyP blev yderligere karakteriseret ved anvendelse af ekstraktionsprocedurer og kolorimetriske bestemmelser. Ved kombination af resultaterne fra disse metoder og NMR kunne det fastslås, at små mængder af langkædet og granulær polyP også var tilstede i det eksterne mycelium, når det var forsynet med store mængder fosfat. Variation i fosfatpuljer, polyP-mængde og polyP-kædelængde mellem forskellige AM svampearter blev undersøgt for at bidrage til forståelsen af AM svampearternes varierende evne til at forsyne værtsplanten med fosfat. Resultatet af denne undersøgelse viste, at der er forskelle mellem AM svampearter i størrelsen af fosfatpuljer i det eksterne mycelium og i effektiviteten af translokering af fosfat til roden.

Abbreviations

δ	Chemical shift
ACPase	Acid phosphatase
ALPase	Alkaline phosphatase
AM	Arbuscular mycorrhizal
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
B_0	Stationary external magnetic field
BAS	Branched absorbing structures
C	Carbon
cyt	Cytoplasmic / cytoplasm
D_2O	Deuterium oxide
DAPI	4′,6-diamidino-2-phenylindole
EDTA	Ethylene diamine tetraacetic acid
ELF	Enzyme labeled fluorescence
FB	Fast blue salt
FID	Free induction decay
fw	Fresh weight
h	Hours
M_0	Net magnetization
MDP	Methylene diphosphonic acid
MES	2-(N-morpholino)-ethane sulphonic acid
min	Minutes
NMR	Nuclear magnetic resonance
NDP	Nucleoside diphosphate
NTP	Nucleoside triphosphate
P	Phosphate
PD	Phenol detergent
PC	Phenol chloroform
PCA	Perchloric acid
P_i	Inorganic orthophosphate
polyP	Polyphosphate
$polyP_{cen}$	Central P_i residues in polyP chain
$polyP_{pen}$	Penultimate P_i residues in polyP chain
$polyP_{ter}$	Terminal P_i residues in polyP chain
PPGK	Polyphosphateglucokinase
PPK	Polyphosphatekinase
ppm	Parts per million
PPP	Pentose phosphate pathway
PPX	Exopolyphosphatase
pyroP	Pyrophosphate
r.f.	Radio frequency
S.D.	Standard deviation
S/N ratio	Signal-to-noise ratio
s	Seconds
T_1	Longitudinal relaxation time
T_2	Transverse relaxation time
TCA	Trichloroacetic acid
UDGP	Uridine diphosphoglucose
vac	Vacuolar / vacuole
wk	Week

Chapter 1 - General introduction

1.1 Scope and structure of the thesis

The general introductory chapter of this thesis is an attempt to first briefly introduce the organism of interest, the arbuscular mycorrhizal (AM) fungus, and secondly to elucidate the AM fungal role in nutrition. Our current knowledge about the mechanisms by which AM fungi contribute to plant nutrition will be introduced concerning mainly the recently published work on mechanisms for the uptake, translocation and release of phosphate (P). The methods used for the work presented in this thesis are described in Chapter 2 and my contribution to the understanding of these poorly understood mechanisms follows in Chapter 2 and the subsequent chapters. My work will be concerned with P metabolism exclusively and further introductory comments to the experimental work presented here are given in the beginning of Chapter 2. Chapter 3 is a reprint of a paper reproduced by kind permission from the copyright holder and Chapter 4 is prepared as a paper manuscript which will shortly be submitted for publication in a shortened form. Chapters 3 and 4 can be read separately from the rest. This has the consequence that introductory comments, notation etc. are described several times. Attempts have been made in order to customize notation; still, there may be slight differences that should cause no confusion. A general discussion concerning all the results presented here, conclusions and future perspectives are given in Chapter 5. The list of references has been collected into one list placed at the end of the thesis, however the references in the paper included as Chapter 3 are also given within the chapter.

1.2 Arbuscular mycorrhizal fungi

AM fungi form symbiotic associations with the roots of a wide range of land plants. AM fungi are of very ancient origin (fossil fungal hyphae suggest more than 500 million years b.p., Redecker *et al.*, 2000); they are the most common and widespread type of mycorrhizal fungi and belong to the order Glomales (Smith and Read, 1997). AM fungi are obligate biotrophic symbionts and the plants forming AM include herbs, grasses and many woody plants. The symbiosis between AM fungi and plants is characterized by bi-directional nutrient transport between the symbionts (Smith and Read, 1997). The symbiosis is normally mutualistic with the AM fungus receiving an indispensable supply of fixed carbon (C), in return for improved inorganic nutrient (mainly P) uptake by the host plant.

Plants show only minor defense reactions in response to colonization by AM fungi (Gianinazzi-Pearson *et al.*, 1996; Kapulnik *et al.*, 1996; Harrison, 1999), and the colonization is normally believed to be rather non-specific. In general, it is believed that an AM fungus isolated from one species of host plant will colonize any other species that has been shown to be capable of forming AM. However, much variation in mycorrhizal effect, i.e. plant growth and nutrient uptake, on host plants has been demonstrated; an AM fungus having no effect on a host plant under given nutrient conditions may be beneficial under other nutrient conditions. Or under fixed nutrient conditions one AM fungus may

have no effect on one host plant but large effect on another (Johnson *et al.*, 1997; Smith *et al.*, 2000). Indeed, even parasitic relationships have been demonstrated (Johnson *et al.*, 1997; Burleigh *et al.*, 2002). Furthermore, there are even differences in the extent to which species of plants become colonized by AM fungi under given soil and nutrient conditions, and also in the extent of different fungal species or even between isolates of the same species being able to colonize the roots of the same plant species (Smith *et al.*, 2000; Burleigh *et al.*, 2002). Indeed, colonization by one AM fungus can strongly affect colonization by another AM fungus in the same community. This has to be kept in mind, when generalized conclusions on physiological mechanisms are made.

1.2.1 Establishment of colonization

The lifecycle of AM fungi and by this the colonization of roots, is initiated from hyphae growing from resting spores, infected root fragments or hyphae (Smith and Read, 1997). The spores are the best defined initiators and they appear to be survival structures of the fungi. The large resting spores are unusual in that they are multinucleate and, depending on the species, may contain thousands of nuclei per spore (Bécard and Pfeffer, 1993). Recent genetic analyses suggest that the fungi are asexual and reproduce clonally (Rosendahl and Taylor, 1997). The spores germinate in response to increases in soil temperature or moisture content, and hyphae will begin to grow from the spores; asymbiotic growth is maintained for 1 or 2 weeks (Fig. 1A) (Bago *et al.*, 2000). However, the spore contains only limited amounts of lipid and carbohydrate and further development of the mycelium depends on a successful colonization of a root system; so far, AM fungi have not been cultured in the absence of a plant host. Once a root is reached, an appressoria is formed on the epidermal cell wall; next, the hyphae penetrate the cell wall of the root epidermal cell and form inter- and intracellular mycelium inside the root (Fig. 1B).

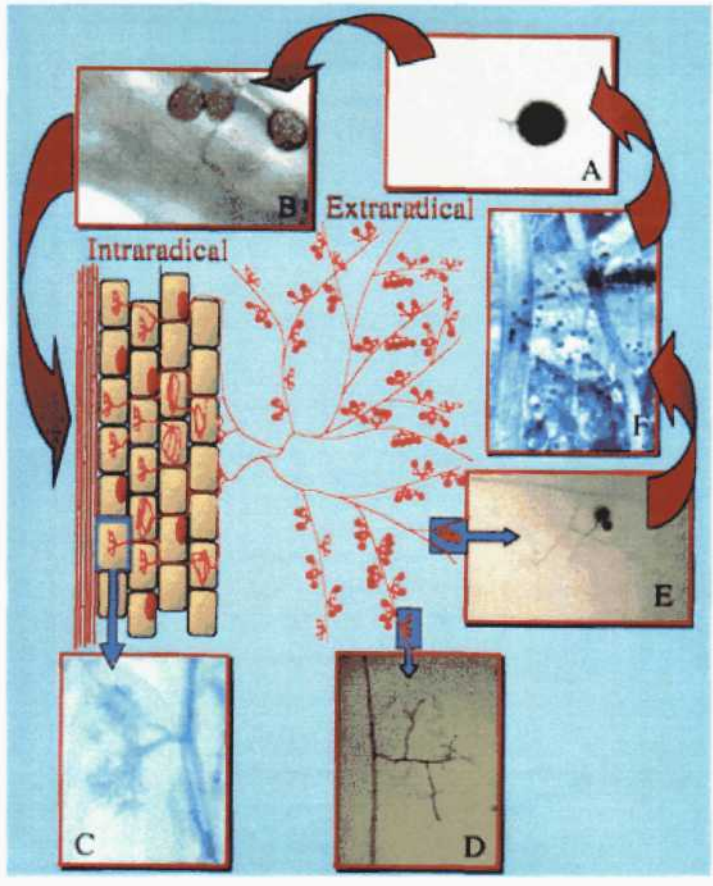

Fig. 1. Lifecycle of AM fungal colonization. A, Spores. B, Germinating spores reaching a root. C, Arbuscules D, E and F, Extraradical mycelium with different characteristics structures: branched absorptive structures (BAS) (D) and spores (E and F) (From Bago *et al.*, 2000).

1.2.2 Morphology of the mycelium

The mycelium has intraradical and extraradical phases, in relation to the host root, with the appressoria or "entry-point" hyphae as the link between them. The intraradical part consists of hyphae and lipid-filled vesicles (Although, 20% of the AM species do not form vesicles, i.e. from the genera *Gigaspora* and *Scutellospora*) in the apoplastic space between the plant cells (intercellular), as well as intracellular vesicles and coils in the exodermal cells as it passes through them (Fig. 1). The intraradical mycelium finally terminates in intracellular highly branched structures named arbuscules within the cortical cells (Fig. 1C) (Smith and Read, 1997; Harrison, 1999; Bago *et al.*, 2000). However, the plant influences the intraradical development of the fungus and a single species of a fungus may show different growth patterns depending on the plant host (Gallaud, 1905; Smith and Smith, 1997). Recently, Cavagnaro *et al.* (2001) showed that these growth patterns could also be determined by fungal identity. The two main types are referred to as *Paris* and *Arum* types. The main characteristics of the two types are as follows:

Paris: Absence of intercellular hyphal growth in the root cortex and presence of many intracellular coils, directly linked to each other from cell to cell. Furthermore, few or absent arbuscules. This type occurs frequently in the plant kingdom, but the type is not well studied.

Arum: Extensive intercellular hyphal growth and development of intracellular arbuscules. Indeed, much experimental work focuses on this type because of their presence in many crop species.

Due to this, all the work referred to in the following thesis focuses on *Arum* type.

The intracellular mycelium does not disrupt the plasmalemma of the host cell, but penetrate the cell walls so that fungal and plant host are in close contact, separated only by their membranes and a narrow plant-derived apoplast (Fig. 2). For the arbuscules this results in a huge increase in the plant-to-fungal contact surface area and, owing to this, the arbuscules have been assumed to be responsible for the C flow from the plant to the fungus together with the release of nutrients such as P and nitrogen from the fungus to the plant (Harley and Smith, 1983; Blee and Anderson, 1998). This has been supported by indirect

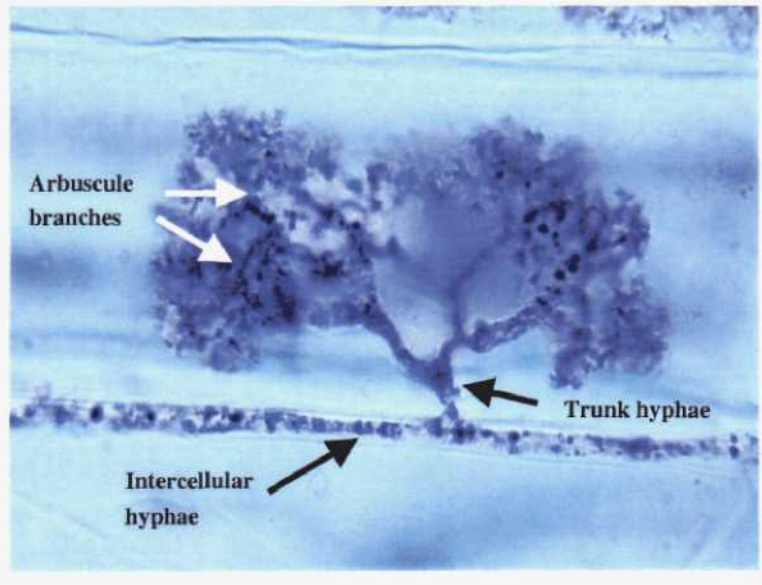

Fig. 2. Metabolically active arbuscule inside a plant root cell. The arbuscule was double-stained with nitroblue tetrazolium and acid fuchsin (Modified from Dickson and Smith, 2001).

evidence for the transfer of P (Rosewarne *et al.*, 1999; Gianinazzi-Pearson *et al.*, 2000; see 1.3.5), however, the site of transfer of C has been questioned since evidence for C transfer at the arbuscules is not available (Gianinazzi-Pearson *et al.*, 1991; Bago *et al.*, 2000). The C transfer site could as well be intercellular hyphae or hyphal coils (Dickson and Kolesik, 1999; see 1.2.3). During the formation of an arbuscule the cell undergoes a number of dramatic changes in organization of organelles; the vacuole is fragmented, the nucleus migrates to a central position within the cell and the number of organelles are increased (Carling and Brown, 1982; Balestrini *et al.*, 1992; Bonfante and Perotto, 1995). Despite the intensive effort expended by both symbionts to develop the arbuscule and the transfer site, the life span of an arbuscule is only a few days, after which it collapses and decays leaving the cell undamaged and capable of hosting another arbuscule (Harrison, 1999).

When the fungus is well established in the root cortex, the extraradical mycelium of the AM fungus extends out into the surrounding soil. Indeed, an extensive hyphal network grows from AM roots (Jakobsen *et al.*, 1992a). The extraradical mycelium forms mainly runner hyphae of several orders (Friese and Allen, 1991) but also characteristic fine hyphae have been described (Nicolson, 1959; Friese and Allen, 1991; Bago, 2000). These fine hyphae have recently been renamed branched absorptive structures (BAS), described for *Glomus intraradices* grown on agar plates (Bago *et al.*, 1998; Bago, 2000) and for *G. mosseae* grown in soil using an inserted membrane technique (Baláz and Vosátka, 2001) (Fig. 1D). Finally, spores are formed completing the fungal lifecycle (Fig. 1E and F). The extraradical mycelium is responsible for the uptake of mineral nutrients and their translocation to the plant. The mycelium also contributes to soil stability by the aggregation of soil particles (Tisdall, 1991; Tisdall *et al.*, 1997), probably mediated in part by glycoproteins produced by the hyphae (Wright and Upadhyaya, 1996). The active extraradical mycelium and intercellular hyphae are coenocytic, but localized loss of activity is associated with retraction of the cytoplasm and formation of cross walls (Bago *et al.*, 1998).

1.2.3 Nutrient exchange between symbionts

An understanding of AM physiology requires that the metabolism of the transferred C and mineral nutrients is understood. The C metabolism of AM fungi has been covered in a number of reviews, the latest by Bago *et al.* (2000), and an overview of the latest results is given here. The mineral nutrients supplied by the fungus, in this thesis only concerning P, will be further described in the following section 1.3.

An important unanswered question about AM fungi is why they do not complete their life cycle in the absence of symbiosis with a host root. In contrast to ectomycorrhizal fungi that can develop and complete their lifecycle without a host plant enabling studies and comparisons of their metabolism both under symbiotic and asymbiotic conditions, AM fungi are fully dependent of the host plant for C supply. AM fungi stop growing unless they establish a functional symbiosis with a host root, the fungus cannot take up C from any other structure or source than from the root interior (Shachar-Hill *et al.*, 1995; Bago *et al.*, 1999; Pfeffer *et al.*, 1999; Smith *et al.*, 2001). This has been a serious hindrance to studying AM metabolism, and as a result, much less is known about the metabolism of AM than

about ectomycorrhizas. However, despite the failure so far to culture AM fungi axenically, they can be grown in sterile culture with transformed roots (monoxenic culture) (Bécard and Fortin, 1988), and the development of this *in vitro* culture system to the split-dish system (St-Arnaud *et al.*, 1996) has facilitated further examinations. Intensive studies using AM roots produced in pots or the divided *in vitro* system, together with ^{13}C-labelling and nuclear magnetic resonance (NMR; see 1.3.6) (Shachar-Hill *et al.*, 1995; Pfeffer *et al.*, 1999) have revealed that the intraradical fungal structures absorb sugars as hexoses (glucose and fructose) effectively from the root in the symbiotic state. The sugars are then metabolized to small amounts of carbohydrates (trehalose and glycogen) which serve to buffer the intracellular concentration of glucose for use in the oxidative pentose phosphate pathway (PPP) for production of pentose for nucleic acid synthesis. However, the studies mentioned do not exclude that the flux through the trehalose and glycogen pools is high, with a high activity in the common metabolic pathway as a result. The finding of trehalose and glycogen is consistent with the known presence of the enzyme glucose phosphate dehydrogenase, which initiates the PPP from glucose-6-phosphate, in intraradical hyphae of *Gi. margarita* (Saito, 1995). This study also revealed hexokinase activity in intraradical hyphae, an enzyme that catalyzes phosphorylation of glucose to glucose-6-phosphate, which is the first step of glycolysis. Furthermore, large amounts of neutral storage lipids (triacylglycerides) are synthesized by the fungus within the root and lipids are then proposed to be stored or translocated to the extraradical mycelium for storage and metabolism (Pfeffer *et al.*, 1999) (Fig. 3). These studies suggest that there is little or no lipid synthesis in the extraradical mycelium, and therefore the metabolism of C is highly specialized and very different in the extraradical and intraradical mycelia. Indeed, a recent study using *in vivo* two-photon laser-scanning microscopy showed movement of lipid bodies, supposed to be responsible for translocation of storage lipids, within the extraradical mycelium of *G. intraradices* and *Gi. margarita* (Bago *et al.*, 2002). However, the export of lipids from intraradical to extraradical mycelium does not exclude the export of both lipids and small amounts of carbohydrate, as discussed by Bago *et al.* (2000).

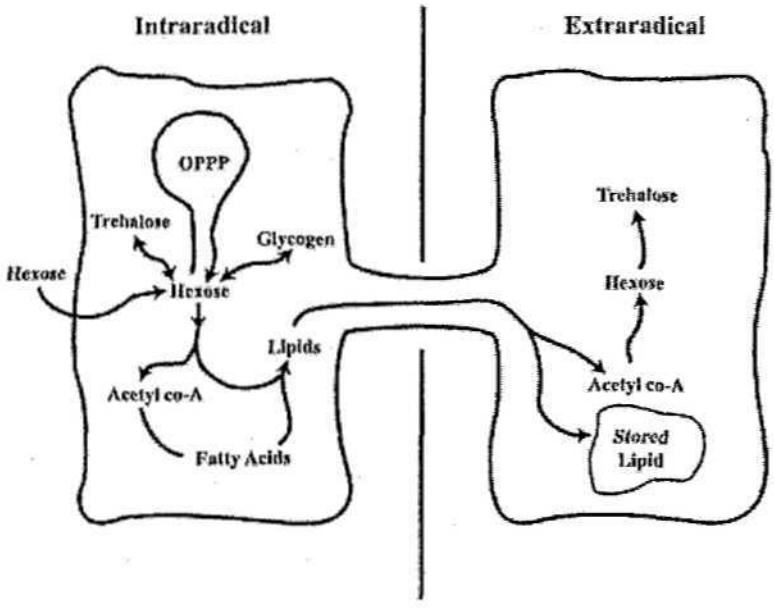

Fig. 3. Proposed model for major fluxes of carbon in the fungus in the symbiotic state of AM mycorrhizae (From Pfeffer *et al.*, 1999). OPPP; oxidative pentose phosphate pathway. This model illustrates what is known about the main fluxes of C in symbiotic AM fungi, however, it also illustrates what is omitted. As described, only few enzymes involved in the different metabolic pathways have been identified, and molecular characterization of the plant and fungal C transporters are limited (Bago *et al.*, 2000).

Evidence from a study of hexokinase activity involved in glucose phosphorylation suggests the occurrence of glycolysis and PPP in the extraradical hyphae (Ezawa *et al.*, 2001a; see 1.3.4).

Unfortunately, the mechanism by which the C effluxes from the host root and is triggered and regulated is still unclear. No plant or fungal sugar transporters involved in such efflux have yet been identified (Harrison, 1999; Bago *et al.*, 2000). However, at the level of gene expression, Harrison (1996) demonstrated induced expression of a plant hexose transporter in mycorrhizal *Medicago truncatula* roots that is probably involved in C uptake in cortical cells near intraradical hyphae (Harrison, 1999). Hence, the plant seems to retain ability to absorb glucose from the interfacial apoplast and compete with the colonizing fungus (Smith *et al.*, 2001). Although the arbuscules represent a large area of interface between the symbionts and traditionally has been assumed to be the site of C transfer from host to fungus (Blee and Anderson, 1998), the observation that the arbuscular membrane lacks ATPase activity has led to the suggestion that C uptake might occur via the intercellular hyphae, whose membranes have been observed to have a high ATPase activity and thus are energized for active transport processes (Gianinazzi-Pearson *et al.*, 1991). However, it is known that fungi can have both active and passive sugar transport systems, and consequently it is unclear whether uptake of C by the AM fungus requires an active transport mechanism similar to those of plant transporters, or whether concentrations of C at the interfaces could be sufficient to permit uptake by facilitated diffusion. So, whether arbuscules are the site of C transfer is a matter of debate (Gianinazzi-Pearson *et al.*, 1991; Smith and Read, 1997; Dickson and Kolesik, 1999; Bago, 2000; Bago *et al.*, 2000; Smith *et al.*, 2001).

During AM fungal sporulation and completion of the fungal lifecycle a large number of spores are formed, with as many as 14000 to 38000 per root estimated in monoxenic cultures (Bago *et al.*, 2000). Because 45% to 95% of the AM spore C pool is neutral lipid, the spores constitute a major sink for the C provided by the host plant (Bago *et al.*, 2000). The studies just discussed do not consider the C metabolism in asymbiotic stages of the fungal lifecycle, the germinating spores. Yet, this part of the C metabolism has also been investigated intensely recently (Saito, 1995; Bago *et al.*, 1999). This stage of fungal development exhibits characteristics of both intraradical and extraradical symbiotic hyphae. Labeling with ^{13}C and NMR experiments have shown that germinating spores can take up small amounts of hexose to be metabolized to for instance trehalose; however the synthesis of storage lipids in the asymbiotic phase is greatly reduced (Bago *et al.*, 1999). Instead, most C is transported to AM fungal spores as triacylglycerides (storage lipids) from the extraradical mycelium and sugars are presumably mostly derived from stored lipids. These triacylglycerides are used in the common pathways, with significant C-fluxes through gluconeogenesis, the glyoxylate cycle, the tricarboxylic acid cycle, glycolysis, the PPP and most or all of the urea cycle (Saito, 1995; Bago *et al.*, 1999; Bago *et al.*, 2000), to sustain growth.

To sum up, the studies just described have demonstrated what is known about C metabolism in AM fungi, i.e. most about main fluxes of C, and this section also points out that more experimental work is needed to fully understand this part of the symbiosis. However, it has been beyond the scope of my

work to investigate C metabolism in AM fungi, and from here the focus of this thesis will purely be on P metabolism and transport in AM fungi.

1.3 Phosphate metabolism and transport in AM fungi

1.3.1 Phosphate in soil

P is an essential nutrient for all organisms, being present in nucleic acids, phospholipids and metabolic compounds like adenosine triphosphate (ATP), and it is required in relatively large amounts. Plants absorb inorganic orthophosphate (P_i, $H_2PO_4^-$ ions) from the soil solution, which is present at very low concentrations, and P_i is therefore often the nutrient that is most limiting to plant growth (Richardson, 2001). Much of the normally rather large total amount of P in soil is present in poorly soluble forms as inorganic minerals (especially Ca, Fe and Al salts) or organic P-derivatives (from dead plant-parts, microorganisms and animals) that the plant can not immediately access. In addition, much P_i is adsorbed onto soil surfaces (Fig. 4).

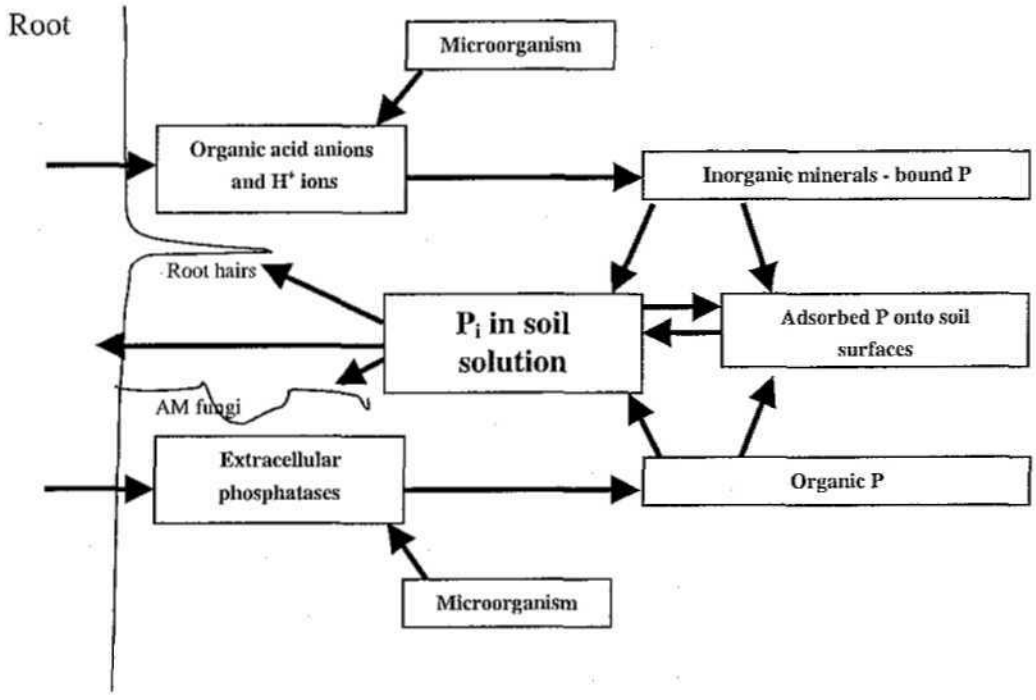

Fig. 4. Diagram of the various P pools and the balance between them in soil.

The total P content of soils averages approximately 0.05% by weight, but that of the soil solution is much lower, around 1% (< 10 μM) of the total soil P is dissolved (Barber, 1984; Smith and Read, 1997). In natural mineral soil P is predominantly found as inorganic compounds, though inaccessible to plants due to precipitation with other ions or binding to soil surfaces. On the other hand, in most

agricultural soils, most of the soil P is found in organic form, and here the P mobilization faces a completely different challenge, as described below.

The availability of P_i to the plant is limited by the slow rate of diffusion of P_i through the soil; the high affinity of P_i to different soil particles makes P_i virtually immobile in soil. The rate of diffusion of P_i ions vary with the P_i content of soil, the buffering capacity and the tortuosity of the diffusion pathway (Smith and Read, 1997). Plants continuously remove P_i from the soil solution close to the root, and since the diffusion of P_i is slow, a steep concentration gradient of P_i towards the root is developed, and an area surrounding the root may be completely drained of soluble P_i. This area without accessible P_i is usually referred to as the P-depletion zone (Nye and Tinker, 1977) (see Fig.8). The size of this zone depends on the rate of diffusion of the P_i, however in non-mycorrhizal plants this depletion zone extends to a distance of approximately 10 mm from the root surface (Li *et al.*, 1997). The formation of a depletion zone limits the P_i uptake of the plant. Plants have developed a range of mechanisms that influence the availability of soil P_i, in order to supply P_i at a rate that is adequate for optimal plant growth (Barber, 1984). These include the rate of root growth, total root length, abundance and distribution of root hairs and the kinetics of P_i uptake at the root surface (Liu *et al.*, 1998a,b; Chiou *et al.*, 2001; Richardson, 2001). In addition, biochemical processes of either plant or microbial origin that occur at the root surface further influence the availability of soil P_i to plants (Marschner, 1998; see below). However, the absorptive surface of the root system can also be greatly enlarged by colonization with AM fungi (Li *et al.*, 1991; Jakobsen *et al.*, 1992a,b).

AM fungi can increase the absorptive surface of the root system via the extensive growth of the extraradical mycelium beyond the P-depletion zone and explore the soil outside the depletion zone for nutrients (Li *et al.*, 1991; Jakobsen *et al.*, 1992a,b). This, together with a much faster hyphal translocation to the plant compared to the slow diffusion of P_i in soil and further a possible access to small soil pores inaccessible to roots, makes mycorrhizal roots much more P_i uptake effective than non-mycorrhizal roots. Furthermore, AM fungi are believed to be a part of improved drought resistance (Meddich *et al.*, 2000) and increased salt tolerance (Al-karaki, 2000) shown by AM colonized plants. However, it has often been speculated, whether these are secondary effects of improved plant P status made by the AM fungus colonization (Smith and Read, 1997).

Mobilization of phosphate by AM fungi

The ability of microorganisms to bring insoluble inorganic and organic P into solution is of great importance for the P cycling in the natural environment. Traditionally, AM fungi have been considered to play a mainly indirect role in the release of P_i from soil mineral complexes and organic compounds (Joner *et al.*, 2000a). However, there is recent evidence that the AM fungi might mineralize organic P and then make organic P available to the host plant (Joner *et al.*, 2000b; Koide and Kabir, 2000).

Inaccessible inorganic P-minerals may become available to plants due to changes in pH (P bound by ionic bonds), metal ion concentration, chelating of the metal ion by organic acid anions, water

potential and the nature of the soil colloids. P_i is most readily available at around pH 6.5. At lower pH, the decreasing solubility of Fe-P and Al-P controls the solution concentration, whereas at higher pH decreasing solubility of Ca-P becomes important (Barber, 1984; Smith and Read, 1997). Plants and fungi are principally able to change pH of the rhizosphere locally, due to efflux of protons by H^+-ATPases accompanying uptake of nitrogen as ammonium, and thus acidifying the soil (Marschner, 1998). Also, plants and some fungi may excrete organic acid anions, which chelate Fe and Al and hence make P_i more available. Some AM fungi seem to have the ability to change pH (Bago and Azcón-Aguilar, 1997; Yao *et al.*, 2001), but reliable proof of the efficiency and importance of AM fungi in pH changes of the soil remains unproven. Ectomycorrhizal fungi has been shown to excrete organic acid anions into the rhizosphere (Cumming *et al.*, 2001), however whether AM fungi do is not known as far as I am aware.

In most agricultural soils, organic P comprises 20-85% of the total P, of which the largest fraction appears to be phytin and its derivatives (Jennings, 1995). Organic P-compounds may be utilized by plants after mineralization and subsequent release of P_i. The covalent nature of the organic bound P means that release of P_i must be via a more specific mechanism than for instance pH changes. Thus, enzymes with phosphatase activity are involved in the extracellular reactions releasing P from soil organic P. Acid and alkaline phosphatases (esterases, ACPase and ALPase, respectively), which catalyze hydrolytic cleavage of the covalent C-O-P ester bond of organic P present in soil and release P as plant-available P_i, may originate from plant and soil microorganisms (Yadav and Tarafdar, 2001). However, the question about any function of extracellular phosphatases of AM fungi has been controversial because experiments have not been performed in the absence of other soil microorganisms (Joner and Johansen, 2000). The development of the split-dish *in vitro* system (St-Arnaud *et al.*, 1996) overcomes this problem, and results from studies of *G. intraradices* indicate that this fungus can hydrolyze organic P, and further, that the resultant P_i can be taken up and transported to host roots (Joner *et al.*, 2000b; Koide and Kabir, 2000). The issue is well reviewed by Joner *et al.* (2000a), where the rather conflicting results of changes in phosphatase activity as affected by presence of AM fungi are discussed. In short, existence of extracellular AM phosphatases does not seem to be of a great quantitative importance for the P nutrition, since AM fungal biomass in soil is very low. The role of AM fungal phosphatases in mineralization of organic P in soil is still rather unclear (Joner *et al.*, 2000a).

1.3.2 Mycorrhizal diversity and P nutrition

As a result of colonization with AM fungi, mycorrhizal roots in general achieve higher inflows of P_i than non-mycorrhizal roots, and are capable of maintaining a relatively high rate of uptake over much longer periods (Smith *et al.*, 2001). In some AM symbiosis the fungal contribution to P_i uptake may be significantly higher than that of the plant itself (Li *et al.*, 1991; Pearson and Jakobsen, 1993; Marschner and Dell, 1994). Furthermore, it is found that plants down-regulate their high-affinity root P transporter in an AM symbiosis, indicating that colonized plants rely heavily on P transport via the fungus during an AM symbiosis (Liu *et al.*, 1998a,b). Indeed, many AM-dependent plants show a poor soil P_i uptake when not colonized by an AM fungus. However, reports have demonstrated negative

effects of high soil P_i levels on AM formation (Mosse, 1973; Abbott *et al.*, 1984). The improved P_i uptake generally results in increased plant growth. But it is well documented that AM fungi differ in their effectiveness to supply P_i to their host and hence in their ability to enhance plant growth (Jakobsen *et al.*, 1992a,b; Pearson and Jakobsen, 1993; Smith *et al.*, 1994; Dickson *et al.*, 1999; Smith *et al.*, 2000). The growth benefit of plants in response to colonization by different AM fungi can be quite variable, ranging from dramatic increases in growth to neutral and even pathogenic reactions (Johnson *et al.*, 1997; Burleigh *et al.*, 2002). In addition, AM fungi can influence plant community composition by differently affecting the growth of different plant species (Johnson *et al.*, 1997) and mycorrhizal colonization may influence plant community diversity (Grime *et al.*, 1987; Van der Heijden *et al.*, 1998; Hartnett and Wilson, 1999; O´Connor *et al.*, 2002).

Indeed, many plant species are highly colonized by AM fungi and many plants are dependent on a successful symbiotic relationship for sufficient P_i supply, especially when soil P_i is low. It is also clear that AM fungi can translocate P_i from soil to plant root. However, our current knowledge about the mechanisms by which P_i is taken up, translocated and released towards the host plant by the AM fungi is still incomplete, as shown in the following sections concerning the recently published work on these mechanisms.

1.3.3 Uptake of P_i by extraradical AM hyphae

The extraradical mycelium of AM fungi is extremely efficient at acquiring P_i from soil. However, the fungi must absorb P_i in an active, energy dependent process across membranes since the P_i uptake occurs against a steep concentration gradient. The average concentration of dissolved P_i in the soil solution is usually in the range of 0.5 to 10 μM (Barber, 1984), compared to a likely concentration in the cytoplasm of AM fungal hyphae of 5 to 10 mM (Smith *et al.*, 2001). Studies concerning the molecular mechanisms of AM fungal P_i uptake have lagged somewhat behind, due to the obligate symbiotic nature of the AM fungi and problems in separating the two organisms in the symbiosis; it has been difficult to separate uptake and translocation. However, the development of the split-dish *in vitro* system (St-Arnaud *et al.*, 1996) has again proven to be very useful for further investigations, molecular studies of AM fungi have exploded during the last years.

The first kinetic investigations of P_i uptake in AM fungi were performed on hyphae from germinating spores of *Gi. margarita* (Thomson *et al.*, 1990). These studies suggested presence of active, high- (K_m 1.8 to 3.1 μM) and passive, low-affinity (K_m 10.2 to 11.3 mM) P transport systems similar to those described previously in *Neurospora crassa* (Burns and Beever, 1979). Later, a high-affinity P transporter, that shares structural and sequence similarity with the high-affinity proton coupled P transporters from yeast (Pho84) and *N. crassa* (Pho5) was cloned from *G. versiforme* (GvPT), with expression limited to extraradical mycelium (Harrison and van Buuren, 1995). Due to the high sequence homology to the genes encoding P transporters in yeast and *N. crassa*, the protein encoded by *GvPT* was predicted to be an integral membrane protein. The function of the protein encoded by *GvPT* as a functional P transporter was confirmed by complementation of a yeast P transport mutant, and P transport activity showed a K_m of 18 μM. This value is actually too high to cope with rapid

uptake from soil P_i concentrations of less than 10 μM, however GvPT was named a high-affinity P transporter due to its homology to Pho84 and Pho5 (Harrison and van Buuren, 1995). The high K_m value might be an artifact of the kinetic measurements being made in a yeast system (see below). Recently, the expression and regulation of a homologue transporter gene from the extraradical mycelium of the AM fungus *G. intraradices* (*GiPT*) cultured in the split-dish *in vitro* system was analyzed (St-Arnaud *et al.*, 1996; Maldonado-Mendoza *et al.*, 2001). P_i concentrations typical of those found in the soil solution resulted in expression of *GiPT*, and the study indicated that the P status of the mycorrhizal root influenced P_i uptake by extraradical hyphae and *GiPT* expression; a high P status of the mycorrhizal root resulted only in a slight induction of *GiPT*. It was suggested that the lower expression of *GiPT* was due to a source-sink effect such that P_i efflux at the arbuscule feedback regulates P_i uptake in the extraradical hyphae, a reasonable thought based on the current knowledge. The two P transporter homologues mentioned are the only type of P transporters found so far, but considering the very high efficiency of AM fungi for P_i uptake in a variety of conditions, it is likely that more than one uptake system exists, as suggested by Thomson *et al.* (1990). As will be demonstrated in the following, many investigations of the P metabolism in the fungi indicate that the various AM fungi species have different P metabolism, therefore the uptake mechanisms should not be generalized at this stage.

The uptake of P_i across the fungal plasma membrane via a P transporter requires energy. Recent work suggest that uptake of P_i occurs by electroneutral proton co-transport via the high-affinity P transporter and a plasma membrane-bound P-type H^+-ATPase (found in *G. mosseae* (GmHA5, Ferrol *et al.*, 2000 and an unpublished homologue from *G. intraradices* (GiHA5), Nielsen, 2001) as is the case for plants. The H^+-ATPase establishes the proton gradient across the fungal plasmalemma needed for the proton symport system. Expression analyses show co-regulation with the described P transporter (GiPT) suggesting a mechanism of P_i uptake as described (Nielsen, 2001).

In a recent physiological study by Schweiger and Jakobsen (1999), the kinetic parameters for P_i uptake of extraradical hyphae of *G. invermaium* were estimated. Results were based on shoot P content and root length of mycorrhizal and non-mycorrhizal *Trifolium subterraneum* plants grown at 10 levels of P_i including a number of assumptions. In this study a K_m value of 0.17 μM was estimated, a value 100 times smaller than obtained for the P transporters (18 μM; see above) but only 10 times smaller than the K_m of the high-affinity P uptake system described by Thomson *et al.* (1990) (1.8-3.1 μM; see above). However, the calculations were based on data from plants grown in soil with a high P-fixing capacity (less P_i available), which could explain the higher affinity for uptake sites for P_i than previously found. The P_i inflow into hyphae estimated from inflow to colonized parts of the roots due to hyphal uptake of P_i and total hyphal length per meter colonized root has been found to be between 0.6 and 19.2×10^{-15} mol $m^{-1}s^{-1}$ depending on AM fungal species and time of growth (Jakobsen *et al.*, 1992a; Schweiger and Jakobsen, 1999). Combined with the reported mean diameter of external AM hyphae of 2.6 μm, these values corresponded to P_i flux into hyphae of between 0.2 and 2.4×10^{-9} mol $m^{-2}s^{-1}$ (Jakobsen *et al.*, 1992a), an influx much higher than influx into non-mycorrhizal roots (8.97×10^{-11} mol $m^{-2}s^{-1}$; Schweiger and Jakobsen, 1999). An earlier reported value of inflow of P_i to hyphae

from soil was 2.25×10^{-13} mol $m^{-1}s^{-1}$ (Sanders and Tinker, 1973), two orders of magnitude higher than the values estimated by Jakobsen *et al.* (1992a). However, the differences can be explained by differences in experimental growth system. Accordingly, hyphae are very effective in absorbing P_i from the soil, especially at low soil P_i concentrations.

1.3.4 Metabolism and translocation of P in extraradical AM hyphae

Once P_i has been taken up by the extraradical mycelium, it is accumulated and translocated. AM fungi release significant amounts of P_i to their plant host besides the use of P_i in their own metabolism and translocation distances up to 7 cm have been observed (Jakobsen *et al.*, 1992b). P_i entering the cytoplasm of the AM fungus may be incorporated into phosphorylated primary metabolites, structural molecules and nucleic acids. Furthermore, transport of excess of P_i into the vacuole for storage and formation of polyphosphate (polyP) may be a major part of the mechanism by which the fungus controls the cytoplasmic P_i concentration (probably 5-10 mM as in most organisms, Smith *et al.*, 2001) and maintain P_i homeostasis in the long term (Jennings, 1995; Mimura, 1999) together with a possible localization for the P supposedly transported to the host plant.

It is widely accepted that P_i excess taken up into the AM extraradical hyphae is subsequently translocated to the vacuoles and to some extent condensed into polyP and translocated to the intraradical hyphae (Callow *et al.*, 1978; Cox *et al.*, 1980; Cooper and Tinker, 1981; Smith and Read, 1997). The presence of large amounts of P in the AM fungal vacuoles suggests that a transport system for P across the tonoplast exists in AM fungi. However, no vacuolar P transporters or accompanying vacuolar ATPases have been detected yet and the precise location of the polyP found in AM has not yet been elucidated. Furthermore, the function of polyP in controlling cytoplasmic P_i concentration has recently been questioned (Smith *et al.*, 2001).

Knowledge about P transport across the tonoplast in plants and fungi in general is limited and attempts to measure the mechanism of P transport across the tonoplast in plants have not succeeded. It is known from plants that the concentration of P_i in the vacuoles can vary considerably, in contrast to the cytoplasmic concentration, and the sharp regulation of P_i concentration in the plant cytoplasm suggests that a P tonoplast transporter exists at least in plants (Mimura, 1999).

The use of the vacuole as a reservoir for cytoplasmic P_i also applies in yeast (Ogawa *et al.*, 2000). It is known that yeast cells accumulate large amounts of P_i as polyP that is stored in the vacuole. Cytoplasmic P_i homeostasis in yeast and other polyP-accumulating fungi and algae seems dependent on the synthesis and the degradation of polyP and may therefore be a more complicated process than in higher plant cells (Mimura, 1999; see below). Therefore, another possible mechanism of regulating the cytoplasmic P_i concentration in the AM fungus could be that the P_i uptake is directly coupled to synthesis of polyP as in the case of yeast (Ogawa *et al.*, 2000; see below).

Synthesis of polyP

Inorganic polyPs are linear polymers of from three to greater than 1000 P_i residues linked by high-energy phosphoanhydride bonds (Fig. 5). PolyP is prominent in many organisms, especially so in the vacuoles of yeast, where it may represent 10-20% of the cellular dry weight (Kornberg *et al.*, 1999). As much as 37% of the total P in yeast (*Saccharomyces cerevisiae*) can be stored as polyP (Ogawa *et al.*, 2000). However, the polyP content is dependent on the P_i supply, and it is known that yeast accumulates a large amount of polyP in vacuoles under conditions of high P_i preceded by a period of P_i starvation, referred to as the "polyP overplus" phenomenon (Harold, 1966; Ogawa *et al.*, 2000). Whether this is also the case for AM fungi will be investigated in the experimental part of this thesis.

