Systemic effects of mycorrhization on root and shoot physiology of *Lycopersicon esculentum*

Inaugural-Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Juli 2006

Aus dem Institut für Chemie und Dynamik der Geosphäre (ICG-III)
des Forschungszentrums Jülich

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: JuProf. Dr. I. Janzik

Koreferent: Prof. Dr. U. Schurr

Tag der mündlichen Prüfung: 6.11.2006

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Abbreviations

ABA	Abscisic acid
ANOVA	Analysis of variance
bp	Base pairs
CC	Non-mycorrhizal control splitroot pot
CHS	Chalcone synthase
CS	Chorismate synthase
Cys	Cysteine
DAHP	3-Deoxy-D-arabino-heptulosonate-7-phosphate
DAHPS	DAHP-synthase
DW	Dry weight
E	Extinction
E4P	Erythrose-4-phosphate
EPSPS	5-Enolpyruvylshikimate 3-phosphate synthase
γ-ECS	γ-Glutamylcysteine synthetase
Fru	Fructose
FW	Fresh weight
Glc	Glucose
G.i.	Vesicular-arbuscular mycorrhizal fungus *Glomus intraradices*
Glu	Glutamate
Gly	Glycine
G6P(DH)	Glucose-6-phosphate (dehydrogenase)
GSH	Glutathione, reduced
GSH-S	Glutathione synthetase
GSSG	Glutathione, oxidised
HK	Hexokinase
HR	Hypersensitive response
Inv	Invertase
ISR	Induced systemic resistance
JA	Jasmonic acid
LA	Leaf area
Le	*Lycopersicon esculentum*, Tomato
LT	Leaf temperature
mC	Half mycorrhizal splitroot pot

mC-C	Non-mycorrhizal half of mC
mC-m	Mycorrhizal half of mC
mm	Fully mycorrhizal splitroot pot
myc	Mycorrhizal, mycorrhization
O_3	Ozone
OD	Optical density
P_i	Inorganic phosphate
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PEP	Phosphoenol-pyruvate
PGI	Phosphogluco-isomerase
PR	Pathogenesis related
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
SA	Salicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SLW	Stomatal conductance
Suc	Sucrose
TP	Dew point
TR	Transpiration rate
VAM	Vesicular-arbuscular mycorrhiza
VOC	Volatile organic compound

Abstract

In a splitroot system, the influence of mycorrhization of tomato plants with the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices* on physiology and shikimate pathway transcription was investigated to distinguish between local effects in the mycorrhizal roots and systemic effects in the shoot and in the non-mycorrhizal part of a half-mycorrhizal root.
Mycorrhization caused a growth depression and reduced concentrations of elemental carbon and carbohydrates in mycorrhizal and half mycorrhizal roots compared to controls. The two parts of the half mycorrhizal root showed the same low carbon concentration, indicating a systemic effect on carbon availability in the root and the great sink strength of the fungus. Despite, in a developed symbiosis the elevated nitrogen concentration in shoots and roots of mycorrhizal plants, with higher concentrations in the mycorrhizal part of the half mycorrhizal roots, indicated a better supply of mycorrhizal roots and shoots with nutrients, on the cost of nitrogen supply of the non-mycorrhizal part of the root. Although increased nitrogen levels could lead to increased amino acid synthesis, the biosynthesis pathway for the three aromatic amino acids, the shikimate pathway, was not regulated in this later stage of the symbiosis. However, elevated shikimate pathway transcripts in mycorrhizal roots in the early stage of the symbiosis were demonstrated for the first time. This indicates an involvement of the shikimate pathway in early defence responses against the fungus and an influence of changes in carbon status and sugar metabolism on the pathway.
A more detailed look to the entry enzyme of the shikimate pathway in plants revealed that one of its two isoforms (DAHPS2) was upregulated by mycorrhization. This one was also induced by short-term ozone exposure, whereas the other was unaffected under the investigated conditions. Furthermore, an influence of mycorrhization on the shoot reaction to ozone was found. Dependent on the mycorrhization rate, an additional treatment with ozone caused additive DAHPS induction of the second isoform in shoots. VOC emissions and glutathione concentrations were only elevated in shoots of non-mycorrhizal plants after ozone exposure, indicating changes in root-shoot interactions involving signalling cascades. Neither early jasmonic acid or hexenal induction nor later methyl-salicylate emissions seem to be relevant in the regulation of DAHPS in response to ozone. Moreover, ozone alone did not only induce the shikimate pathway in shoots, but there was also an isoform specific induction of DAHPS transcripts in roots after ozone treatment, what would require a fast transduction of a shoot signal to the roots.
Whether the signalling from shoot to root after ozone exposure is mediated by the same compounds as the root to shoot signalling in the mycorrhizal symbiosis still remains unclear. Furthermore, the different affected pathways and substances may be influenced by different signalling cascades, reflecting the various re-programming in plant metabolism during interactions with belowground symbionts and aboveground environmental parameters.

Zusammenfassung

Der Einfluss von Mykorrhizierung mit *Glomus intraradices* auf Physiologie und Shikimatweg-Transkription von Tomatenpflanzen wurde in einem Splitroot-System untersucht, um zwischen lokalen Effekten in der mykorrhizierten Wurzel und systemischen Effekten im Spross und in nicht mykorrhizierten Wurzelteilen einer halb-mykorrhizierten Wurzel zu unterscheiden.

Mykorrhizierung führte zu vermindertem Wachstum und reduzierten Kohlenstoff- und Kohlenhydrat-Konzentrationen in voll- und halb-mykorrhizierten Wurzeln im Vergleich zu Kontrollpflanzen. Auch die beiden Seiten der halb-mykorrhizierten Wurzel zeigten dieselben niedrigen Kohlenstoff-Konzentrationen, was auf einen systemischen Einfluß in der C-Verfügbarkeit und einen hohen C-Bedarf des Pilzes hindeutet. Dagegen waren in der voll etablierten Symbiose die Stickstoff-Konzentrationen in Wurzeln und Sprossen mykorrhizierter Pflanzen höher als in Kontrollen. In der mykorrhizierten Wurzelhälfte von halb-mykorrhizierten Wurzeln waren die Konzentrationen ebenfalls höher als in der nicht mykorrhizierten Hälfte. Das deutet auf eine bessere Nährelement-Versorgung durch den Pilz hin, auf Kosten der Versorgung der nicht mykorrhizierten Wurzeln. Trotz der verbesserten Nährstoff- und insbesondere der Stickstoffversorgung, die potentiell zu einer vermehrten Aminosäure-Synthese führt, war der Syntheseweg der drei aromatischen Aminosäuren, der Shikimatweg, in der voll etablierten Symbiose nicht transkriptionell reguliert. Allerdings konnte in der frühen Phase der Symbiose zum ersten Mal eine erhöhte Menge an Shikimatweg-Transkripten aufgrund der Mykorrhizierung gezeigt werden. Dies legt eine Beteiligung des Shikimatwegs an der pflanzlichen Abwehr zu Beginn der Symbiose und einen Einfluss von Änderungen im Zuckermetabolismus auf den Shikimatweg nahe. Eine genauere Analyse des Eingangsenzyms des Shikimatwegs zeigt, dass eine der zwei bekannten Isoformen (DAHPS2) durch Mykorrhizierung induziert war. Diese Isoform wurde ebenfalls durch einen Ozonpuls induziert, wohingegen die andere Isoform unter den untersuchten Bedingungen nicht reguliert war.

Außerdem hatte die Mykorrhizierung einen Einfluß auf die Reaktion des Spross auf Ozon. Die zusätzliche Behandlung von mykorrhizierten Pflanzen mit Ozon führte, abhängig vom Mykorrhizierungs-Grad, zu einer zusätzlichen Induktion der DAHPS2 im Spross. Ozon löste nur in Sprossen nicht-mykorrhizierter Pflanzen eine Induktion von VOC-Emissionen und erhöhte Glutathion-Konzentrationen aus, was auf veränderte Spross-Wurzel-Interaktionen und deren Signalkaskaden in mykorrhizierten Pflanzen hindeutet. Weder Jasmonsäure- noch Hexenal- oder Methylsalicylat-Emissionen scheinen für die Regulation der DAHP-Synthase nach Ozonexposition relevant zu sein. Ozon allein induziert nicht nur den Shikimatweg im Spross, sondern auch isoform-spezifisch die DAHP-Synthase in Wurzeln, was eine schnelle Signalvermittlung zwischen Spross und Wurzeln erfordert.

Ob die Signalweiterleitung vom Spross in die Wurzel nach Ozonbehandlung von denselben Substanzen vermittelt wird wie in der Mykorrhiza-Symbiose von den Wurzeln in den Spross, ist nicht geklärt. Dass mehere Stoffwechselwege und Substanzen reguliert wurden, unterstreicht, dass durch Wurzel-Symbiosen und oberirdische Umweltparameter zahlreiche Umprogrammierungen im pflanzlichen Stoffwechsel ausgelöst werden.

1 Introduction

Dynamics of leaf and root growth is dependent on endogenous control and environmental impact. Both factors influence carbon partitioning, because a coordinated carbon flux is necessary for maintenance of growth. The resulting carbon allocations are controlled by both sink demand and source control of photosynthate production (Andersen, 2003). Partitioning between the competing sinks is determined by the relative sink strength, which is influenced by abiotic and biotic factors (Biemelt & Sonnewald, 2006). Thus, coordination between root and shoot is necessary to control the carbon partitioning and nutrient acquisition. CO_2 assimilation is dependent on the nitrogen supply of the shoot (Khamis *et al.*, 1990) and the nitrate uptake is dependent on a continuous flow of carbohydrates to the root (Rufty *et al.*, 1981). Assimilation of nitrogen and sulphur includes reactions that are among the most energy-requiring reactions in living organisms and thus are strongly regulated at several levels (Taiz & Zeiger, 2002).

Source-sink interactions are not only important for normal growth and development, but may also play a role in plant-microbe interactions (reviewed in Biemelt & Sonnewald, 2006). Not only plant pathogens such as bacteria, fungi or viruses, but also mycorrhizal fungi evolved strategies to change plant metabolism to their own benefit. The mycorrhiza symbiosis provides a great carbon sink in roots and therefore has impact on shoot carbon metabolism and the balance between carbon flow into primary and secondary metabolism.

Ozone is another factor varying C allocation. Current levels of ozone are capable of altering the timing and quantity of carbon flux to soils (Andersen, 2003), affecting interactions with the rhizosphere and thus root associated microorganisms and symbionts. Short ozone pulses could result in a short-term export stop of carbohydrates and induce accumulation of N-rich secondary compounds. The shikimate pathway is a pathway which could ensure the described coordination between carbon and nitrogen metabolism, which allows the plant to react to varying environmental conditions as it leads to the synthesis of nitrogen rich compounds as well as phenylpropanoids and other carbon-rich secondary metabolites, which lack nitrogen (Coruzzi & Bush, 2001; Walch-Liu *et al.*, 2005).

1.1 The mycorrhiza provides a strong C-sink in roots

Mycorrhiza is a very old symbiosis between soil-borne fungi and the roots of higher plants. Plants benefit from the symbiosis by a better supply with nutrients like phosphate and nitrogen, while the fungus is supplied with carbon by the plant. This symbiosis has a strong influence on the plant metabolism and its carbon-nutrient-balance. The first bryophyte-like land plants in the early Devonian (400 million years ago) had already endophytic

associations resembling vesicular-arbuscular mycorrhiza (VAM). It is suggested that these mycorrhizal fungi assisted in their colonisation of land (reviewed by Brundrett, 2002; Harrison, 2005). These associations occur in terrestrial ecosystems throughout the world and have a global impact on plant phosphorus nutrition. Trappe (1994) defined mycorrhizas as "dual organs of absorption formed, when symbiotic fungi inhabit healthy absorbing organs (roots, rhizomes or thalli) of most terrestrial plants and many aquatics and epiphytes". He also suggested that mutualistic functioning of these associations should be a defining criterion of the term mycorrhiza, which was first mentioned to the peculiar association between tree root and ectomycorrhizal fungi (Frank, 1885). The VAM fungi are obligate biotrophs and depend on the plant for supply of carbon. Until now VAM have been found in a wide range of habitats (Strack *et al.*, 2003), mainly in the roots of angiosperms, gymnosperms and pteridophytes but also in some mosses and lycopods (Smith & Read, 1997).

On the basis of morphological criteria, how the fungal mycelium relates to root structures, there are two major mycorrhizal groups: the endomycorrhizas and the ectomycorrhizas. The endomycorrhizas are again subdivided into three groups: the ericoid, the orchidaceous and the VAM. The VAM fungi belong to 6 genera and were first described by Nägeli (1842). Their taxonomy is based mainly on morphological characteristics of the spores. *Glomus* is thought to be the most abundant genus among soil fungi (Marschner, 1995) and was first described by Tulasne & Tulasne (1844). VAM are characterised by formation of branched haustorial structures (arbuscules) within the cortex cells of the plant and by a mycelium which extends into the surrounding soil. In crop plants, arbuscules, the major site of nutrient exchange between fungus and host plant, are short-lived structures in the root cortex, which senesce 3-4 days after production (Bonfate-Fasolo, 1986). This means that new arbuscules are formed throughout the whole symbiosis. In addition, many but not all VAM form lipid-rich storage organs (vesicles) within the plant roots.

The first published experimental mycorrhization of tomato was possibly done by Mosse (1956) with *Endogone* in open pot experiments. This species was re-named *Glomus mosseae* later. Another species often used in experiments on VAM is *Glomus intraradices,* first isolated in Florida and described by Schenck & Smith (1982).
It is well known from many experiments with different fungi and plants that a given VAM fungus may have totally different effects depending on the affected plant species (van der Heijden *et al.*, 1998). In general, the plant growth response to VAM colonisation depends on the balance between a suppressor effect - due to the fungal requirements of mainly carbon

for the production and maintenance of the fungal biomass - and the benefits of the symbiosis associated with a better nutritional status of the plant and other secondary effects.

A short period of general defence of the plant against the invading fungus is described for the beginning of the symbiosis. Aromatic secondary compounds derived through the shikimate pathway are known to be involved in these defence reactions (reviewed by Gianinazzi-Pearson, 1996; Garcìa-Garrido & Ocampo, 2002; Strack *et al.*, 2003). Especially, phenylpropanoids accumulating during the symbiosis are involved in the plant fungus interaction. Furthermore, this accumulation induced by mycorrhizal fungi influences the interaction of the plant with other organisms. Lignin in mycorrhizal roots for example is thought to play a role in resistance against *Fusarium* in tomato roots (Dehne & Schönbeck, 1979). Soluble and wall-bound phenols, depending on the presence of symbiosis, play a role in pathogen resistance in *Allium* especially near young arbuscules (Grandmaison *et al.*, 1993). Levels of phenylalanine ammonia-lyase (PAL) transcript were elevated in mycorrhizal *Medicago* roots (Harrison & Dixon, 1993).
There are, however, also influences of VAM on aromatic metabolites which are not defence-related. In onion, tyrosine and phenylalanine concentrations were higher in mycorrhizal roots (Tawaraya & Saito, 1994), although amino acid and sugar contents in root exudates were reduced by mycorrhization in *Leucocephala* (Bansal & Mukerij, 1994). Another example is the increase in levels of flavonoid secondary metabolites and mRNA transcripts encoding proteins involved in their synthesis during mycorrhization in *Medicago trunculata* (Harrison & Dixon, 1993).
Taken together, these data show that the plant aromatic metabolism in roots seems to play different roles in the development of the VAM symbiosis. On one hand, it is involved in general defence mechanisms at the beginning of the symbiosis. On the other hand, it seems to have a central role also after the establishment of the mycorrhizal symbiosis by supporting a balance between primary and secondary metabolism. Despite these major roles of the aromatic metabolism, regulation of the shikimate pathway itself, in which many secondary compounds are synthesised from the three aromatic amino acids, has not been investigated during mycorrhization.

1.2 The shikimate pathway, an interface between carbon and nitrogen metabolism

The shikimate pathway was discovered as the biosynthetic route to the aromatic amino acids phenylalanine, tyrosine and tryptophan through the classical studies of Bernhard Davis,

David Sprinson and their collaborators (Herrmann, 1995). It is a pathway which branches into many secondary compounds and it is estimated that up to 20 % of the fixed carbon goes through it (Haslam, 1993).