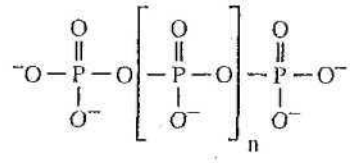

Fig. 5. Molecular structure of a linear polyP (From Kornberg *et al.*, 1999).

The extent to which polyP is synthesized depends on the other processes that are occurring inside the fungus. In prokaryotes, polyP synthesis requires the expenditure of energy and the involvement of ATP mediated by polyphosphatekinase (PPK) (polyphosphatase), which also catalyses the reverse reaction (Jennings, 1995):

$$ATP + (polyP)_n \leftrightarrow ADP + (polyP)_{n+1}$$

However, to my knowledge, ATP has not been detected in significant amounts in vacuoles nor has PPK-like activity. Therefore another mechanism for synthesis of polyP in eukaryotes should be expected.

Regulatory mechanisms for polyP synthesis

Not much is known about the regulatory mechanisms for synthesis of polyP in fungi in general. However, regulation of P_i accumulation and polyP metabolism has to some extent been studied in yeast. Recently, several genes and proteins involved in the polyP synthesis in yeast have been characterized by DNA microarray technology (Ogawa *et al.*, 2000). At least five genes code for proteins involved in the accumulation of polyP, and there is evidence for the formation of a complex for polyP synthesis on the vacuolar membrane by four of these proteins (Phm1-Phm4). In addition, the last protein Phm5 is suggested to be associated with or be a polyphosphatase (polyP-synthetase) (Fig. 6). Furthermore, the results by Ogawa *et al.* (2000) suggested that vacuolar ATPase activity is not strictly essential for polyP synthesis, as previous work had shown, but an analogous activity producing the proton motive force across the vacuolar membrane (tonoplast) is needed. In comparison, an active transport of P_i into the vacuoles of an AM fungus and subsequent or simultaneously synthesis of polyP would maintain a concentration gradient of P_i between cytoplasm and vacuole as maintained for the uptake from soil to the AM fungal cytoplasm, for as long as the transport across the tonoplast was

energized. The work of yeast also revealed the paradox of the increasing ability of the cell to convert P_i into polyP in response to P_i starvation, and it is suggested that this may represent a strategy for accumulating and holding precious P_i (Ogawa *et al.*, 2000). Consequently, polyP accumulation is required, presumably as a sink, to sustain a high rate of long-term uptake of P_i in yeast. Indeed, an *in vivo* ^{31}P NMR investigation of P metabolism and polyP dynamics in yeast in response to stress (Castrol *et al.*, 1999) revealed that P_i uptake and polyP synthesis appeared to be regulated in concert. Consequently, if this theory is true also for AM fungi, it could explain why no convincing results of polyP synthesis have appeared from work with PPK of this enzyme being present in AM fungi for synthesis of polyP.

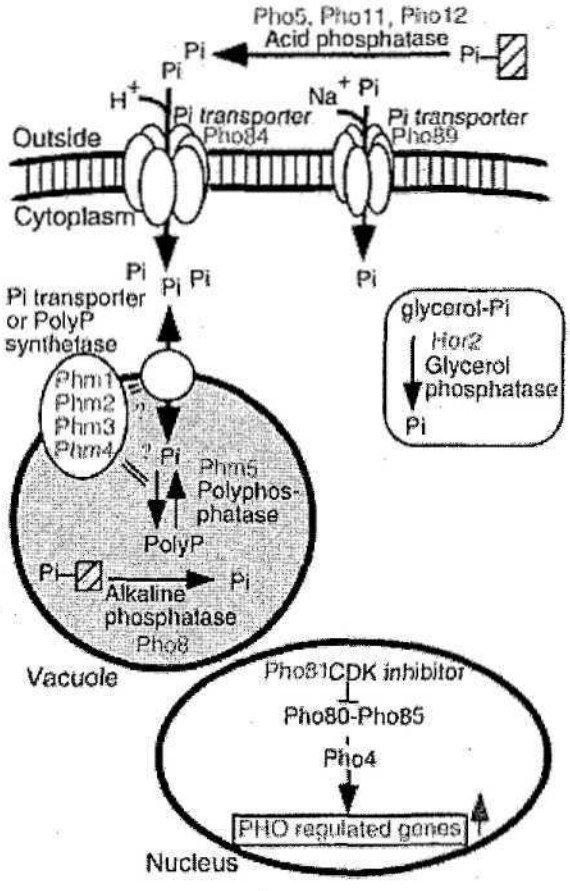

Fig. 6. Proposed P_i uptake and storage system in yeast (*S. cerevisiae*). When yeast encounters conditions of P_i starvation, the low P_i signal initiates Pho81 activity, which suppresses Pho80-Pho85 kinase activity. This inhibition results in an active Pho4 protein, which is localized to the nucleus where it acts as a specific transcriptional activator of PHO-regulated genes. The P_i starvation signal triggers increased production of at least four types of phosphatases, which can contribute to increased levels of P_i; the nonspecific ACPases Pho5, Pho11, Pho12, which are localized in periplasmic space; the nonspecific ALPase Pho8, which is localized to the vacuole; the glycerol phosphatase Hor2 and the putative polyphosphatase Phm5 which is localized in the vacuole. In addition, P_i starvation induces the expression of genes encoding P transporters, Pho84 (homologue to GvPT and GiPT; Harrison and van Buuren, 1995; Maldonado-Mendoza *et al.*, 2001) and Pho89. Finally, the polyP accumulation proceeds as explained. CDK inhibitor; cyclin-cyclin dependent protein kinase inhibitor. (Modified from Ogawa *et al.*, 2000).

Possible functions of polyP in AM fungi

As a proposed reservoir for P_i as one of the possible functions for polyP, the polymer enjoys a clear osmotic advantage over P_i (Mimura, 1999). A stable level of P_i essential for metabolism and growth can be ensured by a reservoir in which polyP can be converted to P_i by associated exopolyphosphatases (PPX; see below). In addition, polyP can be a high-energy P_i alternative to ATP as a phosphagen, since the bond between residues has a high free energy of hydrolysis equivalent to that for the terminal P_i group of ATP (Kulaev and Vagabov, 1983; Kornberg *et al.*, 1999). Indeed, some bacteria can utilize polyP as an ATP substitute via polyphosphateglucokinase (PPGK), a polyP-phosphotransferase which transfers the terminal P_i residue of polyP to glucose, producing glucose-6-phosphate. Glucose phosphorylation is the first step of glucose metabolism through glycolysis and the PPP, both of which occur in the fungi (see 1.2.3). Capaccio and Callow (1982) detected PPGK activity in the AM fungus *G. mosseae*. However, the stationary-phase levels of polyP are not thought to be a physiologically significant energy source. A recent study by Ezawa *et al.* (2001a) investigated the role of polyP as a phosphagen in spores, extra- and intraradical hyphae of *G. etunicatum* and *G. coronatum*. Hexokinase, which utilizes ATP as a phosphagen, was active in all components of both fungi, suggesting that glucose can be metabolized generally through glycolysis and PPP. Activity of PPGK was detected in the spores and intraradical hyphae of both fungi but was negligible compared with that of hexokinase. Thus, they concluded that it is unlikely that polyP is a major phosphagen in glucose metabolism of the fungi, indeed, the presence of a polyP-phosphotransferase would be expected if polyP is a phosphagen. However, keeping the detection of PPGK activity in *G. mosseae* in mind, again it should be stressed that general conclusions of mechanisms in AM fungi should be proposed rather carefully, since various fungi may behave differently. So whether or not polyP is a phosphagen in AM fungi can not be determined.

Recent literature and investigations presented in this thesis suggest that supply of P_i to the mycelium increases the amount of polyP that can be detected by toluidine blue staining of *G. etunicatum* and *G. coronatum* (Ezawa *et al.*, 2001b) and by *in vivo* ^{31}P NMR spectroscopy of *G. intraradices* (see following chapters). The amount of polyP in the intraradical and extraradical hyphae of *Gi. margarita* has been estimated from successive extractions with trichloroacetic acid (TCA) (acid soluble, short-chain polyP), ethylene diamine tetraacetic acid (EDTA) (long-chain polyP) and phenol-chloroform (PC) (granular polyP) (Solaiman *et al.*, 1999; Solaiman and Saito, 2001). These studies suggested that in the intraradical hyphae, most of the polyP was present as short-chain and long-chain forms, whereas, in the extraradical hyphae, most of the polyP was present as long-chain or granular forms. They explain these results as the possible outcome of hydrolysis of long-chain polyP into shorter chains by endopolyphosphatase (see below) in intraradical hyphae, though this has not been verified. The same work showed that polyP only contributed to between 5.4 and 17.3% of the total P, and that polyP therefore may be quantitatively less important than previously thought (Solaiman *et al.*, 1999; Smith *et al.*, 2001). As a result, the content of polyP does not seem large enough to provide a mechanism of controlling cytoplasmic P_i concentrations and reducing osmotic stress (see above). In addition, a study of the polyP contents in *G. manihotis* and *Gi. rosea* using 4′,6-diamidino-2-phenylindole (DAPI) for staining of polyP failed to detect any polyP in the extraradical mycelium of

G. manihotis, whereas *Gi. rosea* accumulated considerable amounts (Boddington and Dodd, 1999). However, the DAPI method has been shown to be very non-specific, and it is doubtful whether the DAPI staining reveals polyP (personal communication T. Cavagnaro 2002). The presence of insoluble polyP granules has often been speculated (Cox *et al.*, 1975; Cox and Tinker, 1976; Solaiman *et al.*, 1999), however, whether or not polyP granules are present in all AM fungi is a matter of debate. Orlovich and Ashford (1993) used freeze-substitution to illustrate that polyP was present in soluble form stabilized by K^+ ions in the ectomycorrhizal fungus *Pisolithus tinctorius* and that granules were probably an artefact of specimen preparation. Nevertheless, Bücking and Heyser (1999) recently showed that not all polyP granules were artifacts caused by the preparation procedure. Different species of fungi might have different P metabolism, possible due to differences in life-cycle strategies, as proposed by Boddington and Dodd (1999). The differences found in the various fungi mentioned highlight the importance of studying the development and function of a number of different species of AM fungi.

However, the presence of polyP in many AM fungi is undoubted, and it seems reasonable that a main function for the synthesis of polyP is to store large amounts of P_i to be further translocated and transferred to the host plant. Being a major transport molecule is in my point of view a very important function of polyP and it will be further discussed in the following sections concerning the possible mechanisms of translocation of P_i or polyP in AM fungi and the possible ways of breakdown of polyP.

Mechanisms of translocation of P in AM fungi

Whether or not the major form of P is as P_i or polyP, there is no doubt that large amounts of P are delivered to the interfaces between fungus and plant. The rates of P flux along extraradical hyphae have been measured in several studies to be between 1.3×10^{-3} mol m^{-2} s^{-1} in monoxenic cultured hyphae grown in Phytagel (Nielsen *et al.*, 2002), 3.8×10^{-4} mol m^{-2} s^{-1} in hyphae grown in soil (Sanders and Tinker, 1973) and 2-20 $\times 10^{-6}$ mol m^{-2} s^{-1} in hyphae grown in soil-agar (Cooper and Tinker, 1978). All values were calculated per unit cross sectional area of hyphae. Indeed, the amount of P translocated in AM hyphae is too large for passive diffusion to be responsible over the distances measured (Pearson and Tinker, 1975; Harley and Smith, 1983). Pressure-driven bulk flow is another possible translocation mechanism. If intracellular bulk flow occurs then it will override any translocation for which the driving force is a concentration gradient, and all nutrients will move in the same direction down a pressure gradient, regardless of concentration (Harley and Smith, 1983). Bi-directional translocation in the same hyphae is not then theoretically possible, and it is hard to envisage different nutrients being moved in different directions in different hyphae of the same mycelium (Harley and Smith, 1983). Furthermore, as stated in Nielsen (2001), such a mechanism must involve a flow of cytoplasm. And as revealed in the following chapters, together with previous results from AM fungi (Callow *et al.*, 1978; Cox *et al.*, 1980) and ectomycorrhizas (Ashford, 1998; Ashford *et al.*, 1999), there is evidence for the majority of P being present in vacuoles as P_i and polyP. As a result, pressure-driven bulk flow cannot be responsible for the main translocation of P in AM fungal mycelium. Harley and Smith (1983) discussed the evidence for another translocation mechanism that involved the directional movement of P as polyP in discrete compartments (e.g. vacuoles), powered by

cytoplasmic streaming. Bi-directional cytoplasmic streaming has been observed in extraradical AM mycelium (Cox *et al.*, 1980; Cooper and Tinker, 1981; Giovannetti *et al.*, 2000; Nielsen *et al.*, 2002) and may be involved in P translocation. However, in ectomycorrhizas, directional movement of P-rich (including polyP) pleiomorphic motile tubular vacuoles has been proposed as the transport system (reviewed by Ashford, 1998). Several studies have revealed that vacuoles have a high degree of plasticity and are rarely single compartments, but are part of a microtubule-connected, highly mobile system (Ashford *et al.*, 1994; Cole *et al.*, 1998; Hyde *et al.*, 1999). Indeed, there is recent evidence for tubular vacuoles in AM fungi (Uetake *et al.*, 2002). This work demonstrated the uptake and concentration of a putative vacuolar specific dye into the tubular system, examined by confocal microscopy of germ tubes and extraradical hyphae of *Gi. margarita*, suggesting existence of vacuoles in a tubular system. Furthermore, observed velocities of various organelles presumably including vacuoles measured from extraradical hyphae in monoxenic cultures are comparable with the mentioned P flux-rates (Giovannetti *et al.*, 2000; Nielsen *et al.*, 2002), suggesting translocation of P by AM fungi in vacuoles in a motile tubular vacuole system similar to ectomycorrhizas. Indeed, even though neither Giovannetti *et al.* (2000) nor Nielsen *et al.* (2002) was able to demonstrate the nature of the translocated organelles conclusively to be vacuoles, the work by Uetake *et al.* (2002) indicates the structures to be mainly tubular vacuoles. In addition, the existence and localization of microtubules in AM fungi have been verified and examined by Timonen *et al.* (2001). They found microtubules distributed longitudinally within the mycelium, linking extra- and intraradical mycelium together. In conclusion, net directional translocation of P in polyP form could occur via transport in a system of motile tubular vacuoles, however the characterization of polyP (proportion of total P and size) needs further investigation.

Breakdown of polyP and delivery of P_i to the symbiotic interface

Once a vacuole containing P_i or polyP has been translocated to the intraradical mycelium, the P_i must exit the fungal vacuole to reach the plant. Consequently, any polyP must be broken down in the vacuoles or transported across the tonoplast to the cytoplasm and a mechanism must exist for the exit of P from vacuoles. For a possible explanation of the differences in vacuole loading (extraradical) and vacuole unloading (intraradical), the two parts of the AM fungi must have somewhat different P metabolism, and as described, polyP is believed to play a central role here. There is no reason to assume other than that polyP is in some kind of equilibrium with vacuolar P_i and that synthesis and breakdown of polyP therefore indirectly regulate the equilibrium of vacuolar P_i and cytoplasmic P_i.

PolyP can be utilized as a substrate for transferases like PPK and PPGK (see above) and by hydrolases. There are two types of polyP-hydrolyzing enzymes (anhydrases): endopolyphosphatase cleaves the internal linkages of polyP, shortening chain length; whereas PPX hydrolyses terminal residues and releases P_i. Several PPX-type activities have been observed in vacuoles, cytoplasm and cell envelope in yeast (Kornberg *et al.*, 1999). As expected, the extraradical and intraradical mycelia of AM fungi appear to have somewhat different polyP metabolism. Capaccio and Callow (1982) detected PPX-type activity with maximum activity at pH 5 in intraradical hyphae of the AM fungus *G. mosseae*, where polyP hydrolysis is therefore likely to occur. The study by Ezawa *et al.* (2001b)

showed clear differentiation of the polyP metabolism between the intra- and extraradical hyphae of two AM fungi, *G. etunicatum* and *G. coronatum*. Both fungal species have at least two different PPX-type enzymes, which differed in activity between intra- and extraradical hyphae. The two enzymes have different pH optima; high activity of one PPX-type enzyme was observed at pH 5.0 in the intraradical hyphae, whereas the activity of the other PPX-type enzyme was much higher at 7.5 in the extraradical hyphae, though the extraradical hyphae of *G. etunicatum* showed very low activity. Thus, extraradical hyphae not only synthesize polyP, but have also polyP-hydrolyzing activity. If this hydrolyzing activity is extracellular, it would be interesting in relation to hydrolysis of the possible polyP-pool in the soil, arising from the decomposition of microorganisms, as suggested in the thesis by Nielsen (2001). The results on substrate specificity by Ezawa *et al.* (2001b) showed higher substrate specificity with long-chain polyP at pH 7.5, whereas activity at pH 5.0 showed higher substrate specificity with short-chain polyP, in agreement with the results of Solaiman *et al.* (1999) suggesting longer polyP chains in extraradical hyphae compared to intraradical hyphae. Ezawa *et al.* (2001b) suggested dominance of acidic hydrolyzing activity in intraradical hyphae, and they proposed that the acidic PPX-type activity was an ACPase. The work indicated that the acidic PPX-type activity played an important role in the hydrolysis of polyP in vacuoles of intraradical hyphae prior to P_i release to the interface between the fungus and the plant. However, due to the use of crude extracts, the precise identity of the enzyme activities is obscure, apart from contributing to polyP breakdown. Without subcellular fractioning, the supposed PPX-type activities have not been proved to be vacuolar.

Nevertheless, based upon these findings, Ezawa *et al.* (2001b) suggested that in intraradical intercellular hyphae polyP concentration is maintained by the dynamic and regulated balance between synthesis and hydrolysis, with vacuolar H^+-ATPase energizing polyP synthesis and constitutively expressed PPX-type activity (possible an ACPase) responsible for hydrolysis. Whereas, in arbuscules, net hydrolysis of polyP may be increased due to inactivation of vacuolar H^+-ATPase, and this may trigger release of P_i into the apoplast (Fig. 7). However, besides being sure of presence of polyP and some kind of polyP-hydrolyzing activity, this model remains very speculative. Further characterization of the involved enzymes is required.

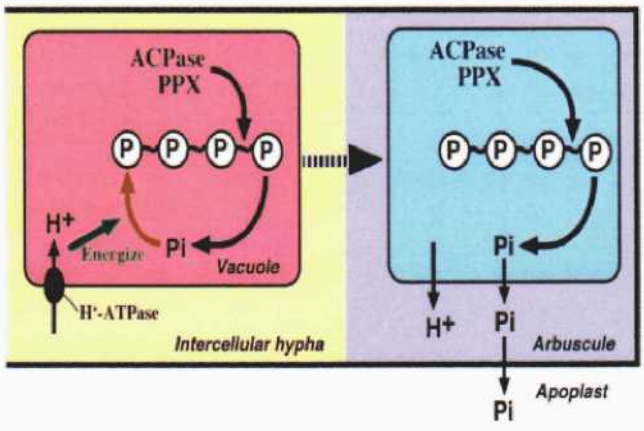

Fig. 7. Hypothetical model of polyP metabolism in the intraradical hyphae of AM fungi. ACPase, acid phosphatase; PPX, exopolyphosphatase. Red arrow: polyP synthesis, dark blue arrow: polyP hydrolysis (From Ezawa *et al.*, 2001b).

If the source of P_i for transfer to the apoplast is provided by net hydrolyses of polyP within the arbuscule, as suggested by Ezawa *et al.* (2001b), this may cause a potential pH problem. Hydrolysis of polyP generates H^+ ions and lowers pH, and this could create a problem for local pH regulation in both the vacuoles and the cytoplasm of the fungus. However, results of Guttenberger (2000) could be interpreted as acidification of the arbuscule itself, with the arbuscule sealed off from the trunk hyphae by a cross wall (Smith *et al.*, 2001), then supporting the idea of H^+ release during hydrolysis of polyP. Indeed, a recent study by Dickson and Smith (2001) demonstrated the presence of cross walls in arbuscular trunk hyphae after loss of metabolic activity. In this study it was suggested that P_i could be trapped in the arbuscule, with the development of the cross wall preventing back flow of P_i to the intercellular hyphae. Still, this hypothesis also remains very speculative and the precise location of the acidic compartment has to be confirmed before the location of polyP hydrolysis can be determined.

Other intracellular phosphatases in AM fungi

Intracellular ALPase activity has been shown in both extraradical (Zhao *et al.*, 1997; Vosatka and Dodd, 1998; Boddington and Dodd, 1999; Kjøller and Rosendahl, 2000; van Aarle *et al.*, 2001) and intraradical AM fungal mycelium (Gianinazzi-Pearson and Gianinazzi, 1978; Tisserant *et al.*, 1993; Ezawa *et al.*, 1995; Saito, 1995; Boddington and Dodd, 1999) of various species of AM fungi. ACPase activity has also been demonstrated in both extraradical (van Aarle *et al.*, 2001) and intraradical AM mycelium (Gianinazzi *et al.*, 1979; Ezawa *et al.*, 1995; Saito, 1995; Ezawa *et al.*, 2001b). In the intraradical mycelium both ALPase and ACPase activity has been localized in the vacuoles of intercellular hyphae and arbuscules (Gianinazzi-Pearson and Gianinazzi, 1978; Tisserant *et al.*, 1993; Ezawa *et al.*, 1995; Saito, 1995). Accordingly, ACPases (Ezawa *et al.*, 2001b; see above) and ALPases (Gianinazzi-Pearson and Gianinazzi, 1978; Tisserant *et al.*, 1993; Ezawa *et al.*, 1995) have been thought to be involved in polyP breakdown. The ALPase activity in intraradical hyphae has been found to be related to the stimulation of the growth of plants when infected by AM fungi (Tisserant *et al.*, 1993; Kojima *et al.*, 1998) and Tisserant *et al.* (1993) proposed that the fungal ALPase could provide a useful marker for analyzing the symbiotic efficiency of AM fungi. In addition, studies have shown that there are differences between species of fungi in the localization of intraradical ALPase activity, and this difference might reflect different sites for P_i transfer, as discussed for C (Ezawa *et al.*, 1995; see 1.2.3). However, Larsen *et al.* (1996) found no correlation between intraradical ALPase activity and fungal P transport, suggesting ALPase activity not related to P metabolism. Finally, Ezawa *et al.* (1999) found an ALPase in arbuscules of *G. etunicatum* with apparent substrate specificity for glucose-6-phosphate and none for pyrophosphate compounds such as ATP and polyP, indicating that the ALPase may be involved in the metabolism of C-compounds, and thereby release P_i into the apoplast. The study by Solaiman and Saito (2001) of P_i efflux from intraradical hyphae of *Gi. margarita* supported this hypothesis. They found that addition of glucose enhanced P_i efflux from intraradical hyphae, and proposed that glucose increased polyP hydrolysis and further glucose was being phosphorylated to glucose-6-phosphate. This glucose-6-phosphate was then hydrolyzed with ALPase with increased P_i efflux as a result. However, how glucose stimulates polyP hydrolysis was not discussed, and the breakdown of polyP is therefore still the critical point in the release of P_i from the vacuoles in the intraradical hyphae which is needed for direct transfer of P_i to the apoplast or via

an intermediate C-compound. In conclusion, the role of ALPase has not been satisfactory determined with respect to P metabolism and polyP breakdown. The strong indications found by Ezawa *et al.* (2001b) that a non-specific intraradical ACPase was involved in hydrolysis of polyP suggests that ACPase activity is more likely involved in P_i transfer from intraradical mycelium to host. However, the function of phosphatases in the P metabolism in AM fungi is not clear and more detailed studies of ALPases and ACPases in different species of AM fungi are needed.

ALPase activity in the extraradical mycelium might be an effective marker for metabolic activity in studies of AM fungi (e.g. Zhao *et al.*, 1997; Vosatka and Dodd, 1998; Kjøller and Rosendahl, 2000). Several colorimetric staining techniques have been developed, as it will be further demonstrated in the following chapters with emphasis on the enzyme-labeled-fluorescence (ELF) (van Aarle *et al.*, 2001). The ELF method has been shown to be much more sensitive for staining of phosphatases than for example the Fast blue salt (FB) staining method (van Aarle *et al.*, 2001). ELF precipitated in AM fungi with both acid and alkaline buffers, suggesting both ALPase and ACPase activity, respectively (van Aarle *et al.*, 2001). Young spores showed higher precipitation than older spores, however, this could be a penetration effect or the fact that old spores are full of lipid. Specific staining of AM fungal structures in roots was observed when alkaline buffer was used, not with acid buffer, and by this, the ELF substrate could be used as an indicator of metabolically active fungal tissue. The work by van Aarle *et al.* (2001) revealed a patchy distribution of the ELF precipitates in the extraradical mycelium, probably due to a high precipitation in the vacuoles, as expected when vacuoles are believed to be the main P storage and transfer organelle. However, care should be taken in relating ALPase to P metabolism (see above). FB staining showed no effect of increasing P addition on the ALPase activity in the extraradical mycelium of *Gi. rosea* and *G. manihotis* (Boddington and Dodd, 1999). Instead, low levels of ALPase activity were observed in the extraradical mycelium and auxiliary cells of *Gi. rosea* during early development of this fungi. This coincided with accumulation of polyP in the auxiliary cells. The ALPase activity increased in the auxiliary cells only after a decline in polyP accumulation, indicating a negative feedback mechanism at this state of development of the fungi.

1.3.5 Transfer of P_i from AM fungus to plant

Once P_i has been translocated to the symbiotic interface, it has to be transferred to the plant root cells in order for the symbiosis to be functional. P_i transfer between fungus and host plant is assumed to occur across the arbuscule-plant cell interface. Plant P transporters which are induced by P starvation have been shown to be down-regulated in mycorrhizal *M. truncatula* roots, indicating that these transporters are not involved in symbiotic P_i transfer but in P_i uptake from the soil (Liu *et al*, 1998b; Chiou *et al.*, 2001). Monovalent cations stimulated the passive efflux of P_i from hyphae of the ectomycorrhizal fungus *Pisolithus tinctorius* (Cairney and Smith, 1993), and a similar mechanism might be present in AM fungi. Indeed, the fact that the host plant experiences a high sink strength combined with a high source of P_i in the fungus, could be responsible for the transfer of P_i in a passive efflux towards the plant (Smith *et al.*, 2001). Furthermore, the fungal P transporter GvPT seemed not to be expressed in the intraradical fungal structures (Harrison and van Buuren, 1995), this would reduce the reabsorption of P_i by the fungus from the arbuscular apoplast and promote net P_i transfer

towards the plant (Rosewarne *et al.*, 1999). However, it is predicted that AM fungi contain an efflux mechanism (or mechanisms) to release P_i from the arbuscule and that plants posses a plant uptake system to transport P_i into the cortical cell (Smith and Smith, 1990). P_i flux across the symbiotic interfaces has been estimated to be between 3 and 15×10^{-9} mol m^{-2} s^{-1}, assuming that the arbuscules provide the main interface for transfer (Cox and Tinker, 1976; Dickson *et al.*, 1999), and based on these findings, the AM fungi likely have some type of specialized efflux mechanism operating in the arbuscular membrane to permit sufficient P_i efflux to the arbuscular interface. Nevertheless, the mechanism by which the fungus is induced to release P_i at the arbuscular interface remains circumstantial.

Indeed, there is some indirect evidence that P_i transfer from the fungus to the plant may occur at the arbuscular interface; a high-affinity plant P transporter gene (*LePT1*), which is responsible for P_i uptake driven by a pH gradient across plasma membrane, was expressed in arbuscule-containing cells of tomato (Rosewarne *et al.*, 1999). And a possible plasma membrane H^+-ATPase which generates a pH gradient across the plant plasma membrane has been found on the periarbuscular membrane in root cells of transgenic tobacco in symbiosis with *G. fasciculatum* (Gianinazzi-Pearson *et al.*, 1991; Gianinazzi-Pearson *et al.*, 2000). Recently, a P transporter gene (*StPT3*) in potato has been identified (Rausch *et al.*, 2001) and the molecular work indicated expression of the gene in root sectors when mycorrhizal structures are formed. The work gave rise to the hypothesis that the protein encoded by *StPT3* functions as an AM symbiosis specific P transporter active in the periarbuscular membrane. The protein exhibits high homology to GvPT responsible for the P_i transport across the plasmalemma in the extraradical mycelium of an AM fungus, and it is proposed that the StPT3 protein mediates P_i acquisition in the root-fungal interface similar to GvPT in the extraradical hyphae.

To summarize the just described work on P metabolism by the AM fungi, a hypothetical model is presented here (Fig. 8).

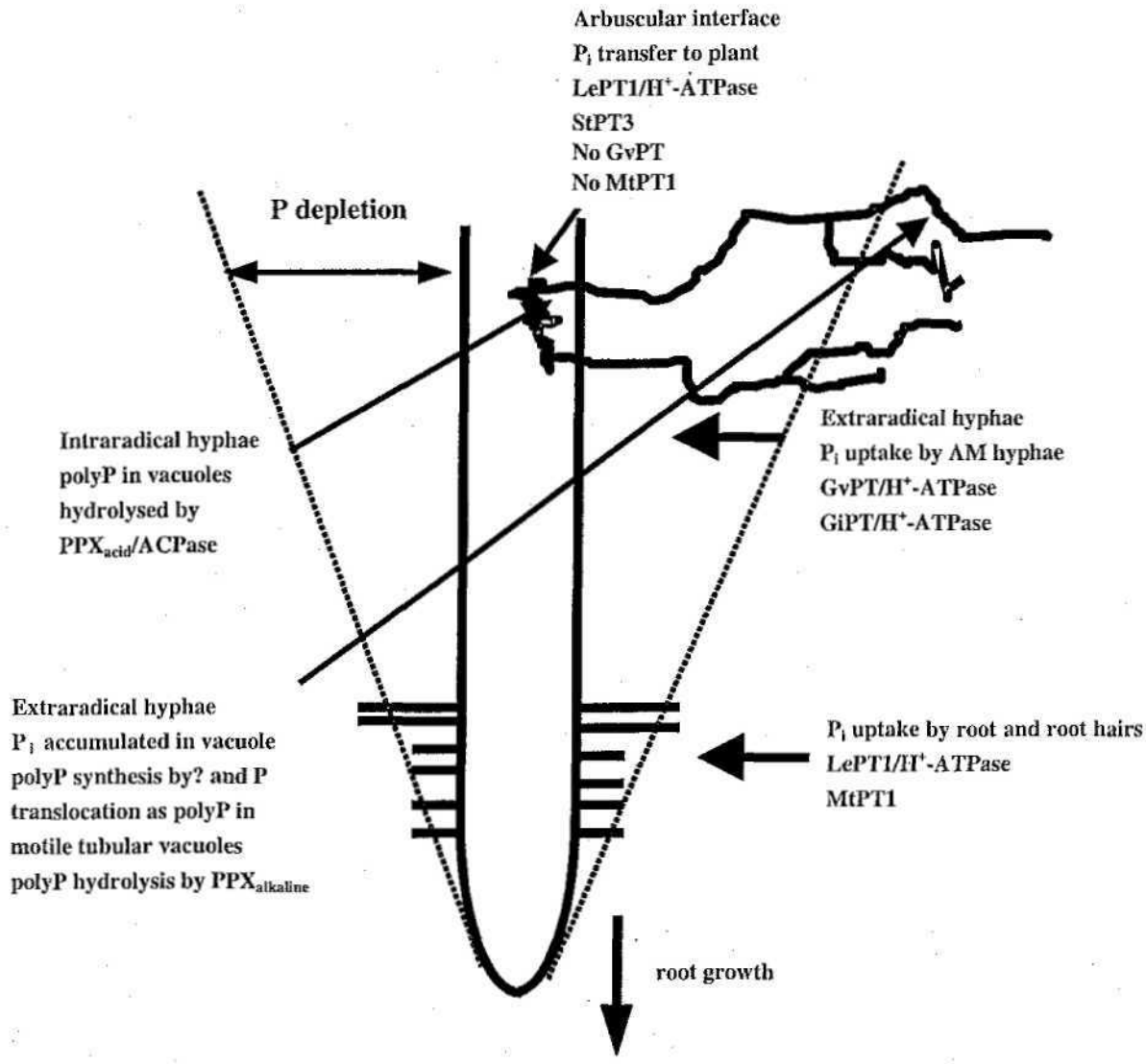

Fig. 8. Hypothetical model of P metabolism by AM fungi (Modified from Smith *et al.*, 2001)

1.3.6 *In vivo* ^{31}P NMR for the study of P metabolism

As it can be seen from the above sections some of the most important questions about AM fungi concern the metabolism of the nutrients transferred between the symbionts. More detailed information on metabolism and transport is required. Approaches applied to understand the P metabolism include investigations of enzymes involved in P and polyP metabolism (Gianinazzi-Pearson and Gianinazzi, 1978; Tisserant *et al.*, 1993; Ezawa *et al.*, 1995; Saito, 1995; Ezawa *et al.*, 2001a,b), detection of polyP using extraction procedures and polyacrylamide gel electrophoresis (Callow *et al.*, 1978; Solaiman *et al.*, 1999), studies of P transport by hyphae using radiotracer techniques (Cooper and Tinker, 1978; Jakobsen *et al.*, 1992a,b; Schweiger *et al.*, 1999) or phosphoimaging (Nielsen *et al.*, 2002) and characterization both of fungal and plant P transporters (Harrison and van Buuren, 1995; Liu *et al*, 1998a,b; Rosewarne *et al.*, 1999; Chiou *et al.*, 2001; Maldonado-Mendoza *et al.*, 2001;

Rausch *et al.*, 2001). However, in several of the investigations mentioned, the P metabolism has only been examined with invasive methods, i.e. with possible interfering of the metabolic processes or enzyme activities involved. For creating a link between results of fungal metabolism obtained using destructive methods and real-life biological processes, there is a need for studies using non-invasive and non-destructive techniques. For this purpose, non-destructive and non-invasive *in vivo* ^{31}P NMR spectroscopy is a unique analytical method for the study of P metabolism in fungal and plant tissue (Ratcliffe, 1996).

NMR has contributed significantly to the understanding of the pathways and regulation of C metabolism (see 1.2.3) and P metabolism in mycorrhiza (reviewed by Pfeffer *et al.*, 2001). Because NMR can identify metabolites and further gives information on the amounts, forms and locations of various metabolites it has been a useful method in studying metabolism and transport in both ectomycorrhizal and AM fungi. Although the physiological questions asked in both ectomycorrhizal and AM fungi are frequently the same, these two symbioses are very different. Ectomycorrhizal fungi can grow in a free-living state so it is possible to follow and compare their metabolism both under symbiotic and asymbiotic conditions. In contrast, the obligate nature of the AM fungus has been a serious hindrance to study this particular system. However, *in vivo* ^{31}P NMR spectroscopy is very suitable for studying the dynamic behavior of P uptake and to investigate any polyP metabolism in mycorrhizal tissue. ^{31}P NMR spectroscopy has been used for *in vivo* studies of P metabolism of ectomycorrhizal fungi cultured under axenic conditions. When P_i was added to ectomycorrhizal fungi, polyP signals were found in the ^{31}P NMR spectra (Martin *et al.*, 1983; Martin *et al.*, 1985; Ashford *et al.*, 1994; Gerlitz and Werk, 1994; Martin *et al.*, 1994; Gerlitz and Gerlitz, 1997; Martins *et al.*, 1999). NMR studies of mycorrhizal roots also have been carried out with emphasis on ectomycorrhizas, as in the studies of intact mycorrhizal red pine roots (MacFall *et al.*, 1992), living mycorrhizal beech root tips (Loughman and Ratcliffe, 1984) and mycorrhizal beech and pine roots (Gerlitz and Werk, 1994; Gerlitz and Gerlitz, 1997). In contrast, there have been very few *in vivo* ^{31}P NMR investigations of AM fungi, with just one study of AM roots of leek and germinating spores (Shachar-Hill *et al.*, 1995), and no published NMR work on P metabolism in the extraradical mycelium of AM fungi besides the work presented in this thesis. The principles of NMR and the applications of ^{31}P NMR spectroscopy to plant systems are further described in the following chapters.

1.4 Summary and objectives of the thesis

Experimental evidence of how P is utilized and translocated in the AM fungi and transferred to plants is still limited. The uptake, translocation and transfer of P by the extraradical mycelium of the AM fungi have been studied extensively, and a model of the overall mechanisms has been widely accepted. It is believed that P_i in the soil solution is absorbed by the extraradical mycelium via an AM fungal P transporter energized by a P-type H^+-ATPase (Harrison and van Buuren, 1995; Ferrol *et al.*, 2000; Maldonado-Mendoza *et al.*, 2001). The P_i entering the cytoplasm of the AM fungus may be incorporated into phosphorylated primary metabolites, structural molecules and nucleic acids. It is assumed that P_i excess taken up into the AM extraradical hyphae is subsequently transferred to the vacuoles and partly condensed into polyP. The P-containing substances such as polyP are then

believed to be translocated to the intraradical hyphae in vacuoles in a motile tubular system similar to that of ectomycorrhizas (Smith and Read, 1997). Recent studies of the vacuolar system in AM fungi have confirmed the presence of tubular vacuoles and microtubules (Timonen *et al.*, 2001; Uetake *et al.*, 2002). Once translocated to the symbiotic interface inside the root, the polyP has to be hydrolyzed and the released P_i subsequently transferred to the plant root cells to achieve a mutualistic symbiosis. This transfer is believed to occur at the arbuscular interface, which is in agreement with the recent discovery that plant P transporters are expressed in root cells containing arbuscules (Rosewarne *et al.*, 1999; Rausch *et al.*, 2001). In addition, incubation of extracted intraradical mycelium of *Gi. margarita* in glucose increased the efflux of P_i and polyP content in the hyphae decreased simultaneously, indicating a role for polyP in the exchange of C and P_i between symbionts (Solaiman and Saito, 2001). Accordingly, polyP is considered to have an important role in the P translocation process. In addition, polyP as a storage form enjoys a clear osmotic advantage over P_i and synthesis of polyP may be a major part of the mechanism by which the fungus controls the cytoplasmic P_i concentration (Mimura, 1999). PolyP has been detected in AM fungi by cytochemical methods (Cox *et al.*, 1975; Cox *et al.*, 1980; Boddington and Dodd, 1999; Ezawa *et al.*, 2001b), by extraction methods followed by polyacrylamide gel electrophoresis (Callow *et al.*, 1978; Solaiman *et al.*, 1999) and by NMR (Shachar-Hill *et al.*, 1995). However, the amount, size and major role of polyP present in the extraradical and intraradical hyphae is a matter of debate. Several investigations suggest the presence of rather long-chain polyP or granules especially located in the extraradical mycelium (Callow *et al.*, 1978; Solaiman *et al.*, 1999), supporting the idea that polyP metabolism in extraradical and intraradical hyphae may be different. Further, the extraradical mycelium of the AM fungus *G. manihotis* seems not to accumulate polyP in comparison with high amounts of polyP in the extraradical mycelium of *Gi. rosea* (Boddington and Dodd, 1999), suggesting differences in polyP metabolism between species.

In conclusion, the presence of polyP in many species of AM fungi is well documented, but the characterization of the polyP and the mechanisms involved in its metabolism are not clear. Staining methods with variable specificity or invasive methods have commonly been used to identify polyP in previous investigations, such that artifacts of specimen preparation could possibly have interfered with the polyP chain length, as discussed by Orlovich and Ashford (1993). Non-invasive and non-destructive techniques are required in order to obtain more detailed information of P pools and polyP content in AM fungi. For this purpose, *in vivo* ^{31}P NMR spectroscopy is a unique analytical method.

The main objective of the present work was **to use ^{31}P NMR spectroscopy, *in vivo* and on extracts, for investigating P metabolism in mycorrhizal and non-mycorrhizal roots and in extraradical mycelium** of various species of AM fungi, and by that way contribute to the understanding of the P and polyP metabolism and translocation in AM fungi. **The potential use of *in vivo* ^{31}P NMR spectroscopy for the study of P pools and their dynamics in AM fungi and roots was evaluated.** I wanted to investigate the **dynamics of polyP synthesis** in AM fungi, and determine **how fast polyP was synthesized, which chain lengths could be detected** and **in which compartment the polyP was located**. Chain lengths of polyP where further investigated by the use of extraction procedures followed by colorimetric measurements and ^{31}P NMR. Several AM fungal species were included in

the study to investigate aspects of fungal diversity in the overall polyP content and P transport capacity of the fungi. Finally, the active state of P metabolism in the mycorrhiza was confirmed by means of ELF staining for ALPase activity.

Chapter 2 - Methods and preliminary experimental work

2.1 Outline of experimental work carried out

This chapter is an introduction to the development and evaluation of methods used in the present work, which aimed to use ^{31}P NMR as a non-invasive approach for the investigation of the P metabolism and dynamics in AM fungi. The project looked very straightforward at the early start: The chosen compartmented growth system for production of extraradical mycorrhizal hyphae had already been used with success and the expertise with *in vivo* NMR equipment was already implemented to some extent for other purposes (see Scharff, 2001). However, several technical problems during the initial phase had to be solved first. The optimization of the growth system for production of sufficient and biologically active plant and fungal material is described in section 2.2 and in Chapter 3. The development of an experimental *in vivo* NMR setup that was capable of maintaining the biological material in a physiologically vital state during the NMR experiments is described in sections 2.3, 2.4 and Chapter 3. Chapter 2 also includes an introduction to the fundamental principles of NMR spectroscopy, a more specific introduction to *in vivo* ^{31}P NMR applications and a description of other methods used for further investigation of the P metabolism in AM fungi.

Experiments were then carried out as described in the following.

A time-course ^{31}P NMR investigation of the formation of P pools in differently P-treated AM hyphae and mycorrhizal roots was carried out in order to characterize the incorporation of P_i into various P species within the extraradical mycelium and the mycorrhizal roots. The results of this are presented in Chapter 4.

The results obtained were rather different from published work in the field (e.g. Solaiman *et al.*, 1999; Ezawa *et al.*, 2001b), and attempts were made to further characterize the polyP detected. In particular the rather conflicting results of polyP chain length were pursued and investigated by the use of extraction procedures and colorimetric measurements, which is described in section 2.5 and Chapter 4. Furthermore, ALPase-type activity in the extraradical hyphae and mycorrhizal roots was characterized by the ELF method (van Aarle *et al.*, 2001), in order to localize aspects of P metabolism. Results from this are included in sections 2.5, 2.6 and Chapter 4.

A ^{31}P NMR investigation of four P-treated AM fungi was carried out in order to further investigate the possible variation among AM fungal species in their overall P transport capacity and by this contribute to the understanding of diversity of AM fungi. The fungi included were *G. intraradices*, *G. mosseae*, *S. calospora* and *Gi. rosea*, and results from this study are presented in section 2.6.

2.2 Plant material and growth systems

The plant chosen for the work described in this thesis was cucumber (*Cucumis sativus* L. cv. Aminex, F1 hybrid), in majority of the work grown in symbiosis with the AM fungus *Glomus intraradices* Schenck & Smith (BEG 87 or DAOM 197198). Clover (*Trifolium subterraneum* L.) was also tested for maximal production of extraradical mycorrhizal hyphae. However, symbioses between cucumber and *G. intraradices* produced the highest amount of extraradical mycelium and the growth of both plant and fungus was overall largely reproducible. Other species of AM fungi used included *Scutellospora calospora* (Nicol. & Gerd.) Walker and Sanders isolate WUM 12 (BEG 43), *Glonus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe isolate V296 (isolated by M. Vestberg) and *Gigaspora rosea* Nicolson & Schenck (BEG 9), as presented in the section 2.6.

An experimental compartmented growth system suitable for production of pure extraradical mycelium was developed and described by Johansen *et al.* (1996). This growth system was used with a few adjustments and optimizations for possible harvest of a high amount of young biologically active hyphae without large numbers of spores. A detailed description of the growth system, overall experimental design and harvest procedure is given in Chapters 3 and 4; however, the principles and optimization procedures will be presented here. The cucumber plants were grown in an irradiated (10 kGy, 10 mV electron beam) 1:1 soil and sand mixture (here called 'soil') in a mesh-bag which prevent root penetration but allow free passage of AM fungal hyphae. Basal nutrients minus P were added to the soil (see Chapters 3 and 4) and the final soil had an extractable P content of 11 $\mu g\ P\ g^{-1}$ as obtained with 0.5 M $NaHCO_3$ (Olsen *et al.*, 1954). The mesh-bag was filled with 700 g (Table 1; Chapter 3) or 725 g (section 2.6; Chapter 4) soil into which was incorporated 100 g (Table 1; Chapter 3) or 75 g (section 2.6; Chapter 4) inoculum from a *T. subterraneum* L. pot culture. The mesh-bag was filled with 800 g of soil when experiments included non-mycorrhizal controls. The extraradical hyphae grow into 2200 g washed, autoclaved quartz sand surrounding the mesh-bag and could easily be collected from the sand by aqueous suspension and subsequent decanting onto a sieve. Root material could be collected from the mesh-bag after washing away the soil and sand. The pots were watered daily to 60% water holding capacity throughout experiments, except for one or two days before final P treatment at the end of experiments. Soil nitrogen was supplemented periodically as an aqueous solution of 0.36 M NH_4NO_3.

AM fungal mycelium was required in rather large quantities because of the relatively low sensitivity of the *in vivo* NMR measurements (see 2.3). In order to meet this demand for mycelium, plants had to be grown for 10 wk, but at this time a high spore abundance was also observed. This was undesirable because the metabolism of AM fungi differ between spores and hyphae in the symbiotic stage (Pfeffer *et al.*, 2001; Bago *et al.*, 1999; see 1.2.3). The experimental procedure therefore had to be modified in order to obtain spore free mycelium. This was achieved by replacing the sand outside the mesh-bag with fresh sand after some weeks of growth. Mycelium subsequently developed in the new sand during the following growth period. Various growth periods before and after the sand was changed were tested for their influence on hyphae production. The results are given in Table 1, which show that a 4 wk pre-establishment phase followed by 2-3 wk allowing for hyphal growth into the new sand was

sufficient for the production of the required amounts of spore free mycelium. These conditions were applied for subsequent experiments.

Table 1. Experimental conditions for maximal production of extraradical AM hyphae (*G. intraradices*) grown in symbiosis with cucumber in compartmented pots. The overall experimental design was as described above and in Chapter 3. An aqueous solution of 0.36 M NH_4NO_3 was supplied to pots weekly after two wk of growth, 50 mg each wk. No P_i was added to the pots.

No.[#]	Plant age (no sand change) (wk)	Plant age before sand change (wk)	Period after sand change (hyphae age) (wk)	Mean amount of hyphae per pot (g fw)[$] (S.D.)[†]
1	10			0.45 (0.02)
2		6	3	0.46 (0.30)
3		5	3	0.64 (0.20)
4		4	3	1.08 (0.03)
5		4	2	0.55 (0.06)

[#]The results were obtained in different experiments. No. 1 contained duplicate pots within the same experiment. No. 2, 3 and 4 were duplicate pots within one experiment. No. 5 contained three replicate pots within one experiment.

[$]fw; fresh weight including some adhering sand

[†]S.D.; standard deviation

In the case of P-treatment, KH_2PO_4 in aqueous solution could be supplied either directly to the root compartment at any time during growth or exclusively to the extraradical hyphae by supplying the P_i-solution to the sand along the edge of the pot in the hyphal compartment some time after the sand had been changed. P_i ions would move approximately 1.3 cm d^{-1} in pure water, but this will be lower in sand because of a reduced cross-sectional area for diffusion due to sand particles and because of a tortuous diffusion pathway in sand. I tested the diffusion of P_i supplied at the edge of the pot in the hyphal compartment from pots hosting non-mycorrhizal cucumber, and no P signals could be detected in the corresponding ^{31}P NMR spectra of non-mycorrhizal roots when P_i was added just before, and only traces of P signals could be detected when P_i was added 24 or 48 h before harvest (results not shown). This indicated that P_i added to the hyphal compartment did not diffuse significantly to the root compartment in two days.

Various growth media for the hyphae were also tested for their suitability to allow for rapid and gentle harvest of hyphae. Fine river sand (particles smaller than 1 mm) and sand with a larger particle size (particles 1-3 mm) were compared, and river sand was preferred due to difficulties in cleaning the hyphae from the larger particle size sand, resulting in much more adhering of sand to the hyphae.

In some of the subsequent experiments the amount of mycelium, which could be harvested from the pots, showed an undesirable variation between pots. Preliminary tests of a monoxenic *in vitro* cultivation system were performed in order to investigate an alternative experimental setup for

producing hyphae for the NMR measurements. The monoxenic system is a system from which all other organisms except fungus and plant are excluded and they can be established either in standard petri dishes (Bécard and Fortin, 1988) or in compartmented petri dishes (St-Arnaud *et al.*, 1996). The 9 cm petri dishes used for my experiment were divided in two by a plastic barrier, 7 mm high, where the extraradical mycelium was allowed to cross, allowing for a compartment with hyphae but no roots. The monoxenic cultures consisted of *G. intraradices* (DAOM 197198) and Ri T-RNA transformed carrot (*Daucus carota* L.) root. The cultures were established as described by Nielsen *et al.* (2002). P_i was supplied to the hyphal compartment the day before harvest as 2 ml 7 mM KH_2PO_4 (0.56 mM KH_2PO_4 in hyphal compartment). Fungal structures were extracted from the medium using slow stirring in 10 mM citrate buffer and hyphae were collected on a 50 µm stainless sieve and subsequently transferred to an NMR tube containing M medium (Bécard and Fortin, 1988) at pH 5.5 including 10% D_2O. The ^{31}P *in vivo* NMR measurements were performed on 15 mg fw mycelium collected from three petri dishes. The resulting spectrum is presented in section 2.6.

The main advantage in using the monoxenic system instead of the compartmented pot setup is the easy handling of the system during growth, the ability to continuously monitor each experimental unit for hyphal growth and since not space consuming, the possibility of growing more cultures than eventually needed while the mycelium is generated. This offers the possibility of obtaining more similar cultures; i.e. same amounts of hyphae to be selected for further subjection to P-treatment and time-course studies. In addition, the hyphal compartment can contain a gel-free nutrient solution allowing for much easier harvest of the mycelium (Maldonado-Mendoza *et al.*, 2001). However, further development of the setup and elaborate analysis of mycelium from monoxenic cultures has been beyond the scope of my work and will instead be suggested as a future perspective.

2.3 *In vivo* NMR spectroscopy

2.3.1 Fundamental theory of NMR

To provide a suitable background for the later presentation and discussion of *in vivo* NMR experiments, an introduction to the important principles will be presented in the following section. The method has been widely used, and its application to plant systems in general as well as to plant-microbe symbioses has already been mentioned briefly in Chapter 1 and is discussed in the review part of Chapter 3. A detailed introduction to the theory and applications of NMR can be found in various textbooks and recent reviews (Macomber, 1988; Martin, 1991; Sanders and Hunter, 1993; Pfeffer and Shachar-Hill, 1996; Bligny and Douce, 2001; Köckenberger, 2001; Pfeffer *et al.*, 2001; Ratcliffe and Shachar-Hill, 2001) on which the following sections are also based.

General principles

NMR spectroscopy is the manifestation of atomic nuclear spin angular momentum, and many isotopes, among these the biologically relevant ^{1}H, ^{13}C, ^{15}N, ^{17}O, ^{23}Na and ^{31}P, have nuclear magnetic moments, reflecting the existence of a nuclear spin that is characterized by the spin quantum number I.

For simplicity, only nucleus with I = ½ are described in the following (i.e. not ^{17}O and ^{23}Na). The magnetic dipole axes of the nuclei are usually randomly ordered. However, when exposed to a stationary external magnetic field (B_0), this field will interact with the magnetic moments of the nuclei. The nuclear spin I = ½ will, in the applied magnetic field, generate two energy levels or spin states (Fig. 9) and align the magnetic dipole axes parallel or anti-parallel with the external field, i.e. the magnetic dipole axes lose their degeneracy in the presence of a magnetic field.

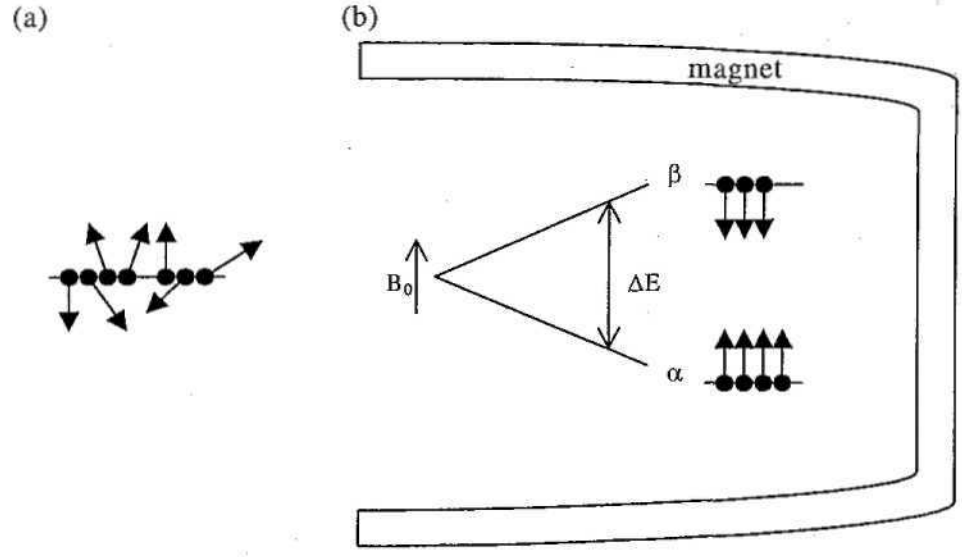

Fig. 9. Energy level and population diagram for spin I = ½ (a) outside and (b) inside a magnetic field B_0.

The energy levels of the two spin states are separated by an amount ΔE, which is field dependent (Eq. 1):

$$\Delta E = h \gamma B_0 / 2\pi \qquad \text{Eq. 1}$$

where h is Planck's constant and γ is the magnetogyric ratio of the nucleus (Table 2). The magnetogyric ratio is a proportionality constant that describes the spin state energies of a given nucleus in an external magnetic field.

Table 2. Nuclear properties of selected biologically relevant nuclei (modified from Macomber, 1988).

Isotope	I	Relative natural abundance (%)	Magnetogyric ratio $\gamma / 10^6$ (rad T^{-1} s^{-1})
^{1}H	1/2	99.985	267.512
^{13}C	1/2	1.108	67.264
^{15}N	1/2	0.37	-27.107
^{17}O	5/2	0.037	36.27
^{23}Na	3/2	100	70.761
^{31}P	1/2	100	108.29

A new thermal equilibrium is achieved in which the population of nuclei with the magnetic dipole axes aligned parallel with B_0 (lower energy, α state) is slightly bigger than the population of the nuclei with axes aligned anti-parallel (higher energy, β state) (Fig. 9). The relative distribution of the population of nuclei in the two energy states is given by the Boltzmann distribution (Eq. 2):

$$P_\beta / P_\alpha = \exp(-\Delta E / k\,T) \qquad \text{Eq. 2}$$

where P is the fraction of the population of nucleus in each state, T is the absolute temperature and k is the Boltzmann constant. As a result of this uneven distribution of the nuclear spin, a weak net magnetization (M_0) of the sample aligned parallel to B_0 arises. The net magnetization will move around the main field with a characteristic angular resonance precession frequency, called the Larmor frequency ω, which is a function of γ and B_0 (Eq. 3; see Fig. 10):

$$\omega = \gamma B_0 \qquad \text{Eq. 3}$$

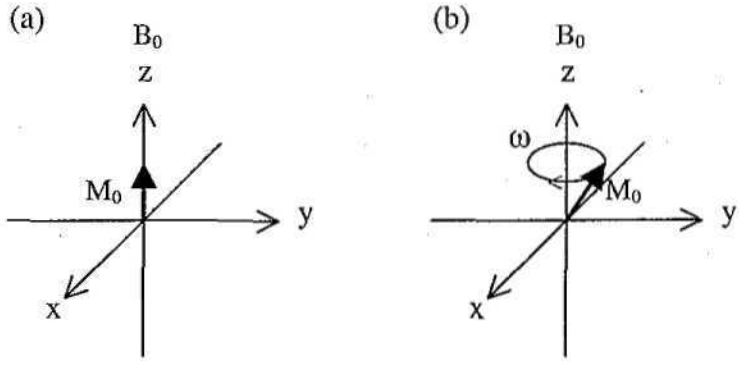

Fig. 10. The behavior of the net magnetization M_0 (a) at equilibrium, and (b) after perturbation by a pulse.

The angular Larmor frequency can be transformed into linear frequency (ν) by division by 2π. Indeed, the resonance frequency for a particular nucleus in a given applied magnetic field is unique and it depends on its local chemical environment. Since the main magnetic field is shielded by surrounding electron clouds, the effective magnetic field acting on a nucleus depends on the chemical nature of the group and the chemical environment in which it is bound. Therefore, the identification of the chemical nature of a compound by its resonance lines is based on this principle.

If the net magnetization is perturbed by a radio-frequency (r.f.) pulse (see Fig. 10), it moves in the transverse plane with the Larmor frequency and it can be measured through the induction of an oscillating current in a coil surrounding the sample. After suitable amplification and analysis, this current would be the NMR signal. However, after perturbation, the system will behave like any other system and return to equilibrium. Consequently, the magnetization created by the pulse and the associated voltage decay to zero and the time-dependence of the signal is recorded by the spectrometer

in the form of a digitized free induction decay (FID). The FID is converted into a frequency domain spectrum by Fourier transformation, producing a plot of intensity (signal) against frequency, referred to as the spectrum. However, there are a number of sources for experimental noise that affect the sensitivity of the spectrum. The signal-to-noise (S/N) ratio is proportional to $B_0^{7/4}$ and to the number of nuclei within the sample. All NMR signals can be characterized by the S/N ratio, intensity, frequency, line-shape and relaxation times of the signal, and these quantities are all affected by the physical and chemical environment of the magnetic nucleus. To understand how these quantities lead to information about biological systems, it is necessary to have some understanding of them, and of factors that affect them, and so some of the key points are summarized in the following section.

The NMR signal

In many cases the **signal-to-noise** ratio in the spectrum from a single FID is inadequate to define a signal, reflecting the insensitivity of the NMR technique. In short, with regard to the S/N ratio, the following factors have to be considered:

- the natural abundance of the nucleus under study and its relative sensitivity (Table 2)
- the magnetic field strength of the spectrometer
- the isotopic enrichment when possible (^{13}C, ^{15}N, ...)
- the intracellular concentration of investigated metabolites
- the sample volume (as large as possible)
- the width of signals and possible broadening due to paramagnetic ions and field inhomogeneity
- the relaxation times
- the possibility of repeating experiments and the total accumulation time (see below)

The natural abundance of an isotope (see Table 2) is one of the factors that determine whether the number of nuclei within a biological sample is large enough to be detected. The ^{31}P isotope is 100% naturally abundant and therefore in this sense favorable to observe. The sensitivity also depends on the field strength of the magnet, the higher the field strength, the greater the sensitivity. However, by repeating the experiment n times and Fourier transforming the sum of n FIDs it is possible to improve the S/N ratio by a factor of $\sqrt{n}$. Each FID is usually referred to as a scan or transient and this time-averaging process is essential for many *in vivo* NMR studies. By operating at a high-field strength for *in vivo* studies, and by time averaging the data, the sensitivity of the experiment can be increased to a level that permits the detection of millimolar metabolite concentrations.

Signal **intensities** are important because they can be related to the tissue content of the metabolites that produce the signals. However, it is important to realize that the observed intensity has a direct relation to the tissue content only under certain conditions, making quantitative analyses possible (see below).

The return of the net magnetization to equilibrium is characterized by the two first order **relaxation** processes with time constants T_1 and T_2 for an identical ensample of nuclei. T_1 is known as the spin-

lattice (or longitudinal) and T_2 is known as the spin-spin (or transverse) relaxation time. The spin-lattice relaxation process is responsible for the re-establishment of thermal equilibrium and restoration of the initial population distribution between two spin states by an exchange of energy between the excited nuclei and their environment. Spin-spin processes, on the other hand, involve a mutual exchange of spin energy, and the net effect of this energy transfer is to cause a loss of phase coherence in the transverse plane. The recovery of the z component of the net magnetization, characterized by T_1, is usually slower than the decay of the transverse component of the net magnetization in the xy plane, characterized by T_2, and in neither case is the recovery instantaneous. Pulse sequences are available that allow these quantities to be measured. In these experiments, the pulse sequence is designed to produce a magnetization for detection that reflects the operation of either T_1 or T_2 relaxation pathways.

The transverse and longitudinal relaxation processes also have important influence on the detection of the NMR signal. Transverse relaxation reduces the magnitude of the magnetization that can be detected in the xy plane and is responsible, along with inhomogeneities in the magnetic field, for the characteristic decay of the time domain signal (the FID). For practical purposes one often uses an experimental time constant T_2^*, which includes the effects of both the intrinsic spin-spin relaxation and also the magnetic field inhomogeneities; i.e. T_2^* takes into account the line broadening caused by the inhomogeneity of the magnetic field. Longitudinal relaxation can also affect observed intensities, since it is this process that re-establishes the net magnetization along the B_0, and T_1s of the system need to be considered whenever time-averaging is used to improve the quality of the spectrum (see below). Adding up FIDs to improve the overall S/N ratio works efficiently only if the magnetization has recovered sufficiently between the pulses that excite the signal. If the recycle time is too short and the pulses are applied too frequently, then the magnetization becomes saturated and the contribution of the corresponding signals to the spectrum is reduced. As a result of this effect, establishing the optimum data acquisition conditions is an important practical consideration in obtaining *in vivo* NMR spectra.

Consequently, the optimal pulse angle also has to be considered when discussing fundamentals about NMR; i.e. what is the appropriate amount of excitation to be provided by the r.f. pulse? A safe strategy is to use 90° pulses, each followed by a delay of five times the greatest T_1 of all the NMR signals in the spectrum. This ensures that there is no saturation and that none of the NMR signals can be missed. However, this procedure is very time-consuming, and therefore the possibility of using a higher pulse repetition rate together with a smaller pulse angle, thus reducing the time needed for the measurement, has been investigated. The optimal pulse angle α_{opt} for obtaining maximum signal, which has become known as the Ernst angle, is defined in terms of its cosine by the Ernst equation (Eq. 4):

$$\cos \alpha_{opt} = \exp (-t_r / T_1) \qquad \text{Eq. 4}$$

where t_r is the pulse repetition time (recycle delay). For routine purposes it is reasonable to use a 45° pulse and a T_1 / t_r ratio of about 2. However, it is difficult to combine several T_1 values for an optimal signal for all nuclei in a spectrum. Indeed, to be able to use the optimal pulse angle and repetition time requires that the different T_1s are known in the biological system. Typical T_1 values for P_i in vacuoles

and polyP in fungi are very short, see Table 3. Based on this information I have chosen to use a recycle delay of 0.5 s and 45° pulse angle in the *in vivo* spectra, which according to the Ernst equation is the most efficient combination for nuclei with a T_1 of 1.4 s. This value should make sure that the P metabolites in the fungi are fully relaxed between scans and that the spectra can be used quantitatively. However, the relative levels of P_i in roots should be used with some caution due to possible underestimation, since the T_1 values for P_i in roots are somewhat larger than values from pure fungi. Typical values of T_1s for ^{31}P metabolites in plants and fungi are given in Table 3, with most T_1s around 0.01-5 s, however with a lot of variation.

Table 3. Literature values of *in vivo* and *in vitro* T_1s for ^{31}P metabolites

Biological system	T_1 (s) (S.D.)				
	P_i (cyt)[§]	P_i (vac)[§]	ATP[*] α β γ	$PolyP_{cen}$[#]	Ref.
Maize root tips	2.78 (0.31)	3.58 (0.5)	0.39 (0.05) 0.21 (0.02) 0.35 (0.06)		1
Maize root tips	1.8	5.1			2
Soybean nodules aerobic (O_2) anaerobic (N_2)	 0.95 (0.19) 0.95 (0.20)	 0.77 (0.08) 3.98 (0.69)			3
Soybean root tissue		6.30 (0.93)			3
Hebeloma arenosa red pine roots (ectomycorrhizal roots)		0.4		0.06	4
H. crustuliniforme culture (ectomycorrhizal fungus)		0.15 (0.04)		0.03 (0.003)	5
Cenococcum graniforme culture (ectomycorrhizal fungus)		0.14 (0.03)		0.004 (0.002)	5
Growth medium[†]		0.65		0.79 (0.09)	5
Laccaria bicolor culture (ectomycorrhizal fungus)				0.07	6

References: 1) Pfeffer *et al.*, 1986; 2) Lee *et al.*, 1990; 3) Pfeffer *et al.*, 1992; 4) Macfall *et al.*, 1992; 5) Martin *et al.*, 1985; 6) Martin *et al.*, 1994

[§]Inorganic orthophosphate in (cyt); cytoplasm or (vac); vacuoles

[*]ATP; α-, β- and γ-phosphate of adenosine triphosphate

[#]$PolyP_{cen}$; central (cen) P_i residues in a polyP chain; i.e. except terminal (ter) and penultimate (pen) P_i residues

[†]*In vitro* value measured in modified Pachlewski´s medium including K_2HPO_4 or standard polyP of average chain length 17, see reference 5

The energy of an NMR transition is determined by the strength of the magnetic field and by the magnetogyric ratio of the nucleus (see above). The resonance **frequency** is linearly dependent of the field strength, and the precise frequency of an NMR signal depends on the exact field strength experienced by the nucleus. An immediate consequence of this is that inhomogeneities in the field increase the width of the NMR signal, since nuclei that are otherwise identical experience slightly different field strengths in a non-uniform field and thus have slightly different resonance frequencies.

The local field experienced by a magnetic nucleus is modified by the interaction of the applied field with the local electron density, and therefore the resonance frequency of a nucleus depends on its chemical environment. Thus, each chemical environment gives rise to a different resonance frequency, the so called chemical shift, and this effect, which is central to the NMR technique, allows separate signals to be observed for different metabolites in *in vivo* spectra. The chemical shift (δ) of each resonance is defined by Eq. 5:

$$\delta = 10^6 (\nu_i - \nu_s) / \nu_s \qquad \text{Eq. 5}$$

where ν_i is the frequency of the resonance of interest and ν_s is the resonance frequency of the signal corresponding to a standard compound. This definition allows the resonance frequency of a signal to be expressed in a form independent of field strength of the magnet used to record the spectrum, and the chemical shift is expressed in parts per million (ppm).

Magnetization in the transverse plane relaxes exponentially and Fourier transformation of an exponentially decaying time-domain signal produces a resonance with a Lorentzian **line-shape**. There is a direct relation between the width of this line at half-height and the transverse relaxation time (Eq. 6), such that the faster the decay in the time domain (shorter T_2^*), the broader the line in the frequency domain.

$$\Delta\nu_{1/2} = (\pi T_2^*)^{-1} \qquad \text{Eq. 6}$$

This has important practical implications because the broader the line, the harder it is to detect, and the greater the chance of overlap between adjacent lines to give a poorly resolved spectrum.

As already mentioned, one source of line broadening is the lack of uniformity in the magnetic field. This situation is worse for *in vivo* measurements than for simple solution NMR experiments because of variations in the magnetic susceptibility across a biological sample. Air, both inside and around a tissue sample, is a particular problem because the difference in magnetic susceptibility between air and aqueous tissue material is large enough to cause significant field gradients at tissue-air interfaces. Vacuum infiltration of an aqueous medium eliminates the air spaces and solves the problem, but this it not always desirable physiologically, since physiological perturbations should be minimized (see 2.3.2).

As stated, another cause of broad lines is the existence of efficient transverse relaxation mechanisms. T_2 rates increase as the mobility of the spin system decreases and so NMR signals become broader as molecular mobility is restricted. Increasing molecular weight, molecular mobility decreases and as a result it is usually only the freely mobile, low molecular weight metabolites that are detected by *in vivo* NMR. Paramagnetic ions, for example Mn^{2+}, and pure oxygen O_2, can also cause efficient transverse relaxation and broadening lines. Indeed, identifying the cause of the line broadening leads to information about intracellular environment of the compound that gives rise to the NMR signal. In some cases line broadening can cause a signal being undetectable (nuclei present in the sample but not detected in the NMR experiment are said to be NMR-invisible) and this too may be informative (Bental *et al.*, 1990). This often occurs for large molecules such as long-chain polyPs and phospholipids.

One final point about NMR line-shapes concerns the problem of making accurate intensity measurements. A significant fraction of the total intensity is included in the "wings" of the signal. However, integration across the total spectral width of a signal is impossible and, in practice, the limits for the integration are determined by the S/N ratio of the signal and by the extent to which it overlaps other peaks in the spectrum.

2.3.2 The airlift system

The physiological significance of an *in vivo* NMR experiment depends on the state of the tissue during NMR measurements. The aim must be to minimize the physiological perturbations arising from the experimental procedure and to maintain the tissue in a physiologically reasonable and controllable state throughout the experiment. Consequently, in order to be able to study P metabolism of living hyphae and roots by ^{31}P NMR spectroscopy, a system for maintaining the biological tissue in a physiologically healthy state while in the NMR tube had to be constructed, and this will be described in the following section.

In general, maintaining the biological tissue in a physiologically healthy state implies a continuous supply of oxygen and nutrients as well as removal of waste products. This can be done in a so-called perfusion system, where an oxygenated buffer solution is circulated down in the NMR tube through the tissue (Roby *et al.*, 1987). Such a system had already been implemented in our laboratory by A. M. Scharff (Scharff, 2001), and I started out by using this. However, while functioning well with roots and N_2 fixing root nodules, this system could not be used for hyphae. The excised hyphae tended to pack around the exit tube and block the flow or even escape into the tubes circulating the buffer. Attempts were made to immobilize the hyphae. This included rolling the hyphae loosely in a mesh, like a cake roll, or embedding the hyphae into agarose threads, as successfully done for various cell types (Rasmussen *et al.*, 1993; Lundberg *et al.*, 1994). However, it turned out to be extremely difficult to use these methods. The roll tended to limit the S/N ratio in the spectrum and it was difficult to have enough hyphae in the detection volume to maintain signals. Furthermore, the size of the hyphae made it impossible to embed them into agarose without cutting them into small pieces and hence making the validity of '*in vivo*' measurements doubtful.

Instead, another circulation system called the airlift system (Fox *et al.*, 1989) was considered for oxygenating an NMR sample. This system combines direct air bubbling into the suspension buffer and further slow circulation of the buffer within the NMR tube. In order to obtain information of this system, I visited the *in vivo* NMR plant group of R. G. Ratcliffe in Oxford. During this visit, it became clear that the airlift system was the right way of handling *in vivo* NMR for AM fungi. Such a very simple system was constructed, and was used for all *in vivo* experiments described in this thesis. In the airlift system a flow of gas emerges in the suspending buffer within the confines of a narrow airlift tube and, as it escapes from the tube, it sets up a circulation of the medium (Fig. 11).

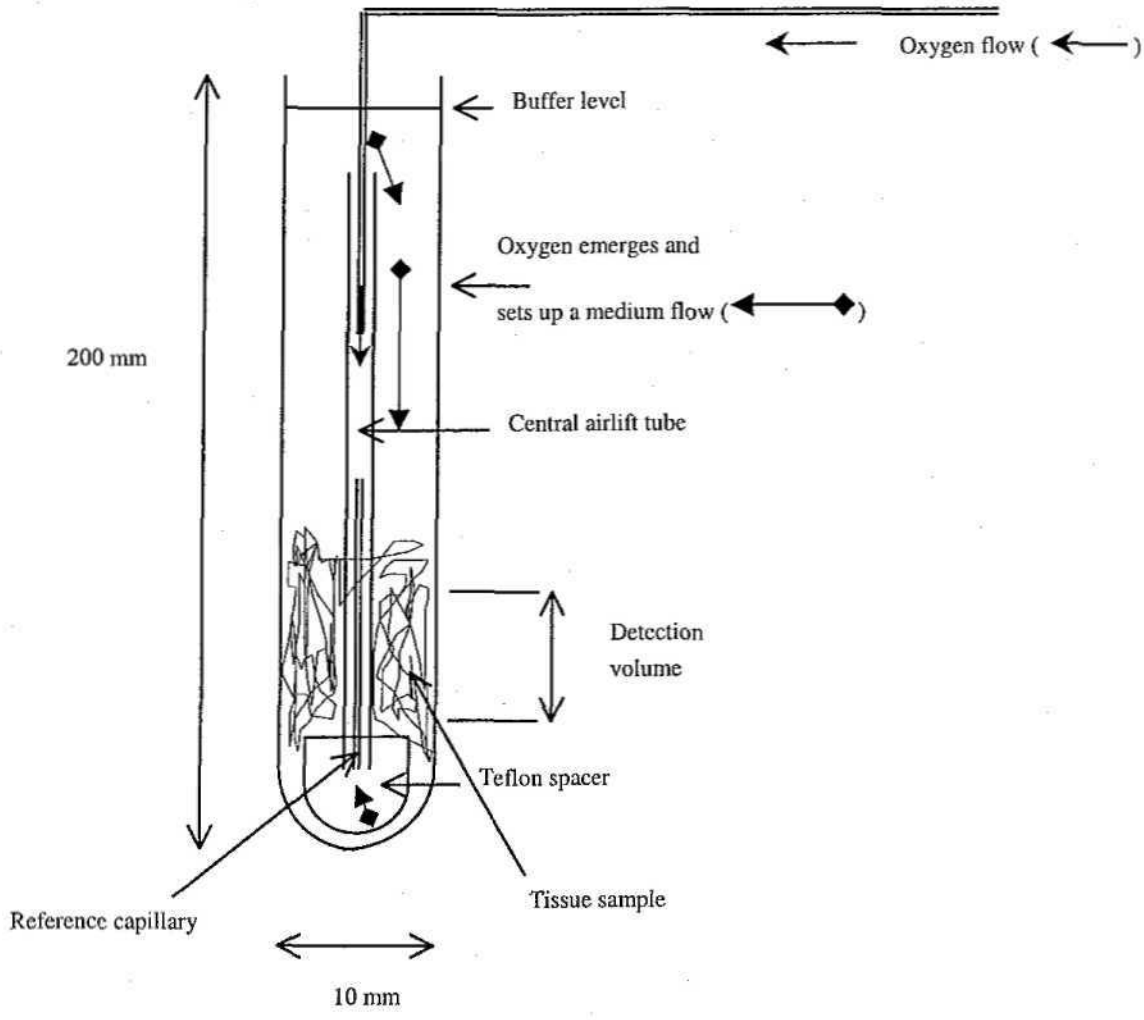

Fig. 11. Schematic drawing of the airlift system used for studying P pools and metabolism in AM fungi and roots by ^{31}P NMR spectroscopy.

This arrangement works well with excised tissues, which can be oxygenated with an airlift system as effectively as with a perfusion system, and it is particularly convenient for oxygenating cell suspensions (Fox *et al.*, 1989; Fox and Ratcliffe, 1990). There is no possibility of the gas flow causing line broadening because the gas emerges above the region that generates the NMR signal. The versatility of the airlift system can be further increased by incorporating a system of tubes for changing the suspending buffer in the NMR tube. By modifying the composition of the suspending

buffer (e.g. introducing P_i, removing oxygen or C source) it is possible to perturb the metabolism of the living system and to monitor the spectral changes caused by these changes simultaneously; thereby obtaining several successive spectra from the same sample (Bligny and Douce, 2001). However, since extraradical AM fungal hyphae are not able to take up C in any form (see Chapter 1) and since the P uptake and metabolism of AM fungi was dramatically effected after excision of mycelium from its C source (see 2.4.5), circulation of a C source seemed meaningless and was not included in my simple airlift system.

Approximately 0.1 – 0.2 g fw hyphae could be contained within the NMR tube, more compressed hyphae resulted in very poor shimming (see 2.4.1) of the sample and therefore not satisfying S/N ratio.

2.3.3 Information available from *in vivo* ^{31}P NMR spectroscopy

NMR spectroscopy permits a non-invasive detection of a wide range of ions and metabolites in functioning tissue, and so metabolic mechanisms can be followed in real time as they take place within a living tissue (Ratcliffe, 1996). It is the low molecular weight components of the cell that are usually observed, and to be detectable these ions and metabolites must be freely mobile and present in a sufficient quantity to exceed the detection threshold for the magnetic isotope of interest. Indeed, the NMR technique can detect only those metabolites that are present at concentrations of about 0.1 mM or above. For this reason, NMR is believed to be rather insensitive, and should be regarded as a method that is complementary to, rather than competing with, other methods that are available for studying metabolism. In general terms, *in vivo* NMR spectroscopy provides an analytical method for identifying and quantifying particular ions and metabolites. It provides methods for measuring intracellular pH, for probing the subcellular compartmentation of certain ions, and for following the flux through metabolic pathways (Ratcliffe, 1996).

Ultimately what can and cannot be achieved by the technique depends on the magnetic properties of the nucleus that is the source of the NMR signal, and since the properties of the biologically interesting magnetic nuclei differ greatly (see Table 2) there is corresponding variation in the applications of the different nuclei *in vivo*. The magnetic properties of ^{31}P favour the detection *in vivo* of a small subset of the phosphorylated metabolites that occur in cells. ^{31}P NMR spectroscopy has been used extensively to investigate metabolic processes in plants (e.g. Roberts *et al.*, 1980; Martin *et al.*, 1983; Rolin *et al.*, 1989; Lee *et al.*, 1990; Aubert *et al.*, 1998). The ^{31}P isotope has a 100% natural abundance and although the sensitivity of the ^{31}P nucleus is less than the ^{1}H nucleus, it is usually possible to obtain informative *in vivo* ^{31}P NMR spectra in a few minutes. A typical ^{31}P NMR spectrum has signals from a number of important phosphorylated metabolites, including sugar esters, P_i, nucleoside triphosphates (NTP) and, in the case of many algae and fungi, polyPs. However, the information that can be derived from a ^{31}P NMR spectrum extends beyond the simple measurement of these metabolites under different physiological conditions. Firstly, the pH difference between the cytoplasm and vacuole, together with the slow exchange of P_i across the tonoplast, leads to separate signals for the cytoplasmic and vacuolar P_i pools in the ^{31}P NMR spectra of most plant tissues (Ratcliffe, 1987). Since the pH dependence of the P_i chemical shift can be calibrated, the detection of

the two P_i signals leads to information on the cytoplasmic and vacuolar pH values (Roberts *et al.*, 1980). Secondly, signals arising from the α, β and γ phosphates of NTP and α and β of nucleoside diphosphates (NDP) provide information about the metabolic activity. Therefore, it is possible to monitor the metabolic state of the organism while the experiment is running, and in this way ensure that enough oxygen and nutrients are supplied (Roberts, 1987). Thirdly, the method is a powerful technique for investigating time dependent phenomena and it is therefore possible to visualize the kinetic behavior of P uptake and storage and to investigate any PolyP metabolism in the tissue.

2.4 The study of P pools in AM fungi by ^{31}P NMR spectroscopy

The adaptation of a general ^{31}P NMR method for the study of P pools in AM fungi *in vivo* turned out to be a challenge. The real advantage of ^{31}P NMR spectroscopy is the ability to study metabolic processes as they occur inside the living tissue. Much effort was used to optimize the physiological status of the excised extraradical mycelium inside the NMR tube. But the fact that extraradical mycelium cannot utilize an exogenous C supply was a serious experimental limitation which markedly impaired the P_i uptake of excised hyphae. Any P treatment therefore had to be performed while the symbiosis was still functioning and *in vivo* ^{31}P NMR was used to examine the P pools remaining after severance of the hyphae from the roots and their dynamics in AM fungi and roots.

All the ^{31}P NMR spectra presented were recorded on a Varian Unity Inova 600 MHz spectrometer using a 10-mm-diameter broadband probehead unless otherwise stated. Growth conditions, buffer composition and NMR acquisition parameters are given in figure captions.

2.4.1 Acquisition of *in vivo* ^{31}P NMR spectra

As already described in the section concerning NMR principles, it is extremely important to maximize the S/N ratio by optimization of NMR acquisition parameters. The main factors that affected the signal intensities in the various spectra presented in this thesis were i) sample volume, ii) intracellular concentration of metabolites and iii) line broadening due to paramagnetic ions or field inhomogeneity.

Because of the inherent insensitivity of NMR, it is desirable to use as large amounts of material as possible, such that the S/N ratio in the spectrum is maximized. This may mean dense packing, and consequently a negative effect on sample oxygenation. Small amounts of sand particles adhering to the harvested and washed hyphae could not be avoided and this could cause field inhomogeneity or even contamination due to paramagnetic ions and subsequent line broadening.

The possible contamination due to a high amount of paramagnetic ions was examined by obtaining ^{31}P NMR spectra of the washing water from the hyphal collection and cleaning procedure. As it can be seen from Fig. 12, little line broadening of the signal from P_i was seen, suggesting paramagnetic contamination to be very low. The broad lines seen in the various spectra must be due to other disturbances of the magnetic field. Indeed, experiments comparing different amounts of hyphae inside the NMR tube suggested that the tissue should not be packed too tight in order to maximize the quality

of the spectra at the same time as achieve maximal oxygenation of the hyphae (results not shown). Approximately 0.15 g fw hyphae and roots could be contained within the NMR tube, however due to the placement of the Teflon-spacer in the bottom of the NMR tube (see Fig. 11) almost all tissue was within the volume of the NMR detection coil. Therefore tissue amount within the NMR tube corresponded approximately to the amount of tissue actually measured.

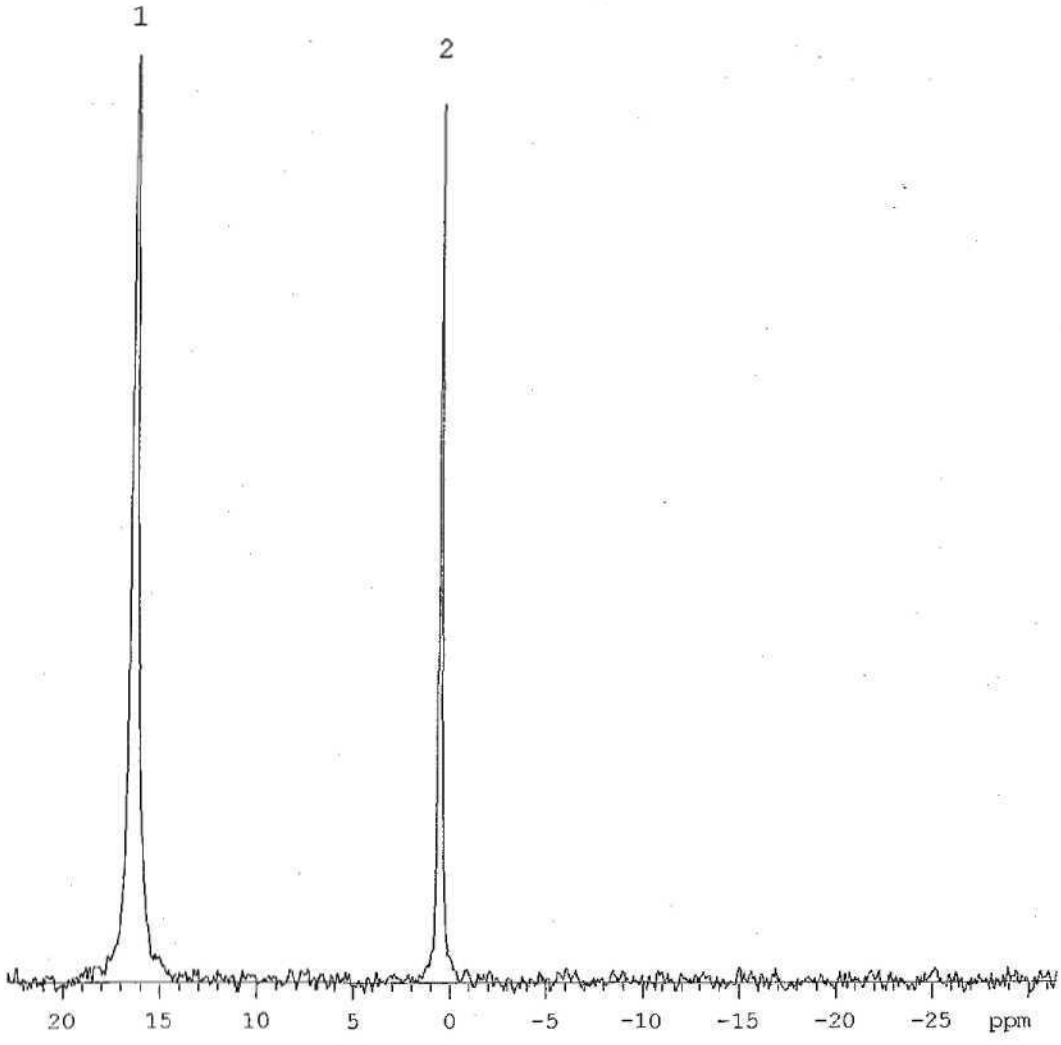

Fig. 12. ^{31}P NMR spectrum of the washing water from sand, containing hyphae which has been treated before harvest with 100 mg P_i applied via watering with a 0.32 M KH_2PO_4 solution. The water used for the spectrum was collected during the hyphal collection and cleaning procedure. The signals were assigned to (1) MDP (methylene diphosphonic acid) present as an aqueous solution of 100 mM at pH 7.5 in a capillary inside the NMR tube and used as the chemical shift reference at 16.38 ppm. The chemical shifts are quoted on the scale that puts the signal from 85% orthophosphoric acid at 0 ppm. (2) P_i present in the washing water at pH 6.40. The NMR acquisition parameters were 90° pulse angle (53 μs), 0.59 s acquisition time, 2.5 s recycle delay, proton decoupling by Waltz-16 composite pulse sequence, 15000 Hz sweep width, 1200 transients and 20 Hz line broadening. Total acquisition time was 61 min. The line-width of the P_i signal was 14 Hz before applied line broadening.

The locking and shimming of the magnetic field also needs to be considered when obtaining *in vivo* NMR spectra. During data accumulation it is essential to prevent the magnetic field and the r.f. transmitter frequency from changing independently of each other. They must therefore be locked together to ensure that the field-frequency ratio is kept constant. Normally NMR spectrometers are equipped as standard with deuterium (^2H) lock, in which the ^2H NMR signal is used as the lock signal.