The condensation of phosphoenol pyruvate (PEP, derived from the glycolysis) and erythrose 4-phosphate (E4P) to DAHP (3-Deoxy-D-arabino-heptulosonate-7-phosphate) catalysed by the DAHP-synthase (DAHPS) is the first step of the shikimate pathway (Fig 1-1). The 6-step synthesis of chorismate is known as the prechorismate pathway.

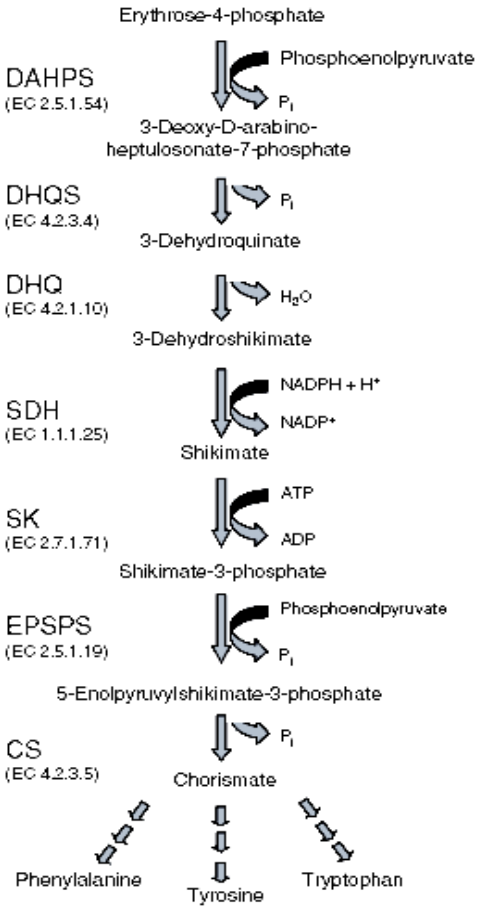

Fig 1-1. Enzymes and metabolites of the shikimate pathway (Janzik *et al.*, 2005).

In plants, the third and fourth step is catalysed by a single, bifunctional enzyme. The 6-step synthesis of chorismate is known as the prechorismate pathway.

Chorismate is the last common metabolite for the synthesis of the three aromatic amino acids (Fig 1-1) and many derived substances. Shikimate, an intermediate of the prechorismate pathway, was discovered by Eykmann (1891) and was isolated first from the fruits of *Illicium religiosum* (japanese: shikimi-ki). To date the shikimate pathway has been discovered only in apicomplexan parasites (Roberts *et al.*, 1999), plants, bacteria and fungi but is not abundant in animals. It is therefore an important target for herbicides and antibiotics and is of great scientific interest (detailed reviews by Herrmann & Weaver, 1999;

Jensen, 1985; Weaver & Herrmann K.M., 1997; Schmid & Amrhein, 1995; Herrmann, 1995; Schmid & Amrhein, 1999).

The biochemical reactions are identical in bacteria, fungi, and plants. In bacteria, all enzymatic activities are separable from each other and the shikimate pathway serves almost exclusively to synthesise the aromatic amino acids. At least in some fungi, the activities of the 2nd to the 6th step reside in a single polypeptide, called *arom* complex. In contrast, in plants all steps are catalysed by individual peptides, except for the 3rd and 4th steps which are catalysed by a bifunctional enzyme. The sequences of plant shikimate pathway enzymes show significantly greater similarity to bacterial than to fungal enzymes (Gasser *et al.*, 1988).

In *Escherichia coli,* three DAHPS isoenzymes are differently regulated by feedback inhibition of the three aromatic acids and through transcriptional control (Ogino *et al.*, 1982). But none of the plant DAHP synthases is inhibited by any of the three aromatic amino acids. However, DAHPS was found to be activated by tryptophan in carrot cells (Suzich *et al.*, 1985) and apparently plays a regulatory function in lignin biosynthesis (Jones *et al.*, 1995).

In plants, all shikimate pathway enzymes have N-terminal extensions characteristic for chloroplast transit sequences (Herrmann & Weaver, 1999), but a controversial question remains as to the presence of an additional cytosolic pathway (reviewed by Schmid & Amrhein, 1995; Schmid & Amrhein, 1999). The enzymatic activity measured in the cytosol might be due to enzymatically active precursors. Futhermore, the enzyme associated with the cytosolic DAHPS acitivity does not seem to be very specific for the condensation of PEP and E4P. Chloroplasts, in which processes take place induced by light and photosynthesis, are more plausible sites for the regulation of the enzymes of the shikimate pathway. In fact, light has been shown to up-regulate the DAHPS in parsley cell cultures (Henstrand *et al.*, 1992) and in *Arabidopsis* DAHPS has been shown to be activated by the ferredoxin/thioredoxin system (Entus *et al.*, 2002). Most of the here described shikimate pathway regulations have been described for cell cultures or shoots.

The shikimate pathway is located in plastids. Non-green tissue like roots and flowers contain the greatest amount of mRNA for the inducible isoenzymes of the pathway (Weaver & Herrmann K.M., 1997; Görlach *et al.*, 1994). In these organs a direct link to photosynthesis or light is not relevant for carbon re-routing and thus in these organs other regulatory mechanisms may occur. It is likely that a regulation in roots depends much more directly on the metabolic status. Changes in metabolic needs occur during adaptation to changing environmental conditions in shoots and roots and the involved biosynthetic pathway has to be regulated respectively. Enzymes of the shikimate pathway have been shown to respond to nitrogen and amino acid starvation, what affects root and shoot development (Weaver &

Herrmann K.M., 1997). The mycorrhizal symbiosis, as already mentioned, has a great impact on the nutritional status of the plant, especially nitrogen. In addition, phenylpropanoid or flavonoid biosynthetic enzymes are also involved in the initial stages in mycorrhizal symbiosis. It still remains to be demonstrated, whether the shikimate pathway itself responds to mycorrhizal symbiosis.

In the following paragraphs the steps catalysed by DAHPS, 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) and chorismate synthase (CS) are discussed in more detail, as they were found to be affected in this study. These three reactions together with the 2nd one catalysed by 3-Dehydroquinate synthase are the only reactions that release inorganic phosphate (P_i).

At least activities of two **DAHPS** isoforms have often been demonstrated. One of these is stimulated exclusively by Mn^{2+} whereas the other requires either Co^{2+}, Mg^{2+} or Mn^{2+} for full activity. In tobacco, spinach and parsley, the Mn^{2+}-stimulation has been assigned to the chloroplast whereas the Co^{2+}-requiring isoenzym appears to be located in the cytoplasm, summarised by Schmid & Amrhein (1995). The cytosolic enzyme may not be a specific DAHPS because it can use substrates other than erythrose 4-phosphate (Doong *et al.*, 1992). In tomato, however, two DAHPS genes coding putative plastidic isoforms but differing completely in their organ-specific expression have been found (Görlach *et al.*, 1993). The first isoform was mainly expressed in cotyledons and detected also in leaves, stems and flowers, but barely in roots. In contrast, the second isoform is mainly expressed in roots and stems. Likewise, two to three different DAHPS genes have been identified in other solanacaeous species. For these genes a higher divergence has been found between the respective gene pairs within the same organism compared to the corresponding genes in different plant species. Additionally to light (see above), enzymatic activity of DAHPS is inducible by wounding (Dyer *et al.*, 1989), elicitor treatment (Görlach *et al.*, 1995) and ozone (Janzik *et al.*, 2005).

In tomato, one **EPSPS** gene has been analysed in detail, while the 2nd one was detected only in southernblot analysis. The expression of the tomato EPSPS gene can be induced by different elicitors, including fungal elicitors (Görlach *et al.*, 1995). The basal levels of transcript vary only slightly between the organs and the expression pattern is similar to that of shikimate kinase and the two chorismate synthase genes (Görlach *et al.*, 1994).

The last common step before branching into the three amino acids is catalysed by the **CS** that requires flavin for its reaction. In tomato, three plastidic isoenzymes were identified, which are derived from only two genes. Different splicing of the 2nd isoform leads to two different transcripts (Braun *et al.*, 1996). Whether these isoforms fulfil different functions is

still unclear. Current knowledge about their reaction mechanism and function is reviewed by Macheroux *et al.* (1999).

Up to now only little is known about the coordinated regulation of the prechorismate pathway in response to environmental stimuli in roots. Görlach et al. (1995) and Janzik *et al.* (2005) described a coordinated regulation in response to fungal elicitors and ozone in cell culture and shoots, respectively. They argued that this induction is needed to supply the high amount of accumulated secondary metabolites in response to ozone. Therefore, the signalling that occurs in response to ozone in shoots and also might influence shikimate pathway regulation on roots, should be described in more detail below.

1.3 Ozone alters carbon flow

Ozone (O_3) is a naturally occurring molecule with 1-3*10^{-6} Vol-% in the atmosphere. Tropospheric ozone is a significant air pollutant that has been shown to reduce crop yield. Ozone was discovered by Christian Friedrich Schönbein, who named it after the greek word for smell (*ozein*) (Schoenbein, 1840). Only in 1959 it was found to be phytotoxic and to cause "weather flecks" on tobacco leaves (Heggestad, 1991). The toxicity is due to the high reactivity of ozone. It is a strong oxidant and, once having entered the leaves via the stomata, oxidises a variety of cellular targets either directly or indirectly through the production of secondary reactive species like superoxide, H_2O_2 and hydroxyl ions (Heath, 1980; Mehlhorn *et al.*, 1990; Smirnoff, 2005). The local defence observed in O_3-treated leaves is similar to those produced during the hypersensitive response in incompatible plant-pathogen interactions, reviewed by Grace (2005), and thus might also influence reactions, which occur in the early phase of mycorrhizal development in the roots. However, ozone has also been found to induce very specific reactions (Foyer *et al.*, 1994). Ozone is believed to initiate an oxidative burst, which causes the formation of reactive oxygen species (ROS), leading to the induction of defence genes by activating a signalling cascade. Some varieties seem to be more sensitive than others. For example the tobacco cultivar Bel W3 (Heggestad, 1991), as well as the tomato variety "Roma" is very sensitive. Within 15-48 h after the onset of ozone exposure (5 h, 200 ppb), up to 60% of the leaf area of "Roma" were damaged (Wohlgemuth, 2002), which might indicate strong defence reactions and accumulation of shikimate pathway derived compounds. The symptoms studied so far are mainly caused by short high-level exposures, leading to lesions and visible cell death. Chronic, long-term ozone exposure of relatively low concentrations, on the other hand, results in e.g. reduction in photosynthesis and growth, and hence lower yields, decreased pathogen tolerance and other alterations, reviewed by Jaspers *et al.* (2005). The role of ozone as an abiotic elicitor of

plant defence reactions is summarised by Sandermann *et al.* (1998) and Schraudner et al. (1996).

The ozone response and especially the lesion development is modulated by salicylic acid (SA) and jasmonic acid (JA) (Rao *et al.*, 2002; Pasqualini *et al.*, 2002) as well as other signalling molecules. Jaspers *et al.* (2005) reviewed the role of plant hormones in lesion development. The role of absisic acid (ABA) lies mostly in the regulation of stomatal conductance. SA, ethylene and JA are involved in the oxidative cell death cycle. ROS cause accumulation of SA and cell death. This leads to ethylene production and propagation of cell death. JA is the antagonistic component in this system and functions in the containment of cell death, see also Sasaki-Sekimoto *et al.* (2005). It has been discussed that the key signal SA is not exclusively synthesised via phenylalanine-ammonia-lyase (PAL), but also from the upstream metabolite chorismate (Wildermuth *et al.*, 2001). Chorismate itself is the precursor for the three aromatic amino acids (Chap 1.2) as well as for a great variety of aromatic secondary metabolites. Notably, with regard to the oxidative cell-death cycle, all signalling compounds can form volatile metabolites. Ethylene is volatile itself; SA and JA become volatile when methylated.

Together with the regulative role of SA in response to ozone already implicated, many aromatic metabolites are increased in response to ozone (reviewed by Sandermann *et al.*, 1998; Langebartels *et al.*, 2002). Many of them are synthesised via the shikimate and phenylpropanoid pathways, which have been shown to be stimulated by ozone (Paolacci *et al.*, 2001; Grace, 2005; Janzik *et al.*, 2005). The ozone-induced oxidative burst coincides with an increase in PAL expression and synthesis of phenolic compounds. Such an increase of PAL transcripts and enzyme activity under ozone exposure was reported for several herbaceous species (Sharma & Davis, 1994; Bahl *et al.*, 1995; Guidi *et al.*, 2005; Booker & Miller, 1998).

Depending on experimental conditions and analysed species, increased, enhanced, and unchanged levels of **glutathione** have been reported for ozone treated leaves (reviewed by Foyer & Rennenberg, 2000). However, like ozone itself, induction of glutathione precedes induction of gene transcription for the phenylpropanoid pathway in the interaction between powdery mildew and oat or barley (reviewed by Noctor *et al.*, 2002). Yet, given the different signal transduction pathways, it is likely that increases in glutathione concentration alone are not responsible for the elicitation of the defence response. Much more the thiole-disulfide status seems to be crucial in particular for expression of pathogenesis related (PR) proteins, which are induced by SA and involved in Systemic acquired resistance (SAR) (Wingate *et al.*, 1988; Foyer *et al.*, 2005a). Glutathione has many functions in plants and the reduced form (GSH) is a product of primary sulphur metabolism, as it acts as a transport and storage

form of reduced sulphur. Glutathione (γ-Glu-Cys-Gly) is synthesised in two ATP-dependent steps, catalysed by γ-Glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSH-S). Under most conditions, the activity of γ-ECS limits the rate of glutathione synthesis (reviewed by Foyer & Rennenberg, 2000). Glutathione is a precursor of phytochelatins, acts as substrate for the GSH-S-transferase, plays a role in cell division and protects proteins from denaturation. These and other roles are reviewed by Noctor *et al.* (2002). Many functions, like cell defence, signalling and gene expression, depend on the concentration and/or redox state of the glutathione pool (Noctor *et al.*, 2002).

In addition to the functions listed above, glutathione is probably best known as antioxidant. The response of the glutathione pool observed under stress is an increase in the GSSG/GSH ratio, followed by an increase in the total glutathione concentrations (Tausz *et al.*, 2004). Further, glutathione is the substrate of the dehydroascorbate-reductase to reduce the re-oxidised ascorbate in the Halliwell-Asada cycle (Foyer & Halliwell, 1976), which is an effective detoxification system for H_2O_2.

1.4 Systemic effects of ozone and mycorrhization

Ozone does not only affect local defence and antioxidant status, but has also deep impact on plant growth and development. This in turn affects the source-sink balance and carbon allocation to the roots, reviewed by Andersen (2003). Mycorrhization, which also alters the source-sink balance, likely interacts with the response to ozone exposure. Leaf sensitivity to ozone is increased in mycorrhizal tomato plants and increased phosphorus nutrition has been shown to increase leaf injury (McCool & Menge, 1984). In sugar maple, long-term ozone exposure reduces energy costing structures, such as arbuscules, and increases storing structures like vesicles (Duckmanton & Widden, 1994). The increase in below-ground storage structures is discussed to be the reason for the mycorrhizal plants being more sensitive to ozone stress (Duckmanton & Widden, 1994), indicating that ozone exposed mycorrhizal plants may be more susceptible to infections by pathogenic fungi.

Along with systemic effects of ozone treatment to mycorrhizal symbiosis, there are also systemic effects of mycorrhization on the shoot. Mycorrhization could modify disease-symptom development and gene expression in the leaves of symbiotic plants. Leaves of mycorrhizal plants infected with leaf pathogens showed a higher incidence and severity of necrotic lesions than those of non-mycorrhizal controls (Shaul *et al.*, 1999).