However, deuterium oxide (D_2O) was not added to the buffer solution used in the *in vivo* spectra obtained in Oxford (Fig. 13; Chapter 3). Therefore, locking the spectrometer frequency to the D_2O signal was not possible. In order to optimize the magnetic field homogeneity in the sample inside the magnet (i.e. shim the magnet) it was necessary to maximize the integral of the proton signal from water in the sample and the shape and size of the water FID. In contrast, in the *in vivo* spectra obtained with the Varian 600 MHz spectrometer, 10% of D_2O was added to the buffer solution in order to lock the magnetic field and r.f. transmitter frequency to each other. Carefully shimming of the magnetic field homogeneity in the sample improved the S/N ratio. The line width at half height of the proton signal from water was used to measure the field homogeneity. This value should be as little as possible, values of 50 and 30 Hz could usually be obtained for hyphae and roots, respectively.

2.4.2 Assignment of ^{31}P NMR signals

After the first ^{31}P NMR spectra were obtained, the focus of my work was to assign the observed NMR signals and thereby identify the various metabolites in hyphae and roots. Valid assignments of signals in a spectrum are an essential first step in any *in vivo* NMR study. The strategy I used for the assignment of peaks was as follows: The signals were assigned to the corresponding chemical shift values and further identified to P metabolites by comparison with ^{31}P spectra of living plants, algae and fungi, which were obtained from the literature (Table 4). Also spectra of P standards and of tissue-extracts (i.e. *in vitro* measurements) can be helpful for the assignments. Spectra of extracts normally contain sharp lines in contrast to *in vivo* spectra and hence overlapping of signals from different metabolites is considerably reduced (Bligny and Douce, 2001). I used all three approaches in order to identify the various *in vivo* P signals (Figs. 13 and 14; Table 5).

As can be seen from Table 4, reported chemical shift values sometime vary according to different literature sources and they depend on the exact pH, concentration of metabolites, ionic strength, concentration of multivalent cations, chelating agents such as EDTA and temperature. The most commonly NMR-observed *in vivo* ^{31}P NMR metabolites are P_i and various P-esters, including NTP, and in addition, polyP residues in fungi and algae.

Table 4. Values of *in vivo* ^{31}P chemical shifts in ppm of P metabolites in plants, algae and fungi obtained from the literature

Biological system (reference)	P-mono-esters[¤]	P_i (cyt)	P_i (vac)	NTP[*] α β γ	UDPG[£] α β	PolyP (ter)[#] and pyroP	PolyP (pen)[#]	PolyP (cen)[#]
Pinus serotina (pond pine) **roots** (Ayling and Topa, 1998)	3-5	2.65	0.89	-10 -18 -5	-11 -12			
Ulva lactuca green **macroalgae** (Lundberg *et al.*, 1989)		2.6	1.3	-9.9 -18 -5		-6	-19	-21
Suillus bovinus (pine seedling) **ectomycorrhizal roots** (Gerlitz and Werk, 1994)	5	2.6	1	-10 -18 -5				-22
Various **ectomycorrhizal fungi**[†] (Martin *et al.*, 1983)			2			-9		-21
Paxillus involutus **ectomycorrhizal fungus** (Grellier *et al.*, 1989)			1					-21
Suillus bovinus **ectomycorrhizal fungus** (Gerlitz, 1996)		1.3	0.7	-10 -18 -5		-6		-22
Pisolithus tinctorius **ectomycorrhizal fungus** (Ashford *et al.*, 1994)		1.03					-21.8	-23.0
Laccaria bicolor **ectomycorrhizal fungus** (Martin *et al.*, 1994)						-6.2	-20	-22.3
G. etunicatum (leek) **AM roots** (Shachar-Hill *et al.*, 1995)	~4	~2	~0.8	-10 -19 -5	-10.5 -12			-22
G. etunicatum germinated **AM spores** (Shachar-Hill *et al.*, 1995)			~2			-6.5	-19	-22
Solanum tuberosum (potato) transgenic **leaves** cpPPK-48 (van Voorthuysen *et al.*, 2000)	4-5	~2.4	~0.7	-10 -19 -5	-10.5 -12			-23
Aspergillus niger (saprophytic fungi) (Hesse *et al.*, 2000)	4.9	3.0		-9.9 -18.6 -4.9	-10.6 -12.3	-5.8	-19.7	-22.5

All chemical shifts are quoted on a scale that puts the signal of 85% orthophosphoric acid at 0 ppm

[¤]P-monoesters include glucose-6-phosphate, mannose-6-phsophate, glycerol-3-phosphate, phosphoethanol-amine, 3-phosphoglycerate and fructose-6-phsophate

[*]NTP; α-, β- and γ-phosphate of nucleoside triphosphate

[£]UDPG; α- and β-phosphate of uridine-diphosphoglucose

[#]ter; terminal polyP residues, pen; penultimate polyP residues, cen; central polyP residues, pyroP; pyrophosphate

[†]Results obtained from *Cenococcum graniforme*, *Hebeloma crustuliniforme* and *H. cylindrosporum* and determined from shown spectra

For the continuity, the spectra included in Chapter 3 are also shown below (Fig. 13). All the expected P metabolites, which are normally observed in plant tissue, were present in both AM and non-mycorrhizal roots (Fig. 13a and b; Table 5; Chapters 3 and 4). However, only P_i and polyP could be detected in the spectra of pure AM hyphae (Fig. 13c; Table 5; Chapters 3 and 4). P_i and polyP resonances were also the only significant resonances in most spectra of ectomycorrhizal mycelium (Table 4; Loughman and Ratcliffe, 1984; Martin *et al.*, 1985). The assignments were further confirmed by comparison with recorded ^{31}P NMR spectra of extracts of hyphae (Fig. 13d and e; Table 5; Chapter 3; see also 2.5.1) and synthetic polyP with a given average chain length (Fig. 14; Table 5). Comparisons of the two extraction procedures are discussed in Chapter 3. The main difference in the spectra recorded from the two extraction procedures was the separation of the relevant P signals and the signal size. The perchloric acid (PCA) extraction procedure (Fig. 13d) seemed to hydrolyze some polyP in comparison with extraction by the phenol-detergent (PD) method (Fig. 13e). Comparison of the chemical shift values of P pools in AM hyphae obtained by extracts or *in vivo* showed almost identical chemical shift values. For the purpose of determining the average chain length of the polyP found (see 2.4.4), the integrated areas for the terminal, penultimate and central polyP resonances were measured. The ^{31}P NMR spectra of synthetic polyP with a given average chain length (Fig. 14) were also obtained in order to investigate the upper limit of NMR-detectable polyP, i.e. the maximum average chain length that can be observed in an NMR spectrum. The largest commercial available polyP with an average chain length of 75 P_i residues can easily be seen in the spectrum (Fig. 14e).

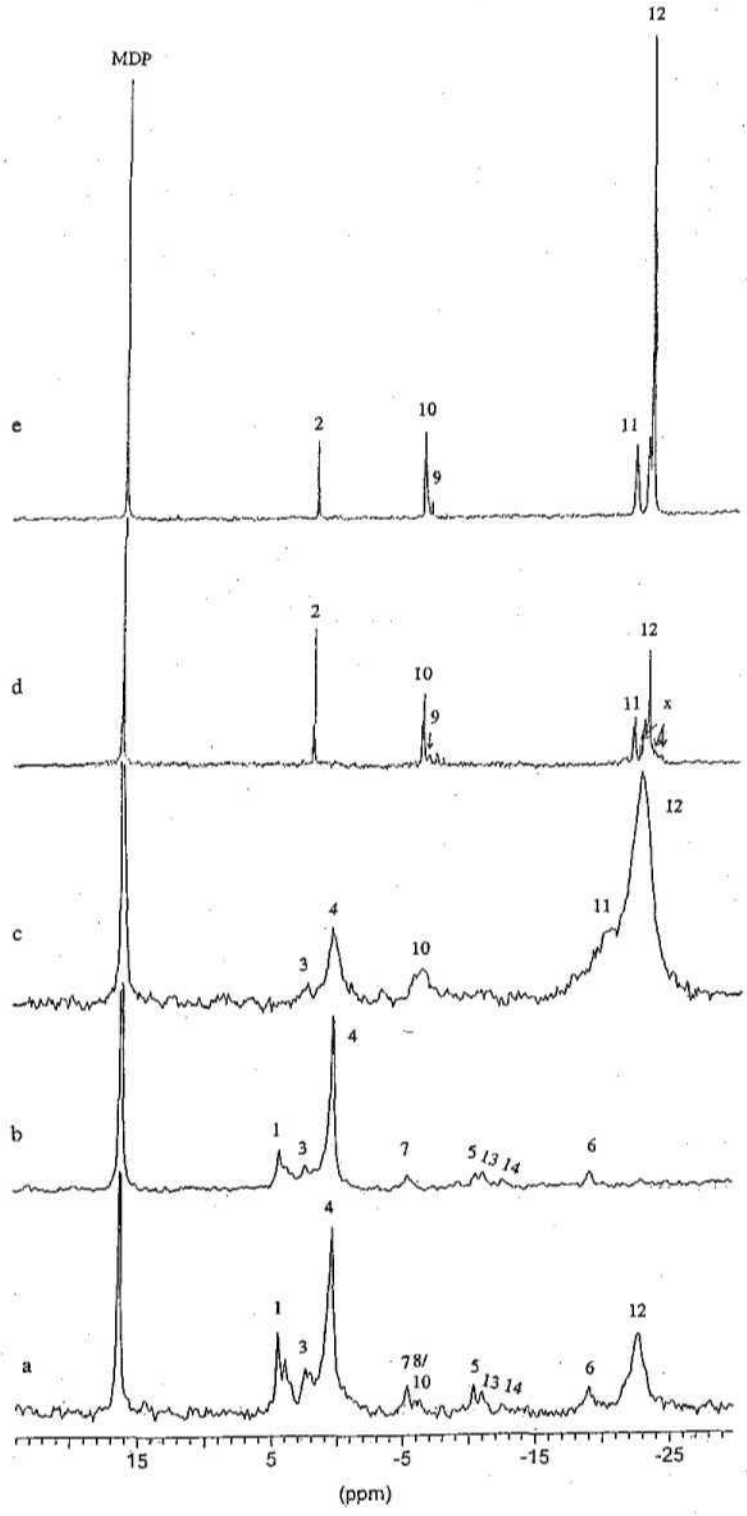

Fig. 13. The data presented in this figure were also included in Chapter 3. ^{31}P NMR spectra of (a) seven-wk-old *G. intraradices* mycorrhizal cucumber roots measured *in vivo*, (b) seven-wk-old non-mycorrhizal cucumber roots measured *in vivo*, (c) excised three-wk-old *G. intraradices* hyphae measured *in vivo*, (d) a neutralized PCA extract of three-wk-old *G. intraradices* hyphae and (e) a neutralized PD extract of three-wk-old *G. intraradices* hyphae. The chemical shifts of ^{31}P were measured relative to the signal of MDP at 16.38 ppm (pH 7.5), and the chemical shifts are quoted on the scale that puts the signal from 85% orthophosphoric acid at 0 ppm. The MDP (100 mM at pH 7.5) in the *in vivo* spectra was kept in a capillary centred in the NMR tube. Numbers refer to assignments given in Table 5; x is unidentified P. Overall experimental setup, P-treatment, harvest, extraction procedures, NMR acquisition parameters and *in vivo* buffer composition were as described in Chapter 3. The *in vivo* spectra were recorded during my stay in Oxford on a Bruker CXP300 spectrometer using a double-tuned $^{13}C/^{31}P$ 10-mm-diameter probe head.

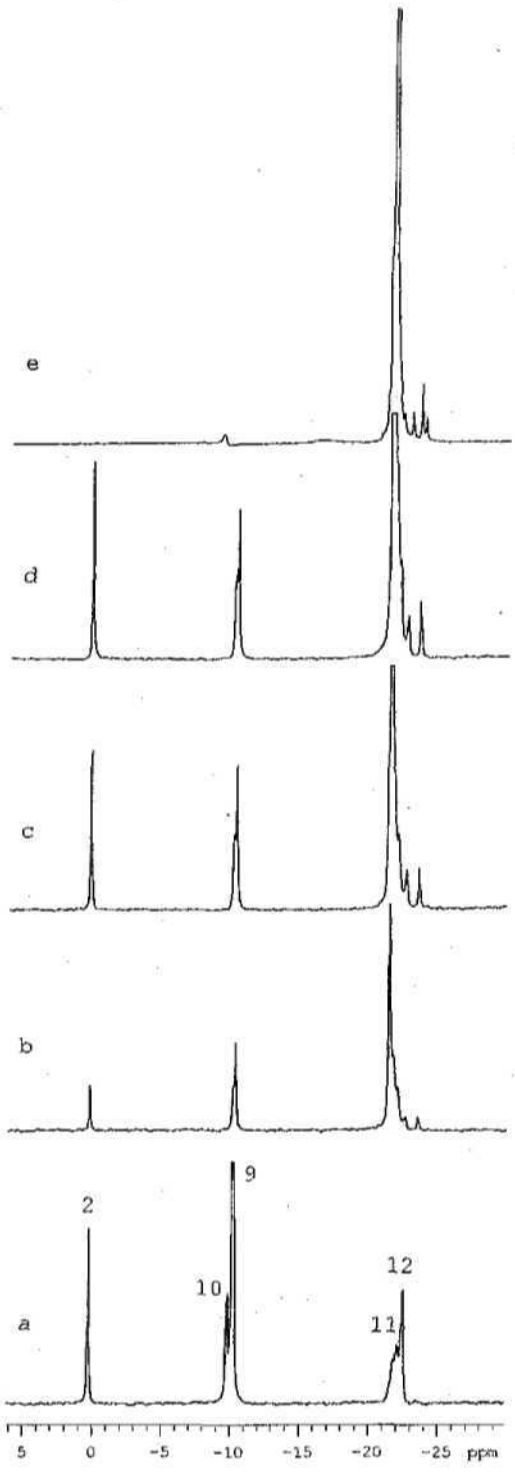

Fig. 14. ^{31}P NMR spectra of synthetic polyP (a) type 5; a standard solution of polyP with chain length 5 pH 4.8, (b) type 15; chain length 15 pH 4.5, (c) type 25; chain length 25 pH 4.5, (d) type 35; chain length 35 pH 4.4 and (e) type 75+; chain length 75+ pH 4.5. The polyP standards were synthetic polyP glasses with average chain length as stated obtained from Sigma Chemical Co. In each solution, approximately 5 mg of the polyP standard (the amount of type 75+ was higher, but not precisely determined) was dissolved in 2.7 ml H_2O and 0.3 ml D_2O with 0.1 M Na_2EDTA added. Chemical shifts of ^{31}P were measured relative to the signal at 16.38 ppm from MDP (100 mM at pH 7.5) contained in a capillary included in the NMR tube and are quoted relative to the resonance of 85% phosphoric acid at 0 ppm. The resonance of MDP is outside the shown *spectral window. Numbers refer to assignments given in* Table 5. *In spectra a, c, d, e signals were cut of in order for the* spectra to be present in one figure, but the true signal areas were determined in order to estimate average chain length (see Table 6). The NMR acquisition parameters were 90° pulse angle (53 μs), 0.59 s acquisition time, 2.5 s recycle delay, proton decoupling by Waltz-16 composite pulse sequence, 15000 Hz sweep width, 1200 transients and 20 Hz line broadening. Total acquisition time was 61 min.

Table 5. *In vivo* and *in vitro* ^{31}P chemical shifts in ppm of P metabolites in AM hyphae and mycorrhizal roots.

No.†	P metabolite		Chemical shift* (ppm)					
			AM hyphae *in vivo*	AM hyphae PCA extract pH 7.5❖	AM hyphae PD extract pH 7.5#	Non-mycorrhizal roots *in vivo*§	Mycorrhizal roots *in vivo*	PolyP standard 15▼ pH 4.5
1	Phosphomonoesters#					4.5	4.4§^	
2	P_i	*in vitro*		2.6	2.6			0.2
3		*in vivo* (cyt)	(3.0)£§			2.9	2.7§ / 2.3^	
4		*in vivo* (vac)	1.0§ / 0.4^			0.8	0.8§ / 0.3^	
5	NTP	α				-10.4	-10.4§^	
6		β				-19	-19§^	
7		γ				-5.3	-5.3§^	
8	NDP	β					-5.9?§§	
9	PyroP			-5.7	-5.7			-10.3
10	PolyP	terminal	-5.9§/-6.4^	-5.5	-5.2		-5.4§/-6.4^	-10.2
11		penultimate	-20§/-20.1^	-20.8	-20.5		-20§^	
12		central	-22.4§/-23^	-21.8	-21.6		-22.7§/-22.9^	-21.4
13	UDPG	α				-11	-11§^	
14		β				-12.5	-12.5§^	

†Numbers refer to annotations in ^{31}P NMR spectra in Figs. 13 and 14

*All assignments are based on comparisons with literature values. Chemical shift values are quoted relative to the resonance of 85% phosphoric acid at 0 ppm. NMR acquisition parameters are given in either Chapter 3, Chapter 4 or the legend of Fig. 14

❖The neutralized PCA extract was prepared as described by Roby *et al.*, 1987

#The neutralized PD extract was prepared as described by Callow *et al.*, 1978 and modified by Ashford *et al.*, 1994

§Chemical shift values determined from spectra recorded during my stay in Oxford on a Bruker CXP300 spectrometer using a double-tuned ^{13}C/^{31}P 10-mm-diameter probe head (Chapter 3)

^Chemical shift values determined from spectra recorded on the Varian spectrometer (Chapter 4)

▼The polyP standard 15 was a synthetic polyP glass with average chain length 15 obtained from Sigma Chemical Co.

#Phosphomonoesters include glucose-6-phosphate, mannose-6-phsophate, glycerol-3-phosphate, phospho-ethanolamine, 3-phosphoglycerate and fructose-6-phosphate

£The signal for cytoplasmic P_i could not always be detected in excised hyphae

§Large overlap of this signal with signals for γ-NTP and terminal polyP; most of the signal are due to these two metabolites

The polyP observed in various fungi can be identified on the basis of three signals, as seen from Tables 4 and 5. However, care should be taken especially in the assignment of the terminal polyP residues, since the chemical shift of this signal is highly pH dependent, i.e. the chemical shift may be influenced by the nature of the intracellular environment (see 2.4.3). Another problem arises from the

overlap of the signal from the terminal polyP residue with the signal from pyrophosphate, largely seen in the spectra of synthetic polyP (Fig. 14).

Finally, it should be emphasized that as pot grown *G. intraradices* hyphae or mycorrhizal/non-mycorrhizal cucumber roots were not treated with additional P_i at any time during growth, no or very small P signals could be detected in the *in vivo* ^{31}P NMR spectra of the excised tissue (Fig. 15). The concentrations of the P metabolites that were present in the tissue were therefore below the detection threshold. This was probably caused by the age of the plants and the limited amount of soil P_i available at the time of harvest (as compared to an initial content of extractable soil P_i of 11 μg P g^{-1}; see 2.2). The P_i was at the time of harvest probably severely deficient in the soil and instead diluted in the plant.

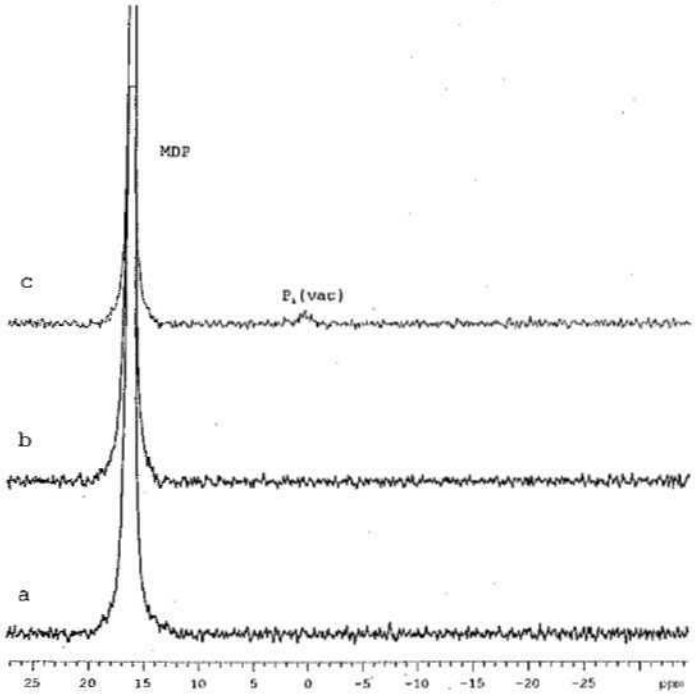

Fig. 15. *In vivo* ^{31}P NMR spectra of not additionally P-treated (a) three-wk-old non-mycorrhizal cucumber roots, (b) three-wk-old mycorrhizal cucumber roots and (c) three-wk-old excised *G. intraradices* hyphae. The roots were harvested from pots where the extraradical mycelium and roots were not separated. These pots contained 400 g 1:1 soil and sand mixture (w/w) into which was incorporated 100 g *G. intraradices* inoculum (see Chapter 3) in the mycorrhizal pot. Basal nutrients minus P were mixed in the soil and growth was maintained as described in Chapter 3. The total addition of N was 100 mg; pots were harvested after three wk of growth. The excised hyphae were harvested from compartmented pots as described in Chapter 3 (hyphae three wk old, plants seven wk old). The chemical shifts of ^{31}P were measured relative to the signal at 16.38 ppm from MDP (100 mM at pH 7.5) kept in a capillary centred in the NMR tube, and the chemical shifts are quoted on the scale that puts the signal from 85% orthophosphoric acid at 0 ppm. Harvest, NMR acquisition parameters and *in vivo* buffer composition were as described in Chapter 4, except that 14400 transients were used given a total acquisition time of 2 h 4 min and applied line broadening was only 20 Hz.

2.4.3 pH dependency of P chemical shifts

The measurement of cytoplasmic and vacuolar pH has been one of the most important applications of *in vivo* ^{31}P NMR and the method has been used extensively in studies of cytoplasmic pH regulation of algae and plants (e.g. Fox and Ratcliffe, 1990; Küsel *et al.*, 1990; Fox *et al.*, 1995). Several other methods are available to measure the intracellular pH in living cells (micro-pH electrodes, fluorescent

dyes, the distribution of weak acids or bases) (Kurkdjian and Guern, 1989; Guern *et al.*, 1991). However, NMR is the only non-invasive method for determining the intracellular pH.

NMR signals from protonated metabolites are often pH dependent when the pH of the surrounding medium is close to the pK_a of the ionisable group. To be useful, an *in vivo* NMR pH probe has to have a pK_a that is within approximately 1 pH unit of the intracellular pH. P_i, a ubiquitous ion with a pK_a of 6.8 for the species $H_2PO_4^-$ (placed at 0.58 ppm), is an excellent probe for the cytoplasmic pH (typically around 7.4 in a well-oxygenated tissue) and it can also be used to put an upper limit on the vacuolar pH (often in the range 4.5 to 5.5). Still, in order to measure intracellular pH using ^{31}P NMR, the *in vivo* ^{31}P chemical shifts are compared with a titration curve of P_i. Such a calibration curve is not simple to make, all the methods available for measuring intracellular pH are affected by uncertainties in the construction of appropriate calibration curves. Roberts *et al.* (1981) explored this problem in some detail and showed that uncertainties about the solute composition of cytoplasm and vacuole (especially total ion strength and free Mg^{2+} concentration) made changes in pH more accurately measured than absolute pH values.

Calibration curves of measured chemical shifts for P_i in two different solutions, whose ionic compositions were intended to be as close as possible to the cytosol and vacuole, respectively, were constructed as suggested by Spickett *et al.* (1993), in order to apply the NMR technique for measuring pH in AM fungi and roots (Fig. 16). All pH values estimated in this thesis were obtained using these calibration curves or similar curves used in R. G. Ratcliffe´s laboratory (used for the pH values estimated in Chapter 3).

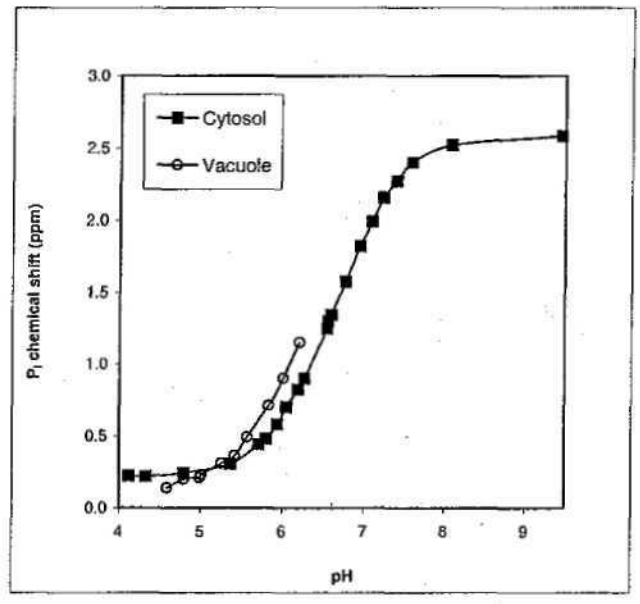

Fig. 16. Calibration curves of P_i chemical shifts as a function of pH in different solutions. The cytosolic calibration solution consisted of 100 mM KCl, 5 mM $MgSO_4$, 2 mM NaH_2PO_4 and 3 mM Na_2HPO_4. The vacuolar calibration solution contained 20 mM KCl, 50 mM $MgCl_2$, 10 mM citric acid, 5 mM malic acid and 5 mM NaH_2PO_4. pH was regulated with KOH and HCl. Chemical shifts of P_i were measured relative to the signal from MDP (100 mM at pH 7.5) contained in a capillary included in the NMR tube and are quoted relative to the resonance of 85% phosphoric acid at 0 ppm. The calibration curves were constructed in collaboration with A. M. Scharff.

The pH values in the cytoplasm and vacuole of mycorrhizal cucumber roots were estimated to be 7.4 and around 5, respectively, from the P_i chemical shifts at 2.3 ppm and 0.3 ppm in the representative ^{31}P spectrum of mycorrhizal cucumber roots presented in Chapter 4. The vacuolar pH value is at the lower end of the calibration curve where the pH dependence of the P_i signal is weak and is therefore difficult to measure precisely. Similar estimates were obtained for the corresponding pH values of the two cellular compartments in non-mycorrhizal cucumber roots (see Chapter 3).

The pH value in the cytoplasm of excised *G. intraradices* hyphae was estimated to be 7.4 from the spectrum obtained in Oxford, but as already mentioned, the signal for the P_i in the cytoplasm could not always be detected. The value agreed with the previously reported cytoplasmic pH of around 7.5 in intercellular hyphae (Ayling *et al.*, 1997). The vacuolar pH value in excised *G. intraradices* hyphae was estimated to be around 5.5, from the chemical shift at 0.4 ppm found in the various ^{31}P spectra of excised hyphae presented in Chapter 4. This value was consistent with acidic vacuoles, in agreement with the results obtained by Ezawa *et al.* (2001b).

Another important pH probe present in the biological system used in this work is the polyP, and information on the subcellular distribution of the polyP can sometimes be deduced from the ^{31}P NMR spectrum. The chemical shift of the ^{31}P signal from the terminal P_i residues is pH dependent and therefore comparison of the chemical shift value with a calibration curve similar to the ones for P_i using polyP standards instead of P_i can indicate the location of the polyP. In a study of the marine macroalgae *Ulva lactuca* (Lundberg *et al.*, 1989) and of the ectomycorrhizal fungus *Laccaria bicolor* (Martin *et al.*, 1994) the chemical shift value from the terminal P_i residues indicated that the polyP was located in the vacuoles. In order to predict the compartment containing the polyP, a calibration curve of measured chemical shifts for the terminal polyP residues in a solution similar to the one used for the vacuolar P_i calibration curve was constructed (Fig. 17, 50 mM $MgCl_2$). However, the chemical shift of signal from the terminal P_i residues and the pK_a value is also very sensitive to the binding of Mg^{2+} (MacDonald and Mazurek, 1987; Lundberg *et al.*, 1989). Therefore a calibration curve of measured chemical shifts for the terminal polyP residues in a solution with less Mg^{2+} was constructed (Fig. 17, 2 mM $MgCl_2$), as suggested by Martin *et al.* (1994). A concentration of 50 mM $MgCl_2$ was too high a concentration of Mg^{2+} for the calibration curve to be useful in the area of a chemical shift value of –6.4 ppm for the terminal P_i residue obtained in *in vivo* NMR spectra of excised hyphae (see Table 5). However, at a concentration of 2 mM $MgCl_2$ the chemical shift of terminal P_i residues predict a pH of 6.0. This acidic pH value supported a vacuolar compartmentation of the NMR-observable polyP. The results obtained here indicate a lower concentration of Mg^{2+} in the fungal vacuoles than contained in the

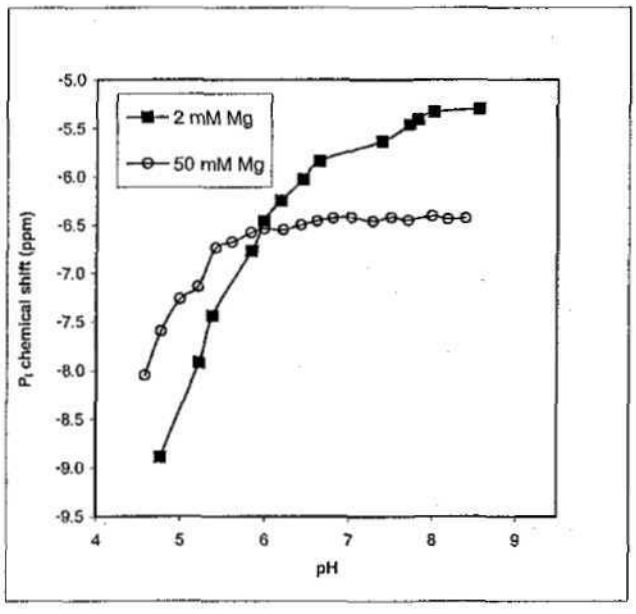

Fig. 17. Calibration curves of terminal polyP residues chemical shift as a function of pH in different solutions. The *50 mM Mg calibration solution corresponded to the vacuolar* calibration solution used in Fig. 16, except that 5 mM polyP standard with average chain length 15 obtained from Sigma Chemical Co. was included instead of NaH_2PO_4. The 2 mM Mg calibration solution contained 5 mM PolyP with average chain length 25 obtained from Sigma Chemical Co., 2 mM $MgCl_2$ and 100 mM KCl, as suggested by Martin *et al.* (1994). pH was regulated with KOH and HCl. Chemical shifts of terminal PolyP were measured relative to the signal from MDP (100 mM at pH 7.5) contained in a capillary included in the NMR tube and are quoted relative to the resonance of 85% phosphoric acid at 0 ppm.

vacuolar calibration solution suggested by Spickett *et al.* (1993), however this needs further investigation.

Like terminal P_i residues in the polyP chain, pyroP is also highly sensitive to the concentration of Mg^{2+}. The dependency of the chemical shift values for pyroP of both pH and Mg^{2+} concentration are seen in Fig. 18. Only low amounts of pyroP can be observed in the *in vivo* spectra presented here, however the polyP standards commercially available contains large amounts of pyroP. Since data for the pyroP calibration curves were included in the spectra obtained to construct the calibration curves of terminal polyP, the calibration curves of pyroP are included as Fig. 18.

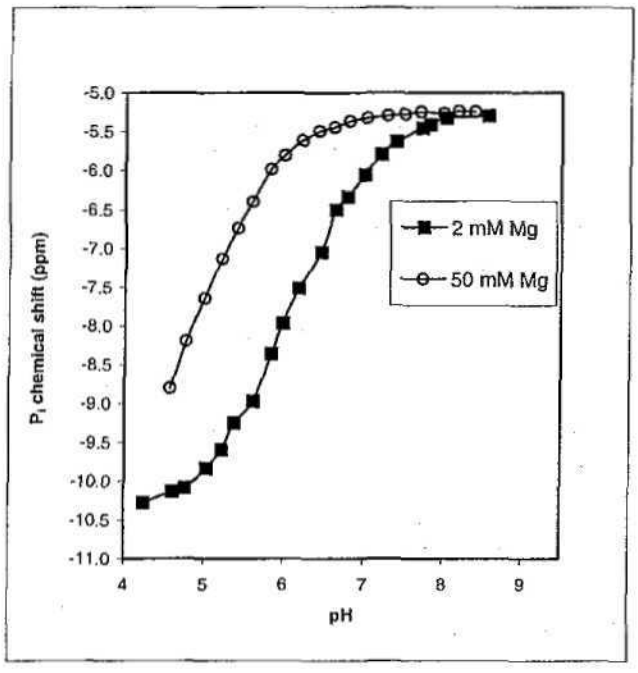

Fig. 18. Calibration curves of pyroP chemical shift as a function of pH in different solutions. The calibration solutions, pH regulation and NMR parameters were similar to the ones used in Fig. 17.

2.4.4 Monitoring the dynamic behavior of P by ^{31}P NMR

In principle, *in vivo* ^{31}P NMR spectroscopy should be very suitable for studying the kinetic behavior of P uptake and physiology of the fungus and for investigation of polyP metabolism in the mycorrhizal tissue (see above). PolyPs, which occur in many microorganisms, give rise to a characteristic set of ^{31}P NMR signals that have been detected *in vivo* in many living systems (see Table 4), including yeast (Castrol *et al.*, 1999), *Aspergillus niger* (Hesse *et al.*, 2000), axenically cultured ectomycorrhizal fungi (e.g. Martin *et al.*, 1983, 1985, 1994; Ashford *et al.*, 1994; Gerlitz and Werk, 1994; Gerlitz and Gerlitz, 1997; Martins *et al.*, 1999) and mycorrhizal root tissues (Loughman and Ratcliffe, 1984; MacFall *et al.*, 1992). In contrast, there have been very few *in vivo* ^{31}P NMR investigations of AM fungi, with just one study of AM roots of leek and germinating spores (Shachar-Hill *et al.*, 1995) besides the work presented in this thesis.

Indeed, in the mentioned studies, the presence of polyP was demonstrated by the detection of an NMR signal around –22 ppm in the ^{31}P NMR spectrum arising from the central P_i residues in the polyP chain. Signals from P_i residues located at different places in the polyP chain can be detected and the

ratio of the areas of the terminal ($polyP_{ter}$), penultimate ($polyP_{pen}$) and central ($polyP_{cen}$) P_i residues can be determined. These measurements allow the average chain length of the detectable polyP to be calculated using the formula

$$2(polyP_{ter} + polyP_{pen} + polyP_{cen}) / polyP_{ter} \quad \text{(Eq. 7)}$$

It is normally relatively easy to estimate the polyP average chain lengths of extracts and standards containing polyP. Based on the integrated areas of the terminal, penultimate (when detectable) and central P_i residues in the spectra presented in Fig. 14, the average polyP chain length of synthetic polyP standards were estimated. Results of this are presented in Table 6.

Table 6. Estimated average polyP chain length of synthetic polyP glasses obtained from Sigma Chemical Co.

Type polyP	Average chain length estimated from ^{31}P NMR spectra*
5[$]	6
15[†]	14
25[†]	22
35[†]	34
75+[£]	nd

*NMR acquisition parameters are given in the legend of Fig. 14

[$]Much pyroP present in the spectrum, however terminal and penultimate P_i residues could be assigned and integrated, given a ratio between $polyP_{ter} : polyP_{pen} : polyP_{cen} = 2 : 2 : 2$

[†]Some pyroP present in the spectra, and heights of the various peaks were used to verify calculation, given ratio between $polyP_{ter} : polyP_{pen} : polyP_{cen} = 2 : 2 : 10$ for type 15, $polyP_{ter} : polyP_{pen} : polyP_{cen} = 2 : 2 : 18$ for type 25 and $polyP_{ter} : polyP_{pen} : polyP_{cen} = 2 : 2 : 30$ for type 35

[£]*The signal of central P_i residues is too large in comparison with the signal for terminal P_i residues to determine* true areas

nd: Not detected

The calculated average polyP chain lengths of the synthetic polyP glasses were close to the expected values. However, the synthetic polyP glasses with chain length below 35 P_i residues all contain large amounts of pyroP, which makes it difficult to estimate accurately the areas of signals for terminal P_i residues. This shows that overlapping signals can influence results obtained from areas of signals. Furthermore, the results also show that the estimation of average polyP chain length of long-chain polyP using ^{31}P NMR is impossible, since the signals for terminal and penultimate P_i residues are too small to be separated from the noise.

The appearance of the polyP signals in *in vivo* ^{31}P NMR spectra is very variable, reflecting the expected heterogeneity in the endogenous polyP pools within the tissue, and this can complicate the interpretation of the spectra. In some cases, the signals are narrow, indicating that they are derived from freely mobile compounds of low molecular weight, and in these cases it is usually possible to

estimate the average chain length of the polyP. In other cases the signals can be broad, even to the point of being undetectable from the base line of the spectrum, and several factors may be responsible for this, including immobilization by precipitation or binding to membranes, a high degree of polymerization and interaction with paramagnetic ions (Bental *et al.*, 1990). For this reason, ^{31}P NMR should be suitable for investigating polyP dynamics within the tissue. It would thus be possible to study whether the AM fungus with access to high amounts of P_i will first synthesize mobile short-chain polyP (NMR-visible) for translocation of P and subsequently immobile long-chain or granule polyP for storage of P (NMR-invisible), as has been demonstrated for the ectomycorrhizal fungus *Suillus bovinus* (Gerlitz and Werk, 1994). Furthermore, it can be hypothesized that synthesized polyP will be transformed to P_i under P deficiency. And it can be studied how P and polyP metabolism responds to stress like changes in extraradical pH (Gerlitz and Gerlitz, 1997), concentration of toxic ions (Gerlitz, 1996; Castrol *et al.*, 1999) and osmotic chock (Bental *et al.*, 1990).

Values of average chain length of polyP in extraradical mycorrhizal hyphae estimated from ^{31}P NMR spectra of various fungi and quoted in the literature are given in Table 7. The average chain lengths reported are normally relatively short.

Table 7. Literature values of average polyP chain length for polyP in mycorrhizal fungi

Fungi	Average chain length estimated from ^{31}P NMR spectra	Reference
ectomycorrhizal fungi†	11	(Martin *et al.*, 1985)
Pisolithus tinctorius **ectomycorrhizal fungus**	15	(Ashford *et al.*, 1994)
Laccaria bicolor **ectomycorrhizal fungus**	25-30	(Martin *et al.*, 1994)
Germinated **AM** *G. etunicatum* spores	5	(Shachar-Hill *et al.*, 1995)

†*C. graniforme* and *H. crustuliniforme*

Values for average chain length of polyP in extraradical hyphae of various AM fungi estimated from ^{31}P NMR spectra obtained *in vitro* (extracts) and *in vivo* and from mycorrhizal cucumber roots estimated from *in vivo* ^{31}P NMR spectra obtained in this study are presented in section 2.6 and in Chapters 3 and 4. The obtained average chain lengths were primarily short (< 20 P_i residues).

2.4.5 Monitoring of the physiological status by ^{31}P NMR

A ^{31}P NMR spectrum of a sample can usually provide an important practical indicator of hypoxic metabolism in biological systems (Roberts and Xia, 1996). An inadequate oxygen supply will usually result in weak NTP signals, a strong cytoplasmic P_i signal and a cytoplasmic pH below the range of 7.4-7.6 (i.e. acidification of the cytoplasm), which is typical of well-oxygenated higher plant tissue (Fox and Ratcliffe, 1990). The supply of oxygen to a tissue can normally be optimized by monitoring these parameters and it was easy to demonstrate that the roots used for NMR measurements in the

present work were in an well-oxygenated state, since all the NMR signals needed were present in the spectra (Fig. 13; Chapters 3 and 4). As discussed in Chapter 3, small variations in the chemical shift of cytoplasmic P_i and corresponding pH values of the cytoplasm in cucumber roots could be explained by oxygen deprivation of the tissue during experiments. However, as the lowest measured cytoplasmic pH value in roots was 7.2, both mycorrhizal and non-mycorrhizal cucumber roots could easily be kept metabolically active and well-oxygenated in the airlift system while experiments were performed.

However, as can be seen from the *in vivo* ^{31}P NMR spectra of excised hyphae (Fig. 13; Chapters 3 and 4), no or only very small signals for NTP or P_i in the cytoplasm were detected in extraradical mycelium. This can be explained by a very low concentration of the metabolites in the cytoplasm, i.e. below the detection threshold, or a very low amount of cytoplasm in the hyphae (see Chapters 3 and 4). But from a more practical point of view, the missing signals meant that it was difficult to measure the metabolic activity and to judge the possibility of performing P_i uptake and storage investigations on excised hyphae. Instead, I used other approaches to assess the metabolic status of the excised fungi.

First, the efficiency of P_i uptake by excised *G. intraradices* hyphae which had received no P_i before their excision from the roots was investigated by incubating the excised hyphae in oxygenated buffer (buffer composition as described in Chapter 4) including 1 mM KH_2PO_4 for 15 or 120 min. *In vivo* ^{31}P NMR spectra were obtained of the excised hyphae immediately after incubation had finished (Fig. 19a and b). Secondly, the turn-over of P pools in the mycelium was studied in a P-adequate sample. *In vivo* ^{31}P NMR spectra of the excised hyphae were obtained just after harvest of the P-pretreated hyphae and subsequently after 12 h in oxygenated P-free buffer (Fig. 19c and d).

The two spectra of AM fungi which had received no P before their excision from the roots and subsequently were incubated in P buffer (Fig. 19a and b) indicated polyP (as central polyP at –22.6 ppm) as well as vacuolar P_i (at 0.4 ppm) in similar amounts within one treatment (as obtained from integrated areas). The two spectra were very similar, although the areas of the two signals were both slightly larger after 120 min incubation. The experiment indicated that the excised hyphae take up P_i and accumulates polyP to some extent. But the ratio between amounts of polyP and vacuolar P_i was much smaller compared to a P-adequate sample (Fig. 19c) and to differently P-treated AM hyphae (P-treated before harvest; see Chapter 4). It was indicated that the nutrient uptake and transport was not functioning optimally in excised hyphal tissue, and the termination of hyphal organic C uptake could be partly responsible for this.

The examination of the turn-over of P pools in excised AM hyphae by *in vivo* ^{31}P NMR (Fig. 19c and d) showed the expected signals for vacuolar P_i and polyP, but the amount of polyP was much smaller after 12 h in oxygenated buffer than immediately after excision. In contrast, the vacuolar P_i signal increased during the 12 h. That is, polyP is to some extent degraded to P_i during the 12 h. Published work on polyP in ectomycorrhizal fungi (Martin *et al.*, 1985) showed that polyP serves as a P reserve and that polyP is degraded to P_i during P starvation. The work by Martin *et al.* (1985) also showed that the degradation rate of the polyP varied among species of ectomycorrhizal fungi. A similar effect

could be the explanation for the turn-over of polyP seen in *G. intraradices*. But the increased area of vacuolar P_i cannot fully explain the degradation of polyP; some P was lost and had become NMR-invisible during the 12 h P starvation. For this reason, this experiment should be repeated.