1.5 Goal of this work

Goal of this study was to investigate metabolic and physiological changes in mycorrhizal tomato plants, which are caused by better nutrient supply and by alterations in carbon flow between shoot and root. Directly linked to the carbon status is the biosynthesis of secondary aromatic compounds (Grace & Logan, 2000; Fritz *et al.*, 2006). Therefore, an adjustment of the carbon status in mycorrhizal plants is supposed to lead to an altered balance of aromatics. In relation to the control the mycorrhizal plants are expected to starve less on nutrients, which could also influence the carbon and secondary metabolism locally and systemically in roots and shoots. To enhance this difference, mycorrhizal and non-mycorrhizal plants were undernourished because under phosphate deficiency conditions plants take the greatest advantage out of mycorrhization, as reviewed by Clark & Zeto (2000).

The influence of mycorrhization on primary and aromatic secondary metabolism is well known (Chap 1.1). It was aim of this study to investigate if the upstream pathway, which provides aromatic amino acids and branches into biosynthetic pathways of many secondary metabolites, is regulated by mycorrhization as well. To distinguish between effects in mycorrhizal and non-mycorrhizal parts of the root, the influence of *Glomus intraradices* on the shikimate pathway was investigated in tomato plants grown in splitroot pots with both sides non-inoculated, both sides inoculated, and only one side inoculated.

In order to determine whether changes in the shikimate pathway transcription were correlated with changes in primary or secondary metabolism, the influence of mycorrhization on primary metabolism and nutrient supply was monitored in parallel by measuring carbohydrate and nutrient concentrations of shoots and roots.

To get an idea about local and systemic changes in carbon flow between primary and secondary metabolism as well as between shoot and root, mycorrhizal plants were additionally treated with ozone, which is an effective inducer of aromatic secondary metabolites on shoot level (Langebartels *et al.*, 2002). Vesicular-arbuscular mycorrhizal (VAM) fungi are strong permanent root C-sinks, which influence defence reactions to pathogens in shoots. As diseases caused by foliar pathogens often increase in mycorrhizal plants (Shaul, 1999), shikimate pathway transcription might be also influenced in shoots of mycorrhizal plants. PR1 transcription levels, volatile organic compound (VOC) emissions and glutathione concentrations were determined in ozone treated, mycorrhizal plants to get hints about possible signalling mechanisms and to investigate the influence of mycorrhization on defence reactions and secondary compounds.

2 Material and Methods

Material

2.1 Technical equipment

All technical equipment utilised is listed in tab. 2-1.

Tab 2-1. List of techniqual equipment.

Equipment	Type	Manufacturer	Location
Autoclave	2540EL	Systec	Wettenberg
	FVS_X	Fedegari, Tecnomara AG	Zürich
Ball mill	MN200	Retsch	Haan
Centrifuge	Centrifuge Allegra 25R, Rotor TS-5.1-500	Beckmann Coulter	Unterschliessheim-Lohhof
	Centrifuge 5415 C and 5417 R, Standardrotor F 45-30-11	Eppendorf	Hamburg
	Vacuum centrifuge, Concentrator 5301	Millipore	Schwalbach
Electrophoresis systems	OWL seperationSystem	VWR	Darmstadt
Fluorescence Imaging System	LAS3000	Raytest	Straubenhardt
Hybridisation	Hybridisation Incubator 7601	GFL	Burgwedel
	Hybridizer HB-1000	UVP Inc	Upland, CA
Incubation shaker	Bühler Incubator hood: TH 15	Johanna Otto GmbH	Hechingen
Microscope	Photomikroskop Typ III	Zeiss	Köln
PCR machine	Robo-Cycler® Gradient 96, temperature cycler	Stratagene	Heidelberg
pH meter	Accumet model 15	Fisher scientific	Schwerte
Photometer	$Uvikon_{XL}$	Bio-Tek Instruments	Neufahm
	Ht2	Anthos MIkrosysteme	Krefeld
Shaker	Model 260300	Boekel Industries Inc	Feasterville, USA
	BioDancer	New Brunswick Scientific	Nürtingen
Scales	Explorer weighing scales	OHAUS	Giessen
	Fine weighing scales AE160	Mettler	Giessen
	PG503-S DeltaRange®	Mettler Toledo	Giessen
	Type 1712	Sartorius GmbH	Göttingen
Thermoblock	Thermomixer Comfort	Eppendorf	Hamburg
UV linker	Stratalinker® 1800	Stratagene	Heidelberg
Videodocumentation	UVT-28MP ICU-1	Herolab	Wiesloh
Water purification system	Milli-Q Gradient A10	Millipore	Schwalbach

2.2 Chemicals and enzymes

All chemical substances and enzymes utilised are listed in tab. 2-2.

Tab 2-2. List of used chemical substances and enzymes.

Substance	**Manufacturer**
3-chloro acetic acid (TCA)	Sigma-Aldrich
3-morpholino-1-propansulfonic acid (MOPS)	Merck, Roth
Acetic acid	Merck
Adenosine triphosphate (ATP)	Sigma
Agarose	USB, Roth, Bio-Rad
Ampicillin	Fluka
Aqua Roti Phenol	Roth
Bromphenol blue	Sigma-Aldrich, Bio-Rad
CDP-Star®	Roche
Chloroform	Merck
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck
DIG Wash and Block Buffer Set	Roche
DTNB	Sigma-Aldrich
Ethanol 99,8 % (EtOH)	KMF Laborchemie Handels GmbH
Ethidium bromide	Sigma-Aldrich
Ethylene diamine tetra-acetic acid (EDTA)	Sigma-Aldrich
Formaldehyde	KMF Laborchemie Handels GmbH
Formamide	Merck
Glycerol	Merck
Glutathione	Sigma-Aldrich
Hydrogen peroxide (H_2O_2)	KMF Laborchemie Handels GmbH
Imidazole	Merck
Isoamyl alcohol	Merck
Lactic acid	Merck
Lithium chloride ($LiCl_2$)	Merck
Magnesium chloride x $4H_2O$ ($MgCl_2$)	Merck
Methylene blue	SERVA
N-(2-Hydroxyethyl)-piperazin-N´-(2-ethanesulfonic acid) (HEPES)	Roth
Natrium Acetate	Merck
Natrium hydroxide (NaOH)	Merck
Nicotinamid adenine dinucleotide phosphate (NADP)	Roche
Phenol chloroform isoamylalcohol (PCI)	Roth
Phenol, TE-saturated	Fluka
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Potassium hydroxide (KOH)	Merck
Rotisol	Roth
Sodium dodecyl sulfate (SDS)	Roth
SSC (20x): 3 M NaCl, 0.3 M Sodium citrate, pH 7.0	Eppendorf

Tris (ultrapure)	Roth
Trizol	Invitrogen
Trypane blue	Merck
Xylencyanol	Bio-Rad
Enzymes	
α-amylase, order-no. A 3176	Sigma-Aldrich
Amyloglucosidase, order-no. 102 857	Roche
Glucose-6-phosphate-dehydrogenase (G6PDH), order-no. 127 671	Roche
Glutathionereductase, order-no. G 3664	Sigma-Aldrich
Hexokinase (HK), order-no. 426 362	Roche
Invertase (Inv), order-no. I 4504	Sigma-Aldrich
M-MLV Reverse transcriptase	Promega
Phosphoglucoisomerase (PGI), order-no. 128 139	Roche
Taq-Polymerase	Fermentas
T4 DNA ligase	Promega

2.3 Organisms

Plant material

Tomato (*Lycopersicon esculentum* "Roma")

The tomato species "Roma" is an important crop plant of the solanacaeous family, which is commercially mycorrhized in horticulture. Tomato seeds `Roma` were obtained from Kiepenkerl (Samenshop 24, Aurich).

Marigold (*Tagetes patula* L. "Bonita del Sol" (Walz))

Marigold plants were used as host plants for inoculum production (Chap 2.4.1). With seeds, obtained from Prof. H. Bothe, University of Cologne, the mycorrhization of *Tagetes* was started. To reduce the danger of external infection, the plant species for inoculum production was different from the test plant species. Marigold can be easily infected by *G. intraradices* and prevents the tomato plants from infection with the pathogen *Pythium* through the inoculum (von Alten *et al.*, 1990).

Mycorrhizal fungus

Tomato and marigold plants were inoculated with the arbuscular mycorrhizal fungus of the genus "*Glomus*" (*Glomus intraradices* Schenk & Smith INVAM Sy167, isolated in Syria). The self culturing of the fungus was started with inoculum obtained from Prof. H. Bothe, University of Cologne, containing *Glomus intraradices* spores (Chap 2.4.1).

Bacteria

For transformation and cloning of cDNA-fragments, *Escherichia coli* DH5α (Clontech, Heidelberg) was used with the following genotype: F^- ϕ80d*lac*ZΔM15 Δ(*lac*TYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_{k-}, m_{k+}) *pho*A *sup*E44 λ *thi gyr*A96 *rel*A1

The utilised recombinant plasmids are listed in Tab 2-8.

Methods

2.4 Plant cultivation and growth conditions

Tomato and marigold plants were grown between May 2004 and November 2005 in a greenhouse ("PhyTec") with high transparency and permeability for UV-B radiation. Due to the micro structured surfaces of the selected glass quality of the greenhouse, the passing light is scattered and the cultivation area is illuminated equally. Thus the plants were cultivated under conditions similar to ambient conditions.

With additional lamps (400 W), which were turned on at a light intensity below 20 kLux, a 12 h light/12 h dark cycle was ensured. The temperature was mostly between 18-27°C, dependent on seasonal changes. The average relative humidity was about 54%, the average CO_2 concentration was about 440 ppm.

After the third harvest three weeks after the transfer in single or splitroot pots, tomato plants were fertilised 1-2 times per week with 50 ml of Hoagland solution (Hoagland & Arnon, 1938) per pot (2x 50 ml in the splitroot system). If not specially fertilised, the plants were automatically watered three times per day, each time with 65 ml tap water per pot. All pots were placed on a grating to avoid damming.

2.4.1 Marigold cultivation and inoculum production

The plants were germinated and grown in 1.6 l pots containing a 7:1:1:1 mixture of expanded clay:soil:sand:dry inoculum. About 20 seeds per pot were sown. After four weeks, they were weekly fertilised with 50 ml of Hoagland solution per pot.

Tab 2-3. Physical and chemical properties of expanded clay.
The analysis was done by Fibo Exclay Germany.

Water uptake		100 ml/l
pH		7.2
Salt content (KCl)		0.32 g/l
Soluble parts of:	Ca^{2+}	67 mg/l
	Na^{+}	15 mg/l
	Mg^{2+}	12 mg/l
Phosphate binding		146 mg P_2O_5/l

After 2-3 months the root ball was either directly used as fresh inoculum (Chap 2.5) or dried for about three weeks. To allow the fungus to accumulate storage compounds and produce spores, the shoots were cut off only after drying. The dried root ball is durable for about one year (Dehne & Backhaus, 1986) and was used to re-inoculate the marigold plants (see above, this paragraph). The successful mycorrhization was controlled by trypane blue staining of the roots (Chap 2.6).

The inoculum was produced with the expanded clay technique (2-4 mm, Lamstedt clay, Fibo Exclay, Tab 2-3). Because of the porous surface, fungal spores and mycelium can bind to the clay particles (Dehne & Backhaus, 1986), which exhibit an effective propagation unit (Feldmann & Idczak, 1992).

2.4.2 Tomato pre-cultivation

Tomato seeds `Roma` were sown in a multipot tray containing soil (Einheitserde ED73, Ferdinand Irnich GmbH&Co.KG, Jülich), which was always kept damp. The below described experiments were started by transferring three weeks old seedlings to 1.6 l single pots or splitroot-pots (Chap 2.5).

2.5 Experimental setup and harvest

The influence of mycorrhization on root and shoot physiology was investigated in a splitroot system, in order to detect also systemic effects of the fungus on the non-mycorrhizal control root of a half mycorrhizal plant. Systemic effects of mycorrhization on pathogen resistance (Cordier *et al.*, 1998; Shaul *et al.*, 1999) and on carbon partitioning (Koch & Johnson, 1984; Wang *et al.*, 1989; Lerat *et al.*, 2003) were investigated earlier. In this system, systemic effects within roots and shoots on the shikimate pathway and on physiology were

investigated. The influence of ozone itself as well as the combination of ozone and mycorrhization on the shikimate pathway and on carbohydrates was investigated in single pot experiments.

Tab 2-4. Overview of the experiments shown and discussed in the following chapters.
w. a. i.= week after inoculation; myc=mycorrhization. CC: both splitroot pots non-mycorrhizal; mC: half mycorrhizal splitroot system; mm: both root parts mycorrhizal. VOC: volatile organic compound.

	Treatment	**Exp. replicates**	**Harvest (w. a. i.)**	**Plants per harvest**	**Analysis**	**Ref. in results:**
Single pot	Ozone	1	6	2 (control, O_3)x 7	Transcript (Glutathione)	Chap 3.6
	Myc	2	4.5	2 (control, myc)x 7	Elemental C, N, P, S	Chap 3.2 Tab 3-1
	Myc & ozone	2	myc: 1 & 3 myc&O_3 : 2 & 4	4x 5-7 (no O_3: control&myc. O_3: control&myc) 2x 6 (VOC)	Transcript Carbohydrates Glutathione VOCs	Chap 3.7.2 Chap 3.7.3 Chap 3.7.4 Chap 3.7.1
Splitroot pot	Myc	3	1-7	3 (CC, mC, mm)x 5-7	Biomasses Transcript Carbohydrates Glutathione	Chap 3.1 Chap 3.4 Chap 3.3 Chap 3.5
	Myc	1	3 & 7	3 (CC, mC, mm)x 6-7	Elemental C, N, P, S	Chap 3.2

Shoots and roots were harvested once a week from the first up to the 7th week after inoculation, starting with the transfer of seedlings from a multipot tray into the 1.6 l single (Chap 2.5.2/3) or splitroot pots (t=0) (Chap 2.5.1). By harvesting control plants before harvesting the half-mycorrhizal and mycorrhizal plants always in the same order, diurnal effects were minimised.

After weighing shoots and roots of single plants, material of 5-7 plants per harvest were pooled. The liquid N_2-frozen plant material was used for transcriptional analysis, sugar/starch analysis and determination of glutathione content (Tab 2-4). Additionally, shoots and roots were harvested and dried for 3 weeks at 75°C. After determination of the dry weight of these plants, the shoots and roots were milled for C-, N-, P- and S-analysis (Tab 2-4).

2.5.1 Splitroot experiments: mycorrhization

Two 1.6 l pots were stuck together and a 5 cm PVC-border was adhered around (Fig 2-1). The splitroot pot was filled with a 8:1:1 mixture of expanded clay:soil:sand (CC). 3-4 weeks old tomato seedlings were transferred to the middle of these pots. In mycorrhizal pots one

part of expanded clay was substituted by fresh inoculum (mm, Chap 2.4.1). In mixed pots one pot was filled with the substrate mixture without inoculum (mC-C) and the other pot was filled with a substrate mixture containing inoculum (mC-m). To prevent the non-mycorrhizal half of the pot from cross infection with fungi, the control pots (mC-C) were placed on a separate base, which had holes pasted up with mesh tissue roots cannot grow through.



Fig 2-1. Tomato plant in a splitroot pot, one week after transfer from a multipot tray.

In the mycorrhizal splitroot experiment the fresh weight of the aboveground and belowground part was determined separately before freezing the plant material in liquid nitrogen. During the first two harvests the whole shoot was harvested, because otherwise the amount of material would have been insufficient. In the 3rd and 4th week only the epicotyl was collected. In the 5th to the 7th week after inoculation only green, fully expanded leafs were frozen. The cotyledons and yellowish leafs were always discarded, because the metabolism might be different in these parts and the amount of shikimate pathway transcripts is different in cotyledons compared to other organs (Görlach *et al.*, 1994).
The whole root ball was cleaned under running water, dried with cellulose cloth and weighed. Samples from different parts of the root were collected and pooled for the estimation of the mycorrhization rate. The remaining root material was frozen in liquid nitrogen for further analysis.

2.5.2 Single pot experiments: ozone

For ozone fumigation experiments, the three weeks old pre-cultivated tomato seedlings (Chap 2.4.2) were transferred to 1.6 l single pots filled with a 8:1:1 mixture of expanded clay:soil:sand. If plants were inoculated, one part of expanded clay was substituted by fresh inoculum (Chap 2.4.1). 6 weeks after the transfer into 1.6 l pots, the plants were fumigated with 200±20 ppb ozone and harvested at different times 2-30 h after onset of ozone fumigation.

For ozone experiments, the tomato plants were transferred into plexiglas-chambers with a total volume of 630 l. Air flow was set to 30 l min^{-1}. Temperature was set to 25°C, photon flux density near the leaves was about 300 µmol m^{-2} s^{-1} and the relative humidity about 40%. Ozone was generated in a glass cell by photolysis of molecular oxygen with a UV light source (Double Bore lamp, Jelight Company, CA, USA). For ozone exposure the ozone containing flow from the ozone generator was mixed with the air entering the chamber. The ozone concentration (200 nl l^{-1}) in the chamber was kept constant by measuring the concentration with an ozone analyser (Ozone monitor 450, Advanced Pollution Instrumentation, San Diego, USA).

The stomata were considered to be open, because the plants were watered at least half an hour before starting the fumigation experiment. Therefore, ozone could easily enter the leaf. After ozone fumigation, the plants were re-transferred into the green-house and remained there until they were harvested.

At indicated times after onset of ozone the 4th, 5th and 6th leaves were harvested. The first primary leaf was defined as leaf 1, so the higher the number the younger the leaf. The plants were mainly in an 8-leaf-stadium at the time of harvest. The harvested leaves were fully expanded source leaves with developed stomata, which are considered to react most to ozone exposure. Roots were harvested as described in Chapter 2.5.1.

2.5.3 Single pot experiments: mycorrhization and ozone

For investigation of the combination of mycorrhization and ozone, plants were transferred into 1.6 l single pots as described in Chap 2.5.2. The ozone exposure and harvest was done as described in Chap 2.5.2. In the 1st and 2nd week the whole epicotyl was harvested in order to get enough plant material for transcriptional analysis. In the 3rd and 4th week the oldest yellowish leaf and the youngest not expanded leaves were discarded before freezing in order to detect metabolically active source leaves. Two and four weeks after starting the experiment, shoots and roots of mycorrhizal and non-mycorrhizal plants were additionally harvested. But instead of freezing them into liquid nitrogen, they were dried to weight constancy. After determining the dry weight, they were milled, in order to analyse concentrations of nutrient elements (Chap 2.7.1).

2.6 Determination of mycorrhization rate

Staining of mycorrhizal roots

For the identification of fungal structures and for quantification of the symbiosis (Phillips & Hayman, 1970) representative segments of the root system were cleared for 20 min in a 10% (w/v) KOH solution at 90°C. Roots were washed with tap water and then stained in 0.05% (v/v) trypane blue solution (in glycerol:lactic acid, 1:1) for one hour. Excess stain was removed by soaking over night in 50% (v/v) glycerol/H_2O. The roots were then stored in 50% (v/v) lactic acid/H_2O until determination of mycorrhization rates.

Figure 2-2 shows trypane blue stained arbuscules, vesicles and hyphae of *Glomus intraradices* in marigold and tomato roots.

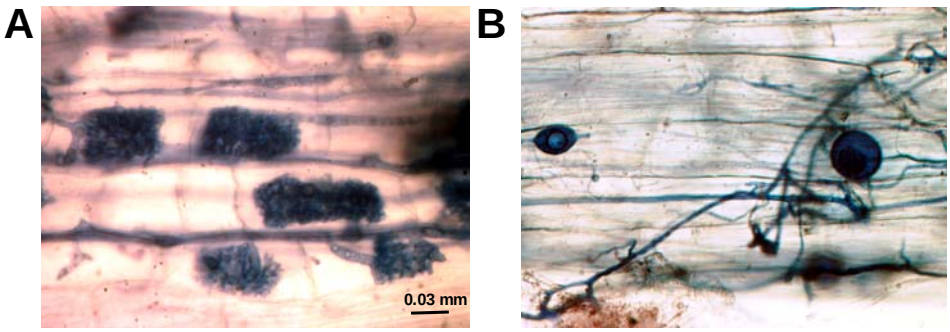

Fig 2-2. Stained fungal structures of *Glomus intraradices* in marigold and tomato plants.
A (enlargement: 10x25): arbuscules and intraradical hyphae in marigold roots. B (enlargement: 10x25): vesicles and hyphae in tomato roots.

Estimation of mycorrhization rate with the microscope

The percentage of mycorrhizal colonisation of arbuscules was visually determined similar to the line intersect method (Ambler & Young, 1977).

Stained roots were spread on a microscope slide and observed with an enlargement of 63x. In every root segment within one window it was checked if mycelium, arbuscules or vesicles occurred. A segment was considered as mycorrhizal if one of the structures was abundant, the segments containing arbuscules were considered to determine the mycorrhization rate. Non-mycorrhizal control roots were also stained and investigated under the microscope. To get statistically significant results, at least 100 microscope windows per sample were observed. The percentage of mycorrhizal root segments to the quantity of observed segments is a dimension of the total mycorrhization rate.

2.7 Metabolite analysis

2.7.1 Element analysis

For carbon, nitrogen, sulphur and phosphorus analysis, the dried plant material was ground in a ball mill (Retsch, Type MM 2). The quantitative analysis of C, N, S was done with a CHNS-analyser (System LECO, CHNS-932), P was analysed with a spectrometer (Thermo IRIS). All analysis was done by M. Michulitz, H. Engel, H. Lippert and N. Merki in the Central Division of Analytical Chemistry (ZCH) of the Research Centre Jülich.
Phosphorus was analysed with a HNO_3/H_2O_3 micro wave extraction. 50 mg material was diluted in an end volume of 14 ml. For carbon and sulphur analysis the sample was burned in flowing oxygen. The CO_2 and SO_2 combustion gases were measured by selective IR detectors. After corresponding absorption of these gases, the content of the remaining nitrogen was determined by thermal conductivity detection.

2.7.2 Concentrations of sugar, starch and chlorophyll

Extraction

Chlorophyll and soluble sugars were extracted at 80°C. About 60 mg of plant material ground in liquid nitrogen was extracted in 400 µl 80% ethanol, 2 mM HEPES for 15 min. To make sure that the powder was uniformly extracted, the sample was shaken at 1300 rpm with a thermoblock (Eppendorf). The sample was centrifuged 1 min at RT and the supernatant was stored at 4°C. The extraction was repeated once with 400 µl 50% ethanol, 2 mM HEPES and twice with 200 µl 80% ethanol, 2 mM HEPES until the leaf powder was pale. The supernatants were pooled and filled to 1.5 ml with 80% ethanol ($V_{extract}$).

For starch analysis, the insoluble extracted plant material left over from the ethanol extraction was autoclaved for 90 min with 500 µl water. 100 µl of the autoclaved sample ($V_{incub\text{-}aliquot}$) was incubated in 400 µl 50 mM NaAc buffer containing amyloglucosidase and α-amylase (pH 4.9, prepared as described below) over night at 37°C. By this procedure the starch is cleaved into glucose. After extraction the samples were stored at -80°C until determination of glucose concentrations.

NaAc buffer: 200 µl amyloglucosidase and 2 µl α-amylase were centrifuged for 2 min at 13.000 rpm. The pellet was dissolved in 10 ml 50 mM Na-acetate-buffer, pH 4.9.

Chlorophyll

Immediately after extraction, the chlorophyll (a+b) concentration of the pooled ethanolic extracts was measured according to Arnon (1949). After brief vortexing, 400 µl of the extract ($V_{aliquot}$) was diluted 1:1 to a final volume of 800 µl ($V_{cuvette}$) and centrifuged for 4 min at 13000 rpm. The extinction (E) was measured against 80% EtOH at λ=652 nm. The chlorophyll concentration was estimated as follows with 1 mg chlorophyll ml^{-1} having an E of 34.4:

$$c_{chl}(mg * g^{-1}FW) = \frac{E * \frac{V_{extract}(\mu l)}{V_{aliquot}(\mu l)} * V_{cuvette}(\mu l)}{34.4(ml * mg^{-1} * cm^{-1}) * 1cm * FW(mg)}$$

Soluble carbohydrates

Glucose (Glc), fructose (Fru) and sucrose (Suc) concentrations were analysed with a coupled enzyme assay according to Jones *et al.* (1977). The method is based on the $NADP^+$-dependent conversion of glucose-6-phosphate (G6P) to 6-Phosphogluconat by the enzyme glucose-6-phosphate dehydrogenase (G6PDH) and detected via the reduction of $NADP^+$ to $NADPH+H^+$. This reduction is detected quantitatively via the increase of the optical density at λ=334 nm. Following the reaction of G6P with pre-existing G6PDH, enzymes converting glucose, fructose and sucrose to the detectable G6P were sequentially added.
One molecule hexose corresponds to one produced molecule of $NADPH+H^+$, one molecule of sucrose to two $NADPH+H^+$. The analysis was done using a multiple photometer (ht II, Anthos Mikrosysteme GmbH).

The enzymes were prepared as follows: 160 µl **G6PDH**, 240 µl hexokinase (**HK**) and 120 µl phosphoglucoisomerase (**PGI**) were centrifuged (4 min, 13000 rpm, RT). Each pellet was dissolved in 200 µl TRIS buffer (100 mM, 10 mM $MgCl_2$, pH 8.1). 1/4 of a 1.5 ml reaction tube was filled with invertase (**Inv**). The solid was dissolved in 100 µl TRIS buffer.
In each well of a 96-well-microtiter plate 160 µl master mix was mixed with 20 µl extract. Per microtiter plate the master mix consists of:

15.5 ml 100 mM imidazole buffer (pH 6.9, 5 mM $MgCl_2$)
480 µl ATP (60 mg ml^{-1}, prepared in imidazole buffer)
480 µl $NADP^+$ (36 mg ml^{-1}, prepared in imidazole buffer)
200 µl G6PDH (prepared in TRIS buffer)

After the reaction has reached a plateau, 2 µl of HK, PGI and Inv was added successively. Soluble carbohydrate concentration was determined as follows with

- Molar extinction coefficient of $NADPH^+$: ε=6.22 ml*µmol^{-1}*cm^{-1}

- Specific factor of the photometer: 2.85 cm^{-2}

$$c_{hexose}(\mu mol * g^{-1}FW) = \frac{\Delta mOD * \frac{V_{extract}(\mu l)}{V_{aliquot}(\mu l)}}{6.22(ml * \mu mol^{-1} * cm^{-1}) * 2.85(cm^{-2}) * FW(mg)}$$

The sucrose concentration is calculated by dividing c_{hexose} by a factor of 2.

Starch

The starch concentration was determined in glucose equivalents by adding HK as described above, with 100 mM TRIS buffer (pH 8.1, 10 mM $MgCl_2$,) instead of imidazole buffer.

$$c_{starch}(\mu molGlc * g^{-1}FW) = \frac{\Delta mOD * \frac{V_{extract}(\mu l)}{V_{aliquot}(\mu l)} * \frac{V_{incub}(\mu l)}{V_{incub-aliquot}(\mu l)}}{6.22(ml * \mu mol^{-1} * cm^{-1}) * 2.85(cm^{-2}) * FW(mg)}$$

2.7.3 Glutathione assay

Total glutathione was determined according to Gronwald *et al.* (1987) except for the modifications described below. The glutathione recycling system by Ellman´s Reagent (5,5´-Dithiobis(2-nitrobenzoic acid), DTNB) and glutathione reductase is a highly sensitive method for the detection of glutathione (Fig 2-3).

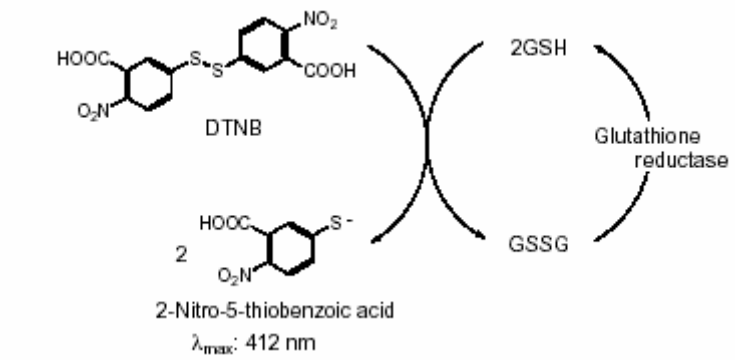

Fig 2-3. Principle of total glutathione detection.
The colorimetric reaction is stable and the OD increases linearly for 5 to 30 min (Dojindo total glutathione quantification kit, technical manual).

250 mg of plant material (FW) was ground with 500 µl 5% TCA in a 4°C-pre-cooled mortar and pestle. After addition of 1 ml 0.1 M K-P-buffer, pH 8.0 the sample (V_{total}=1.5 ml) was

centrifuged 10 min (5000 rpm at 4°C). 40 µl leaf extract (V_{sample}) was added to the reaction medium which consists of 2.38 ml buffer 1, 0.2 ml buffer 2 and 0.2 ml buffer 3. If root extract was measured, 120 µl was added to 2.26 ml buffer 1, 0.2 ml buffer 2 and 0.2 ml buffer 3. The components were equilibrated in a one-way plastic cuvette at 25°C for 100 sec before initiating the reaction by adding 0.2 ml (1 unit) yeast glutathione reductase (leading to $V_{cuvette}$=3.02 ml). The change in absorbance was measured at 412 nm for 8.3 min and the slope (ΔOD per min) between 5 and 8.3 min was determined to calculate the total glutathione concentration according to the calibration equation (see below).

0.1 M K-P-buffer: 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 was mixed to a pH of 7.5 and 8.0.

Buffer 1: 0.1 M K-P-buffer containing 5 mM EDTA, pH 7.5.

Buffer 2: 1 mM NADPH prepared in buffer1.

Buffer 3: 6 mM DTNB prepared in 0.1 M K-P-buffer, pH 7.5.

Glutathione reductase: was diluted in 0.1 M K-P-buffer, pH 7.5 to a final concentration of 1 unit per 200 µl.

Calibration curve using kinetic method

A 200 mM glutathione stock solution was prepared in 0.1 M K-P-buffer, pH 8.0 and diluted with the same buffer to 250 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.13 µM, 1.56 µM. 20 µl of the dilution was added to the cuvette instead of plant extract (see above). The assay was accomplished as described above. The rate of change at OD_{412} between 300 and 500 sec (slope = ΔOD*min^{-1}) of each dilution was determined and plotted against the concentrations (µM) of the calibration solutions in order to create a calibration curve with the following equation:

$$glutathione(\mu M) = \frac{slope - 0.00004}{0.0028}$$

The glutathione concentration was calculated according to the following equation:

$$glutathione(\mu mol * g^{-1}FW) = glutathione(\mu mol * l^{-1}) * \frac{V_{cuvette}(\mu l)}{V_{sample}(\mu l)} * \frac{V_{total}(l)}{FW(g)}$$

2.8 Emissions of volatile organic compounds (VOCs)

VOC emissions were studied in continuously stirred tank reactors, described in detail by Wildt *et al.* (1997) and Heiden *et al.* (2003). For these experiments a plant chamber with a volume of 1100 L was used. The air flow was set to 39.5 L min^{-1}. All plants were investigated at a leaf temperature of 24-25°C and a photon flux density of about 400 µmol m^{-2} s^{-1}. After 11 h the light was turned off sequentially within a time period of 1 h. The simulated twilight avoided LOX emissions due to the switching off of the illumination as described by Heiden *et al.* (2003).

Ozone exposure

Ozone was produced as described by Heiden et al. (2003). Ozone concentrations were measured by UV-absorption (Thermo Environmental Instruments, model 49). Absolute H_2O concentrations were determined with aid of a dew point mirror (Walz MTS-MK1). With an assumed water vapour concentration of 0.164% at a dew point of -17 °C, the ΔH_2O between chamber exit and entrance is calculated as follows:

$$\Delta H_2O(\%) = [H_2O]_{out}(\%) - 0.16391261(\%) = EXP(19.044 - 5338.22/TP(°K))(\%) - 0.16391261(\%)$$

Transpiration rate and stomatal conductance

The transpiration rate (TR) is calculated as follows:

$$TR(mol * m^{-2} * s^{-1}) = \Delta H_2O(\%) * 0.01 * \frac{flux(l/\min)}{LA(m^2) * 60(\sec * \min^{-1}) * 24(l * mol^{-1})}$$

The stomatal conductance (SLW) is defined as the quotient of TR divided by the water vapour pressure difference inside the substomatal cavity ($[H_2O]_{SC}$ and air $[H_2O]_{out}$).

$$SLW(mol * m^{-2} * s^{-1}) = \frac{TR}{([H_2O]_{SC}(\%) - [H_2O]_{out}(\%)) * 0.01}$$

with

$$[H_2O]_{SC}(\%) = EXP(19.044 - 5338.22/LT(°K))$$

The concentration of water in the substomatal cavity was calculated from the measured leaf temperatures assuming the relative humidity inside the plants to be 100% (Neubert *et al.*, 1993).