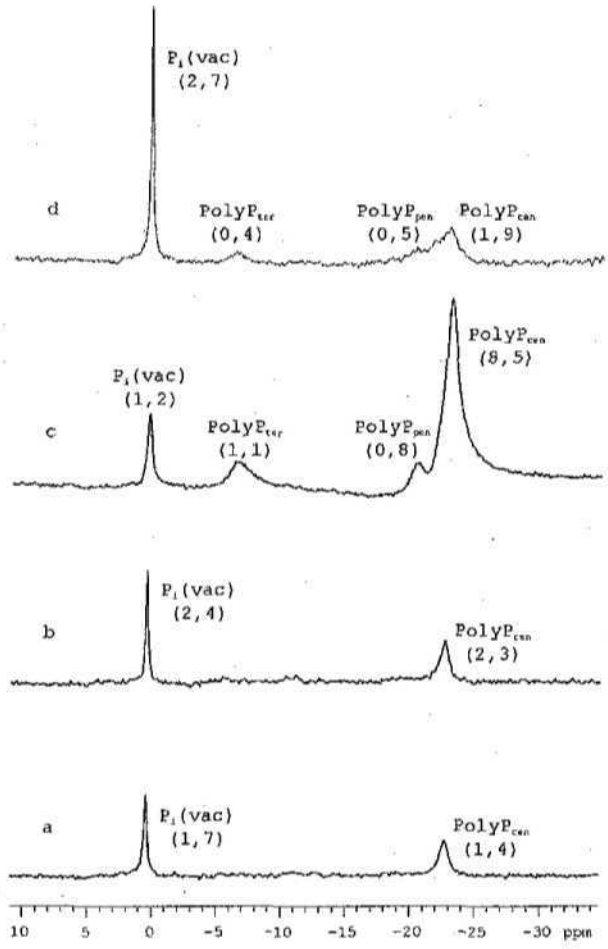

Fig. 19. *In vivo* ^{31}P NMR spectra of excised *G. intraradices* hyphae allowed to grow for two wk after sand change and P-treated differently during growth. a) No P was supplied before harvest; excised hyphae were incubated for 15 min in oxygenated buffer including 1 mM KH_2PO_4. b) No P was supplied before harvest; excised hyphae were incubated for 120 min in oxygenated buffer including 1 mM KH_2PO_4. c) An aqueous solution of 0.32 M KH_2PO_4 was applied to the hyphal compartment of the pot daily during the last week before harvesting; the pot received 100 mg P in total and the spectrum was obtained immediately after harvest. d) Same hyphal sample as spectrum c, but obtained after 12 h in the airlift system. The chemical shifts of ^{31}P were measured relative to the signal at 16.38 ppm from MDP (100 mM at pH 7.5) kept in a capillary, and the chemical shifts are quoted on the scale that puts the signal from 85% orthophosphoric acid at 0 ppm. Signals were assigned to P_i(vac); vacuolar P_i and $PolyP_{ter}$, $PolyP_{pen}$, $PolyP_{cen}$; terminal, penultimate and central P_i residues in polyP, respectively. Integrated areas of signals relative to the MDP signal are included in the figure in brackets. The resonance of MDP is outside the spectral window. The approximate amount of MDP within the detection volume was estimated to be 2 mg (internal diameter of capillary estimated to 1 mm, detection volume 150 mm high). Overall experimental setup and harvest, buffer composition and NMR acquisition parameters were as given in Chapter 4 except that 14400 transients were used given a total acquisition time of 2 h 4 min and applied line broadening was only 20 Hz.

From these experiments, it became clear that the P uptake and metabolism of AM fungi was dramatically affected after excision of the mycelium from its organic C source. Therefore, subsequent experiments were performed with hyphae and roots, which had received different P treatments while the symbiosis was still functionally optimal, i.e. before harvest of the pots. Consequently, subsequent results presented in this thesis (i.e. following sections and chapters) concerns steady state *in vivo* ^{31}P

NMR measurements on plant and fungal tissue which had been previously exposed to various P treatments.

2.5 Other methods used

2.5.1 Extraction procedures

Extraction procedures have been used to confirm *in vivo* ^{31}P NMR assignments and to further characterize the detected polyP in combination with other analytical methods than NMR. In general, it has been customary to consider polyPs in fungi in terms of their solubility in acid (Jennings, 1995). The acid-soluble polyPs are those with average chain length of up to 20 P_i residues; acid-insoluble polyPs have higher chain lengths. However, it is possible to characterize the acid-insoluble polyPs further by their successive solubility in other solvents (Clark *et al.*, 1986), as will be demonstrated. The three extraction procedures used here were: (1) the PCA extraction (Roby *et al.*, 1987) which is the commonly used method for extraction of various P metabolites in tissue. By this procedure, phosphomonoesters, P_i, phytate, ATP, adenosine diphosphate (ADP) and UDPG together with acid-soluble polyP can be extracted. However, since this method omits or possibly hydrolyzes larger size polyP, a second method was used: (2) the PD extraction procedure described by Callow *et al.* (1978) and modified by Ashford *et al.* (1994). This method preferentially extracts polyP and hydrolysis of any polyP is avoided. Comparison of the two extraction procedures by the corresponding ^{31}P NMR spectra are presented in Chapter 3.

During my experimental phase, the focus was directed towards further characterization of the polyP in the extraradical hyphae. A major obstacle in understanding the function for the polyP has been the lack of methods for isolating and determining the sizes of polyPs (Clark *et al.*, 1986). However, a successive polyP extraction procedure (3) was developed and tested for isolation of intact polyP chains from *Propionibacterium shermanii* cells by Clark *et al.* (1986), and modified slightly for the use on AM fungi by Solaiman *et al.* (1999). This method allows isolation of three fractions, each containing polyP of a certain length. The first fraction contains short-chain polyP (less than 20 P_i residues linked together), which is soluble in trichloroacetic acid (TCA), the second fraction contains long-chain polyP, which is soluble at neutral pH in 2 mM EDTA, and the third fraction contains long-chain polyP present as granules extracted by phenol-chloroform (PC). The procedure does not hydrolyze polyP by including [^{32}P]polyP and a 100% recovery was obtained (Clark *et al.*, 1986).

The exact protocols for the extraction procedures can be found in the original literature, and only a flow diagram for each method will be presented here (Fig. 20).

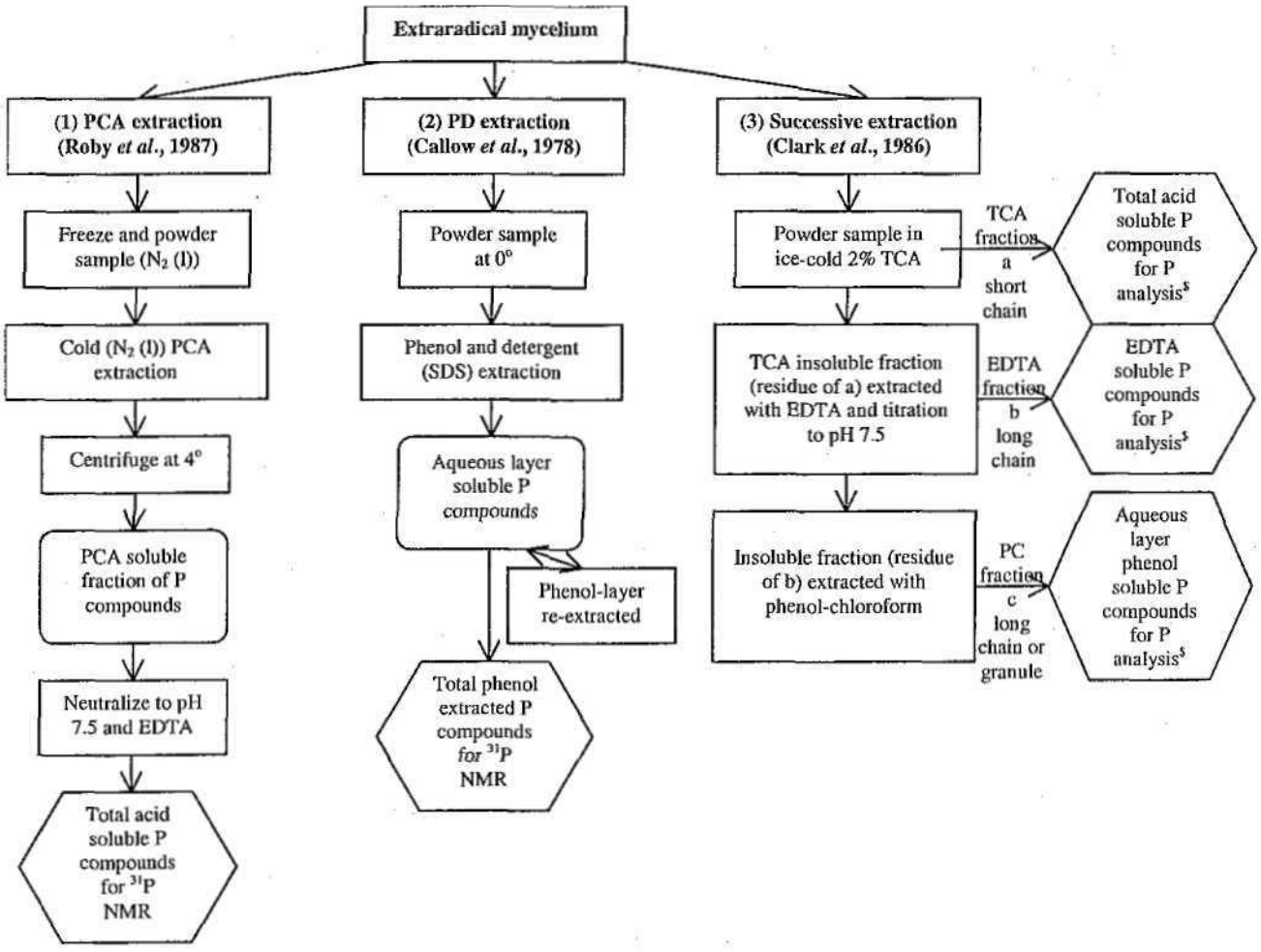

Fig. 20. Overview of the extraction methods used in the present work. ^{5}P analysis of fractions from the successive extraction procedure included use of both ^{31}P NMR spectroscopy and metachromatic reaction of toluidine blue at 530 and 630 nm (see below; Chapter 4).

The successive extraction procedure was carried out on extraradical hyphae that had been used in the time-course study presented in Chapter 4. The extracts were further examined by the use of ^{31}P NMR and colorimetric methods and detailed results of the extraction procedure are presented in Chapter 4. The results of the successive extraction procedure applied to P-treated extraradical mycelium indicated some limitations in extraction of short-chain polyP (TCA soluble). The ^{31}P NMR spectra of the TCA fractions contained no signals for short-chain polyP or no marked increase in the P_i signal, which would have resulted from hydrolysis of polyP. These results were in sharp contrast to the *in vivo* ^{31}P NMR spectra of the same material, where short-chain polyP (approximately 13 P_i residues) was present in much larger amount than vacuolar P_i (see Chapter 4). This could have at least two explanations: The concentration of the TCA used in the extraction process was less than 2% or the TCA was unable to extract short-chain polyP. To investigate this, a TCA extraction of synthetic polyP type 5 in two concentrations was performed. The resulting ^{31}P NMR spectra of TCA-extracted synthetic short-chain polyP are presented in Fig. 21.

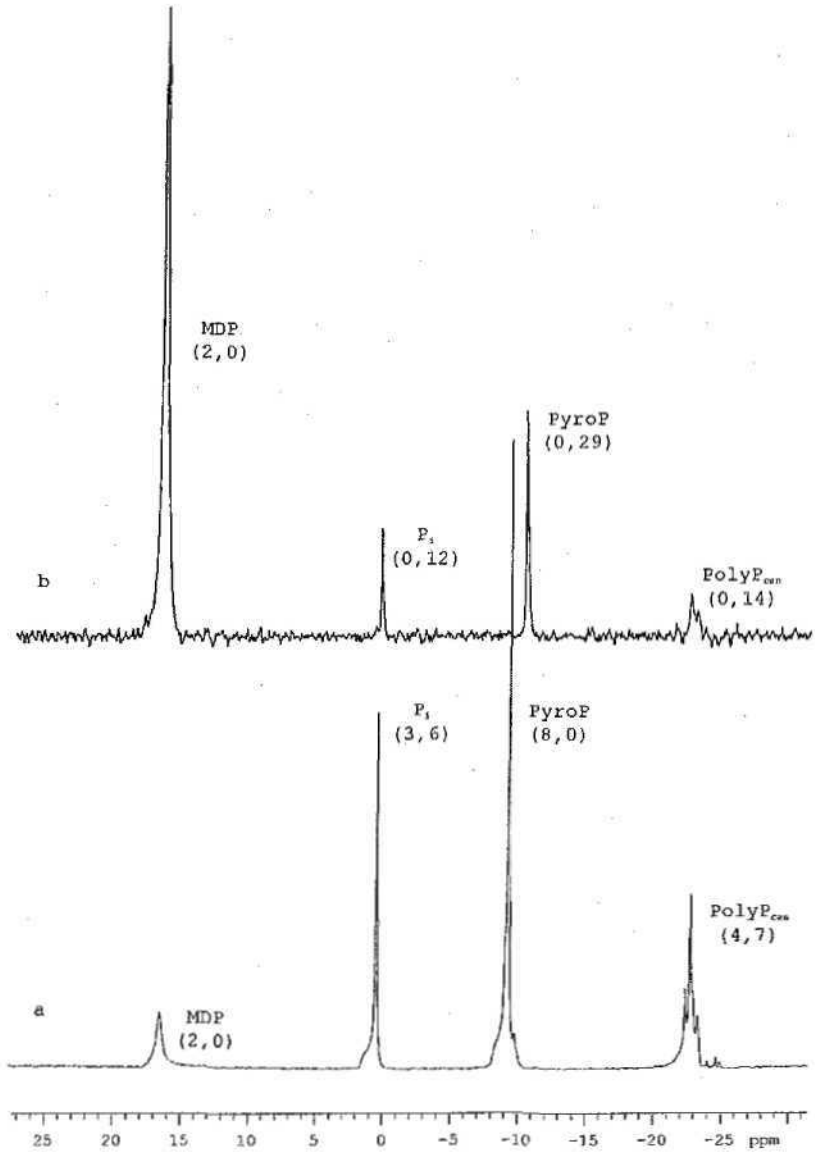

Fig. 21. ^{31}P NMR spectra of TCA extracted synthetic polyP type 5. (a) 10.4 mg polyP type 5 extracted in ice-cold 2% TCA according to Clark *et al.* (1986) and diluted to 3 ml incl. 10% D_2O for the NMR measurements (pH 6.3). (b) 1.06 mg polyP type 5 extracted in ice-cold 2% TCA according to Clark *et al.* (1986) and diluted to 3 ml incl. 10% D_2O for the NMR measurements (pH 5.2). The polyP type 5 was synthetic polyP glass with an average chain length of 5 obtained from Sigma Chemical Co. Chemical shifts of ^{31}P were measured relative to the signal at 16.38 ppm from MDP (100 mM at pH 7.5) contained in a capillary included in the NMR tube and are quoted relative to the resonance of 85% phosphoric acid at 0 ppm. The NMR acquisition parameters were 90° pulse angle (53 µs), 0.59 s acquisition time, 6 s recycle delay, proton decoupling by Waltz-16 composite pulse sequence, 15000 Hz sweep width, 1200 transients and 20 Hz line broadening. Total acquisition time was 2 h and 11 min. Assignments and integrated areas of signals relative to the MDP signal are included in the figure. The difference in S/N-ratio in the two spectra was due to the different scaling in order to show the full signals in the spectra.

Short-chain polyP was extracted by TCA (Fig. 21), but the results indicate that there is a limitation when extracting low amounts of short-chain polyP. Although the difference in concentration of polyP in the two solutions was a factor 10, there was a poor correspondence to the integrated areas of the signals for central P_i residues in the polyP chain as compared to the reference signal (MDP). The ratio between the areas for the MDP and $polyP_{cen}$ signal was 1 : 2.36 when 10.4 mg polyP was extracted (Fig. 21a) but only 1 : 0.07 when 1.06 mg polyP was extracted (Fig. 21b), i.e. the polyP extracted was only one third of the expected at the low polyP concentration. This low concentration was similar to

that detected in excised hyphae by *in vivo* ^{31}P NMR (see Fig. 13c; Chapters 3 and 4). The extracted P in the TCA extracts of synthetic polyP type 5 was hydrolyzed in a 4:1 (v/v) solution of nitric and perchloric acid and total P content was determined by the molybdate blue method (Murphy and Riley, 1962) on a Technicon Autoanalyser II (Technicon Autoanalyzers, Analytical Instrument Recycle, Inc., Golden CO, USA). The total P content in the two extracts corresponded to 9.9 mg polyP type 5 (when 10.4 mg polyP was extracted) and 0.4 mg polyP type 5 (when 1.06 mg polyP was extracted), respectively. This corresponded to a 95% and 38% recovery, respectively, confirming the NMR results. The results of the tests of TCA as a solvent for extraction of short-chain polyP indicated that the missing short-chain polyP in the extracts of extraradical mycelium could be explained by limitations in the extraction procedure. However, the extraction procedure needs to be redone on fresh material to rule out other possible errors due to the chemicals used. This needs further investigation.

2.5.2 Identification of polyphosphate

Besides the characterization of the polyP by ^{31}P NMR spectroscopy, the polyP content in the successive extracts (Clark *et al.*, 1986; *Fig. 20(3)*) was also estimated by a colorimetric method (Solaiman *et al.*, 1999). The polyP content in the extracts was estimated by measuring the absorbance changes due to the metachromatic reaction of polyP with toluidine blue at 530 nm and 630 nm (Griffin *et al.*, 1965; Solaiman *et al.*, 1999). Ten µl of the polyP extract was added to tubes containing 0.75 ml 0.2 M acetic acid and 0.75 ml 30 mg l^{-1} toluidine blue. The amount of polyP was estimated by comparison of the absorption spectra with standard curves produced by using 1 and 5 µg of each of three synthetic polyPs, i.e. type 5 and type 25 polyP for the short-chain and type 75+ polyP for the long-chain polyP. The standard absorption spectra are presented in Fig. 22, together with absorption spectra of extracts of P-treated hyphae (part of figure included in Chapter 4; see Chapter 4 for details in P-treatment).

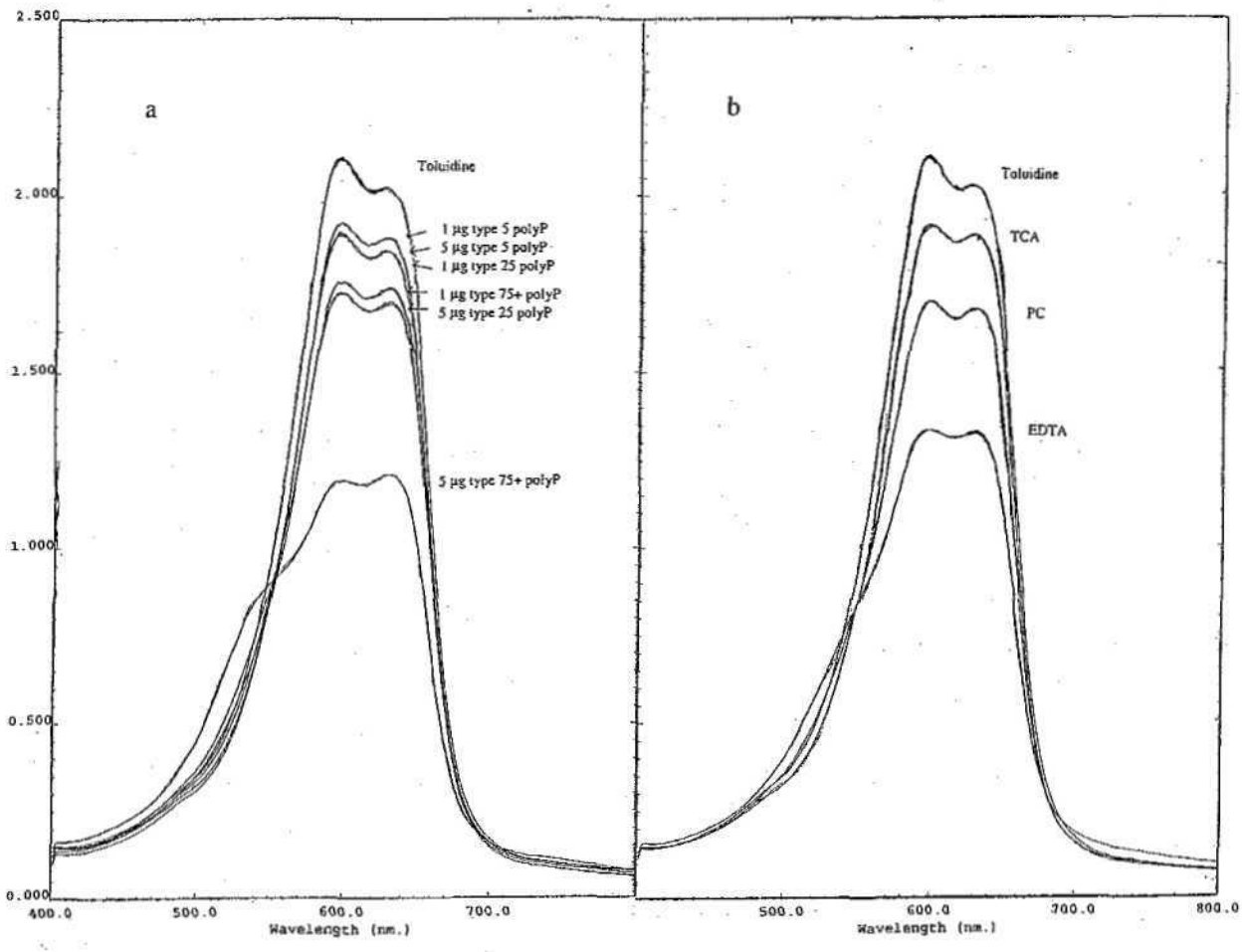

Fig. 22. Absorption spectra of (a) toluidine blue in the absence and presence of synthetic polyP of various chain length and in various amounts as indicated in the figure. The polyP standards were synthetic polyP glasses with average chain length 5, 25 and 75+ obtained from Sigma Chemical Co. (b) toluidine blue in the absence and presence of successively extracted polyP in fractions as indicated in the figure. TCA; polyP extracted by TCA, EDTA; polyP successively extracted by EDTA and PC; polyP lastly extracted by PC. The two-wk-old extraradical mycelium used in the extraction procedure was treated for two wk with 0.7 mM P_i in nutrient solution and additionally 100 mg aqueous P_i prior to harvests (10 to 96 h before harvest, see Chapter 4 for details in P treatment). A similar figure is contained in Chapter 4, however for continuity is it included here as well.

The absorption spectrum of toluidine blue had two maximum values and the absorbance decreased due to formation of the metachromatic complex in the presence of large amounts of long-chain polyP standards (Fig. 22a). No significant metachromatic reaction could be detected in the presence of short-chain polyP or small amounts of long-chain polyP (Fig. 22a). The absorption spectra of the metachromatic reaction of toluidine blue and polyP in the extracts of P-treated hyphae indicated some long-chain and granular polyP in the EDTA and PC fractions, respectively (Fig. 22b). Short-chain polyP in the TCA fraction could not be identified by the colorimetric measurement. Furthermore, no long-chain or granular polyP could be measured by the metachromatic reaction in extraradical mycelium P-treated for shorter periods (see Chapter 4). It is very difficult to convert the metachromasy of polyPs to quantitative measurements, since the intensity of the absorption change varies not only with polyP concentration but also with the average chain length of the polyP. Short-chain polyP cannot be identified using this method and only rough estimates of long-chain polyP concentrations can be obtained as discussed by Lorentz *et al.* (1997). This means that short-chain polyP present in the tissue can be highly underestimated by using this method alone and the method is only valid in

combination with another method like ^{31}P NMR or gel electrophoresis (see Chapter 5 for further discussion).

2.5.3 Phosphatase detection

The ELF substrate method was applied in order to locate the activity of phosphatases in mycorrhizal mycelium. The method has proven to be a suitable and sensitive method for measuring both ACPase- and ALPase-type activity associated with the extraradical mycelium as well as for identification of metabolically active AM fungal structures in roots (van Aarle *et al.*, 2001, van Aarle, 2002; see Chapter 1). Only ALPase-type activity was investigated in this thesis. ALPase-type activity occurs in roots mainly after mycorrhizal colonization and has been proposed as a marker for symbiotic efficiency of mycorrhizal colonization (Tisserant *et al.*, 1993). It was suggested that ALPase is an important enzyme in metabolic processes leading to P_i transfer to the host plant, but the function is not clear (see 1.3.4). The ELF method is based on a phosphatase substrate that fluoresces upon precipitation after enzymatic hydrolysis. The ELF-97 Endogenous Phosphatase Detection kit obtained from Molecular Probes using the included alkaline detection buffer (pH 8) and an incubation time of 30 min was used in the time-course study described in Chapter 4, where the effect of different P treatments on ALPase-type activity was investigated in *G. intraradices*. ALPase-type activity was also investigated in other species of AM fungi; *G. mosseae* and *Gi. rosea* (see 2.6). All samples were observed using a Zeiss Axiovert 35M fluorescence microscope with DAPI filter setting. Micrographs were recorded with a Cool Snap digital microscope camera, using either UV light alone or in combination with visible light.

2.6 Non-published results and their discussion

This section contains results not included in Chapters 3 and 4. These results contribute to meeting the objectives outlined in the Chapter 1 and they inspire to future investigations.

Investigations of other species of AM fungi.

A preliminary study of possible interfungal variations in P pools and ALPase-type activity included ^{31}P NMR and ELF investigations of the AM fungi *S. calospora*, *Gi. rosea*, *G. intraradices* and *G. mosseae*. The ^{31}P NMR spectrum of *S. calospora* was recorded without airlift on a Bruker dpx250 250 MHz spectrometer located at Risø using a 10-mm-diameter broadband probehead and ALPase-type activity was not investigated in this fungus. The aim of the investigation was to detect possible interfungal differences in polyP content, polyP average chain length and ALPase localization, as suggested by previous studies (Ezawa *et al.*, 1995; Boddington and Dodd, 1999; Solaiman *et al.*, 1999). The differences might contribute to the understanding of the observed diversity in the function of different species of AM fungi in their ability to supply P to the hosts (Pearson and Jakobsen, 1993; Smith *et al.*, 1994; Dickson *et al.*, 1999; Smith *et al.*, 2000). Plant and fungus fresh weights and the percent colonized root lengths are summarized in Table 8. Estimates of significance were based on statistics using STATISTICA (StatSoft, Inc. (2001) data analysis software system, version 6, www.statsoft.com) for data analysis. All variables were normally distributed according to Kolmogorov-Smirnov and Lilliefors test for normality. One-way ANOVA with subsequent Bonferroni

Post Hoc tests were used for determining species effects on the measured variables. All replicate pots were used in correlation analysis.

Table 8. Mean values (LSMEAN) and statistics of fresh weight per pot of six-wk-old cucumber plants and two-wk-old extraradical mycelium, and percent colonized root length. The overall experimental design and harvest was as described in Chapter 4 and inoculum were propagated in mycorrhizal *T. subterraneum* L. pot cultures. The experiment contained three replicate pots of each fungus. Pots were watered with nutrient solution including 0.7 mM KH_2PO_4 for the last two wk before harvest, i.e. after the sand had been changed. *In vivo* ^{31}P NMR spectra were obtained of sub-samples of roots and excised extraradical hyphae; in some cases the amounts of extraradical mycelium were too small for NMR studies. No non-mycorrhizal plants were included in the experiment. Values with same letter are not significantly different ($p < 0.05$ ANOVA post hoc Bonferroni tests).

Fungus	*Shoot fw* (g)	*Root fw* (g)	*Hyphae fw* (g)	*Percent colonized* root length (%)[£]
G. intraradices	33.11 a	25.65 a	0.69 ab	86 a
S. calospora	13.72 b	8.46 b	-	31 b
Gi. rosea	14.95 b	8.93 b	0.012 a	50 b
G. mosseae	31.93 a	19.61 a	1.27* b	72 a

The total amount of P added was approximately 20 mg (*G. intraradices*), 6 mg (*S. calospora*), 7 mg (*Gi. rosea*) and 16 mg (*G. mosseae*).

-No extraradical mycelium could be extracted from the hyphal compartment

*Number includes a considerable amount of sand, very sticky hyphae

£Fresh root subsamples were cleared in strong base and stained with trypan blue and the fractions of root lengths colonized by the AM fungus were measured (see Chapter 4).

Cucumber plants colonized by the two *Glomus* species were of similar size at the time of harvest and much larger than the two other species used in the experiment. This was clearly expressed in shoot and root fresh weights (Table 8). The fresh weights of extraradical mycelium that could be harvested from the hyphal compartment differed among AM fungal species. The higher values recorded for *G. mosseae* were partly caused by adhering sand, which was more difficult to remove from this fungus than from the others. No extraradical mycelium of *S. calospora* could be extracted in this experiment, although, a successful extraction of *S. calospora* mycelium had been carried out in a previous experiment at an early stage of my study. The amount of extraradical mycelium of *Gi. rosea* that could be harvested was also low. The percent of root length colonized was highest with *G. intraradices* and lowest with *S. calospora*, and seemed to relate to the amount of extraradical hyphae to some extent (Table 8). The data suggested differences in efficiency of the fungi. The two *Glomus* species were more efficient in colonizing the root, resulting in higher shoot and root fw. However part of the differences may relate to differences in inoculum quality, in particular for *S. calospora*.

In vivo ^{31}P NMR spectra of excised extraradical *G. intraradices*, *G. mosseae* and *Gi. rosea* hyphae and corresponding mycorrhizal roots together with a ^{31}P NMR spectrum of excised extraradical *S. calospora* hyphae obtained without airlift are presented in Fig. 23.

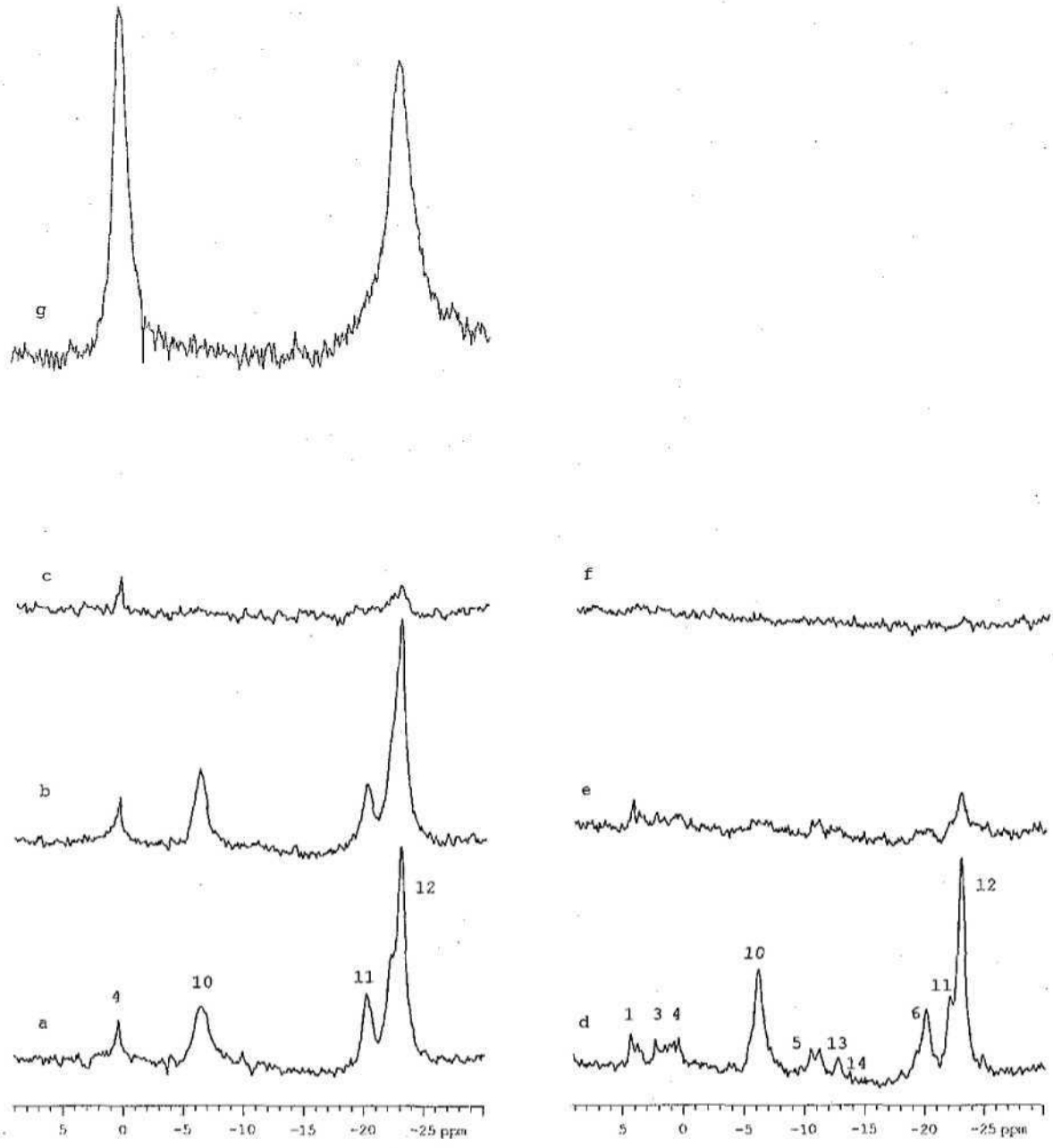

Fig. 23. *In vivo* ^{31}P NMR spectra of two-wk-old excised extraradical hyphae of one replicate of (a) *G. intraradices* (0.09 g), (b) *G. mosseae* (0.13 g) and (c) *Gi. rosea* (0.0013 g) and of the corresponding six-wk-old mycorrhizal roots (0.23 g, 0.21 g and 0.15 g, respectively) (d, e, f). (g) A ^{31}P NMR spectrum of excised ten-wk-old extraradical hyphae of *S. calospora* (0.54 g) obtained without airlift. The experimental setup and harvest of tissue used to obtain spectra a-f were as given in Table 8. The *S. calospora* hyphae were harvested from 10 wk old pots, without change of sand in the hyphal compartment. 100 mg P_i in aqueous solution was supplied to *S. calospora* hyphae 30 min before harvest. Chemical shifts of ^{31}P were measured relative to the signal at 16.38 ppm from MDP (100 mM at pH 7.5) contained in a capillary included in the NMR tube and are quoted relative to the resonance of 85% phosphoric acid at 0 ppm. The resonance of MDP is outside the shown spectral window. Buffer composition and *in vivo* NMR acquisition parameters for spectra a-f were as given in Chapter 4. The excised *S. calospora* hyphae were placed in water incl. 10% D_2O and the ^{31}P NMR spectrum (g) was recorded on a Bruker dpx250 spectrometer located at Risø, with the following NMR acquisition parameters: 30° pulse angle (7.5 μs), 0.28 s acquisition time, no recycle delay, proton decoupling by Waltz-16 composite pulse sequence, 7000 Hz sweep width, 50000 transients and 10 Hz line broadening. Total acquisition time was 3 h 54 min. Numbers refer to assignments given in Table 5.

The two *Glomus* species had rather similar spectra of excised extraradical hyphae (Fig. 23a and b), with a signal for vacuolar P_i and the three signals for polyP. The areas of the signals for vacuolar P_i and central polyP residues were used to estimate the amounts of the two compounds, and the amounts of both vacuolar P_i and polyP were found to be similar in the two *Glomus* species. The average chain length was found to be 11 in both *Glomus* species (cf. equation 7). The spectrum of excised *Gi. rosea* (Fig. 23c) contained signals for vacuolar P_i and for central P_i residues in the polyP chain, but in much lower amounts than in the *Glomus* species. This was probably due to the small sample size of the extraradical hyphae, and the average chain length could not be estimated. The spectrum of excised *S. calospora* (Fig. 23g) contained signals for vacuolar P_i and for central P_i residues in the polyP chain, and in relatively high amounts. No signals for terminal or penultimate P_i residues in the polyP chain could be detected, this indicates a relatively long polyP chain (> 35 P_i residues; see Fig. 14; Table 6). However, the average chain length has only been determined from this single spectrum, and needs to be confirmed from spectra obtained with optimized acquisition parameters. Furthermore, the sample of *S. calospora* mycelium also contained many spores due to the age of the mycelium, and the presence of spores may have influenced the spectrum. ^{31}P NMR spectroscopy carried out on germinating spores of the AM fungus *G. etunicatum* revealed polyP with an average chain length of 5 and a high level of P_i (Shachar-Hill *et al.*, 1995). It was later suggested that the presence of small, mobile polyP units and high P_i levels may reflect the active synthesis of various P metabolites in the germination stage of the AM fungal life cycle (Pfeffer *et al.*, 2001). The large polyP units indicated in *S. calospora* could reflect presence of resting spores, where large amounts of P were stored as long-chain polyP, however this needs further investigation.

The ^{31}P NMR spectra of cucumber roots colonized by the two *Glomus* species (Fig. 23d and e) contained the expected signals for the various P metabolites in mycorrhizal roots. The amounts of the various P metabolites were higher when the roots were colonized with *G. intraradices* than with *G. mosseae*. As the percent colonization was not significantly different between the two *Glomus* species (Table 8) and as the size of the various P pools was similar in the extraradical hyphae of the two fungi (Fig. 23a and b), the root spectra suggested that *G. mosseae* translocated P at a much lower efficiency than *G. intraradices*. No P signals could be detected in the ^{31}P NMR spectra of cucumber roots colonized by *Gi. rosea* (Fig. 23f), and this probably reflects the relatively small signal for vacuolar P_i and polyP in the extraradical mycelium (Fig. 23c).

The localization of ALPase in the extraradical mycelium of *G. mosseae* and *Gi. rosea* and the corresponding mycorrhizal cucumber roots was investigated by the ELF method (see 2.5.3; see Chapter 4 for similar results of *G. intraradices*). For comparison, samples were also investigated in visible light. ALPase-type activity could be observed inside the extraradical hyphae of both *G. mosseae* and *Gi. rosea* (Fig. 24a and e). ALPase-type activity could also be seen in mycorrhizal roots and specific staining of metabolically active fungal tissue was observed (Fig. 24c and g). The investigation indicated that the two species colonize roots differently, since a different distribution of ELF was seen. The pictures of *G. mosseae* roots (Fig. 24g and h) indicate a high degree of colonization at the inner cortex of the root, whereas *Gi. rosea* (Fig. 24c and d) mostly colonized the

outer cortex. However, this could be explained by the way the particular segments of root were colonized, and transverse sections are needed to confirm the different localization of colonization. Such an investigation was not included here.

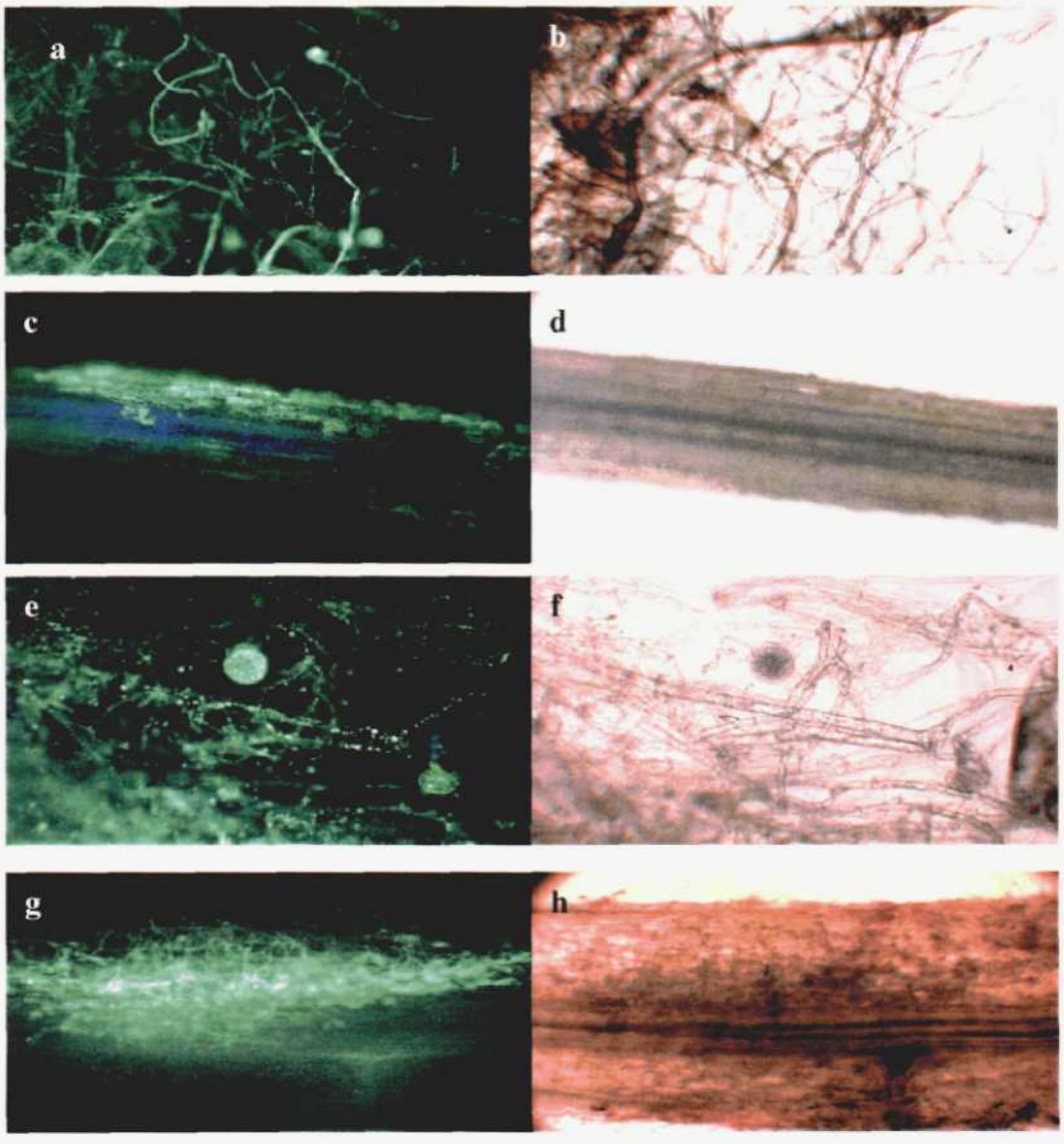

Fig. 24. ALPase-type activity in extraradical hyphae and mycorrhizal roots as visualized by ELF precipitation and epi-fluorescence. (a) Two-wk-old extraradical mycelium of *Gi. rosea* and (b) same tissue sample in visible light. (c) Six-wk-old *Gi. rosea* mycorrhizal cucumber roots and (d) same tissue sample in visible light. (e) Two-wk-old extraradical mycelium of *G. mosseae* and (f) same tissue sample in visible light. (g) Six-wk-old *G. mosseae* mycorrhizal cucumber roots and (h) same tissue sample in visible light. Samples obtained from same pots as used for the ^{31}P NMR spectra presented in Fig. 23.

ALPase-type activity was clearly demonstrated in extraradical mycelium of two species of AM fungi and specific staining of metabolically active fungal tissue could be seen in the corresponding mycorrhizal roots, similar to results obtained previously (Ezawa *et al.*, 1995; Boddington and Dodd, 1999; van Aarle *et al.*, 2001). However, the function of ALPase is not known (see 1.3.4), and any role in polyP breakdown has not been satisfactory determined. All that could be concluded was that the AM fungi were metabolically active.