TP = dew point, LA = leaf area, LT = leaf temperature in °K

Interpretation of the data

A description of the GC-MS system used for VOC analysis was given by Heiden *et al.* (1999). The measured compounds shown in Chapter 3.7.1 were identified by using reference mass spectra from the NBS75k library. For some compounds concentrations were determined using a calibration source. These compounds were used as reference substances (Ref) to calculate the concentrations of other VOC that have similar molecular structure and ionization efficiency as the measured compounds. The concentrations of these compounds were estimated as described by Heiden et al. (2003): The peak area was measured in the single ion mode (SIM). In order to consider the different fractionation of reference substance and substance to be calibrated, the fraction of the peak areas of the respective target peaks over the sum of all peak areas were taken according to the ratio:

$$P - TIC(\%) = \frac{Z_T}{\sum_{i=35}^{Mw+1} Z_i}$$

Z_T is the peak area of the target compound, Z_i is the peak area of the peak with m/z=i. Summation from m/z=35 to Mw+1. m/z=35 is the lower limit measured with the mass spectrometer and Mw is the molecular weight of the respective compound. The peak area is given in counts (cts). The concentration of the respective compounds (x) was estimated with the following equation:

$$ppb_X = cts_X * \frac{P - TIC_{ref}}{p - TIC_X} * \frac{ppb_{\mathrm{Re}\,f}}{cts_{\mathrm{Re}\,f}}$$

For each substance the following reference was chosen and P-TIC estimated with the following major mass.

Tab 2-5. Reference substances and major masses of the analysed VOCs.

Substance	Major mass	Reference substance
E-2-hexenal	55	Z-3-hexenol
α-pinene	93	α-pinene
α-terpinene	93	α-pinene
Ocimene	93	α-pinene
Benzoicacidethylester	105	Methylsalicylate (MeSA)
MeSA	120	MeSA
Trimethyl-tridecatetraene (TMTT)	69	Longifolene

The **emission rates** were determined according to the following equation. The whole leaf area (one sided) was used as normalization factor. This allows direct comparison of the emission rates of compounds from different pathways.

$$flux(mol * m^{-2} * s^{-1}) = \Delta VOC(ppb) * 10^{-9} * \frac{flux(l/\min)}{LA(m^2) * 60(\sec * \min^{-1}) * 24(l * mol^{-1})}$$

Experimental setup

Tomato seedlings were grown as described in Chap 2.5.3. After four weeks, 6 non-mycorrhizal plants were placed in the chamber. After 24 h of acclimatisation VOCs were measured one day before fumigating the plants with ozone (5 h, 200 ppb). After the fumigation period the VOCs were again measured for a period of one day. Afterwards the same experiment was repeated with 6 mycorrhizal plants (ca. 4% mycorrhization). The total leaf area was determined immediately after removal of the plants out of the tank reactors. During the experiment all plants were well watered.

2.9 Molecular biological techniques

2.9.1 RNA isolation from plant material

Total RNA was extracted from frozen leaf and root material. In order to increase the yield and quality, RNA from roots, ozone fumigated shoots and shoots of mycorrhizal plants was isolated following different protocols. The control plants belonging to the respective treatment were isolated following the same protocol as the treated plants.

Roots of mycorrhizal and ozone fumigated plants grown in splitroot and single pots

About 450 mg material was ground in a 4°C-tempered mortar under addition of 500 µl Trizol® (Invitrogen) until no pieces were visible, then 4 ml Trizol were added. After centrifugation (10 min at 4°C, 5000 rpm) the supernatant was extracted in 900 µl chloroform by 30 sec vigorous shaking. The sample was incubated 3 min at room temperature, centrifuged (15 min at 4°C, 5000 rpm) and again extracted in 2.4 ml chloroform. The RNA in the supernatant was precipitated by incubating 15 min with 2.25 ml ice cold isopropanol. The precipitated RNA was washed with 75% ethanol, air dried and dissolved in MilliQ water.

Shoots of ozone fumigated and mycorrhizal plants grown in single pots

The following method was modified from Pawlowski (1994). A 1:1 mixture of RNA extraction buffer (Tab 2-6) und phenol (TE-saturated, pH 8.0, Fluka) was tempered to 90°C. 2 ml of the mixture was prepared per extraction of 500 mg plant material.

Tab 2-6. Components of the RNA extraction buffer for shoots of plants grown in single pots.

Substance	End concentration
$LiCl_2$	100 mM
SDS	1%
Tris-HCl, pH 9.0	100 mM
EDTA	10 mM

About 500 mg material was ground in a 4°C-pre-cooled mortar under addition of 500 µl cold extraction buffer. 2 ml of the extraction buffer-phenol mixture was added and the raw extract was vigorously vortexed. After a 20 min centrifugation step (4000 rpm, RT) the supernatant was extracted twice with the same volume of chloroform. 8 M LiCl was added to a final concentration of 2 M and RNA was precipitated 4-16 h at 4°C. The pellet was resuspended in 300 µl 0.3 M NaAc, pH 5.2 and again precipitated with the 2.5-fold volume of 100% EtOH. The RNA was washed with 75% EtOH and dissolved in MilliQ water.

Shoots of mycorrhizal plants grown in splitroot pots

The following method was modified from Hildebrandt (2000). 4 ml extraction buffer (Tab 2-7) was mixed with the same volume Aqua-Roti-Phenol.

Tab 2-7. Components of the RNA extraction buffer for shoots of mycorrhizal plants grown in splitroot pots.

Substance	End concentration
Na Acetat	100 mM
EDTA	1 mM
SDS	4%
pH 5.0	

About 1 g plant material was thoroughly ground in a 4°C-pre-cooled mortar under addition of 2 ml extraction buffer-phenol-mixture. The remaining 2 ml of the buffer-phenol-mixture was added and the whole sample was centrifuged 10 min at RT (5000 rpm). The supernatant was extracted with the same volume of phenol/chloroform/isoamylalcohol (PCI) by vigorous shaking and vortexing. After a 6 min centrifugation step (RT, 5000 rpm) the supernatant was

again extracted with the same volume of chloroform/isoamylalcohol (24:1). After a 4 min centrifugation step (4°C, 5000 rpm), the RNA in the supernatant was precipitated with the 0.5-fold volume of 8 M LiCl for 30 min (4°C). The pellet was washed with 2 ml 100% Rotisol and 80% Rotisol, dried and dissolved in MilliQ water.

Before checking the quality of the isolated RNA on a TAE gel (Chap 2.9.6), the sample was incubated at 60°C for 10 min and shortly centrifuged at 4°C.

2.9.2 Isolation of bacterial plasmid DNA

Plasmid DNA from *E. coli* cultures grown over night (Chap 2.9.12) was isolated with the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer´s instructions. The DNA was eluted in 50 µl MilliQ water and the concentration was determined spectroscopically (Chap 2.9.5).

2.9.3 cDNA synthesis

First strand cDNA was synthesised from total RNA of tomato using M-MLV reverse transcriptase (Promega) or iScript reverse transcriptase (BioRad) and oligo-dT primer according to the respective manufacturer's instructions.
Fragments of the target genes have been amplified from this cDNA by polymerase chain reaction (PCR, Chap 2.9.10) with gene-specific primers.

2.9.4 Ligation

Fragments of the target genes, eluated from a TAE gel (Chap 2.9.6), were transferred into the cloning vector pGEM-T (Chap 2.9.12.2) with T4 DNA ligase (Promega) according to manufacturer's instructions. The ratio of vector to insert was set to 1:4.

2.9.5 Determination of nucleic acid concentration

The concentration of dissolved nucleic acids was determined by spectrophotometry with an absorption photometer (Bio-Tek Instruments). The extinction was measured at a wavelength of 260 nm. A solution with an absorbance of A_{260}=1 contains ~50 µg DNA/ml or ~40 µg RNA/ml.

The concentration of DIG-labelled probes was estimated via analytical agarose gelelectrophoresis with molecular weight standards. The DNA ladder bands contain known DNA concentrations. Intensities of DNA bands of ladders and probe were compared.

2.9.6 Gelelectrophoresis

50x TAE buffer, pH 7.3: 2 M Tris, 0.9 M Acetic acid, 50 mM EDTA
10x MOPS buffer, pH 7: 400 mM MOPS/NaOH, 100 mM NaAc, 10 mM EDTA
6x DNA loading dye: 30% glycerol, 0.25% bromphenolblue, 0.25% xylenecyanol
11x RNA loading dye: 50% glycerol, 1 mM EDTA, 0.25% bromphenolblue, 0.25% xylenecyanol

Electrophoretic separation of DNA and RNA

For checking the quality or length of DNA or RNA, the samples were separated on agarose containing TAE gels (Sambrook & Russell, 2001) with 1x TAE as buffer. As a standard to estimate the length (bp) of DNA fragments, 10 µl of GeneRuler^{TM} 50 bp DNA ladder (Fermentas) or MassRuler DNA ladder low range (Fermentas) was loaded. After electrophoresis of the gel, it was stained with an ethidium bromide solution (about 1 µg/ml), destained with MilliQ water and photographed under UV light.

RNA for transcriptional analysis was separated according to size by denaturing electrophoresis in agarose gels containing formaldehyde (Sambrook & Russell, 2001). 1x MOPS was utilised as buffer. After washing the gel twice in 10x SSC and once in water for 15 min, it was used for northernblot transfer (Chap 2.9.7).

Isolation of DNA fragments out of a TAE gel

Fragments of target genes were amplified by PCR from cDNA and loaded on a TAE gel. Prior to ligation into a vector, the separated fragment of the right size was eluated out of the gel by the QIAquick® Gel extraction kit (Qiagen) according to manufacturer's instructions.

2.9.7 Northernblot

Transfer technique

The equipment was sterilised in a 3% H_2O_2-solution, glassware was baked 2-3 h at 200°C and destilled water was autoclaved before use.

8-10 µg of each RNA sample, separated on denaturing formaldehyde gels (Sambrook & Russell, 2001), was blotted on nylon membranes (Roche Diagnostics) using a turboblotter (Schleicher&Schuell, BioScience, Dassel) according to manufacturer's instructions. 10x SSC was used as blotting buffer. After blotting over night, the RNA was cross linked with the membrane with a UV stratalinker 1800 (Stratagene) and frozen at -20°C until use.

Staining of the Northernblot membrane

Before prehybridisation, the membrane was stained with trypane blue according to Herrin & Schmidt (1988), to check the regularity of the RNA loading. The membrane was destained with 2x SSC or H_2O and the pattern documented by scanning.

2.9.8 Hybridisation and immunological detection

Hybridisation and immunological detection have been performed with DIG Easy Hyb and the DIG Wash and block buffer set (Roche Diagnostics) according to the manufacturer's instructions with 40 ng DIG-labelled probe/ml hybridisation solution.

The DIG-labelled probes (Chap 2.9.10) were detected by an antibody against the digoxigenin, coupled to an alkaline phosphatase. Incubation with the substrate CDP-Star results in emission of chemiluminescence. The chemiluminescence signal is proportional to the amount of bound probe, which is proportional to the amount of specific mRNA.

For the visualisation of the hybridisation signals by chemiluminescence detection (CDP-Star, Roche Diagnostics), a fluorescence imaging system LAS3000 (Raytest) was used.

Membrane stripping

After hybridisation the membrane can be used again after stripping of the bound probe. According to the DIG Application Manual (DIG Application Manual, Roche Molecular Biochemicals) the membrane was incubated 2x 60 min at 80°C in a solution of 50% formamide, 5% SDS, 50 mM Tris-HCl (pH 7.5). Then it was rinsed 5 min in 2x SCC at RT.

2.9.9 Recombinant plasmids

The competent *E. coli* DH5α cells were transformed (modified from Inoue *et al*., 1990) with the pGEM-T vector (Promega, Mannheim) containing gene specific fragments. pGEM-T is a cloning vector with the genotype Amp^R, *lac*2a.

Bacteria DH5α transformed with the bluescript vector pSK(+) containing fragments of EPSPS and CS1 (Tab 2-8) were obtained from N. Amrhein, ETH, Zürich.

In Tab 2-8 the recombinant plasmids, primers and vectors are listed.

Tab 2-8. Overview of the primers and plasmids,
which were utilised to amplify specific fragments from the recombinant plasmids. The sequence analysis was done with NCBI Blast (www.ncbi.nlm.nih.gov/) and DNAMAN 4.1 (Lynnon BioSoft, Canada).

Gene, Clone#	Full gene name	Accession no.	Primer , annealing temp.	Primer sequences	Length of probe (bp)
Le255**DAHPS1**	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 1	Z21792	5LEDP1 5`-primer T_M=52°C	5´-TGCATTGGTGGATCACG-3´	283
			5LEDP2 3`-primer T_M=52°C	5´-CATGCCTGAGCAAGTTG-3´	
Le377**DAHPS2**	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 2	Z21793	Le5DP2K 5`-primer T_M=53°C	5´-AGCGGTTCATTCGTCTGA-3´	261
			Le3DP2K 3`-primer T_M=53°C	5´-GCTAGAAGCCTTGAAGAACG-3´	
Le260**EPSPS**	5-Enolpyruvyl-shikimate-3-Phosphate-Synthase	M21071	Le5EPSPK 5`-primer T_M=55°C	5´-TCCATCAGCAGCCAATAC-3´	433
			Le3EPSPK 3`-primer T_M=55°C	5´-GTTAAGTTCGCTGAGGTCCT-3´	
Le298**CS1**	Chorismate-Synthase 1	Z21796	5LeCS1K 5`-primer T_M=59°C	5´-TGAATCAGCTGAGGTT-3´	279
			3LeCS1KK 3`-primer T_M=59°C	5´-CCGCTCGGAGTTAAGAATTT-3´	
Le394**ECS**	γGlutamyl-Cysteine-Synthetase	AF017983	LeECS5 5`-primer T_M=52°C	5´-CATGGCCTTGATGTCTCA-3´	392
			LeECS3 3`-primer T_M=52°C	5´-TGCGACCCATGAAGTATG-3´	
Le409**PR1a2**	Pathogenesis related protein	Y08844	LePR1a2K5 5`-primer T_M=52°C	5´-ATCCCGAGCACAAAGCTA-3´	297
			LePR1a2K3 3`-primer T_M=52°C	5´-TCCTGTAGGCAATTGGGT-3´	
	Unspec. primers: pGEM-T		SP6 T_M=52°C	5`-CGATTTAGGTGACACTATAG-3´	164
	Unspec. primers: pGEM-T and pSK(+)		T7 T_M=52°C	5´-TAATACGACTCACTATAGGG-3´	164 (pGEM-T) 165 (pSK)
	Unspec. primers: pSK(+)		T3 T_M=52°C	5`-ATTAACCCTCACTAAAGGGA-3´	165 (pSK)

2.9.10 PCR reaction

All primers were synthesised by MWG Biotech, Ebersberg. The dried primers were dissolved with MilliQ water to a final concentration of 100 µM. All PCR reactions were performed in a Robo-Cycler Gradient (Stratagene).

The PCR reaction was performed in a volume of 50 µl, containing:
1 U *Taq* DNA polymerase (Fermentas)
1x buffer +NH_4SO_4 (Fermentas)
2 mM $MgCl_2$
0.2 mM dNTP
1µM of each of the primers
1 ng plasmid DNA

If cDNA (10 µl) was used as template, no additional dNTP was added. Tab 2-9 shows the standard PCR program.

Tab 2-9. Standard PCR program for specific fragments and DIG probes.
The annealing temperatures were adapted to the used pair of primer.