The results presented in this section suggest that there are differences between species of AM fungi in P pool sizes within the extraradical mycelium and also in effectiveness of translocating the P to the root. The results were somewhat influenced by the low amount of extraradical mycelium of *Gi. rosea* and *S. calospora* which could be harvested and therefore the interfungal differences need further investigation. However, polyP was detected in all species studied, in contrast to the work by Boddington and Dodd (1999). Primarily short-chain length polyP was observed in extraradical mycelium, and this observation is in contrast to the long-chain or granular polyPs detected in other AM fungi by other methods (Cox *et al.*, 1975; Callow *et al.*, 1978; Solaiman *et al.*, 1999). The differences to earlier work could reflect the usefulness of ^{31}P NMR for the study of P pools and polyP dynamics in comparison to extraction procedures, DAPI or toluidine blue staining. The influence of the method for the results is further discussed in Chapter 5. In summary, the important role of polyP in the translocation of P from soil to plant by the AM fungus was demonstrated. It is clear that future investigations of the kind described in this thesis should include several fungi, since interfungal differences in especially polyP metabolism could be measured relatively easily.

In vivo ^{31}P NMR spectra of monoxenic cultured AM hyphae and roots

The compartmented growth system is not always ideal for obtaining a uniform hyphal production (see 2.2; 2.6; Chapter 4). Therefore a preliminary investigation was carried out to study whether monoxenic cultures were useful for studies similar to those presented in this thesis. The P metabolites in 15 mg hyphae collected from three monoxenic cultures of *G. intraradices* were studied by *in vivo* ^{31}P NMR. Surprisingly only polyP signals were seen (Fig. 25). No P signals were seen in ^{31}P NMR spectra of the corresponding mycorrhizal roots (transformed carrots) (results not shown). An explanation of this could be that any P_i in these roots was metabolized fast into immobile and therefore NMR-invisible P metabolites (nucleic acids, phospholipids etc.).

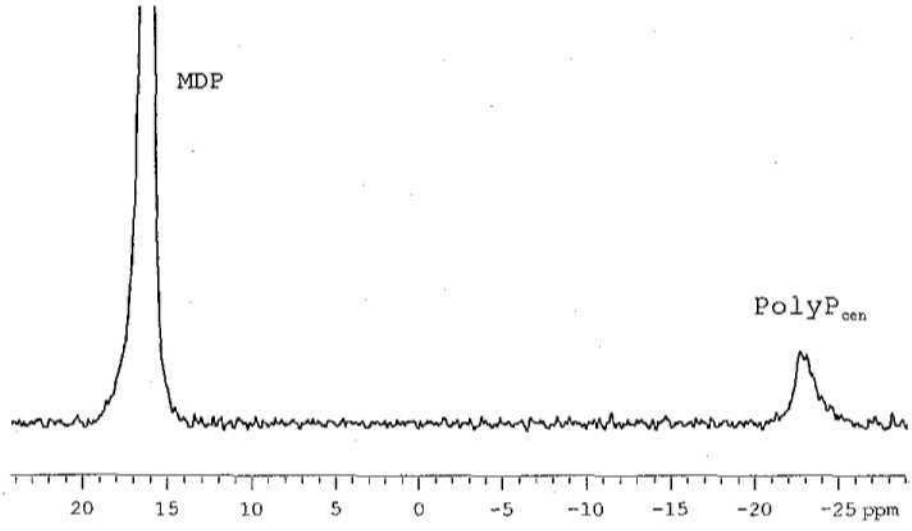

Fig. 25. *In vivo* ^{31}P NMR spectra of excised extraradical monoxenic cultured *G. intraradices* hyphae. The chemical shifts of ^{31}P were measured relative to the signal at 16.38 ppm from MDP (100 mM at pH 7.5) kept in a capillary, and the chemical shifts are quoted on the scale that puts the signal from 85% orthophosphoric acid at 0 ppm. Buffer composition: M medium (see Bécard and Fortin, 1988) including 10% D_2O. *In vivo* NMR acquisition parameters as given in Chapter 4, except for number of transients which were 14400, giving a total acquisition time of 2 h.

The results would also depend on the overall P status of the monoxenic culture, and the suitability of monoxenic cultures for NMR studies of P metabolism in AM fungi should be further investigated. Hyphae can be harvested very easily if the gel medium in the hyphal compartment is replaced by liquid media (Maldonado-Mendoza *et al.*, 2001).

2.7 Summary and conclusions

The work described in this chapter has demonstrated that *in vivo* ^{31}P NMR spectroscopy can be used for the study of P pools and their dynamics in AM fungi. Accordingly, polyP metabolism can be studied easily. However, it turned out to be more challenging to study the P metabolism and translocation in AM fungi *in vivo* than expected at the beginning. The fact that extraradical AM mycelium cannot take up C from any other structure or source than from the root interior was a serious experimental hindrance which markedly reduced the P_i uptake of excised hyphae. As a result, any P-treatment has to be performed while the symbiosis is still functioning and Chapters 3 and 4 concerns *in vivo* ^{31}P NMR measurements on plant and fungal tissue which has been exposed to various P treatments before harvest. Also other methods have been introduced in this chapter and will be further used in the following chapters.

Chapter 3 – ^{31}P NMR for the study of P metabolism and translocation in arbuscular mycorrhizal fungi

Abstract

^{31}P nuclear magnetic resonance (NMR) spectroscopy was used to study phosphate (P) metabolism in mycorrhizal and nonmycorrhizal roots of cucumber (*Cucumis sativus* L) and in external mycelium of the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* Schenck & Smith. The *in vivo* NMR method allows biological systems to be studied non-invasively and non-destructively. ^{31}P NMR experiments provide information about cytoplasmic and vacuolar pH, based on the pH-dependent chemical shifts of the signals arising from the inorganic P (P_i) located in the two compartments. Similarly, the resonances arising from α, β and γ phosphates of nucleoside triphosphates (NTP) and nucleoside diphosphates (NDP) supply knowledge about the metabolic activity and the energetic status of the tissue. In addition, the kinetic behavior of P uptake and storage can be determined with this method. The ^{31}P NMR spectra of excised AM fungi and mycorrhizal roots contained signals from polyphosphate (PolyP), which were absent in the spectra of nonmycorrhizal roots. This demonstrated that the P_i taken up by the fungus was transformed into PolyP with a short chain length. The spectra of excised AM fungi revealed only a small signal from the cytoplasmic P_i, suggesting a low cytoplasmic volume in this AM fungus.

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Page numbers at the top of each page within the paper are from the published version, but to emphasize the unity of the thesis, pages are also numbered consecutively (at the bottom of each page). Paper references are contained within the paper, however also contained in the list of references collected in the bibliography placed at the end of this thesis. Figures within this chapter are numbered independently from the rest of the thesis.

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^{31}P NMR for the study of P metabolism and translocation in arbuscular mycorrhizal fungi

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Key words: Arbuscular mycorrhizal fungi, *Cucumis sativus*, *Glomus intraradices*, NMR spectroscopy, phosphorus transport, polyphosphate.

Abstract

^{31}P nuclear magnetic resonance (NMR) spectroscopy was used to study phosphate (P) metabolism in mycorrhizal and nonmycorrhizal roots of cucumber (*Cucumis sativus* L) and in external mycelium of the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* Schenck & Smith. The *in vivo* NMR method allows biological systems to be studied non-invasively and non-destructively. ^{31}P NMR experiments provide information about cytoplasmic and vacuolar pH, based on the pH-dependent chemical shifts of the signals arising from the inorganic P (P_i) located in the two compartments. Similarly, the resonances arising from α, β and γ phosphates of nucleoside triphosphates (NTP) and nucleoside diphosphates (NDP) supply knowledge about the metabolic activity and the energetic status of the tissue. In addition, the kinetic behaviour of P uptake and storage can be determined with this method. The ^{31}P NMR spectra of excised AM fungi and mycorrhizal roots contained signals from polyphosphate (PolyP), which were absent in the spectra of nonmycorrhizal roots. This demonstrated that the P_i taken up by the fungus was transformed into PolyP with a short chain length. The spectra of excised AM fungi revealed only a small signal from the cytoplasmic P_i, suggesting a low cytoplasmic volume in this AM fungus.

Abbreviations: AM – Arbuscular Mycorrhizal; MES – 2-(N-morpholino)-ethane sulphonic acid; MDP – Methylene diphosphonic acid; *NMR – Nuclear magnetic resonance; NDP – Nucleoside diphosphates; NTP – Nucleoside* triphosphates; P – Phosphate; P_i – Inorganic phosphate; PCA – Perchloric acid; PolyP – Polyphosphate.

Introduction

Arbuscular mycorrhizal (AM) fungi are obligate, mutualistic symbionts, that colonise the roots of most land plants (Smith and Read, 1997). The symbiosis improves the nutrient uptake by the host plant and the AM fungus receives fixed carbon in return. The external mycelium of the AM fungus functions as an extension of the root system, allowing nutrients, such as phosphate (P), to be collected further away in the soil. The P is absorbed by the dense network of external fungal hyphae, then transported to the internal hyphae and transferred to the host (Smith and Read, 1997). Our current knowledge about the mechanisms by which P is taken up and translocated towards the host plant by the AM fungi is limited. Approaches applied to understand these mechanisms include detection of polyphosphate (PolyP) using phenol-detergent extraction and polyacrylamide gel electrophoresis (Callow et al., 1978), studies of phosphorus transport by hyphae using radiotracer techniques (Cooper and Tinker, 1978; Jakobsen et al., 1992a, b; Schweiger et al., 1999), investigations of phosphatase localisation using histo/cytochemical

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staining techniques (Ezawa et al., 1995; Gianinazzi-Pearson and Gianinazzi, 1978; Kojima et al., 1998; Saito, 1995; Tisserant et al., 1993) and characterisation of a fungal P transporter (Harrison and van Buuren, 1995).

^{31}P nuclear magnetic resonance (NMR) has been used for *in vivo* studies of P metabolism of ectomycorrhizal fungi cultured under axenic conditions. PolyP may play an important role in the transportation and storage of phosphorus in the fungus, and when P was added to ectomycorrhizal fungi, PolyP signals were found in the ^{31}P NMR spectra (Ashford et al., 1994; Gerlitz and Gerlitz, 1997; Gerlitz and Werk, 1994; Martin et al., 1983, 1985, 1994; Martins et al., 1999). NMR studies of mycorrhizal roots also have been carried out with emphasis on ectomycorrhizas, as in the studies of intact mycorrhizal red pine roots (MacFall et al., 1992), living mycorrhizal beech root tips (Loughman and Ratcliffe, 1984) and mycorrhizal beech and pine roots (Gerlitz and Gerlitz, 1997; Gerlitz and Werk, 1994). In contrast, there have been very few *in vivo* ^{31}P NMR investigations of AM fungi, with just one study of AM roots of leek and germinating spores (Shachar-Hill et al., 1995), and no published NMR work on P metabolism in the external mycelium of AM fungi. This can be ascribed to the difficulty in obtaining sufficient external mycelium for the NMR analysis.

Our objectives are twofold. Firstly, we review ^{31}P NMR spectroscopy as a method for studying P metabolism, with the emphasis on applications to mycorrhizal fungi and PolyP metabolism. Secondly, we demonstrate the use of ^{31}P NMR spectroscopy, *in vivo* and on extracts, for investigating P metabolism in mycorrhizal and nonmycorrhizal roots of cucumber (*Cucumis sativus* L.) and in external mycelium of the AM fungus *Glomus intraradices*. A compartmented growth system was used to produce external AM hyphae in sufficient quantities for the NMR measurements.

^{31}P NMR spectroscopy

NMR spectroscopy is based on the magnetic properties of the atomic nucleus and many elements have isotopes with such properties. Several biologically important isotopes are readily detectable and the method can be applied to living systems. This is a well developed application of NMR spectroscopy and it is referred to as *in vivo* NMR spectroscopy. *In vivo* NMR is a unique analytical method for the study of plant tissue since it is non-invasive and, therefore, offers the possibility to follow metabolic processes in a tissue in real time (Martin, 1985). ^{31}P NMR spectroscopy has been used extensively to investigate metabolic processes in plants (Lee et al., 1990; Martin et al., 1983; Roberts et al., 1980; Rolin et al., 1989). The ^{31}P isotope has a 100% natural abundance, and although the sensitivity of the ^{31}P nucleus is less than the ^{1}H nucleus, it is usually possible to obtain informative *in vivo* ^{31}P NMR spectra. Different P metabolites in the plant tissue give different signals in the NMR spectrum.

^{31}P NMR experiments provide information about cytoplasmic and vacuolar pH from the pH-dependent chemical shifts of the NMR signals arising from the inorganic phosphate (P_i) located in the two compartments (Roberts et al., 1980). It is, therefore, possible to study the intracellular pH under different physiological conditions. *In vivo* ^{31}P NMR has been used extensively in studies of cytoplasmic pH regulation of algae and plants (Fox and Ratcliffe, 1990; Fox et al., 1995; Küsel et al., 1990). In addition, signals arising from the α, β and γ phosphates of nucleoside triphosphates (NTP) and α and β of nucleoside diphosphates (NDP) provide information about the metabolic activity. Therefore, it is possible to monitor the metabolic state of the organism while the experiment is running, and in this way ensure that enough oxygen and nutrients are supplied (Roberts, 1987).

Phosphate is one of the main nutrients provided to the host plant by the mycorrhizal fungus and, in principle, *in vivo* ^{31}P NMR spectroscopy should be very suitable for studying the P physiology of the fungus. The method is a powerful technique for investigating time dependent phenomena and it is, therefore, possible to visualise the kinetic behaviour of P uptake and storage, and to investigate any PolyP metabolism in the mycorrhizal tissue. ^{31}P NMR spectroscopy has already been extensively used for the study of ectomycorrhizal fungi cultured under axenic conditions, as mentioned in the introduction. In these studies, the presence of PolyP was demonstrated by the detection of an NMR signal around −22 ppm in the ^{31}P NMR spectrum arising from the central P residues in the PolyP chain. Signals from P residues located at different places in the PolyP chain can be detected and the ratio of the intensities of the terminal, penultimate and internal P residues can be determined. These measurements allow the average chain length of the detectable PolyP to be calculated. Eleven P residues have been measured in the PolyP chain in the ectomycorrhizal fungus *H. crustuliniforme* (Martin et al., 1985) and

15 P residues in the ectomycorrhizal fungus *Pisolithus tinctorius* (Ashford et al., 1994). In comparison, PolyP in spores from the AM fungus *Glomus etunicatum* was measured to contain just five P residues (Shachar-Hill et al., 1995). Note that the NMR method only allows the detection of freely mobile PolyP, and that PolyP immobilised by precipitation or binding to membranes does not contribute to the NMR spectrum.

Materials and methods

Biological materials, soil and overall experimental design

The AM fungus, *Glomus intraradices* Schenck & Smith (BEG 87), was used in all experiments and was grown in symbiosis with *Cucumis sativus* L. (Aminex, F1 hybrid). External mycorrhizal hyphae were produced in a compartmented growth system, where the hyphae could be easily extracted from quartz sand. The growth system consisted of a 75 mm diameter 25 μm nylon mesh bag filled with 700 g of an irradiated (10 kGy, 10 MeV electron beam) 1:1 soil and quartz sand mixture (w/w) into which was incorporated 100 g of *Glomus intraradices* inoculum from a *Trifolium subterraneum* L. pot culture. Basal nutrients were mixed into the soil in the following amounts (mg kg^{-1} dry soil): K_2SO_4, 75.0; $CaCl_2 \cdot 5H_2O$, 75.0; $CuSO_4 \cdot 5H_2O$, 2.1; $ZnSO_4 \cdot 7H_2O$, 5.4; $MnSO_4 \cdot H_2O$, 10.5; $CoSO_4 \cdot 7H_2O$, 0.39; $NaMoO_4 \cdot 2H_2O$, 0.18; $MgSO_4 \cdot 7H_2O$, 45.0. The nylon mesh bag was placed in the centre of a pot filled with 2200 g of washed, autoclaved quartz sand (Johansen et al., 1996) (Figure 1). Inoculum was incubated for 1 week in moist (60% of water holding capacity) soil and four pre-germinated seeds were sown in the nylon mesh bag. After the seedlings emerged, the plants were thinned to two per pot and the pots were placed in a growth chamber with a 16/8 h light/dark cycle at 20/16 °C and Osram daylight lamps (HQI T250 W/D 500 μmole m^{-2} s^{-1}). An aqueous solution of 0.36 M NH_4NO_3 was supplied to the pots weekly and the total addition of N was 200 mg per pot. The sand in the outer compartment was replaced with fresh sand 4 weeks after sowing and the hyphae were harvested from this sand after another 3 weeks. An aqueous solution of 0.32 M KH_2PO_4 was applied to the sand compartment of the pots daily during the last week before harvesting; each pot received 100 mg P in total.

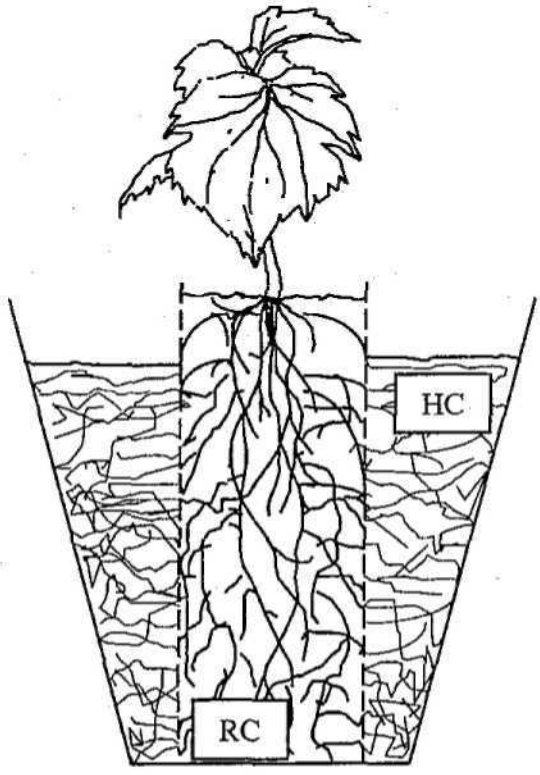

Figure 1. Compartmented growth system, composed of a 25 μm nylon mesh bag (root compartment, RC) and an outer hyphal compartment (HC).

Sample preparation and extraction techniques

External hyphae were collected from the sand by aqueous suspension and subsequent decanting onto a 38 μm sieve. This was repeated four times. The hyphal sample from each pot was placed in water and kept cold. Material used in extraction procedures were not rinsed further, whereas hyphae for *in vivo* ^{31}P NMR experiments were carefully shaken in a buffer containing 10 mM 2-(N-morpholino)-ethane sulphonic acid (MES) and 0.1 mM $CaSO_4$ at pH 6.0 to remove most of the sand trapped in the hyphal sample. The harvest procedure took around 15 min per pot and the rinsing procedure another 20-30 min. The phenol-detergent extraction method described by Callow et al. (1978) and modified by Ashford et al. (1994) was used to prepare extracts of external AM fungi for NMR experiments. Perchloric acid (PCA) was used as an alternative extraction agent. The pH was adjusted to 7.5 in both extracts. Root material was carefully collected from the mesh bag after washing away the soil. Only the first 3 cm of the roots were used for the

^{31}P NMR measurements and the excised root pieces were placed in a buffer similar to the one used for the hyphae, but with 50 mM glucose added. The excised roots were vacuum infiltrated for 3 min and the hypoxic roots were then oxygenated by bubbling oxygen through for 10 min. The samples of hyphae or roots were packed loosely in an NMR tube, using similar masses of material and similar packing in the different experiments.

NMR experiments

The ^{31}P spectra from the extracts were recorded at 242.812 MHz on a Varian Unity Inova 600 spectrometer using a broad band 5-mm-diameter probe head. The spectra were accumulated with a 90° pulse angle, a recycle time of 3.6 s and a total acquisition time of 4.1 h. The spectra obtained *in vivo* were recorded at 121.49 MHz on a Bruker CXP300 spectrometer using a double-tuned $^{13}C/^{31}P$ 10-mm-diameter probe head. The spectra were accumulated with a 45° pulse angle, a recycle time of 0.5 s and a total acquisition of 4 × 30 min in the hyphae spectrum, 9 × 10 min in the mycorrhizal roots spectrum and 12 × 30 min in the nonmycorrhizal root spectrum. All *in vivo* spectra were recorded using an airlift system operating with an oxygen flow rate of *c.* 90 ml min^{-1} (Fox et al., 1989), with the hyphae or roots placed in the same buffer as used during the washing procedure. The chemical shifts of the signals in the ^{31}P NMR spectra were measured relative to the signal from methylene diphosphonic acid (MDP), and the chemical shifts are quoted on the scale that puts the signal from 85% orthophosphoric acid at 0 ppm. The MDP in the *in vivo* spectra was kept in a capillary centred in the NMR tube. Estimates of the cytoplasmic and vacuolar pH were obtained from the chemical shift of the cytoplasmic or vacuolar P_i signal using calibration curves based on the work by Spickett et al. (1993).

Results and discussion

PolyP of a short chain length was seen in actively metabolising external AM fungi for the first time by the use of *in vivo* ^{31}P NMR spectroscopy. The growth system chosen for external AM fungus hyphae production (Johansen et al., 1996) was appropriate for producing sufficient hyphae for the NMR measurements. The wet weight of hyphae extracted from one pot was 0.5 g in average, including a small but non-quantified amount of sand. Approximately 0.2 g of hyphae were used in the *in vivo* ^{31}P NMR measurements.

We compared perchloric acid and phenolic-detergent as extraction agents. The occurrence of PolyP in the extracts of external AM fungi was easily seen in the ^{31}P NMR spectra (Figure 2). Spectra of both extracts showed a signal from the reference MDP at 16.38 ppm. The spectra showed a signal at 2.6 ppm identified as P_i and a signal at −5.2 ppm in the spectrum of the phenol-detergent extract (Figure 2A) and at −5.5 ppm in the spectrum of the PCA extract (Figure 2B) identified as the terminal P residues in the PolyP chain. Also a small signal arising from pyrophosphate was seen at −5.7 ppm in both spectra. A small unidentified signal at −6.5 ppm was seen in the spectrum of the PCA extract. The rest of the visualised signals were all placed around −21 ppm, consistent with the positions previously identified for the penultimate and central P residues of PolyP chains (Ashford et al., 1994; Gerlitz and Werk, 1994; Grellier et al., 1989; Martin et al., 1983, 1985; Shachar-Hill et al., 1995). The signals at −20.5 ppm in the spectrum of the phenol-detergent extract and at −20.8 ppm in the spectrum of the PCA extract were identified as the penultimate P residues and the signals at −21.6 ppm in the spectrum of the phenol-detergent extract and at −21.8 ppm in the spectrum of the PCA extract as the central P residues in the PolyP chain. It was likely that the signal almost inseparable from that of the central P residues at −21.3 ppm in the spectrum of phenol-detergent extract and at −21.5 ppm in the spectrum of PCA extract was the third P residues in the PolyP chain. Again, small unidentified signals were seen at −21.2 ppm, at −22.2 ppm and at −22.6 ppm in the spectrum of the PCA extract indicating several PolyPs with different chain length and therefore possible hydrolysed PolyP in the PCA extract.

The main difference in the spectra recorded from the two extraction methods was the separation of the relevant P signals and the signal size. The separation was clearly more effective when phenolic-detergent was used as extraction agent and much more PolyP was present in this extract compared to the PCA extract. The ratio among the areas of the signals in the spectrum of the phenolic-detergent extract of the external AM fungi for the terminal, the penultimate and the central P (including the third last) residues was 2:2:11, yielding an average PolyP chain length of 15 P residues. This value agrees with the average chain length found in ectomycorrhizas (Ashford et al., 1994; Martin et al., 1985). Calculation of the average chain

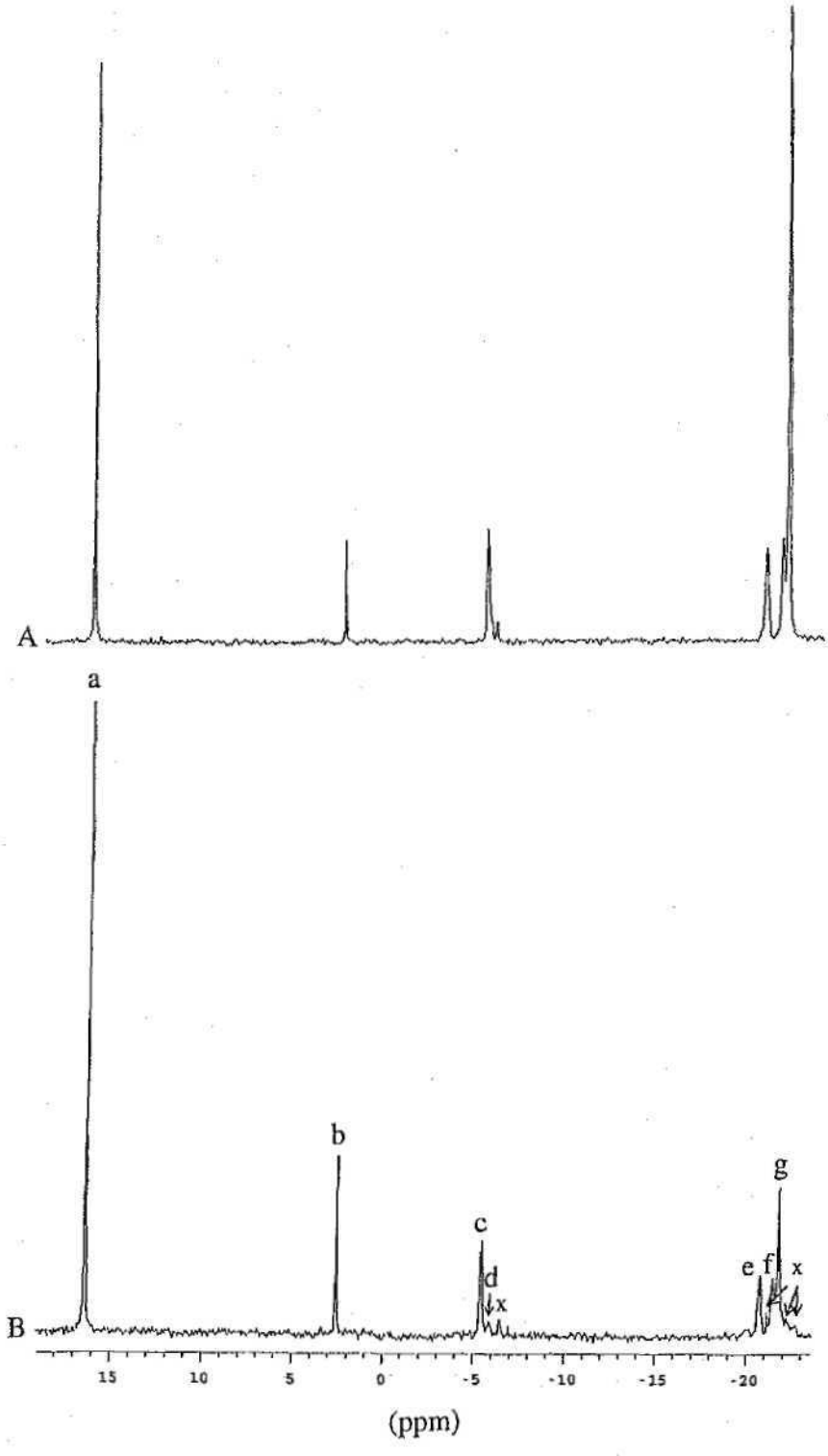

Figure 2. ^{31}P NMR spectra of extracts of *Glomus intraradices* mycelium showing resonances from P_i and mobile PolyP. (A) Extract from *Glomus intraradices* made by phenol-detergent extraction and (B) extract from *Glomus intraradices* made by perchloric acid extraction. Peak assignments are as follows: (a), MDP; (b), P_i; (c), terminal PolyP residues; (d), pyrophosphate; (e), penultimate PolyP residues; (f), the third last PolyP residues; (g), central PolyP residues; (x), unidentified P.

length in the perchloric extract gave a smaller value, with a ratio of 2:2:5 yielding an average chain length of 9. This again showed that the perchloric extraction procedure probably hydrolysed some PolyP.

The *in vivo* ^{31}P NMR spectra of excised roots showed the P-containing metabolites in the living roots of mycorrhizal and nonmycorrhizal *Cucumis sativus* plants (Figure 3). The difference in the signal-to-noise ratio in the two spectra was due to differences in accumulation of spectra. The signal at −22.7 ppm in the mycorrhizal root spectrum corresponded to the central P residues in the PolyP chain (Figure 3B). This signal was not present in the spectrum of nonmycorrhizal roots (Figure 3A). The signals around 4.5 ppm corresponded to several phosphomonoesters. The signal at 2.7 ppm in the mycorrhizal root spectrum (Figure 3B) and at 2.9 ppm in the nonmycorrhizal root spectrum (Figure 3A) arose from cytoplasmic P_i corresponding to a pH of 7.2 and 7.4 for the cytoplasm in the mycorrhizal roots and nonmycorrhizal roots, respectively. The lower cytoplasmic pH in the mycorrhizal roots (Figure 3B) together with the poor lineshape of the signal suggests that the tissue was not as well oxygenated as the nonmycorrhizal roots (Figure 3A). Oxygen deprivation of the tissue could typically be reflected in a shift of the cytoplasmic P_i signal towards a lower ppm value and thus acidification of the cytoplasm. At the same time, the usually unobservable NDP signal could be detected (Fox et al., 1995). The very small signal at around −5.9 ppm in the mycorrhizal roots spectrum (Figure 3B) could be assigned to the βphosphate of NDP, supporting the observation that the mycorrhizal tissue was not uniformly oxygenated. A small contribution to this signal from terminal P residues of PolyP could not be excluded, since this signal would be around this position as well. The assignment of the rest of the signals in the two spectra were as follows (Figure 3): The signal at around 0.8 ppm arose from vacuolar P_i indicating an acidic vacuole with a pH of around 5. The signals at around −5.3 ppm, −10.4 ppm and −19.0 ppm arose from γ, α and βphosphates of NTP and the signals at around −11 ppm and −12.5 ppm from uridine diphosphoglucose (Figure 3). These results corresponded to the study of leek mycorrhizas (Shachar-Hill et al., 1995), except for the amount of cytoplasmic P_i in both the mycorrhizal and nonmycorrhizal root spectrum. In the present work, only a small cytoplasmic P_i signal was seen, indicating a low cytoplasmic volume in this part of the root.

Analysis of excised but living external AM hyphae (Figure 4) indicated a significant amount of PolyP as well as P_i in the vacuoles. The signals in the external AM fungus spectrum were much broader than the signals in the root spectra (Figure 3) and it seemed likely that this was caused by the difficulty of removing all the sand grains from the hyphae. The resolution and signal-to-noise ratio were acceptable after 2 h of spectra accumulation. The signal at 1.0 ppm was identified as the vacuolar P_i corresponding to a pH of 5.6 in the vacuoles (Figure 4). The small signal at 3.0 ppm should be the resonance from the cytoplasmic P_i. This position in the spectra corresponded to a cytoplasmic pH of 7.6, characterising well oxygenated tissue (Loughman & Ratcliffe, 1984), but the fact that this signal was small suggests that the fungus contained only small amounts of cytoplasm. As in the extracts of external AM fungi, the spectrum showed signals at −5.9 ppm and −22.4 ppm, identified as the terminal and central P residues in the PolyP chain. Also the penultimate P residues could be seen, but it was difficult to distinguish this from the central P residues. An approximate average PolyP chain length of 17 was found from the ratio 2:2:13, but it was difficult to measure the correct areas of the signals. However, this chain length seems reasonable in comparison to previous reports from ectomycorrhizal fungi (Ashford et al., 1994; Martin et al., 1985).

Physiologically reliable results from the *in vivo* measurements can be obtained only if the oxygen and substrate supply is sufficient to avoid anaerobic conditions and shortage of nutrients in the NMR tube. Several problems arose for the *in vivo* NMR spectroscopy of the external mycelium of the AM fungus. It was very difficult to keep the AM fungus hyphae homogeneously dispersed in the NMR tube due to the circulation system with liquid flow (known as a perfusion system). The hyphae tended to clot together and stop the flow or escape out in the system of circulation. In contrast, the airlift system in which the liquid medium inside the NMR tube was oxygenated and circulated by air bubbling in the upper part of the NMR tube proved to be very useful (Fox et al., 1989).

The absence of signals arising from NTP in the *in vivo* ^{31}P NMR spectra of the external hyphae of the AM fungus also must be considered (Figure 4). Normally, the metabolic status of the tissue inside the NMR tube is measured by the position of the cytoplasmic P_i signal and the signals arising from NTP. The cytoplasmic P_i signal was very weak (Figure 4), perhaps because of a very low tissue cytoplas-

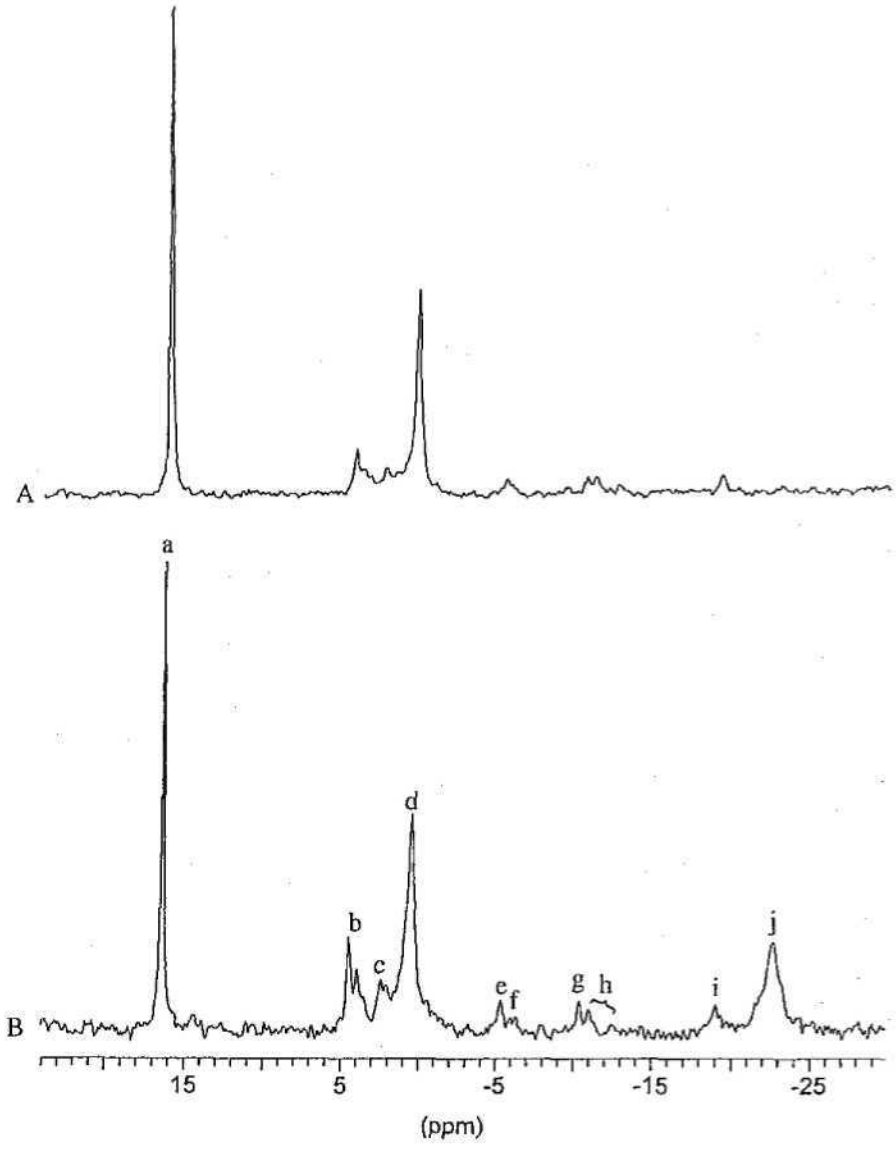

Figure 3. In vivo ^{31}P NMR spectra of excised cucumber roots. (A) Nonmycorrhizal and (B) mycorrhizal with *Glomus intraradices*. Peak assignments: (a), MDP; (b), several phosphomonoesters; (c), cytoplasmic P_i; (d), vacuolar P_i; (e), γ-NTP; (f), β-NDP/terminal PolyP residues; (g), α-NTP; (h), uridine diphosphoglucose; (i), β-NTP; (j), central PolyP residues.

mic content, and the NTP signals were too small to be detected. Therefore it was difficult to measure the metabolic status. However, the signals arising from NTP were also missing in the spectra of extracts (Figure 2), and it is, therefore, possible that NTP levels are very low in 3-week-old external mycelium of *Glomus intraradices*. It is also possible that the cytoplasmic content varies with the age of the AM hyphae similar to the age dependency observed for hyphal activity (Sylvia, 1988).

The identification of soluble PolyP in the excised external AM hyphae of *Glomus intraradices* by *in vivo* ^{31}P NMR provides evidence that this AM fungus stores PolyP of a short chain length. Still, a titration study of the pH dependent chemical shift of the terminal P residues in the PolyP chain is required before a final determination of the compartment for the

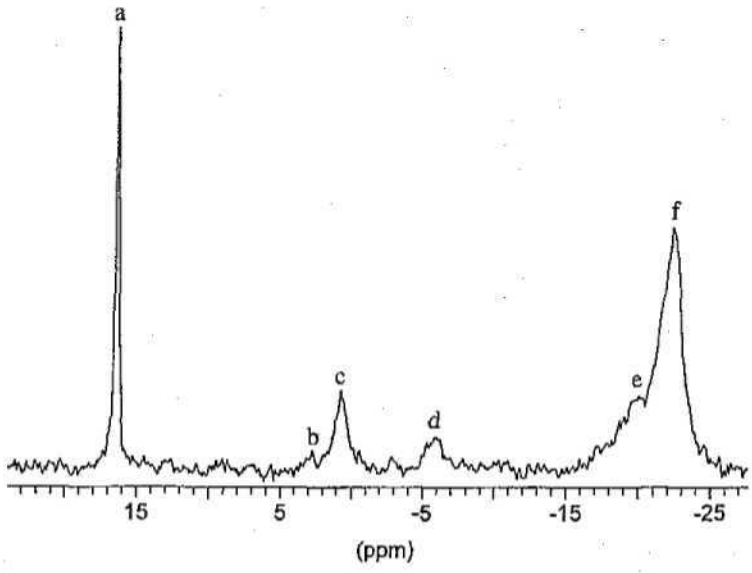

Figure 4. In vivo ^{31}P NMR spectrum of excised *Glomus intraradices* hyphae showing resonances from P_i and mobile PolyP. Peak assignments: (a), MDP; (b), cytoplasmic P_i; (c), vacuolar P_i; (d), terminal PolyP residues; (e), penultimate PolyP residues; (f), central PolyP residues.

PolyP can be predicted (Martin et al., 1994). The identification of PolyP of a short chain length is in contrast to earlier work proposing that PolyP in the AM fungus *Glomus mosseae* existed as large granules which were moved by cytoplasmic streaming and stabilised by Ca^{2+} ions (Callow et al., 1978). The granules were identified after extraction by the phenol-detergent technique including an ethanol treatment and subsequent staining with toluidine blue and were probably artefacts of specimen preparation (Orlovich and Ashford, 1993). Studies using freeze-substitution illustrated that the PolyP was present in soluble form stabilised by K^+ ions in the ectomycorrhizal fungus *Pisolithus tinctorius* (Orlovich and Ashford, 1993). Further characterisation using gel electrophoresis and ^{31}P NMR showed that the PolyP consisted of about 15 phosphate subunits (Ashford et al. 1994). However, the NMR method cannot exclude the presence of granules, since PolyP present as large granules would not have been detected by NMR spectroscopy. The present study demonstrates that the NMR approach overcomes the uncertainties in identifying PolyP after chemical fixation. PolyP of a short chain length may serve as a reservoir pool of P_i inside the hyphae and this is similar to what is seen in several ectomycorrhizal fungi (Ashford et al., 1994; Gerlitz and Werk, 1994; Martin et al., 1983, 1985, 1994). In conclusion, these preliminary studies suggest that the process of P translocation in the external hyphae of arbuscular mycorrhizas may be similar to that of ectomycorrhizas in which the P appears to be transported mainly as PolyP in a motile vacuole system (Ashford et al., 1994, Smith and Read, 1997).

Acknowledgements

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Chapter 4 - Phosphate pools and their dynamics in the arbuscular mycorrhizal fungus, *Glomus intraradices*, studied by *in vivo* ^{31}P NMR spectroscopy

Abstract

In vivo ^{31}P NMR spectroscopy was used for the investigation of phosphate pools in the arbuscular mycorrhizal fungus *Glomus intraradices* and mycorrhizal cucumber roots. A time-course study of differently phosphate-treated extraradical hyphae revealed the appearance of polyphosphate before the appearance of vacuolar inorganic phosphate and further a time lag was observed before the corresponding phosphate metabolites appeared in mycorrhizal roots. The amount of polyphosphate was considerably higher than vacuolar inorganic phosphate and synthesis of polyphosphate might be important for effective phosphate uptake in arbuscular mycorrhizal fungi. The polyphosphate was located in the vacuoles and the measured average chain length of the polyphosphate was short, supporting a role for polyphosphate in the transport of phosphate from soil to host root by arbuscular mycorrhizal fungi. *In vivo* NMR could not detect cytoplasmic inorganic phosphate in the extraradical mycelium possibly because of a small cytoplasmic volume or low concentration of cytoplasmic phosphate.

Publication details

A shorter version of this paper (with fewer details in the introduction and without the alkaline phosphatase measurements) will be submitted for publication in Plant Physiology shortly. Figures and tables within this chapter are numbered independently from the rest of the thesis.

Introduction

As phosphate (P) is an essential nutrient for all organisms, it is required in relatively large amounts and is often limiting to plant growth. It is therefore important for plants to have mechanisms for efficient uptake of this nutrient. Arbuscular mycorrhizal (AM) fungi colonize the roots of most land plants and the symbiosis between AM fungi and plants is characterized by bi-directional nutrient transport between the symbionts (Smith and Read, 1997). The AM fungus receives an indispensable supply of fixed carbon, in return for improved nutrient uptake by the host plant. The extraradical mycelium of AM fungi allows the plant to access inorganic orthophosphate (P_i) in the soil solution beyond the depletion zone formed by the plant around the actively absorbing roots.

The uptake, translocation and transfer of P by the extraradical mycelium of the AM fungi have been studied extensively, and a model of the overall mechanisms has been widely accepted. It is believed that P_i in the soil solution is absorbed by the extraradical mycelium via an AM fungal P transporter energized by a P-type H^+-ATPase (Harrison and van Buuren, 1995; Ferrol *et al.*, 2000; Maldonado-Mendoza *et al.*, 2001). The P_i entering the cytoplasm of the AM fungus may be incorporated into phosphorylated primary metabolites, structural molecules and nucleic acids. It is assumed that P_i excess taken up into the AM extraradical hyphae is subsequently transferred to the vacuoles and to some extent condensed into polyphosphate (polyP). The P-containing substances such as polyP are then believed to be translocated to the intraradical hyphae in vacuoles in a motile tubular vacuolar system similar to that of ectomycorrhizas (Smith and Read, 1997). Recent studies of the vacuolar system in AM fungi have confirmed the presence of tubular vacuoles and microtubules (Timonen *et al.*, 2001; Uetake *et al.*, 2002). Once translocated to the symbiotic interface inside the root, the polyP has to be hydrolyzed and the released P_i subsequently transferred to the plant root cells to achieve a mutualistic symbiosis. This transfer is believed to occur at the arbuscular interface, which is in agreement with the recent discovery that plant P transporters are expressed in root cells containing arbuscules (Rosewarne *et al.*, 1999; Rausch *et al.*, 2001). In addition, incubation of extracted intraradical mycelium of *Gi. margarita* in glucose increased the efflux of P_i and polyP content in the hyphae decreased simultaneously, indicating a role for polyP in the exchange of carbon and P_i between symbionts (Solaiman and Saito, 2001).