Step	Temp (°C)	Min
1x	95	5
35x	95	1
	50-61	1
	72	1
1x	72	5

PCR with DIG-labelled probe

DIG-labelled probes are synthesised via PCR (Tab 2-9) with the PCR DIG probe synthesis kit (Roche) according to manufacturer's instructions. The reaction mix was diluted 10fold and then purified and re-concentrated with Amicon® Microcon® PCR Filter units (Millipore) according to manufacturer's instructions. Finally the probe was dissolved in 20 µl MilliQ water.
The concentration of the DIG-labelled probe was estimated with a TAE gel in comparison to a standard with known amounts of DNA (Chap 2.9.6).

2.9.11 Specifity of the probes

To clone specific fragments of the two DAHPS isoforms, regions of the specific parts of the cDNA were amplified and cloned. The DAHPS1 fragment is located in the 3´-part of the cDNA, the DAHPS2 fragment in the 5´-part.

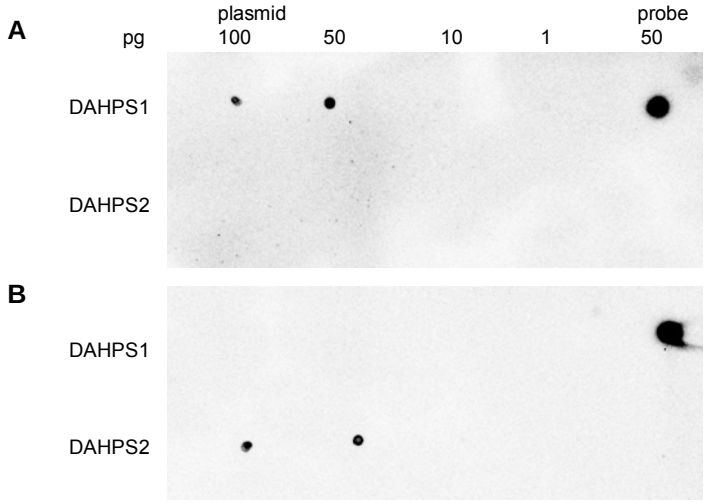

Fig 2-4. Cross hybridisation of DAHPS1 and DAHPS2.
Membrane A was hybridised with probe of DAHPS1, 50 pg probe was taken as control. Membrane B was hybridised with probe of DAHPS2, 50 pg probe of DAHPS2 was taken as control.

Fig 2-4 shows that both probes were specific, quite sensitive and detect down to 50 pg of pure plasmid. The labelled probe as positive control was detected in both cases.
In addition, the sensitivity of the PR1a2 probe was tested against the 2nd PR1 isoform PR1b1 (Tornero *et al.*, 1997) (Fig 2-5).

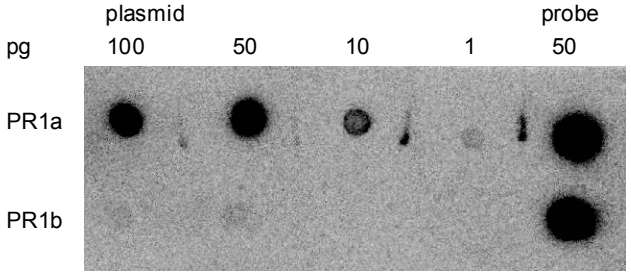

Fig 2-5. Cross hybridisation of PR1a2 and PR1b1
hybridised with PR1a probe with different plasmid amounts to test the sensitivity. Pure probe was taken as positive control.

The PR1a2 probe was isoform specific and highly sensitive. 10 pg of pure plasmid were well detectable, and even 1 pg of plasmid gave signals.

2.9.12 Microbiological methods

2.9.12.1 Growth conditions and cultivation of *Escherichia coli*

Bacteria were grown on LB agar (1.5%) plates with 50 mg/ml ampicillin as selection agent over night at 37°C and could be stored at 4°C for about 2 months, before plating on new plates. Single colonies were grown in 3 ml liquid LB medium (with ampicillin) over night under continuous shaking in order to isolate plasmid DNA.

LB medium (Sambrook & Russell, 2001)
10 g/l tryptone, 10 g/l NaCl, 5 g yeast extract, pH 7.5 with NaOH.

2.9.12.2 Competent cells and transformation

Competent cells of *E. coli* DH5α were prepared according to Inoue *et al.* (1990) and stored at -80°C until they were utilised for transformation.
50 µl of competent cells were incubated with 2 µl recombinant plasmids (Chap 2.9.9) for 20 min on ice, 45-50 sec at 42°C and again on ice for 5 min. 950 µl of SOC medium was added and the cells were incubated for 60 min at 37°C before plating on LB agar plates with 50 mg/ml ampicillin over night (see above).
Successful transformation with the recombinant plasmid was first checked by PCR. Unspecific primers, which bind at the left and right side of the vector cloning site, were used to analyse the size of the inserted fragment. Positive clones were then analysed by sequencing. 2 µg DNA was dried with a vacuum centrifuge (Millipore) and sent to MWG Biotech (Ebersberg) for sequencing.

SOC medium
2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl. After sterilisation 10 mM $MgCl_2$ (sterile filtered), 20 mM $MgSO_4$ (sterile filtered) and 20 mM glucose (sterile filtered) was added.

3 Results

Mycorrhiza is a typically mutualistic symbiosis, i.e. beneficial for both partners. The fungi receive fixed carbon from the host plant. The plant often receives a number of benefits, such as increased nutrient uptake, improved water relations, and resistance to diseases (Smith & Read, 1997; Strack *et al.*, 2003). Therefore a difference in nutrient and sugar status should be detectable between mycorrhizal and non mycorrhizal plants. As the carbon status is directly linked to the biosynthesis of secondary aromatic compounds (Grace & Logan, 2000; Fritz *et al.*, 2006), an adjustment of the carbon status in mycorrhizal plants could lead to an altered balance of aromatics.
To study the influence of mycorrhization on shikimate pathway transcription, nutrient concentration and carbohydrate content, a splitroot system was used as experimental setup with both sides non-inoculated (CC), both sides inoculated (mm) and only one side inoculated (mC).

As plants react differently to mycorrhization in the different stages of the symbiosis, as reviewed by Garcìa-Garrido & Ocampo (2002), the mycorrhization rate has to be determined in parallel with the effects of the treatment during the harvest. In Fig 3-1 typical spreading kinetics of arbuscules within the roots over the whole period of an experiment are shown.
If the plants were fertilised with triple amount of Hoagland solution over the whole experimental period (f, see x-axes in Fig 3-1), the development of the symbioses at the end of the experiment was clearly reduced in the 7th week, but still reached about 10%, showing that also in this experiment nutrient deficiency promotes mycorrhization.

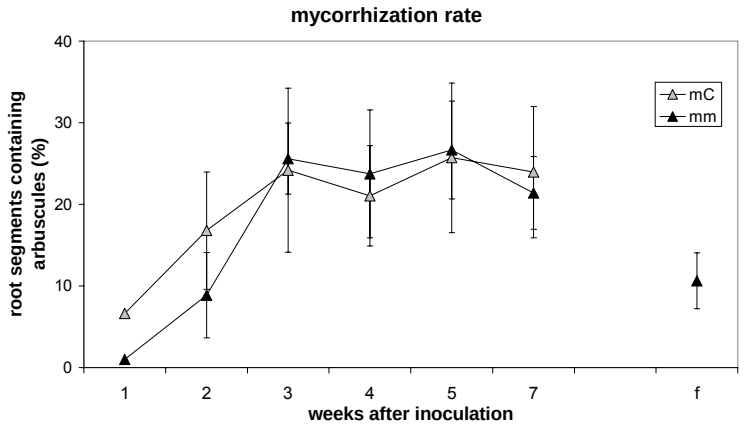

Fig 3-1. Changes in the mycorrhization rate of tomato plants during growth period of 6 weeks.
The mycorrhizal part of the half mycorrhizal root (mC) and the fully mycorrhizal root (mm) is shown (mean value of 7 plants). The values of the first week are visually estimated. The plants were grown in splitroot pots. Harvest and estimation of the mycorrhization rate is described in Chap 2.6.
f = mycorrhization rate in week 7 of plants fertilised with triple amount of Hoagland solution since inoculation.

As shown in Fig 3-1, the fungus developed faster in the mycorrhizal part of half mycorrhizal pots (mC) than in totally mycorrhizal roots (mm). After 3 weeks similar mycorrhization rates (25%) were reached. This means that the number of arbuscules, the site of nutrient and sugar exchange between the symbiosis partners, is twice as high in totally mycorrhizal roots as in half mycorrhizal roots at this time as the biomasses do not differ (Fig 3-2), indicating no significant differences in the number of roots. The faster spreading of arbuscules in half mycorrhizal roots in the first few weeks was confirmed in two further independent experiments, from which shikimate pathway transcripts were determined (Chap 3.4). The number of vesicles - as an indicator of sink strength – also reached the maximum (15-25%) already in the 3rd week after inoculation and the percentage in mC plants was slightly higher in the 2nd week (not shown).

3.1 Biomasses

In order to define the experimental system and to investigate structural effects of mycorrhization with *Glomus intraradices* on tomato plants, first the root and shoot biomasses of mycorrhizal and non-mycorrhizal plants were determined.

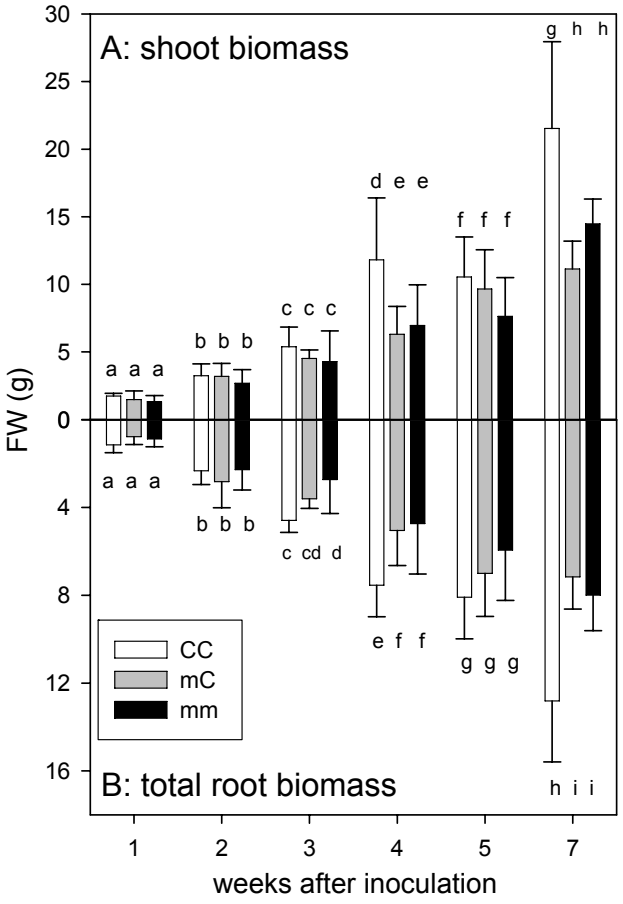

Fig 3-2. Fresh weight (FW) of shoots (A) and roots (B) of tomato plants mycorrhized with *Glomus intraradices*.
The plants were grown in splitroot pots with both sides non-inoculated (CC, white bars), both sides inoculated (mm, black bars) or only one side inoculated (mC, grey bars). Seedlings were 3 weeks old at the time of inoculation. The mean biomass of 7 plants is shown. The error bars represent the standard deviation (SD). Statistical analysis was done according to a one way ANOVA. All pairwise multiple comparisons were done according to Tukey test ($p<0.05$). Different letters indicate significant differences between the treatments within each week. Shoots and roots were tested separately.

After the 3rd week following inoculation the increase in biomass was equally lower in both mC and mm shoots compared to the increase of non-mycorrhizal plants. The root reacted slightly faster, as in the 3rd week the increase in mycorrhizal roots was significantly lower compared to control roots. Only in the 5th week no significant difference but the same trend could be observed between the treatments. The faster development of arbuscules within the half

mycorrhizal roots (Fig 3-1) and thus the greater sink strength in the first 2 weeks had no effect on the growth of shoots and roots in this period (Fig 3-2).

The biomasses of the two root parts of the half mycorrhizal plants were not significantly different from each other except for week 3 (Fig 3-3), which was not in agreement with the data for the total root biomass of fully mycorrhizal plants. From these data, one would have expected greater differences in biomasses between the two halves, especially at the end of the experiment with lower biomasses for the mycorrhizal half. These data show that root biomass is overall affected, also if only one half is mycorrhizal and that the reduction of root biomass is independent of the overall mycorrhization.

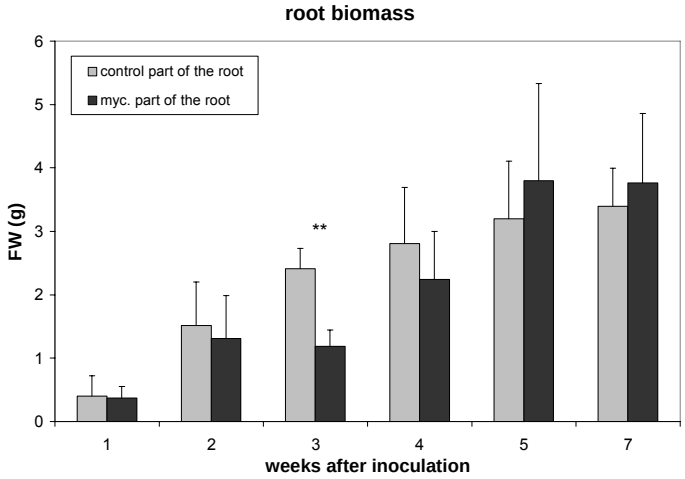

Fig 3-3. Biomasses of both the mycorrhizal and non-mycorrhizal root parts of a half-mycorrhizal plant grown in a splitroot system. The sum of both parts is shown in Fig 3-2 B (grey bars). Asterisks (**) indicate significant differences ($p<0.01$) between the root parts according to a t-test (n=7).

In the splitroot system, mycorrhization of tomato “Roma” with the VAM fungi *Glomus intraradices* did not lead to an improved growth of both shoots and roots. The growth was even reduced after the 3rd week, when the mycorrhization rate nearly reached the maximum. These findings as well as the greater mycorrhization rate in the mC-m root at the beginning of the symbiosis were observed again in two further independent split-root experiments (not shown).

As already mentioned, the plants were grown under deficiency conditions, and the fungus acted as an additional sink. This seems to have led to reduced biomasses of roots and shoots. The shoot-root-ratio (Fig 3-4) gives an idea, whether there is a changed priority for the root or the shoot growth under these conditions.

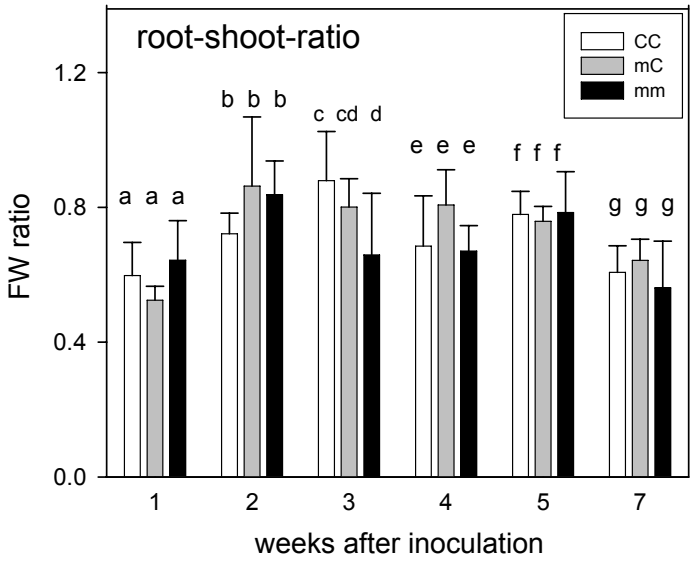

Fig 3-4. Fresh weight (FW) ratio of root to shoot in mycorrhizal and non-mycorrhizal tomato plants. The plants were grown in splitroot pots with both sides non-inoculated (CC, white bars), both sides inoculated (mm, black bars) or only one side inoculated (mC, grey bars). The mean value of 7 plants is shown. Error bars represent the SD. Statistical analysis was done according to a one way ANOVA. All pairwise multiple comparisons were done according to Tukey test ($p<0.05$). Different letters indicate significant differences between the treatments within each week (n=7).