Accordingly, polyP is considered to have an important role in the P translocation process. In addition, polyP as a storage form enjoys a clear osmotic advantage over P_i and synthesis of polyP may be a major part of the mechanism by which the fungus controls the cytoplasmic P_i concentration (Mimura, 1999). PolyP has been detected in AM fungi by cytochemical methods (Cox *et al.*, 1975; Cox *et al.*, 1980; Boddington and Dodd, 1999; Ezawa *et al.*, 2001b), by extraction methods followed by polyacrylamide gel electrophoresis (Callow *et al.*, 1978; Solaiman *et al.*, 1999) and by nuclear magnetic resonance (NMR) (Shachar-Hill *et al.*, 1995; Rasmussen *et al.*, 2000).

A more detailed understanding of the underlying mechanisms for P uptake, translocation and transfer from soil via the fungus to the plant *in vivo* is required. In particular, the metabolism of polyP is still

unclear. PolyPs are linear polymers of three to more than 1000 P_i residues linked by high-energy phosphoanhydride bonds and have been found in many organisms (Kornberg *et al.*, 1999). The polyP metabolic pathways in prokaryotes have been well described. An enzyme of polyP synthesis, i.e. polyphosphatekinase (PPK) which synthesizes polyP from ATP, has been identified and characterized together with several enzymes of polyP utilization. Identified enzymes of polyP utilization include PPK as a phosphotransferase and polyphosphateglucokinase (PPGK), which utilizes polyP as an ATP substitute, i.e. transfers the terminal P_i of polyP to glucose, producing glucose-6-phosphate. Identified enzymes of polyP utilization in prokaryotes also include hydrolases; exopolyphosphatase (PPX), which hydrolyses and releases terminal P_i of polyP and endopolyphosphatase, which cleaves polyP internally to generate shorter chains (Kornberg *et al.*, 1999). In comparison, the polyP metabolism in eukaryotes is poorly understood, no well documented synthesizing enzyme activity has been found so far (i.e. no PPK-like activity has been identified) and only few enzymes of polyP utilization have been identified. The polyP-hydrolyzing enzyme characterized is PPX-type enzymes, identified in vacuoles, cytoplasm and cell envelope in yeast (*Saccharomyces cerevisiae*) (Kornberg *et al.*, 1999). Capaccio and Callow (1982) detected PPGK-type activity in the AM fungus *Glomus mosseae* and demonstrated a possible role of polyP of being a phosphagen. In the same study, PPX-type activity was found in intraradical hyphae, where polyP hydrolysis is therefore likely to occur. Ezawa *et al.* (2001a, b) have detected both PPX-type and PPGK-type activity in the AM fungi *G. coronatum* and *G. etunicatum*. Two different PPX-type enzymes were found, which differed in activity between extraradical and intraradical hyphae (Ezawa *et al.*, 2001b). This demonstrated that polyP metabolism differed between extraradical and intraradical hyphae and that polyP accumulation might be a dynamic balance between synthesis and hydrolysis. Only negligible PPGK-type activity compared with that of hexokinase, which utilizes ATP as a phosphagen, was detectable in the spores and intraradical hyphae (Ezawa *et al.*, 2001a). From this result, it was concluded that polyP was not the main phosphagen for glucose phosphorylation.

The amount, size and major role of polyP present in the extraradical and intraradical hyphae is a matter of debate. Several investigations suggest the presence of rather long-chain polyP or granules especially located in the extraradical mycelium (Callow *et al.*, 1978; Solaiman *et al.*, 1999), supporting the idea that polyP metabolism in extraradical and intraradical hyphae may be different. Further, the extraradical mycelium of the AM fungus *G. manihotis* seems not to accumulate polyP in comparison with high amounts of polyP in the extraradical mycelium of *Gigaspora rosea* (Boddington and Dodd, 1999), suggesting differences in polyP metabolism between species.

Alkaline phosphatases (ALPase) may also be involved in polyP breakdown since cytochemical studies revealed ALPase activity localized in the vacuoles of mature arbuscules (Gianinazzi-Pearson and Gianinazzi, 1978; Tisserant *et al.*, 1993; Ezawa *et al.*, 1995; Saito, 1995). In addition, studies have shown that AM fungi differ in their localization of ALPase activity, and this difference might reflect different sites for P_i transfer (Ezawa *et al.*, 1995). ALPase in the extraradical mycelium might be an effective marker for metabolic activity in studies of AM fungi. However, Ezawa *et al.* (2001b) found a non-specific intraradical acid phosphatase (ACPase) which seemed to be involved in hydrolysis of

polyP, suggesting ACPase more likely to be involved in P_i transfer from intraradical mycelium to host. Enzyme-labeled-fluorescence (ELF) staining has been used for visualization of ALPase and ACPase activity in AM fungal hyphae and AM mycorrhizal roots (van Aarle *et al.*, 2001). ELF substrate is normally slightly fluorescent, but after removal of the P_i group, a bright green fluorescent precipitate is formed, which makes it an appropriate marker for phosphatase activity.

In conclusion, the presence of polyP in many species of AM fungi is well documented, but the characterization of the polyP and the mechanisms involved in its metabolism are not clear. Staining methods with variable specificity or invasive methods have commonly been used to identify polyP in previous investigations, such that artifacts of specimen preparation could possibly have interfered with the polyP chain length, as discussed by Orlovich and Ashford (1993). Non-invasive and non-destructive techniques are required in order to obtain more detailed information of P pools and polyP content in AM fungi. For this purpose, *in vivo* ^{31}P NMR spectroscopy is a unique analytical method (Rasmussen *et al.*, 2000).

The objective of the present study was to characterize the dynamic incorporation of P_i into various P pools within extraradical mycelium and mycorrhizal roots. In addition, we wanted to investigate the dynamics of polyP synthesis, and determine how fast polyP was synthesized, which chain lengths could be detected and in which compartment the polyP was located. For this purpose, a time-course study was carried out to use *in vivo* ^{31}P NMR for investigating the formation of P compounds in differently P-treated hyphae of the AM fungus *G. intraradices* and mycorrhizal cucumber roots. Secondly, chain lengths of polyP where further investigated by the use of extraction procedures followed by colorimetric measurements and ^{31}P NMR. Finally, the active state of P metabolism in the mycorrhiza was confirmed by means of ELF staining for ALPase activity.

Materials and methods

Biological materials, soil and overall experimental design

The AM fungus *Glomus intraradices* Schenck & Smith (DAOM 197198, Biosystematics research centre, Ottawa) was used in all experiments and was grown in symbiosis with *Cucumis sativus* L. (Aminex, F1 hybrid). External mycorrhizal hyphae were produced in a compartmented growth system, where the hyphae could be rapidly extracted from quartz sand. The growth system consisted of a 75 mm diameter 25 μm nylon mesh bag filled with 725 g of an irradiated (10 kGy, 10 MeV electron beam) 1:1 soil and quartz sand mixture (w/w, here called 'soil') into which was incorporated 75 g of *G. intraradices* inoculum from a *Trifolium subterraneum* L. pot culture. Basal nutrients minus P were mixed into the soil in the following amounts (mg kg^{-1} dry soil): K_2SO_4, 75.0; $CaCl_2 \cdot 5H_2O$, 75.0; $CuSO_4 \cdot 5H_2O$, 2.1; $ZnSO_4 \cdot 7H_2O$, 5.4; $MnSO_4 \cdot H_2O$, 10.5; $CoSO_4 \cdot 7H_2O$, 0.39; $NaMoO_4 \cdot 2H_2O$, 0.18; $MgSO_4 \cdot 7H_2O$, 45.0. This final soil had an extractable P content of 11 μg P g^{-1} as obtained with 0.5 M $NaHCO_3$ (Olsen *et al.*, 1954). The nylon mesh bag was placed in the centre of a pot filled with 2200 g of washed, autoclaved quartz sand (Johansen *et al.*, 1996). Inoculum was incubated for one week in

moist (60% of water holding capacity) soil and three pre-germinated seeds were sown in the nylon mesh bag. After seedling emergence, plants were thinned to two per pot and the pots were placed in a growth chamber with a 16/8 hour light/dark cycle at 20/16°C and Osram daylight lamps (HQI T250 W/D 500 μmole m^{-2} s^{-1}). The pots were watered daily and an aqueous solution of 0.36 M NH_4NO_3 was supplied to the pots weekly to provide a total addition of 200 mg N per pot. The sand in the outer compartment was replaced with fresh washed and autoclaved sand four weeks after sowing and the hyphae were harvested from this sand after another two weeks plus the time after additional P treatment. The experiment included 24 pots in total.

P treatments and harvest

The experiment included three series of P treatments (Table 1). A total of 100 mg P was supplied as an aqueous solution of 0.32 M KH_2PO_4 at the outer edge of the hyphal compartment in 20 of the pots at ten different times before harvesting. One set of ten pots had received no P before the supply of 100 mg P at time = 0 h (Table 1, treatment 1) while another set of ten pots had previously received various amounts of P as 0.7 mM P in nutrient solution (pH 6.0, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$; 1 mM NH_4NO_3; 1 mM K_2SO_4; 0.8 mM $MnSO_4 \cdot 7H_2O$; 0.7 mM $Na_2HPO_4 \cdot 2H_2O$; 25 µM Fe(III) NaEDTA; 25 µM H_3BO_3; 5 µM $MnSO_4 \cdot H_2O$; 2 µM $ZnSO_4 \cdot 7H_2O$; 0.5 µM $CuSO_4 \cdot 5H_2O$; 0.1 µM $Na_2MoO_4 \cdot 2H_2O$; 4 nM $CoCl_2 \cdot 6H_2O$) over two weeks (Table 1, treatment 2). Three further pots previously supplied with P received no additional P at time = 0 h (Table 1, treatment 3). One pot received no P at all and served as control. The pots were not watered for two days before final P treatment to ensure a good distribution of the P in the hyphal compartment.

Table 1. P treatment and fresh weights of extraradical mycelium of *G. intraradices* and mycorrhizal cucumber plants. Treatment 1: No P before supply of 100 mg P at time = 0 h. Treatment 2: Hyphae received P during two wk before supply of 100 mg P at time = 0 h. Treatment 3: Hyphae received P during previous two wk, but received no P at time = 0 h.

	Harvest (hours after additional P supply)										
	1	2	5	10	16	24	34	48	72	96	Average value
Treatment 1											
Total P supply (mg)	100	100	100	100	100	100	100	100	100	100	
Shoot (g fw)	30.0	18.6	23.8	25.2	21.3	25.7	25.5	26.1	29.4	33.3	26
Root (g fw)	19.2	11.4	13.6	11.4	12.0	28.5	17.6	17.7	18.5	14.0	16
Root NMR tube (g fw)	0.14	0.09	0.13	0.12	0.16	0.17	0.26	0.17	0.25	0.17	
Hyphae (g fw)	0.08	0.03	0.12	0.03	0.12	0.26	0.73	0.13	0.70	0.07	0.23
Hyphae NMR tube (g fw)	0.08	0.03	0.12	0.03	0.12	0.11	0.12	0.07	0.14	0.07	
Treatment 2											
Total P supply (mg)	125	122	124	124	120	119	120	127	132	130	
Shoot (g fw)	40.9	36.0	41.2	39.9	33.8	30.5	39.0	42.0	53.2	41.3	40
Root (g fw)	32.0	22.6	27.5	30.0	23.2	20.7	18.1	27.3	33.4	22.4	26
Root NMR tube (g fw)	0.31	0.12	0.16	0.20	0.10	0.21	0.32	0.25	0.40	0.11	
Hyphae (g fw)	0.52	0.18	0.39	0.44	0.46	0.26	0.35	0.38	0.51	0.12	0.36
Hyphae NMR tube (g fw)	0.07	0.08	0.09	0.08	0.06	0.08	0.07	0.07	0.08	0.06	
Treatment 3											
Total P supply (mg)	20				24					32	
Shoot (g fw)	39.4				33.3					49.6	41
Root (g fw)	28.9				17.9					24.3	24
Root NMR tube (g fw)	0.16				0.14					0.15	
Hyphae (g fw)	0.38				0.03					0.08	0.16
Hyphae NMR tube (g fw)	0.09				0.03					0.08	

Control pot (No P added, harvested at t = 96 h): Shoot fresh weight 26.3 g, root fresh weight 20.1 g and hyphae fresh weight 0.10 g
fw; fresh weight

External hyphae were harvested from the sand by aqueous suspension and subsequent decanting onto a 38 μm sieve. This was repeated three times for each pot. The hyphal sample from each pot was gently rinsed, first in water and then in a buffer containing 10% D_2O, 50 mM glucose, 10 mM 2-(N-morpholino)-ethane sulphonic acid (MES) and 0.1 mM $CaSO_4$ at pH 6.0 to remove most of the sand trapped in the hyphal sample. The harvest and rinsing procedure of hyphae from one pot lasted about

15 min. Root material was carefully collected from the mesh bag after washing away the soil and the excised root pieces were placed in a buffer similar to the one used for the hyphae. Sub-samples of hyphae or roots were packed loosely in an NMR tube, using similar masses of material (between 0.03-0.14 g fresh weight hyphae and 0.09-0.40 g fresh weight roots, respectively) and similar packing density in the different experiments.

Plant analysis

The total shoot fresh weights were determined. The total fresh weights of both hyphae and roots were determined after NMR analysis after removing excess moisture by pressing the tissue on filter paper. Fresh root sub-samples were cleared in 10% KOH and stained with 0.05% trypan blue by a modification of the method of Murphy and Riley (1962) and the percentage of root lengths colonized by *G. intraradices* were measured in accordance with Giovannetti and Mosse (1980).

In vivo NMR experiments

The *in vivo* ^{31}P spectra were recorded at 242.812 MHz on a Varian Unity Inova 600 spectrometer using a broad band 10-mm-diameter probe head. The spectra were accumulated with a 45° pulse angle (26.5 μs), an acquisition time of 0.064 s, a recycle time of 0.45 s, proton decoupling by Waltz-16 composite pulse sequence, a sweep width of 15.0 kHz, 12000 scans, a total acquisition time of 1h 45 and processed with 30 Hz line broadening. All *in vivo* spectra were recorded using an airlift system operating with an oxygen flow rate of *c.* 90 ml min^{-1} (Fox *et al.*, 1989), with the hyphae or roots placed in the same buffer as used during the washing procedure. The chemical shifts of the signals in the ^{31}P NMR spectra were measured relative to the signal from methylene diphosphonic acid (100 mM MDP, at pH 7.5) contained in a capillary included in the NMR tube, and the chemical shifts were quoted on the scale that puts the signal from 85% orthophosphoric acid at 0 ppm. Assignment of the various P signals in the spectra was done by comparison with ^{31}P NMR spectra of ectomycorrhizal fungi (Martin *et al.*, 1983, 1994; Grellier *et al.*, 1989; Gerlitz, 1996), roots mycorrhizal with ectomycorrhizas (Loughman and Ratcliffe, 1984; MacFall *et al.*, 1992; Gerlitz and Werk, 1994; Martins *et al.*, 1999), AM fungi and roots mycorrhizal with AM (Shachar-Hill *et al.*, 1995; Rasmussen *et al.*, 2000). Estimates of the cytoplasmic and vacuolar pH were obtained from the chemical shift of the cytoplasmic or vacuolar P_i signal using calibration curves made as suggested by Spickett *et al.* (1993). Similarly, a calibration curve of pH versus ^{31}P chemical shift of terminal polyP (synthetic polyP glass of type 25 obtained from Sigma Chemical Co.) was made in order to measure the pH of the compartment in which the polyP was located in as suggested by Martin *et al.*, 1994.

Extraction procedures, colometri and NMR of extracts and standards

^{31}P NMR spectra of synthetic polyP glasses of types 5, 15, 25, 35 and 75+ obtained from Sigma Chemical Co. were recorded in order to investigate the upper limit of NMR-visible polyP, i.e. the maximum average chain length that can be observed in an NMR spectrum. Approximately 5 mg of the synthetic polyP was dissolved in 2.7 ml H_2O and 0.3 ml D_2O with 0.1 M Na_2EDTA added. The EDTA was present to give sharper signals in the NMR spectra (MacDonald and Mazurek, 1987). ^{31}P NMR spectroscopy of the polyP-containing solutions was performed at 242.812 MHz on a Varian Unity

Inova 600 spectrometer equipped with a broad band 10-mm-diameter probe head. The spectra were accumulated with a 90° pulse angle (53 μs), an acquisition time of 0.59 s, a recycle time of 2.5 s, proton decoupling by Waltz-16 composite pulse sequence, a sweep width of *15.0* kHz, *1200* scans, a total acquisition time of 61 min and processed with 20 Hz line broadening. The chemical shifts of the signals in the ^{31}P NMR spectra were measured relative to the signal from MDP (100 mM MDP, at pH 7.5) contained in a capillary included in the NMR tube, and the chemical shifts were quoted on the scale that puts the signal from 85% orthophosphoric acid at 0 ppm.

Different polyP fractions in the hyphae used for *in vivo* ^{31}P NMR investigations were successively extracted with trichloroacetic acid (TCA) (acid-soluble, short-chain polyP), EDTA (neutral-soluble long-chain polyP) and phenol-chloroform (PC) (long-chain granular polyP), based on the method of Clark *et al.* (1986). Extracts were made from extraradical hyphae condensed into five pools (Table 5): 1) Hyphae from treatment 1 with additional P for 1 to 5 h, 2) hyphae from treatment 1 with additional P for 10 to 96 h, 3) hyphae from treatment 2 with additional P for 1 to 5 h, 4) hyphae from treatment 2 with additional P for 10 to 96 h and 5) hyphae from treatment 3. Three replicate samples of each successive extraction were made when enough extraradical hyphae could be harvested. The extracted polyP in aqueous solution was precipitated by adding Tris-HCl (1 M, pH 7.6) to a final concentration of 0.2 M and 2 volumes of acetone. The mixture was frozen at –80°C for more than 15 min, melted and centrifuged for 10 min. The residue was air dried over night, dissolved in water and kept at –20°C until analysis.

The polyP content in the extracts was identified by measuring the metachromatic reaction of toluidine blue at 530 nm and 630 nm, according to Griffin *et al.* (1965) and Solaiman *et al.* (1999). The assay was performed by adding 10 μl of the polyP extract to tubes containing 0.75 ml each of 0.2 M acetic acid and 30 mg l^{-1} toluidine blue. The content of polyP was estimated by comparison of the absorption spectra with standard curves produced by using 1 and 5 μg of each of three synthetic polyP glasses; the polyP chosen were type 5 and type 25 polyP for the short-chain and type 75+ polyP for the long-chain polyP. Synthetic polyP glasses of types 5, 25 and 75+ were obtained from Sigma Chemical Co. PolyP was classified as being present or not in the different fractions (Table 5).

^{31}P NMR spectroscopy of the polyP-containing extracts was performed using the same parameters as used with synthetic polyP, except that the recycle time was 6 s, given a total acquisition time of 2 h 12 min and the spectra were processed with 10 Hz line broadening. Each extract was diluted to 3.1 ml with water containing 10% D_2O.

A TCA extraction of synthetic polyP type 5 was performed at two concentrations (10.4 mg and 1.06 mg polyP type 5), and the resulting extracts were investigated by ^{31}P NMR. Each extract was diluted to 3.1 ml with water containing 10% D_2O and the spectra were obtained using the same parameters as used for extracts of hyphae. The extracted P was hydrolyzed in a 4:1 (v/v) solution of nitric and perchloric acid and total P content was determined by the molybdate blue method (Murphy and Riley,

1962) on a Technicon Autoanalyser II (Technicon Autoanalyzers, Analytical Instrument Recycle, Inc., Golden CO, USA).

Phosphatase activity detection

Extraradical hyphae and mycorrhizal roots stored at -80°C for nine months were assayed for ALPase by the ELF-97 kit (see van Aarle *et al.*, 2001). Only alkaline detection buffer (pH 8, ELF-97 Endogenous Phosphatase Detection Kit, Molecular Probes) was used and an incubation time of 30 min was used in all experiments. The samples were observed using a Zeiss Axiovert 35M fluorescence microscope with DAPI filter setting. Micrographs were recorded with a Cool Snap digital microscope camera, using either UV light alone or in combination with visible light.

Hyphae studied with the ELF-97 assay included (Table 2) no P-treated (control pot), hyphae from treatment 1 with additional P for 34 h, hyphae from treatment 2 with additional P for 48 h and hyphae from treatment 3. Mycorrhizal roots studied by the ELF kit included (Table 2) no P-treated (control pot), roots from treatment 1 with additional P for 2 h and roots from treatment 2 with additional P for 72 h.

Table 2. Extraradical mycelium and mycorrhizal roots studied with the ELF-97 assay.
P treatments as described in Table 1.

P treatment	
Hyphae	Roots
control pot	control pot
1, P for 34 h	1, P for 2 h
2, P for 48 h	2, P for 72 h
3	n.d.

n.d., not determined

Results

Plant growth and mycorrhiza formation

The growth of the plants and the extraradical mycelium was heterogeneous in the investigation and only low quantities of extraradical mycelium could be harvested in some pots (Table 1). The plants received different amounts of P and therefore statistics were not applicable. However, average values were included in Table 1 to give a general indication for growth differences between treatments. Cucumber plants grown in symbiosis with *G. intraradices* that had received no P before supply of 100 mg P (Table 1, treatment 1) were smaller than plants that had received P during previous two wk (Table 1, treatments 2 and 3) at the time of harvest. The difference was expressed in the shoot fresh weight. However also the root fresh weight and the mass of the extraradical hyphae showed the same trends. Further, addition of P at ten different times before harvest (Table 1, treatments 1 and 2) had

only little effect on the shoot, root and extraradical hyphae fresh weight. The proportion of root length colonized by mycorrhizal fungi was 85% ± 7%, independent of P treatment.

Phosphate pools in extraradical mycelium

In vivo ^{31}P NMR spectra were obtained for extraradical *G. intraradices* hyphae harvested at different times from 20 individual pots which were each supplied with 100 mg P at time = 0 h. Previous P supplies to the hyphal compartment were either none (treatment 1, Fig. 1) or 0.7 mM P in nutrient solution for two wk (treatment 2, Fig. 2). Three pots with a previous P supply received no additional P at time = 0 h (treatment 3, Fig. 3). Spectra obtained of hyphae from pots where a marked higher or lower quantity of total hyphae could be harvested within the treatment were labeled in Figs.

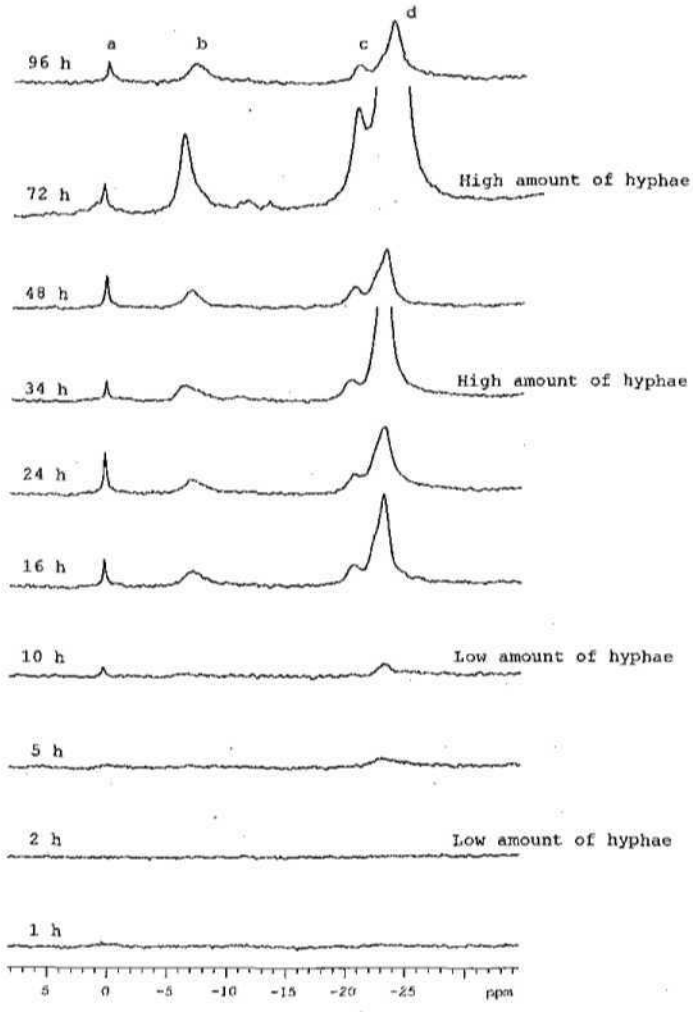

Figure 1. *In vivo* ^{31}P NMR spectra of excised *G. intraradices* hyphae from pots, which had previously received no P, harvested individually ten times after a supply of 100 mg P pot^{-1}. Sample size varied between 0.03 and 0.14 g fw; not all harvested tissue was used in the NMR tube. Spectra were calibrated against reference signal, the reference signal was outside the spectral window. Peak assignments were as follows: (a), vacuolar P_i; (b), terminal polyP residues; (c), penultimate polyP residues; (d), central polyP residues.

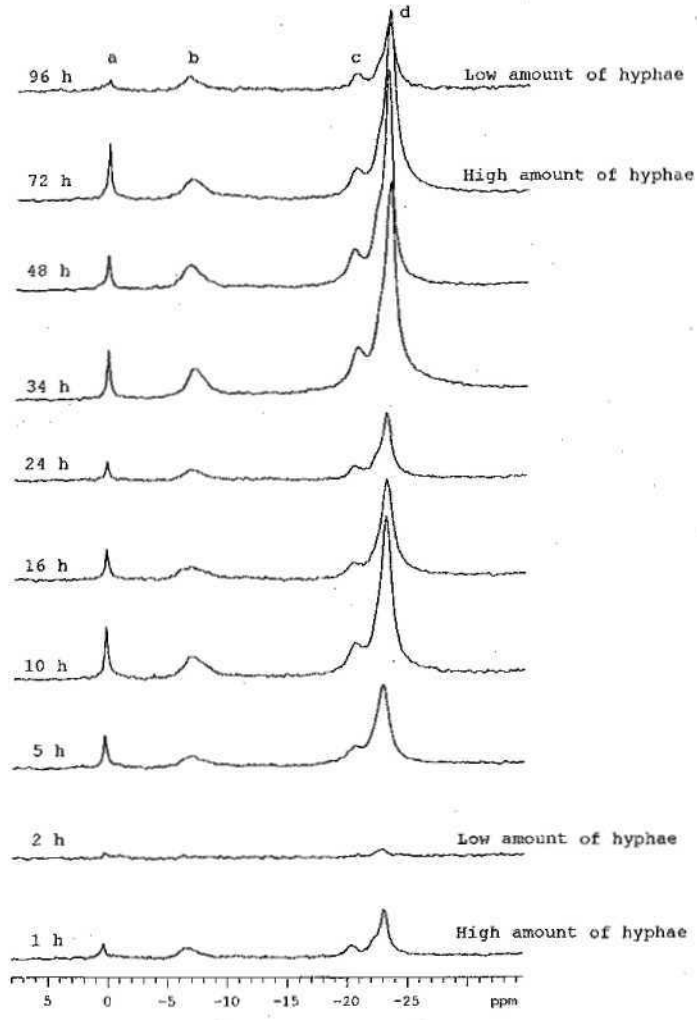

Figure 2. *In vivo* ^{31}P NMR spectra of excised *G. intraradices* hyphae from pots, which had previously received 0.7 mM P in nutrient solution over two wk, harvested individually ten times after additional supply of 100 mg P pot^{-1}. Sample size varied between 0.06 and 0.09 g fw; not all harvested tissue was used in the NMR tube. Spectra were calibrated against reference signal, the reference signal was outside the spectral window. Peak assignments as in Fig. 1.

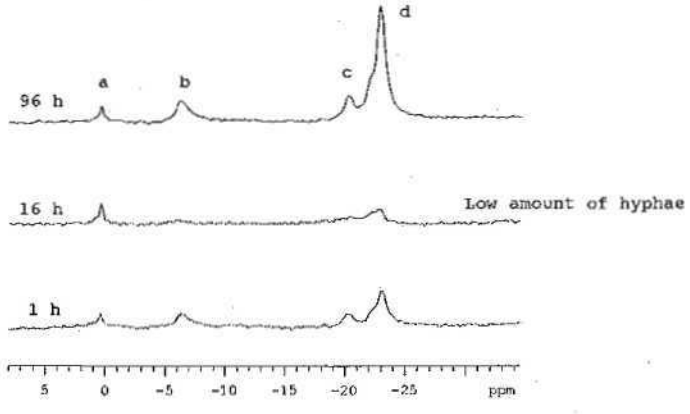

Figure 3. *In vivo* ^{31}P NMR spectra of excised *G. intraradices* hyphae from pots, which had previously received 0.7 mM P in nutrient solution over two wk but received no additional P at time = 0 h, harvested individually at three times. Sample size varied between 0.03 and 0.09 g fw; not all harvested tissue was used in the NMR tube. Spectra were calibrated against reference signal, the reference signal was outside the spectral window. Peak assignments as in Fig. 1.

The signal at –23.0 ppm corresponded to the central P_i residues in a NMR-visible polyP chain. This signal was present in all spectra obtained of hyphae from treatments 2 and 3 (Figs. 2 and 3, peak d) but did not appear in hyphae from treatment 1 until 5 h after the P supply at 0 h (Fig. 1, peak d). The three remaining signals visible in the spectra were assigned to vacuolar P_i (peak a, 0.4 ppm), terminal P_i residues in the polyP chain (peak b, -6.4 ppm) and penultimate P_i residues in the polyP chain (peak c, -20.1 ppm). The vacuolar P_i signal was not detectable until 10 h after P was supplied and the terminal and penultimate P_i residue signals could not be detected until 16 h after P supply within treatment 1 (Fig. 1). No signals were visible in the spectrum of hyphae from the control pot (no P added, spectrum not shown). A vacuolar pH of approximately 5.5 was estimated from the chemical shift value of vacuolar P_i. Harvest times or P treatment did not influence this pH. The chemical shifts of terminal P_i residues in the polyP chain predict a pH of approximately 6.0, as estimated from the pH titration curve for terminal P_i residues (data not shown). This acidic pH value supported a vacuolar compartment for the NMR-visible polyP.

The amounts of vacuolar P_i and polyP were estimated from the areas under the signals for vacuolar P_i and central polyP residues, calibrated against the reference signal. The amounts of vacuolar P_i were considerably lower than amounts of polyP within the same hyphal sample, independent of P treatment (approximately ten times as much polyP as vacuolar P_i) (Figs. 1, 2 and 3). Also, the amounts of vacuolar P_i tended to increase to a constant level in treatments 1, 2 and 3. The amounts of polyP tended to increase after additional P was supplied in both treatments 1 and 2 (Figs. 1 and 2), although a considerable variation was observed in the areas of the signals for central polyP residues. The areas of the signals also varied for central polyP residues in the spectra of hyphae from treatment 3 (Fig. 3). The variation in signal areas corresponded to some degree to the variation in quantity of total hyphae (see also Table 1).

Phosphate pools in mycorrhizal cucumber roots

A time-course of *in vivo* ^{31}P NMR spectra was also obtained for the mycorrhizal roots from the three P treatments. The *in vivo* ^{31}P NMR spectrum of roots from treatment 2, additional P for 24 h was enlarged to facilitate the assignment of the various P signals (Fig. 4). This spectrum contained dominating signals at –22.9 ppm and 0.3 ppm from polyP (peak j) and vacuolar P_i (peak c), respectively, as well as signals from several other P-containing metabolites. The signals around 4.4 ppm corresponded to several phosphomonoesters (peak a) and the smaller signal at 2.3 ppm was attributed to cytoplasmic P_i (peak b). Various signals for nucleic acid triphosphates (NTP, peak d, f and h) at –5.3 ppm, -10.4 ppm and –19.0 ppm were also easily distinguished, together with signals from uridine diphosphoglucose (peak g) at around –11.0 ppm and –12.5 ppm. Signals from terminal (peak e) and penultimate (peak i) P_i residues of polyP could also be detected at –6.4 ppm and –20.1 ppm, however, the signals overlapped the signals from phosphates of NTP.

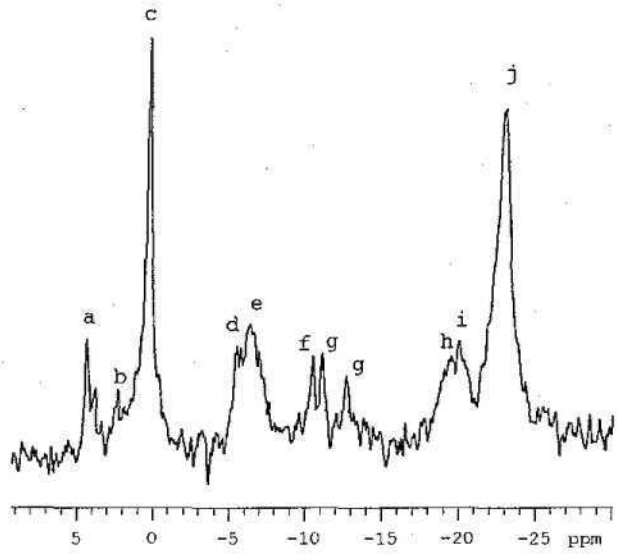

Figure 4. *In vivo* ^{31}P NMR spectrum of mycorrhizal cucumber roots that had previously received 0.7 mM P in nutrient solution over two wk and harvested 24 h after additional P supply of 100 mg P. The spectrum were enlarged in order to show details in the peak assignment. Peak assignments were as follows: (a), several phosphomonoesters; (b), cytoplasmic P_i; (c), vacuolar P_i; (d), γ-NTP; (e), β-NDP/terminal polyP residues; (f), α-NTP; (g), uridine diphosphoglucose; (h), β-NTP; (i), penultimate polyP residues; (j), central polyP residues.

Spectra obtained of roots from pots where a marked higher or lower quantity of total hyphae could be harvested within the treatment were labeled in Figs. 5-7. Signals from the various P-containing metabolites were detectable from the first harvest time (1 h) in the spectra of mycorrhizal roots from treatments 2 and 3 (Figs. 6 and 7). In contrast, no P signals could be detected in the spectra of roots from treatment 1 until 10 h after P supply, when the signal for the central P_i residues in a polyP chain and vacuolar P_i could be identified (Fig. 5). Spectra taken 6 h later (Fig. 5, 16 h) contained all the signals which could be detected in roots that had previously received P. No signals were visible in the spectrum of roots from the control pot (no P added, spectrum not shown).

The positions of the signals for vacuolar and cytoplasmic P_i did not change between harvest times or P treatment; pH was estimated to be 7.4 and approximately 5.3 in the cytoplasmic and vacuolar compartment, respectively. The high cytoplasmic pH values indicate that the tissue was adequately supplied with oxygen (Fox and Ratcliffe, 1990).

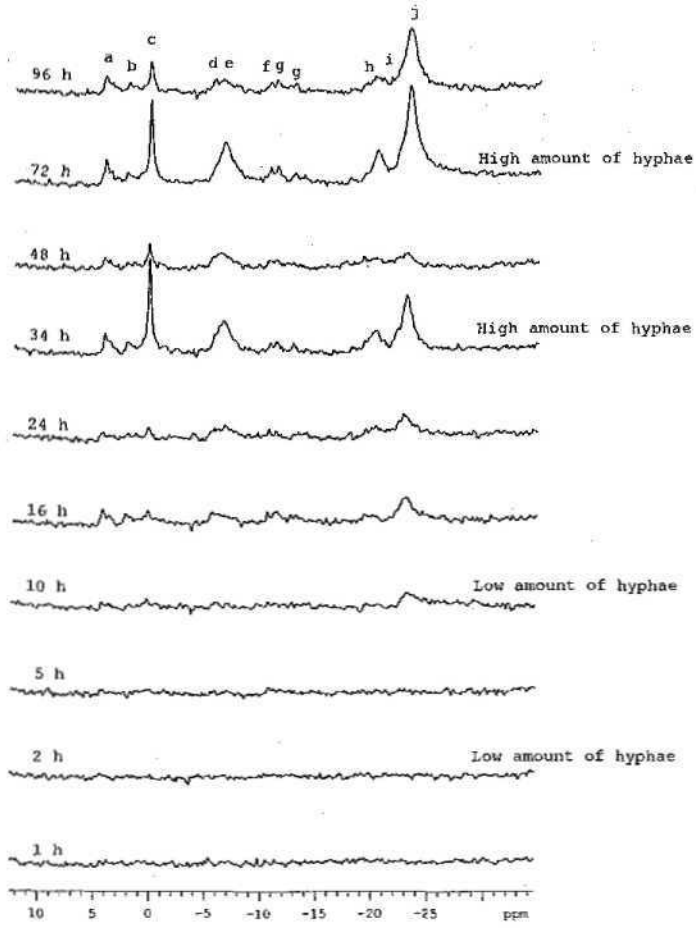

Figure 5. *In vivo* ^{31}P NMR spectra of mycorrhizal cucumber roots from pots, which had previously received no P, harvested individually ten times after a supply of 100 mg P pot^{-1}. Sample size varied between 0.09 and 0.26 g fw; not all harvested tissue was used in the NMR tube. Spectra were calibrated against reference signal, the reference signal was outside the spectral window. Peak assignments as in Fig. 4.

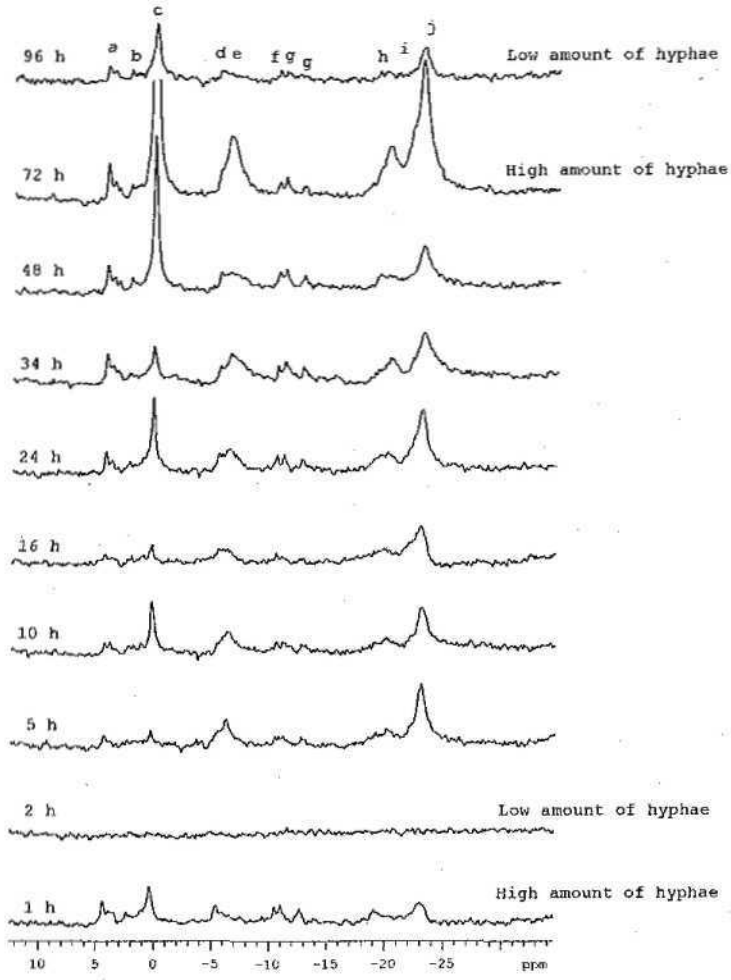

Figure 6. *In vivo* ^{31}P NMR spectra of mycorrhizal cucumber roots from pots, which had previously received 0.7 mM P in nutrient solution over two wk, harvested individually ten times after additional supply of 100 mg P pot^{-1}. Sample size varied between 0.12 and 0.40 g fw; not all harvested tissue was used in the NMR tube. Spectra were calibrated against reference signal, the reference signal was outside the spectral window. Peak assignments as in Fig. 4.

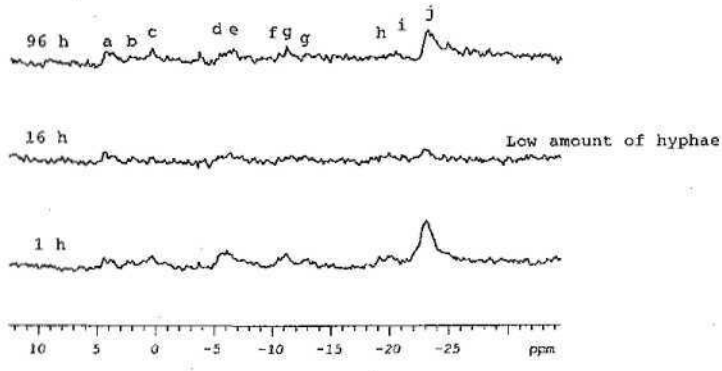

Figure 7. *In vivo* ^{31}P NMR spectra of mycorrhizal cucumber roots from pots, which had previously received 0.7 mM P in nutrient solution over two wk but received no additional P at time = 0 h, harvested individually at three times. Sample size varied between 0.14 and 0.16 g fw; not all harvested tissue was used in the NMR tube. Spectra were calibrated against reference signal, the reference signal was outside the spectral window. Peak assignments as in Fig. 4.

The quantities of the P pools in the roots were estimated in a similar way as done for the hyphae. The amounts of polyP and vacuolar P_i were more similar in the mycorrhizal roots than in extraradical hyphae, and corresponded to the level of vacuolar P_i in the extraradical hyphae. Despite the variation seen in Figs. 5, 6 and 7, some trends could be observed. The amounts of polyP in the mycorrhizal roots tended to increase to a constant level after additional P supply in both treatments 1 and 2 (Figs. 5 and 6). Mycorrhizal roots from treatment 3 contained polyP at this level from the first harvest (Fig. 7). Amounts of vacuolar P_i increased with additional P supply in both treatments 1 and 2, but the variation was high (Figs. 5 and 6). The mycorrhizal roots from treatment 3 contained very low amounts of vacuolar P_i (Fig. 7). Some of the variation in signal areas corresponded to the variation in quantity of *total* hyphae (see also Table 1), especially corresponded high amounts of hyphae to large signals in the spectra.

Average chain length in extraradical hyphae and mycorrhizal roots

The ratio between the areas of the signals in the *in vivo* ^{31}P NMR spectra for terminal, penultimate and central P_i residues in the polyP chain can be used to estimate the average chain length of the polyP chain. The average polyP chain length was calculated in both in extraradical hyphae and mycorrhizal roots using the formula $2(P_{ter} + P_{pen} + P_{cen}) / P_{ter}$, where P_{ter}, P_{pen} and P_{cen} represent the areas of the signals for the terminal, penultimate and central P_i residues in the polyP chain. Some overlapping of the signals for terminal and penultimate P_i residues in the polyP chain with the signals from phosphates of NTP were seen in the spectra of mycorrhizal roots. However, the areas needed for the estimation of the average polyP chain length were obtained by calibrating the overlapping signals with the detached signal of α-NTP. The mean average chain length was 13 P_i residues in the extraradical hyphae and nine P_i residues in the mycorrhizal roots (Table 3).