Also the root-shoot-ratio was not significantly different in all treatments except for week 3, although mycorrhizal plants are significantly smaller. The different mycorrhization rates in the first two weeks between fully and half mycorrhizal plants (Fig 3-1) did not result in differences in the root-shoot ratio. The lower ratio in the 3rd week in mycorrhizal plants was due to the decreased root biomass (Fig 3-2).

The results indicated that growth is equally reduced in both shoots and roots in mycorrhizal plants with no effect on the root-shoot-ratio in the system described here. Furthermore it can be concluded that even the mycorrhization of the half root system alone can lead to a similar pronounced reduction in biomass of shoots and roots, indicating that not the amount of effected roots, but more the general sink strength of the fungus affects plant biomass.

3.2 Elements

Mycorrhizal plants are typically better supplied with nutrients, especially nitrogen and phosphate (Marschner & Dell, 1994). However the investigated mycorrhizal plants grew slower in this system. Possibly under the supplied very low nutrition level the biotic interaction is not symbiotic but parasitic and the fungus is mostly a sink. Another possible explanation for the reduced growth could be enhanced metabolism or cost intensive pre-induced resistance against pathogens, which might be triggered by the fungus and thus could still be of beneficial character for the plant. Therefore the carbon and nitrogen concentration of mycorrhizal and control plants were compared to each other in order to estimate the nutrient and mineral status of mycorrhizal plants.

Carbon and nitrogen have profound effects on plant development, summarised in Krapp *et al.* (2005) and on the interactions between carbon and nitrogen signalling, which affects metabolism, energy and protein synthesis (Palenchar *et al.*, 2004). The various interactions between carbon and nitrogen metabolism will be discussed in this context in Chapter 4.1.
The amount of nitrogen within a tissue can be considered proportional to the amount of protein. A lower C/N ratio of biomass can increase the susceptibility to parasites and the effectiveness of plant defence, because tissues with high protein contents often have a high metabolic activity, which makes them attractive to herbivores. Here, the influence of mycorrhization on the C/N ratio and both carbon and nitrogen concentration are examined.

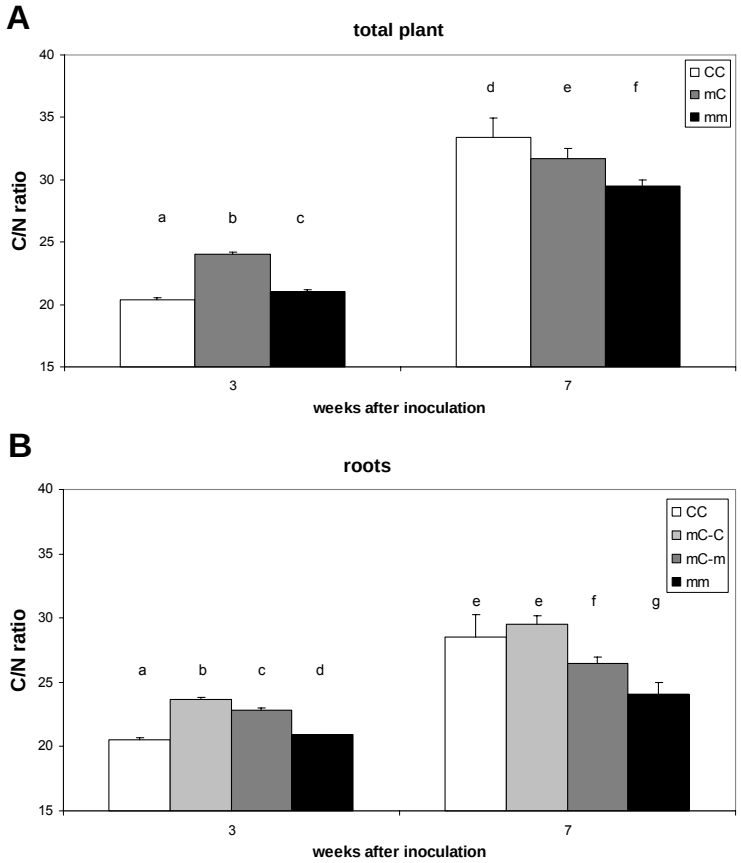

Fig 3-5. C/N ratio of whole plants (A, roots and shoots) and roots (B) of fully-, half- and non-mycorrhizal plants. The plants were grown in splitroot pots with both sides non-inoculated (CC, white bars), both sides inoculated (mm, black bars) or only one side inoculated (mC-C and mC-m, grey bars). The mean value of 7 plants is shown. Error bars represent the SD. Statistical analysis was done according to a one way ANOVA. All pairwise multiple comparisons were done according to Tukey test ($p<0.05$). Different letters indicate significant differences between the treatments within each week.

In the 7th week after inoculation, the C/N ratio was lower in mycorrhizal than in control plants, the C/N ratio of the half mycorrhizal plant was intermediate (Fig 3-5 A). In the 3rd week after inoculation, the C/N ratio in mycorrhizal plants was slightly higher than in controls, with half mycorrhizal plants being the highest. If considered separately, the trend was the same in shoots (not shown) and control and fully mycorrhizal roots in both weeks (Fig 3-5 B). Within the half mycorrhizal roots, the C/N ratio was lower in the mycorrhizal half. This shows that during the symbiosis development there was a change from higher to lower C/N ratios. The C/N ratio can be lowered by an increase of nitrogen or a decrease in carbon. Both high and low C/N ratios could have an effect on plant fitness.

In the next part, the carbon and nitrogen concentrations were investigated separately. The concentrations of carbon per dry weight (DW) in shoots and roots of tomato plants are shown in Fig 3-6.

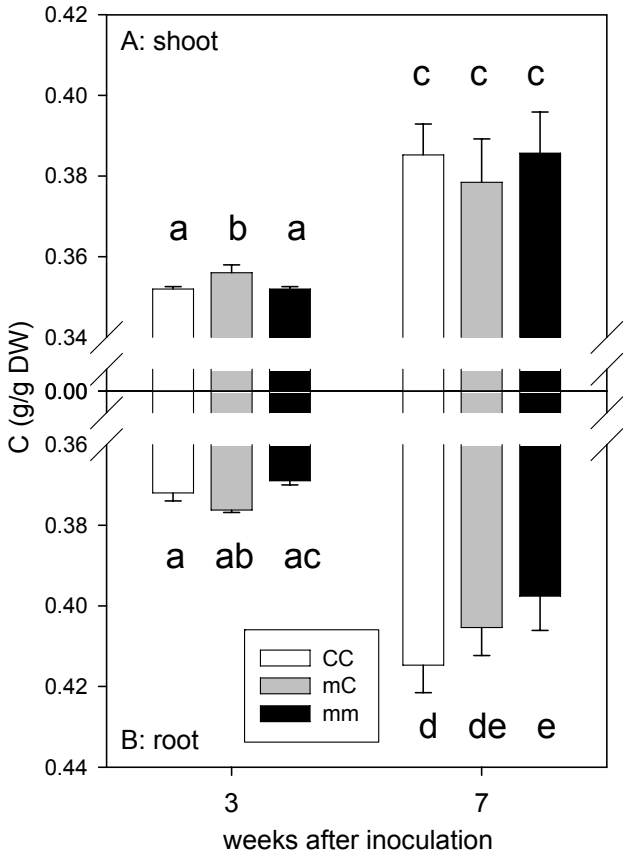

Fig 3-6. Differences in carbon concentration per dry weight (DW) between shoots (A) and roots (B) of mycorrhizal and non-mycorrhizal plants.
7 plants per treatment were pooled, dried and milled. The plants were grown in splitroot pots with both sides non-inoculated (CC, white bars), both sides inoculated (mm, black bars) or only one side inoculated (mC-C and mC-m, grey bars). The mean value of 3 (third week) or 8 (seventh week) measurements is shown. The error bars represent the SD. Statistical analysis was done according to a one way ANOVA. All pairwise multiple comparisons were done according to Tukey test ($p<0.05$). Different letters indicate significant differences between the treatments within each week, with shoots and roots being separately tested.

Mycorrhization had no significant impact on the carbon concentration of the shoot 7 weeks after inoculation at a mycorrhization rate of 20-25%. In contrast, in mycorrhizal roots the carbon concentration was significantly reduced in comparison to control roots in the 7th week. Here, the fully mycorrhizal roots showed a stronger reduction of C content than the half mycorrhizal plants. The generally higher C concentrations in the 7th week compared to the 3rd week are probably due to developmental transitions. In the 3rd week after inoculation, the carbon concentrations of both shoots and roots were highest in the half mycorrhizal plants (mC), while the values of control plants and fully mycorrhizal plants did not differ.

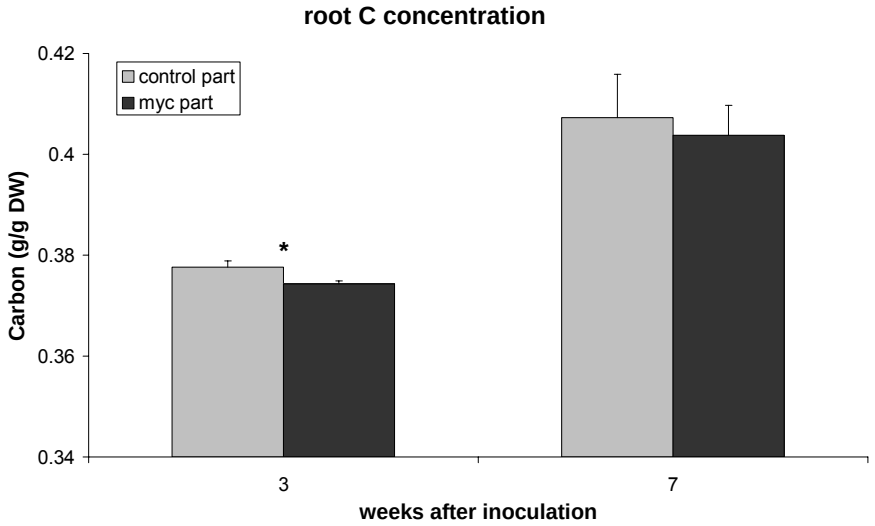

Fig 3-7. Carbon concentration per dry weight (DW) of both the mycorrhizal (myc) and non-mycorrhizal root parts of a half mycorrhizal plant.
7 plants per treatment were pooled, dried and milled. The mean value of 3 (third week) or 8 (seventh week) measurements is shown. The error bars represent the SD. Asterisks (*) indicate significant differences between the grouped bars (t-test, $p<0.05$).

A closer look to the carbon concentration in the different parts of the half mycorrhizal roots (mC) showed that also the root parts of mC plants differed in carbon concentration in the 3rd week (Fig 3-7). In the mycorrhizal part the C concentration was lower, although this small difference can not explain the drastically reduced biomass in the mycorrhizal root part in the 3rd week (Fig 3-3).

Taken together, the C concentration and the overall biomass was lower in roots with a fully established mycorrhiza than in controls, supporting the great sink strength of the fungus during the establishment of the symbiosis. This is in agreement with the finding that the half mycorrhizal roots showed intermediate carbon concentrations between mycorrhizal and control roots. Interestingly the biomass reduction of the half mycorrhizal plants was as pronounced as in fully mycorrhizal plants. This indicates that the biomass reduction is not linear dependent on C concentration or C/N ratio, but must be influenced also by other parameters. In fully mycorrhizal roots, the mycorrhization rate was the same as in half mycorrhizal roots. However, as in the fully mycorrhizal plant both root parts were colonised, the fully mycorrhizal plant had much more fungal carbon sink structures than the half mycorrhizal plant. This might be a reason for the finding of significant effects between control and fully mycorrhizal plants.

Thus, one explanation for the reduced C/N ratio of mycorrhizal plants is the reduced carbon concentration of mycorrhizal roots. Still it is possible that mycorrhizal plants accumulate

higher N concentrations as an additional effect. Therefore, the nitrogen status was also analysed (Fig 3-8, -9).

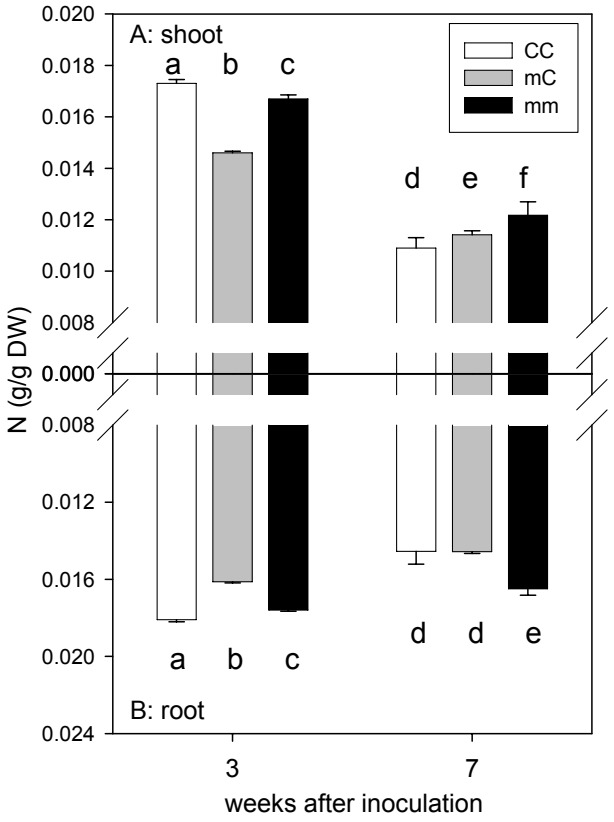

Fig 3-8. Differences in nitrogen concentration per dry weight (DW) between shoots (A) and roots (B) of mycorrhizal and non-mycorrhizal plants.
The plants were grown in splitroot pots with both sides non-inoculated (CC, white bars), both sides inoculated (mm, black bars) or only one side inoculated (mC, grey bars). 7 plants per treatment were pooled, dried and milled. The mean value of 3 (third week) or 8 (seventh week) measurements is shown. The error bars represent the SD. Statistical analysis was done according to a one way ANOVA. All pairwise multiple comparisons were done according to Tukey test ($p<0.05$). Different letters indicate significant differences between the treatments within one week with shoots and roots being separately tested.

In the 3rd week, the nitrogen concentration was lowest in mC shoots and roots (Fig 3-8). In fully mycorrhizal plants, the concentration was only slightly lower than in control plants. In the 7th week, the N concentration was higher in mycorrhizal plants. Half mycorrhizal (mC) plants behaved intermediately for the shoots and showed similar N concentrations like control plants in the roots. Mycorrhization over a longer period seems to have a beneficial effect on the nitrogen concentration at least in the shoot. It will be discussed later, whether this might be due to a better N-acquisition of the plant due to the fungus. However, increases in the root nitrogen concentration should be considered carefully because the method did not distinguish fungal and plant nitrogen.

Yet, a closer look to the nitrogen concentration in the different parts of the half mycorrhizal roots showed that the nitrogen concentration was higher in the mycorrhizal part in the 3rd week (Fig 3-9) and became even higher in the 7th. This is a reverse pattern of what was observed for the carbon concentrations in roots of mm and CC plants (Fig 3-7).