Table 3. Average polyP chain length in extraradical *G. intraradices* mycelium and mycorrhizal cucumber roots. P treatment as described in Table 1.

	Harvest (hours after additional P supply)										
	1	2	5	10	16	24	34	48	72	96	Average value
Treatment 1											
PolyP chain length hyphae	n.d.	n.d.	n.d.	n.d.	10	9	20	9	24	10	14
PolyP chain length roots	n.d.	n.d.	n.d.	n.d.	n.d.	10†	9	6	14	14	11
Treatment 2											
PolyP chain length hyphae	12	n.d.	14	14	11	15	12	15	21	11†	14
PolyP chain length roots	9	n.d.	9	7	8	9	6	6	7	8	8
Treatment 3											
PolyP chain length hyphae	9				n.d.					11	10
PolyP chain length roots	10				n.d.					9	10

n.d., Not determined since no signals for terminal and penultimate P residues in the polyP chain could be detected or the respective areas were to small to be precisely detected

†, Very small signals for terminal and penultimate P present in spectrum, however, areas could be estimated

Further characterization of the polyP

All five synthetic polyP glasses gave rise to NMR spectra containing the expected signals for polyP (spectra not shown). The chain length could be estimated from the ratio between the areas of the signals in the ^{31}P NMR spectra for terminal, penultimate and central P_i residues (Table 4), except for type 75+ polyP. The spectrum of type 75+ polyP contained only one signal for central residues of polyP, indicating a rather long chain, and it was not possible to estimate the precise chain length.

Table 4. Estimated average polyP chain length of synthetic polyP glasses obtained from Sigma Chemical Co.

Type polyP	Chain length estimated from ^{31}P NMR spectrum
Type 5	6
Type 15	14
Type 25	22
Type 35	34
Type 75+	n.d.

n.d., not determined since no signals for terminal and penultimate P residues in the polyP chain could be detected

A combination of extraction procedures, colometric measurements and NMR was performed in order to further investigate the amount and chain length of polyP present in the fungus. PolyP was successively extracted into TCA, EDTA and PC soluble fractions. The absorption spectra of the metachromatic reaction indicated some long-chain and granular polyP in the EDTA and PC fractions, respectively, in extracts of hyphae from treatments 1 and 2 with additional P for 10 to 96 h (Fig. 8, B and C, Table 5). In contrast, the EDTA and PC fractions of the extracts of hyphae from treatments 1 and 2 contained no long-chain or granular polyP in the 1-5 h interval (Table 5, absorption spectra not shown). Short-chain polyP in the various TCA fractions could not be identified by the colorimetric measurements.

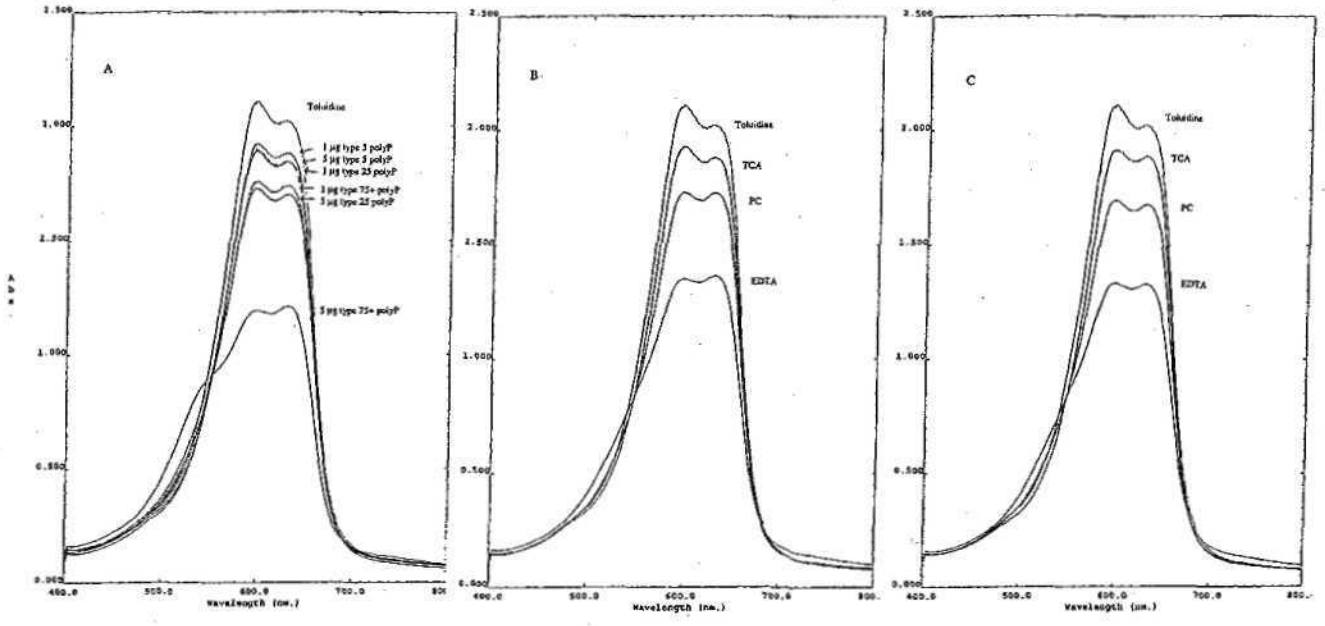

Figure 8. Absorption spectra of (A), toluidine blue in the absence and presence of synthetic polyP of various chain lengths and in various amounts as indicated in the figure. The polyP standards were synthetic polyP glasses with average chain length 5, 25 and 75+ obtained from Sigma Chemical Co. (B), Toluidine blue in the absence and presence of successively extracted polyP in fractions as indicated in the figure. The extraradical mycelium used in the extraction procedure had previously received no P but received 100 mg aqueous P 10 to 96 h prior to harvests. (C), Toluidine blue in the absence and presence of successively extracted polyP in fractions as indicated in the figure. The extraradical mycelium used in the extraction procedure had previously received 0.7 mM P in nutrient solution over two wk and additionally 100 mg aqueous P 10 to 96 h prior to harvests.

Table 5. Successive extracts of extraradical *G. intraradices* mycelium and polyP content in extract fractions measured by the metachromatic reaction of polyP and toluidine blue. P treatments as described in Table 1. + and – indicate presence of polyP or not, respectively.

Treatment	PolyP content		
	TCA (short-chain)	EDTA (long-chain)	PC (granular)
1, P for 1 to 5 h	-	-	-
1, P for 10 to 96 h	-	+	+
2, P for 1 to 5 h	-	-	-
2, P for 10 to 96 h	-	+	+
3	-	-	-

TCA, Trichloroacetic acid fraction of successive extraction
EDTA, EDTA fraction of successive extraction
PC, Phenol-chloroform fraction of successive extraction

^{31}P NMR spectra of the various extract fractions confirmed the presence of polyP in the EDTA and PC fractions of the extracts of hyphae from treatments 1 and 2 in the 10-96 h interval (Fig. 9A and B, spectra of successive extract fractions of hyphae from treatment 2, additional P for 10-96 h shown). The average chain lengths of the polyP present in the EDTA and PC fractions were too long to be measured by NMR, and no signals for terminal or penultimate P_i residues could be identified. Furthermore, the ^{31}P NMR spectra of the TCA fractions contained no signals for short-chain polyP (Fig. 9C), while some P_i could be identified.

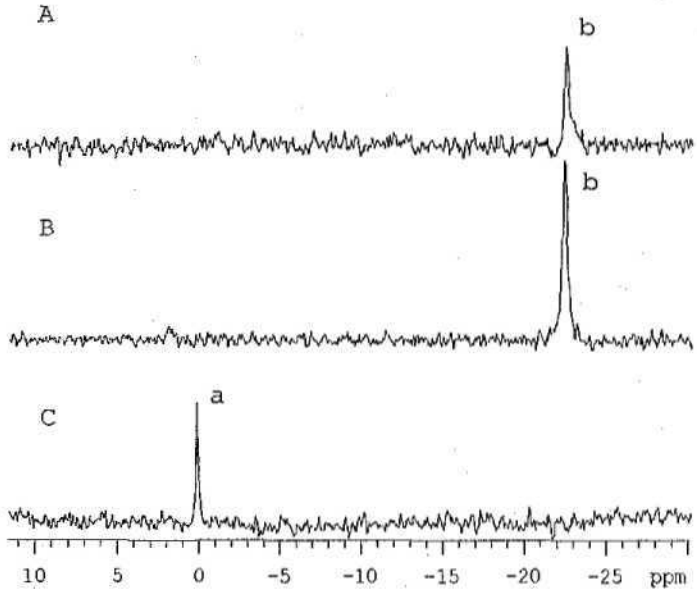

Figure 9. ^{31}P NMR spectra of TCA, EDTA and PC fractions of successively extracted *G. intraradices* hyphae. The hyphae had previously received 0.7 mM P in nutrient solution over two wk and additionally 100 mg aqueous P 10 to 96 h prior to harvests. (A), PC fraction. (B), EDTA fraction. (C), TCA fraction. Peak assignments were as follows: (a), P_i; (b), central polyP residues.

Since no polyP could be found in the TCA fractions of the extracts of hyphae, the TCA part of the successive extraction procedure was tested for recovery of short-chain polyP (results not shown). Both ^{31}P NMR and total P content confirmed that only one third of the expected polyP could be extracted at low polyP concentration.

Alkaline phosphatase activity of the extraradical mycelium and mycorrhizal cucumber roots

Extraradical AM fungal hyphae of *G. intraradices* absorbed the ELF-97 substrate and ALPase activity of the hyphae could be visualized, since a patchy distribution of fluorescent precipitates of the ELF reaction product could be observed inside the hyphae (Fig. 10). The precipitation seemed independent of P treatment and intact spores showed a high precipitation (Fig. 10B).

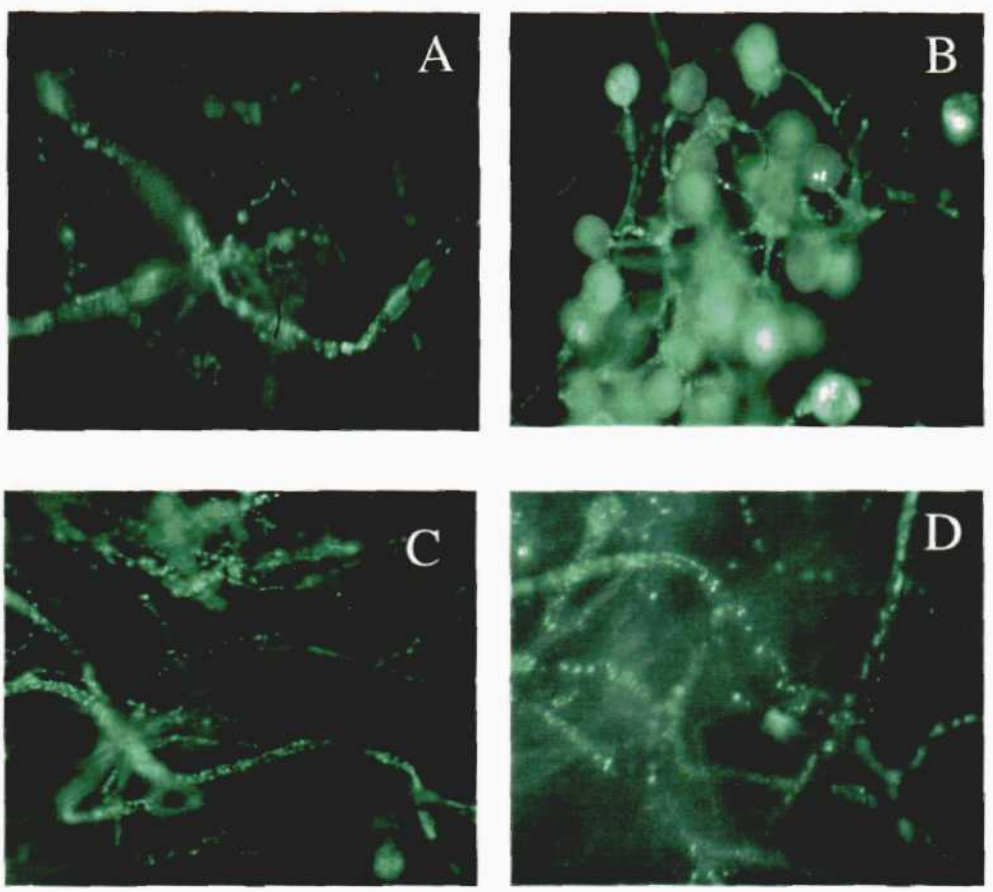

Figure 10. Extraradical *G. intraradices* hyphae growing with cucumber. Location of ALPase activity visualized by ELF precipitation and epi-fluorescence. (A), Activity of hyphae with no P added (control pot). (B), Activity in hyphae and spores from treatment 1, additional P for 34 h (C), Activity in hyphae from treatment 3 (D), Activity in hyphae from treatment 2, additional P for 48 h.

Fluorescent ELF reaction products could also be seen in mycorrhizal roots (Fig. 11). Specific staining of AM fungal structures inside the root was observed, i.e. fungal hyphae and vesicles, and the amount of precipitation was independent of P treatment.

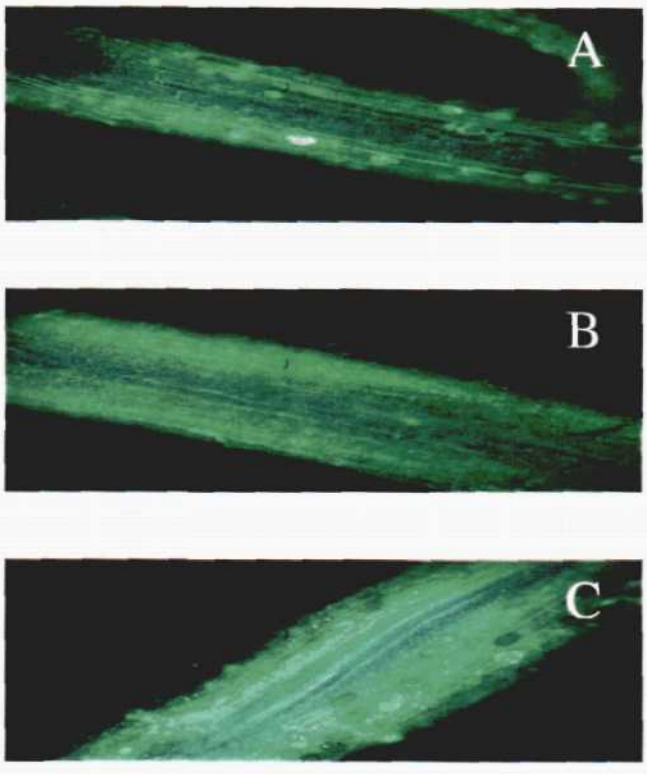

Figure 11. Mycorrhizal cucumber roots incubated in ELF substrate diluted in alkaline buffer. Epi-fluorescence was used to visualize both ALPase activity and fungal structures inside the root. (A), Activity and fungal structures in mycorrhizal roots with no P added (control pot). (B), Activity and fungal structures in mycorrhizal roots from treatment 1, additional P for 2 h. (C), Activity and fungal structures in mycorrhizal roots from treatment 2, additional P for 72 h.

Discussion

In vivo ^{31}P NMR spectra of *G. intraradices* and mycorrhizal cucumber roots

We have successfully used *in vivo* ^{31}P NMR spectroscopy for the identification of P pools in *G. intraradices* hyphae and mycorrhizal cucumber roots with different P treatments. The variation in hyphal amount and distribution in the sand could well have influenced the P_i uptake of the hyphae and roots and this may explain the variation in the data. However, this time-course study revealed a lot of new and important information of the P pools and their dynamics in *G. intraradices*.

When NMR spectroscopy is applied to living tissues, it is generally the mobile, lower molecular weight metabolites that contribute to the spectrum (Pfeffer and Shachar-Hill, 1996). These molecules are visible only if their concentration exceeds the detection threshold and if the resonance intensity is not broadened as a result of immobilization. For *G. intraradices*, the ^{31}P NMR spectra of P-treated, excised extraradical mycelium (Figs. 1 - 3) were characterized by the presence of signals for vacuolar P_i and polyP only. However, this was consistent with ^{31}P NMR spectra acquired from a range of ectomycorrhizal fungi (Martin *et al.*, 1983, 1985; Grellier *et al.*, 1989; Ashford *et al.*, 1994) and AM fungal spores (Shachar-Hill *et al.*, 1995). Absence of NMR-detectable cytoplasmic P_i or NTP in the fungus suggests a small cytoplasmic volume or low concentration of cytoplasmic P_i and NTP, below the detection threshold. Vacuoles are known to store P_i at much higher concentrations than the 5-10

mM which is the common range for the cytoplasm (Klionsky *et al.*, 1990; Smith *et al.*, 2001). Furthermore, the vacuole can occupy a large volume, and multiple vacuoles are common in fungi (Jennings, 1995). A large vacuolar volume in the fungus could actually reduce the viability of cytoplasmic metabolites. Cytoplasmic P_i and NTP signals have successfully been identified in *in vivo* ^{31}P NMR spectra of other fungi, e.g. *Aspergillus niger* (Hesse *et al.*, 2000), yeasts (Nicolay *et al.*, 1982; Castrol *et al.*, 1999), *Neurospora crassa* (Yang *et al.*, 1993) and also in the ectomycorrhizal fungus *Suillus bovinus* (Gerlitz and Werk, 1994), indicating larger proportional cytoplasmic volumes or higher amounts of cytoplasmic P-containing metabolites in these fungi.

All the expected signals for P metabolites, which are normally observed in ^{31}P NMR spectra of mycorrhizal roots, were present in the spectra of P-treated mycorrhizal cucumber roots (Figs. 4 - 7). The chemical shift of the signal for vacuolar P_i in extraradical mycelium was almost identical with the chemical shift of the signal for vacuolar P_i in mycorrhizal root tissue, so it was not possible to distinguish between fungal and root vacuolar P_i. However, the absence of polyP in spectra of non-mycorrhizal roots (Rasmussen *et al.*, 2000) confirmed its fungal origin.

P pools and dynamics in *G. intraradices* and mycorrhizal cucumber roots

This time-course study demonstrated that polyP signals had appeared in P-starved extraradical hyphae already 5 h after P supply. This was before the detection of any signal for vacuolar P_i and the corresponding P species did not appear in the spectra of mycorrhizal roots until hours later (Figs. 1 and 5). These results reflect a much faster uptake of P_i by *G. intraradices* in pot cultures than in monoxenic cultures where $^{32}P_i$ uptake could be detected at 14 but not at 7 h after $^{32}P_i$ supply (Nielsen *et al.*, 2002). In addition, Nielsen *et al.* (2002) saw no time lag between appearance of P in extraradical hyphae and roots. The observed differences may reflect differences in P_i uptake and P_i flux between monoxenic cultured hyphae and hyphae grown in pot systems.

The appearance of polyP before vacuolar P_i is consistent with earlier findings in yeast (*S. cerevisiae*), where P_i uptake and polyP synthesis appeared to be regulated in concert (Castrol *et al.*, 1999). *In vivo* ^{31}P NMR spectroscopy showed that P-starved yeast supplied with P-containing buffer immediately incorporated P_i into large amounts of polyP. This is a previously described phenomenon referred to as the 'polyP overplus' phenomenon (Harold, 1966). Indeed, a genomic expression analysis of yeast (Ogawa *et al.*, 2000) indicated simultaneous synthesis of vacuolar polyP by a vacuolar membrane protein complex and a polyP-synthetase following P_i uptake, and it was suggested that polyP accumulation was required to promote a high rate of P_i uptake over the long term. A similar importance of synthesis of polyP for effective P_i uptake in AM fungi was indicated in the present work. Indeed, changes in polyP content will have a low effect on the osmotic potential of the cell and therefore fast accumulation of polyP will reduce osmotic stress at high internal P_i concentration (Mimura, 1999). The importance of polyP synthesis for maintaining the internal P_i concentration in the mycelium at constant low levels has been investigated for ectomycorrhizal fungi grown in culture (Martin *et al.*, 1994). When the mycelium was cultivated under high P conditions, polyP was synthesized, but when transferred to low P conditions, the stored polyP was hydrolyzed and P_i was

released to maintain the P_i concentration within the fungal cell. Our work indicates a rather constant concentration of vacuolar P_i in extraradical hyphae, supporting synthesis of polyP as a sink for removal of P_i. Preliminary investigations of polyP synthesis and hydrolysis in excised *G. intraradices* extraradical hyphae indicated hydrolysis of polyP after transfer of the mycelium to P free medium (*unpubl. data*).

The appearance of polyP before vacuolar P_i is in contrast to previous results observed for ectomycorrhizas grown in pure culture (Martin *et al.*, 1983, 1985; Grellier *et al.*, 1989). In the latter, intracellular P_i was the most prominent form of P_i accumulated, followed by polyP. However, the relative amounts of polyP varied according to growth conditions (especially P concentration), growth phase and fungal species. Old cultures (36 days) accumulated polyP in higher amounts than young cultures (15 days) (Grellier *et al.*, 1989). In addition, germinating spores of an AM fungus were found to contain high levels of vacuolar P_i relative to polyP (Shachar-Hill *et al.*, 1995), which may reflect differences in P metabolism between growth phases of AM fungi.

Also, the amount of polyP in extraradical *G. intraradices* hyphae seemed to be at least as high or considerably higher than vacuolar P_i, indicating that polyP has an important role in fungal P_i uptake, accumulation and storage. Martin *et al.* (1985, 1994) found that the NMR-observable polyP corresponded to a minimum of 80% of the acid-extractable polyP in actively growing ectomycorrhizal mycelia. In contrast, earlier studies showed that polyP contained only a fraction (between 3% and 17%) of the total P in the mycelium, a fraction comparable to the fraction of P_i (between 14% and 17% of total P). The rest of the total P were present as DNA, phospholipids and other immobile forms of P that do not contribute to the *in vivo* NMR spectra (Martin *et al.*, 1983). Studies of polyP content in AM fungi suggest similar amounts of polyP as detected in ectomycorrhizas. *G. mosseae* contained 16% of the total P as polyP (Capaccio and Callow, 1982) and up to 17% of the total P in extraradical mycelium of *Gi. margarita* may be polyP (Solaiman *et al.*, 1999). However, yeast has been shown to accumulate even larger amounts of polyP, comprising 37% of the total cellular P (Ogawa *et al.*, 2000). Our results established that the polyP pool in extraradical *G. intraradices* hyphae was larger than the pool of P_i, and is therefore the main pool of soluble P metabolites in the mycelium.

A substantial proportion of the NMR-visible P in mycorrhizal roots was of fungal origin, as estimated from the relative areas of the polyP and vacuolar P_i signals, respectively. Assuming that the fungus occupies one-tenth of the host root, similar levels of polyP were present in the intraradical mycelium as observed in the extraradical mycelium.

The characterization of accumulated polyP and consequences for P translocation

The presence of polyP at low P supply and its location in vacuoles supports a role for polyP in the transport of P_i from soil to host root. Almost all polyP is present in vacuoles in eukaryotes such as yeast (Ogawa *et al.*, 2000), ectomycorrhizal fungi (Ashford *et al.*, 1994, 1999; Martin *et al.*, 1994) and other filamentous fungi (Yang *et al.*, 1993). PolyP translocation could occur via transport in a motile, pleiomorphic system of interlinked P-rich tubular vacuoles, as observed in ectomycorrhizas (reviewed

by Ashford, 1998; Allaway and Ashford, 2001). A similar system has recently been demonstrated in AM fungi (Timonen *et al.*, 2001; Uetake *et al.*, 2002), supporting the idea of peristaltic movement of polyP-containing vacuoles in the extraradical mycelium.

It is interesting that the longest polyP chains (> 20 P_i *residues) are found in extraradical hyphae from* pots with marked higher amounts of hyphae (Table 1 and 3, Figs. 1 and 2). However, the observation of primarily short-chain length polyP in the extraradical hyphae (< 20 P_i residues), independent of P treatment, agrees with previous reports of polyP average chain length in mycorrhizas (Martin *et al.*, 1985; Ashford *et al.*, 1994, Rasmussen *et al.*, 2000) and is consistent with polyP translocation in a tubular vacuole system. However, the presence of insoluble polyP granules and especially their influence for long distance transport in a vacuolar system has often been speculated (Cox and Tinker, 1976; Ashford, 1998). PolyP granules have been reported in preparations of mycorrhizas (Cox *et* al., 1975; Ashford *et al.*, 1985; Solaiman *et al.*, 1999), but their occurrence *in vivo* has been questioned (Orlovich and Ashford, 1993). Nevertheless, Bücking and Heyser (1999) recently showed that not all polyP granules were artifacts caused by the preparation procedure.

The successive extraction procedure used here indicated small amounts of long-chain and granular polyP in the extraradical hyphae supplied with high P for more than 10 h (Table 5), as judged from the absorption spectra (Fig. 8) and ^{31}P NMR spectra (Fig. 9) of the extraction fractions. However, the presence of a large amount of long-chain or granular polyP in the mycelium should have influenced the *in vivo* ^{31}P NMR spectra and hence the measured average polyP chain lengths since a signal for synthetic polyP with an average chain length of 75 P_i residues could easily be seen in the ^{31}P NMR spectrum of the compound (spectrum not shown). And such 'granule signals' were not observed.

The average chain length for the polyP contained in the mycorrhizal roots seemed slightly smaller than the corresponding values for the extraradical hyphae. It is possible that longer chain polyP located in extraradical hyphae were more efficient in transporting P towards the root, and that shorter chain polyP in the mycorrhizal root (i.e. intraradical) was the result of hydrolysis, as suggested by Solaiman *et al.* (1999). Variation in polyP chain length between extraradical and intraradical hyphae has been demonstrated in *Gi. margarita* by successive extraction of polyP (Solaiman *et al.*, 1999; Solaiman and Saito, 2001). Most polyP in extraradical mycelium was present as long-chain or granular polyP (66% and 25% of total polyP, respectively), whereas most polyP in intraradical mycelium was present as short-chain or long-chain polyP (40% and 52% of the total polyP, respectively). The results of Solaiman *et al.* (1999) agree with studies of enzymes involved in polyP metabolism. Capaccio and Callow (1982) and Ezawa *et al.* (2001b) both found PPX-type activity with maximum activity at pH 5.0 in intraradical hyphae and Ezawa *et al.* (2001b) found higher substrate specificity with short-chain polyP than with long-chain polyP. Ezawa *et al.* (2001b) suggested dominance of acidic hydrolyzing activity in intraradical hyphae, and they proposed that the acidic PPX-type activity is an ACPase. Indeed, vacuoles have been demonstrated to be acidic in the present study and in previous reports (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001b). However, Ezawa *et al.* (2001b) also detected PPX-type activity with maximum activity at neutral pH in extraradical hyphae with higher substrate specificity

with long-chain polyP than with short-chain polyP, in agreement with Solaiman *et al.* (1999). A dynamic balance between synthesis and hydrolysis of polyP in extraradical hyphae was suggested, with hydrolysis of polyP by alkalization as observed in yeast (Castrol *et al.*, 1999). Hydrolysis of polyP has also been detected in extraradical mycelium (Martin *et al.*, 1994).

The use of ELF substrate was in this study shown to be a sensitive method for microscopic detection of ALPase activity associated with fungal hyphae and mycorrhizal roots, in agreement with van Aarle *et al.* (2001). Indeed, the ELF substrate could be used as an indicator of metabolically active fungal tissue. The proportion of ALPase activity in extraradical mycelium seemed rather independent of P status of the hyphae or mycorrhizal root as observed by Boddington and Dodd (1999), indicating a constitutively active enzyme. However, the role of ALPase has not been satisfactory determined for conclusions about its role in P metabolism and polyP turnover.

Our detection of predominance of short-chain polyP in extraradical mycelium of *G. intraradices*, by means of *in vivo* ^{31}P NMR spectra, could not be confirmed by the alternative approach based on successive extractions. The application of this extraction procedure to P-treated extraradical mycelium indicated some limitations in extraction of short-chain polyP (TCA soluble). Neither the absorption spectra (Fig. 8B and C) nor the ^{31}P NMR spectra (Fig. 9C) of the TCA fractions indicated short-chain polyP. Moreover, we observed no marked increase in the P_i signal in the NMR spectra, which would have resulted from acid hydrolysis of polyP. Therefore the TCA part of the successive extraction procedure was tested for recovery of short-chain polyP. The recovery test confirmed that short-chain polyP was extracted by TCA, but the test indicated that there was a lower limit for extraction of short-chain polyP, and this limit may be lower than the polyP amounts in extraradical hyphae. Only one third of the expected polyP could be extracted at low polyP concentration. This could partly explain why no short-chain polyP could be extracted in the present work, but further investigation is required.

Lack of metachromatic reaction of polyP with toluidine blue does not necessarily mean that there is no polyP. The metachromatic reaction is observed only with longer chain polyP, while short-chain polyP is less sensitive to or cannot be detected by the method (Lorentz *et al.*, 1997). Therefore short-chain polyP present in the tissue extracted by TCA and estimated by the metachromatic reaction can be highly underestimated. However, the differences in polyP chain length between *Gi. margarita* (Solaiman *et al.*, 1999) and *G. intraradices* may reflect differences in polyP metabolism between different fungi.

Concluding remarks

The present work demonstrates that *in vivo* ^{31}P NMR indeed can be applied for the study of P pools and dynamics in AM fungi and associated roots. It was possible to observe the incorporation of P_i directly into a substantial pool of polyP followed by vacuolar P_i in extraradical mycelium of *G. intraradices*. The amount of polyP was considerably higher than vacuolar P_i and it is suggested that synthesis of polyP may be important for effective P uptake and storage in AM fungi. The polyP was located in vacuoles and the measured average chain length was short, supporting a role for polyP in

the translocation of P through AM hyphae. Indeed, polyP was also present in mycorrhizal roots, i.e. in intraradical hyphae. *In vivo* NMR could not detect cytoplasmic P_i in the extraradical mycelium possibly because of a small cytoplasmic volume or a low concentration of cytoplasmic P_i. A time lag was observed before P metabolites appeared in mycorrhizal roots and a substantial pool of NMR-visible P in mycorrhizal roots was of fungal origin.

Chapter 5 – Conclusions and perspectives

Development of methods

The work in the present thesis was focused on the potential use of *in vivo* ^{31}P NMR spectroscopy for the study of P pools and their dynamics in AM fungi. It was demonstrated that the method could be applied for this purpose and the airlift system implemented for oxygenating the tissue while in the NMR tube proved to be the right way of handling *in vivo* NMR of AM fungi. However, it turned out to be more challenging to study the P metabolism and translocation in AM fungi *in vivo* than expected at the beginning. The fact that extraradical AM mycelium cannot take up C from any other structure or source than from the root interior was a serious experimental hindrance which markedly reduced the P_i uptake of excised hyphae. Excised, P-starved hyphae can take up P_i and accumulate polyP to some extent, but the amount of polyP in proportion to P_i was much smaller compared with the proportion in hyphae P-treated before harvest, as estimated from *in vivo* ^{31}P NMR spectra. Consequently, any P treatment had to be performed while the symbiosis was still functioning and *in vivo* ^{31}P NMR was used to examine the P pools remaining after severance of the hyphae from the roots and P pools dynamics in AM fungi and associated roots. The discussion below should be read in conjunction with the discussion section of Chapters 3 and 4.

Importance of polyP

In the present work, polyP was seen for the first time in actively metabolizing extraradical AM fungi by the use of *in vivo* ^{31}P NMR spectroscopy. The *in vivo* ^{31}P NMR spectra of P-adequate excised *G. intraradices* hyphae and mycorrhizal roots contained signals from polyP, which were absent in non-mycorrhizal roots. This demonstrated that the P_i taken up by the fungus was accumulated into polyP and that polyP within the root was of fungal origin. In addition, the amount of polyP in extraradical hyphae was found to be higher than vacuolar P_i and polyP was found to be located in the vacuoles, indicating that polyP has an important role in fungal P_i uptake, accumulation and translocation. The average chain length was estimated to be short (< 20 P_i residues), in agreement with previous reports of polyP chain length in ectomycorrhizas estimated by NMR (Martin *et al.*, 1985; Ashford *et al.*, 1994). This short-chain polyP may serve as a reservoir pool of P_i inside the hyphae, reducing osmotic stress caused by high internal P_i concentration and introducing a source for rapid release of P_i, similar to what is seen in yeast (Castrol *et al.*, 1999; Ogawa *et al.*, 2000) and ectomycorrhizal fungi (Martin *et al.*, 1994). The ^{31}P NMR spectra of P-adequate excised extraradical AM mycelium were characterized by the presence of vacuolar P_i and polyP, and only weak or absent signals for cytoplasmic P_i. This observation suggested a small cytoplasmic volume or a low concentration of cytoplasmic P_i below the detection threshold. Therefore it was difficult to measure the metabolic status of the excised hyphae by *in vivo* ^{31}P NMR spectroscopy. However, ALPase-type activity was observed in excised extraradical hyphae, indicating metabolically active fungal tissue.

It was also observed that polyP accumulated before vacuolar P_i when P-starved *G. intraradices* hyphae were supplied with P_i, revealed in the time-course *in vivo* ^{31}P NMR study presented here. This was

similar to previous findings in yeast (Castrol *et al.*, 1999; Ogawa *et al.*, 2000) and reflected that the "polyP overplus" phenomenon described for yeast (Harold, 1966) also occurs in AM fungi. Therefore it was suggested that polyP synthesis is required to promote a high rate of P_i uptake in AM fungi. A time lag was observed before P metabolites appeared in roots and a substantial pool of NMR-visible P in mycorrhizal roots was of fungal origin. Furthermore, the presence of polyP in the extraradical hyphae even at low P supply and its location in vacuoles supported a role for polyP in the translocation of P by AM fungi, and suggested that short-chain polyP could indeed be translocated in the recently demonstrated motile tubular vacuolar system (Timonen *et al.*, 2001; Uetake *et al.*, 2002). The average chain-length for the polyP contained in the mycorrhizal roots investigated in the time-course study seemed slightly smaller than the corresponding values for the extraradical hyphae, and this could be the result of hydrolysis releasing P_i in the intraradical hyphae before P_i transfer to the host plant.

Primarily short-chain length polyP was observed in extraradical *G. intraradices* hyphae by *in vivo* ^{31}P NMR, independent of P treatment. This observation is in contrast to the long-chain or granular polyPs detected in other AM fungi by invasive methods (Cox *et al.*, 1975; Callow *et al.*, 1978; Solaiman *et al.*, 1999). This difference could also reflect differences between species of AM fungi, but the preparation procedure has been shown to influence the size of polyP (Orlovich and Ashford, 1993). In addition, the work presented here indicated that there was a lower concentration limit for extraction of short-chain polyP by TCA; a method that has been used previously in studies of polyP (Clark *et al.*, 1986; Solaiman *et al.* 1999). In the present work, short-chain polyP was also found in extraradical hyphae of *G. mosseae* as well as *G. intraradices*. PolyP was also detected in extraradical hyphae of *Gi. rosea*, however it was not possible to determine polyP chain-length due to very low amounts of extraradical mycelium in pots containing this fungus. In *S. calospora* (extraradical hyphae and spores) longer chain polyP (> 35 P_i residues) was indicated in an early experiment. However this polyP chain length needs to be confirmed. Accordingly, it would be worth studying P pools and dynamics in a range of AM fungi by *in vivo* ^{31}P, since preparation effects are avoided and average chain lengths easily can be measured. Long-chain polyP (< 75 P_i residues) can be observed by NMR, but due to increasing immobility of longer chain polyP, there is an upper limit for detection of polyP by NMR. Therefore it must be emphasized that short-chain polyP should primarily be characterized by NMR, while longer chain or granular polyPs should be characterized by extraction procedures in combination with colorimetric measurements (metachromatic reaction of toluidine blue and polyP; see below) or gel-electrophoresis. Therefore the methods should be used in combination in future studies of polyP chain size.

PolyP has previously been visualized in extraradical AM mycelium by toluidine blue staining (Ezawa *et al.*, 2001b) and by DAPI staining (Boddington and Dodd, 1999). The metachromatic reaction of toluidine blue is less sensitive to shorter chain than longer chain polyP (Lorentz *et al.*, 1997) and DAPI staining has been shown to be very non-specific. It was not possible to see polyP by DAPI in any fungi studied even though toluidine blue staining showed it was present (personal communication T. Cavagnaro, 2002). Therefore, short-chain polyP can be present without being detected by toluidine blue as stated by Ezawa *et al.* (2001b), and the failure in detecting polyP in *G. manihotis* by

Boddington and Dodd (1999) remains unexplained and requires investigation in a time-course using a combination of methods. In comparison, ^{31}P NMR spectroscopy has in the present work been shown to a valuable method for identifying and semi-quantifying various P metabolites including polyP. No large differences were seen in the size of the P pools in the ^{31}P NMR spectra of extraradical hyphae of *G. intraradices* and *G. mosseae*. However, since percent colonization was not significantly different, but the amounts of the various P pools in roots were higher when the roots were colonized with *G. intraradices* as judged from the ^{31}P NMR spectra, it was suggested that *G. mosseae* translocated P at much lower efficiency than *G. intraradices*. In summary, further studies of polyP metabolism in AM fungi are needed, and the studies need a combination of methods and time-course studies with different P feeding.

ALPase-type activity was demonstrated in the extraradical mycelium of three AM fungi and specific staining of metabolically active fungal tissue could be seen in the corresponding mycorrhizal roots, similar to results obtained previously (e.g. Ezawa *et al.*, 1995; Boddington and Dodd, 1999; van Aarle *et al.*, 2001). ALPase-type activity in *G. intraradices* was independent of P status of the hyphae or mycorrhizal root. However, any function of ALPases in the P metabolism in AM fungi has not yet been elucidated, and the only conclusion from the occurrence of ALPase-type activity in the AM fungi concerned in this study was that the fungal tissue was metabolically active. In addition, studies have demonstrated that extraradical and intraradical fungal vacuoles (Ezawa *et al.*, 2001b) and arbuscules (Guttenberger, 2000) are acidic, supporting the idea that ACPase-type activity is more likely involved in polyP metabolism as suggested by Ezawa *et al.* (2001b). The pH of the extraradical fungal vacuole was in the present work found to be 5.5, supporting acid vacuoles.

Suggestions of further work

The amount of mycelium that could be harvested showed a high degree of variation between pots. The P_i uptake of the hyphae could well have been influenced by the distribution of the extraradical mycelium in the sand and the variation in hyphal amount may explain a great deal of the variation in the results presented in this thesis. Therefore the compartmented growth system would need further development in order to be used in hyphal P uptake experiments, to produce approximately similar amounts of hyphae among pots within an experiment.

In comparison, preliminary results obtained from ^{31}P NMR investigations of extraradical hyphae and mycorrhizal roots harvested from monoxenic cultures (Bécard and Fortin, 1988; St-Arnaud *et al.*, 1996) indicated monoxenic cultures to be useful for studies similar to what is presented here. Hyphae can be harvested very easily from a liquid filled hyphal compartment (Maldonado-Mendoza *et al.*, 2001) and recently Nielsen *et al.* (2002) described the advantages of the methodology compared with classical pot culture systems for P transport investigations. Finally, many species of AM fungi have now been established in monoxenic cultures, allowing easier investigations of interfungal variations of P pools and dynamics in AM fungi. However, the present work demonstrated differences in hyphal P_i uptake and flux between monoxenic cultured hyphae (Nielsen *et al.*, 2002) and hyphae grown in pot systems. In the monoxenic cultures there is no plant using the P, which could explain the differences, indeed the P pool size in extraradical hyphae and roots will depend on the P demands of the host. For

this reason monoxenic systems could be used for ^{31}P NMR investigations of P pools and dynamics in AM fungi, but results obtained from monoxenic cultures should be compared to results obtained from pot systems.

In conclusion, the present work demonstrates that *in vivo* ^{31}P NMR can indeed be applied for the study of P pools and dynamics in AM fungi and polyP has been demonstrated to have an important role for an effective P accumulation and translocation by the AM fungus.

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Bilag 2. Metode for dialogen med ressourceområderne

Erhvervsministeriet (1997:9): *Dialog med ressourceområderne – status februar 1997.*

Boks 1. Metode for dialogen med ressourceområderne[1]

1) Identifikation af relevante erhvervsområder på baggrund af analyser og dialog
Forskellige dele af erhvervslivet, som hver især har specifikke behov for rammebetingelser, inddrages i dialogen. Det er fx:

- erhverv, hvor organisationer og virksomheder ønsker at gå aktivt ind i en dialog om deres rammebetingelser
- erhvervsområder med et betydeligt indre samspil, som dækker en væsentlig del af erhvervslivets beskæftigelse og værdiskabelse
- erhverv, der står overfor store udfordringer, dvs. trusler og muligheder pga. ændring i teknologi, marked eller regulering

2) Referencegruppen udpeger udfordringer og kritiske rammebetingelser og nedsætter arbejdsgrupper
Erhvervsministeriet nedsætter en referencegruppe med virksomheder, organisationer og nøgleministerier inden for det pågældende erhvervsområde. Referencegruppen danner sig et overblik over erhvervets udfordringer på baggrund af dialog og analyser af dets udviklingsvilkår. Referencegruppen identificerer kritiske rammebetingelser og nedsætter arbejdsgrupper.

3) Arbejdsgruppen stiller forslag til erhvevspolitiske initiativer, der forbedrer de kritiske rammebetingelser
I arbejdsgrupperne samler Erhvervsministeriet repræsentanter for virksomheder, organisationer, ministerier og andre aktører med viden om eller muligheder for at påvirke de kritiske rammebetingelser.

Arbejdsgrupperne tager udgangspunkt i de kritiske rammebetingelser og fremsætter på baggrund af grundige drøftelser, analyser og evt. sammenlignende analyser med konkurrentlande forslag til konkrete erhvervspolitiske initiativer, der kan forbedre de kritiske rammebetingelser.

4) Erhvervsministeriet følger implementeringen af forslagene og informerer de relevante aktører og offentligheden
Der er ikke defineret en fast beslutningsgang for de forslag, som er resultat af dialogen. Som hovedregel bæres et forslag videre af de aktører, som bedst kan fremme det. Det kan være en lang proces at gennemføre initiativer.

Nogle forslag vil deltagerne umiddelbart kunne realisere uden nye offentlige bevillinger eller ændringer af offentlige regelsæt. Andre forslag kræver politisk stillingtagen. Disse forslag vil Erhvervsministeriet som hovedregel bære ind i den politiske proces, men andre ministerier vil oftest bringe de forslag videre, der vedrører netop deres ressort.

For at skabe åbenhed i dialogen sørger Erhvervsministeriet for, at erhvervet og offentligheden kan følge arbejdet og resultaterne. Formidlingen sker fx gennem forslagsrapporter fra dialogen med ressourceområderne, hvor reference- og arbejdsgrupperne afrapporterer deres arbejde.

[1] I Ministeriet for fødevarer, landbrug og fiskeri samt i Boligministeriet har dialogen haft en noget anden struktur.