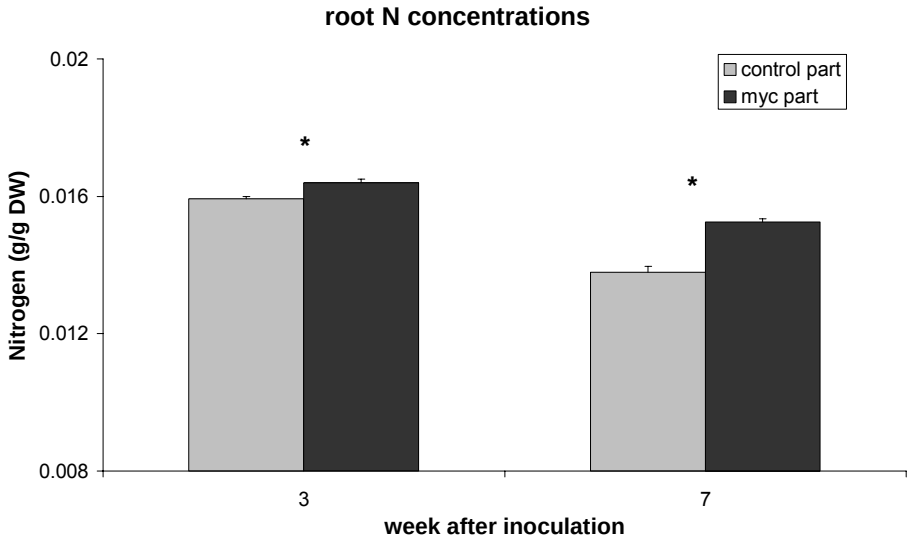

Fig 3-9. Nitrogen concentration per dry weight (DW) of both the mycorrhizal (myc) and non-mycorrhizal root parts of a mycorrhizal plant.
7 plants per treatment were pooled, dried and milled. The mean value of 3 (third week) or 8 (seventh week) measurements is shown. The error bars represent the SD. Asterisks (*) indicate significant differences between the grouped bars (t-test, $p<0.05$).

Another interesting aspect is that despite the fact that there were great differences between the two halves the overall concentration of the splitroot mC roots did not differ from that of the control plant roots 7 weeks after inoculation (Fig 3-8). This implies that the non-mycorrhizal part of the root was not better supplied systemically with nitrogen. There is even a local depletion because the concentration was clearly lower in the control part (0.0138 ±1.80E-04 g/g DW) and higher in mycorrhizal part (0.0152 g/g ±9.50E-05 DW) in comparison with CC (0.0146 ±6.63E-04 g/g DW). The question as to whether the local increase in mC-m is due to nitrogen transport from the fungus to the plant requires further investigations by flux experiments. As this could not be determined in this study, only those effects in the shoot and the whole plant status were regarded as a sign of benefit of mycorrhization.

Mycorrhizal fungi developed efficient nitrogen uptake systems because nitrogen is normally limited in soils and is often abundant as immobile NH_4^+. Under these conditions, mycorrhizal fungi have beneficial effects because they can mobilise NH_4^+ (Clark & Zeto, 2000). Because of the facilitated nitrogen acquisition by VAM, the plant can take up the same or even more nitrogen than control plants with less root mass. The utilised analysis method could not

distinguish between fungal and root nitrogen. However, increased N concentrations in shoots 7 weeks after inoculation in mycorrhizal plants (Fig 3-8) are likely to be due to beneficial symbiosis effects.

Summarised, the differences in the total plant C/N ratio between fully mycorrhizal plants and control plants were caused by two effects. First: increase in shoot and root nitrogen concentration and second: decrease in root carbon concentration. In the 3rd week after inoculation, differences between mycorrhizal (mm) and non-mycorrhizal (CC) plants were first visible in the nitrogen concentration, followed by differences in carbon in the 7th week. In the parts of the half mycorrhizal plant, there were only little changes of both parameters in the 3rd week. In the 7th week, again nitrogen was the decisive factor.

In two further single pot experiments, plants were grown in the greenhouse and harvested 4 weeks after inoculation (Chap 2.5) in order to confirm the results concerning the influence of mycorrhization on element concentrations. Data about phosphorus and sulphur concentrations were additionally listed (Tab. 3-1).
In the first experiment, the symbiosis developed normally (20% myc rate), but the plants were pre-stressed at the transcriptional level (not shown). In the second experiment, the arbuscules could only be observed in 3-4% of the root segments. As a consequence, the reduced growth rate of mycorrhizal plants, as has been seen in the splitroot experiments, could not be observed. The results are shown in Table 3-1. Part of the data of the splitroot experiment described above was also listed for comparison (7th week after inoculation).

Tab 3-1. Concentrations of carbon, nitrogen, sulphur and phosphorus of mycorrhizal (myc) and control (ctrl) plants grown in single pots and harvested 4 weeks after inoculation. Element concentrations were determined as described in Chapter 2-7. The mean of 8 measurements is listed ± standard deviation. These concentrations were compared to the element concentrations of a 4-5 week old tomato plant grown in soil, which was considered as optimally supplied. The 7th week-data of the above-described experiment was also listed again. S*: the sulphur concentrations have to be handled carefully because the concentrations were at the detection limit. For experiment 1&2, the analysis method was modified with V_2O_5 in order to improve the combustion.

Element (g/g DW)	organ	treatment	exp. described above	Experiment 1 (myc rate 20%)	Experiment 2 (myc rate 4%)	Fully supplied
C	shoot	ctrl	0.3852 ±7.67e-3	0.3780 ±0.0065	0.3809 ±0.0054	0.3945 ±0.0075
		myc	0.3857 ±0.0102	0.3741 ±0.0064	0.3765 ±0.0061	
	root	ctrl	0.4147 ±6.80e-3	0.4062 ±0.0111	0.4118 ±0.0074	0.3988 ±0.0076
		myc	0.3976 ±8.52e-3	0.4112 ±0.0088	0.4014 ±0.0079	
N	shoot	ctrl	0.0109 ±4.12e-4	0.0101 ±0.0007	0.0161 ±0.0004	0.0336 ±0.0010
		myc	0.0122 ±5.22e-4	0.0126 ±0.0004	0.0187 ±0.0005	
	root	ctrl	0.0146 ±6.63e-4	0.0135 ±0.0005	0.0147 ±0.0005	0.0277 ±0.0029
		myc	0.0165 ±3.29e-4	0.0167 ±0.0005	0.0162 ±0.0005	
C/N	shoot	ctrl	35.4123 ±1.557	37.5752 ±2.2595	23.652 ±0.4208	11.737 ±0.3026
		myc	31.7053 ±0.8116	29.7901 ±0.4170	20.114 ±0.6594	
	root	ctrl	28.5679 ±1.6584	30.1467 ±0.4812	28.009 ±0.5844	14.517 ±1.5623
		myc	24.1148 ±0.9042	24.6663 ±0.3550	24.737 ±0.4423	
P (%)	shoot	ctrl	0.22 ±0.022	0.4500 ±0.045	0.5100 ±0.051	0.4700 ±0.047
		myc	0.22 ±0.022	0.4900 ±0.049	0.5550 ±0.056	
	root	ctrl	0.165 ±0.017	0.5350 ±0.0535	0.4450 ±0.045	0.3400 ±0.034
		myc	0.155 ±0.016	0.5600 ±0.056	0.5450 ±0.055	
S *	shoot	ctrl	0.00318 ±0.54e-3	0.0073 ±0.0008	0.0079 ±0.0007	0.0042 ±0.0001
		myc	0.0053 ±0.18e-2	0.0094 ±0.0005	0.0097 ±0.0004	
	root	ctrl	0.00283 ±0.17e-3	0.0024 ±0.0001	0.0027 ±0.0002	0.0015 ±0.0001
		myc	0.00293 ±0.21e-3	0.0027 ±0.0001	0.0029 ±0.0001	

In experiment 1&2, the reduced C/N ratio in mycorrhizal plants was mainly caused by higher nitrogen concentrations in the 4th week after inoculation. This fits with the results from the splitroot experiment described above (Fig 3-5 to 3-9). In the second experiment the C/N ratio was much narrower and the N concentration increased less. Perhaps this was attributable to the lower mycorrhization rate. In fully supplied plants, the carbon concentrations were similar, but the nitrogen concentrations were about 3 times higher. Accordingly, the C/N ratio was much lower.

The phosphorus concentration was slightly increased by mycorrhization in experiment 1&2, although not significantly. In roots, the P concentration in mycorrhizal plants was higher than in fully supplied plants. In the splitroot experiment, this trend was observed in the 3rd week. In

the 7th week, on the other hand the P concentration was lower in mycorrhizal roots and remained the same in shoots.
Mycorrhization generally influences the nutrient composition in roots and shoots and seemed to have an enhancing effect on sulphur acquisition, especially in shoots. The crosstalk and interactions between carbon-, nitrogen-, sulphur- and phosphorus-metabolism is discussed in Chapter 4.1.

In the following chapters, the influence of mycorrhization on primary (sugar/starch, glutathione) and secondary (shikimate pathway, VOC) metabolism is investigated to find out whether primary or secondary metabolism is responsible for changes in the C- and N-concentrations or vice versa.

3.3 Sugar and starch

The element analysis did not distinguish root carbon from fungal carbon. VAM store their carbon as lipids and glycerol and transport it in the extraradical mycelium (reviewed by Bago *et al.*, 2000; Bago *et al.*, 2002). So the analysis of sugar and starch concentrations could reflect the carbon flow in the symbiosis. This could in turn explain changes in the C/N metabolism or decreased growth because of the putatively great sink strength of the fungus. In Fig 3-10 changes in sugar and starch concentration are shown during a period of 7 weeks after inoculation in shoots and roots of mycorrhizal and control plants grown in splitroot pots.

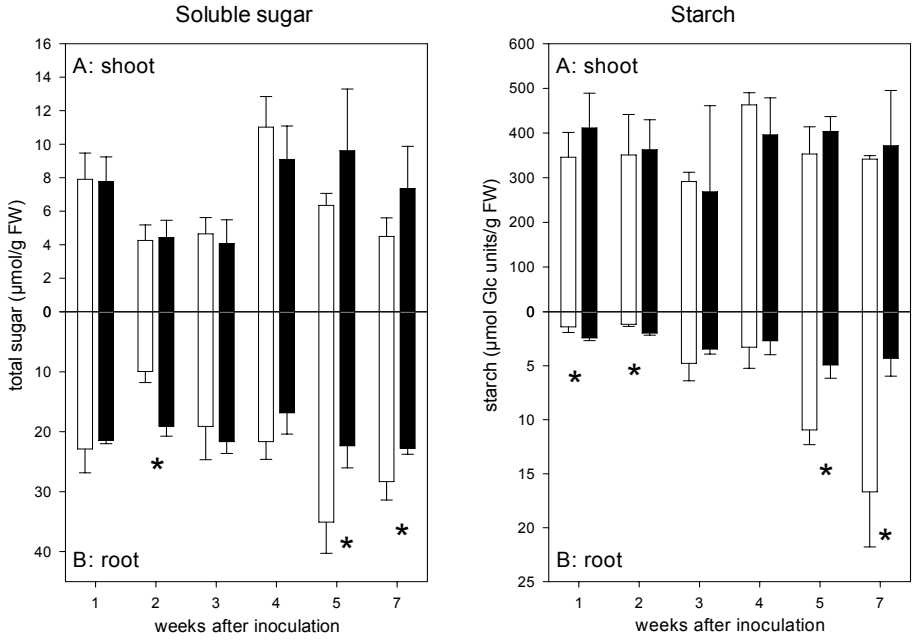

Fig 3-10. Concentrations of soluble sugar (glucose, fructose, sucrose) and starch in shoots and roots of mycorrhizal (black bars) and non-mycorrhizal (white bars) plants.
Material from 7 plants was pooled. The measurement was repeated 3 times, each repetition with 2 replicates. Shoot: There were no significant differences between grouped bars according to t-test. There were also no significant differences in the chlorophyll concentration (data not shown). Root: Asterisks (*) indicate significant differences between the grouped bars according to t-test (*$p<0.05$).

In general, the soluble sugar concentration was higher in roots than in shoots. In contrast, the starch concentration was enhanced in shoots in comparison to roots. Mycorrhization had no statistically significant influence on the shoot sugar and starch concentrations. In roots the sugar and starch concentration in mycorrhizal plants remained the same throughout the 7 weeks whereas the concentration in the control plants increased over time.

In mycorrhizal roots a constant carbon flow to the fungus could explain the low concentrations of root C (Fig 3-6). The higher root soluble sugar concentrations in control plants were due to increased fructose and glucose rather than sucrose concentration (not shown). However, sucrose concentrations are slightly lower in mycorrhizal roots, where

sucrose is converted into hexose before uptake by the fungus and starch levels are low or starch is even absent in arbuscule containing cells (Pfeffer *et al.*, 1999; Bago *et al.*, 2000).
In the established symbiosis 5 and 7 weeks after inoculation, the starch concentrations were lower in the mycorrhizal part of the half mycorrhizal plant compared with the non-mycorrhizal part (Fig 3-11 B), reflecting the differences between CC and mC plants (Fig 3-10). There were no differences in the soluble sugar concentrations between the two root parts (Fig 3-11 A).

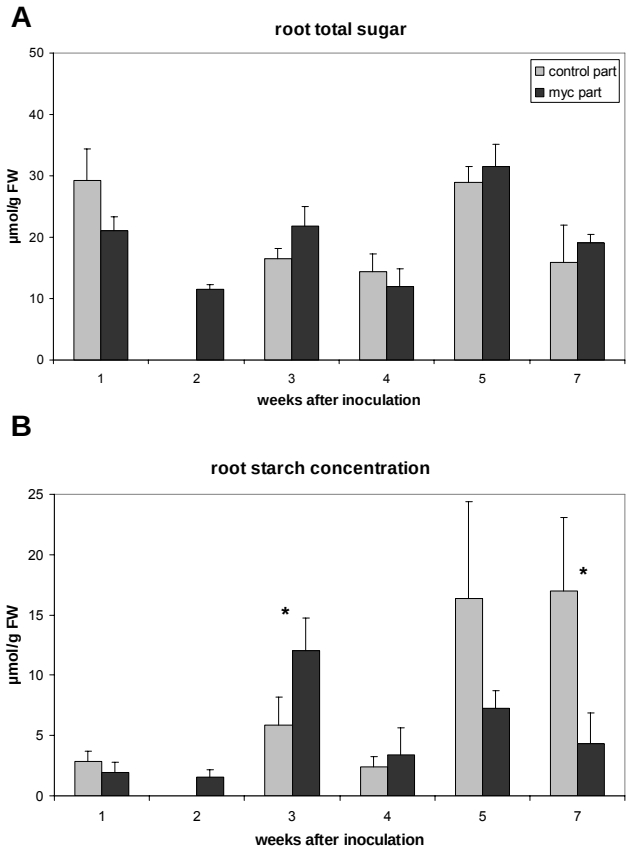

Fig 3-11. Soluble sugar (A) and starch (B) concentration of both the mycorrhizal (dark grey bars) and non-mycorrhizal (light grey bars) root parts of a mycorrhizal plant.
Material from 7 plants was pooled. The measurement was repeated 3 times, each repetition with 2 replicates. Soluble sugars: There are no significant differences between grouped bars according to t-test. Starch: Asterisks (*) indicate significant differences between the grouped bars according to t-test (*$p<0.05$).

Again, as for the nitrogen concentration, there seemed to be no systemic beneficial effect in the non-mycorrhizal part of the half-mycorrhizal root for sugar concentration. The starch concentration was increased 5 and 7 weeks after inoculation in the non-mycorrhizal part, which could mean storage of excess carbon, like it has been observed in roots of control plants. Indeed the starch concentration in the splitroot pot exactly resembles the concentration of the control and mycorrhizal plants respectively. It is possible that the nutrient availability was so limiting that even at the low carbon concentrations the nitrogen may not

have been incorporated into carbon skeletons to build metabolically relevant molecules like amino acids.

In summary, both carbon and carbohydrate concentrations were lower in mycorrhizal roots than in controls, especially 7 weeks after inoculation in the splitroot experiment. To be more specific, in this week mycorrhizal roots contained 0.0171 g less elemental carbon per gram dry weight (DW) and 18.88 µmol less carbohydrates per gram fresh weight (FW) in comparison with control roots.

$$18.88 \mu molGlc * g^{-1}FW \mathrel{\hat{=}} 191.4 * 10^{-6} mol * g^{-1}DW$$

with an average factor of fresh weight per dry weight of FW/DW = 10.14.

$$191.4 * 10^{-6} mol * g^{-1}DW \mathrel{\hat{=}} 0.0138 gC * g^{-1}DW$$

with C_6 = 72 g*mol^{-1}.

Together there is a difference of 0.0138 g/g DW of carbon derived from total non-structural carbohydrates. This is about 80% of the total measured difference in elemental carbon of 0.0171 g. This leads to the conclusion that about 80% of the “missing” carbon corresponds to carbohydrates, which were probably delivered to the fungus that could have stored it as lipids in the extraradical mycelium. The remaining 20% were possibly lost through respiration, exudation or emission. Different emission patterns of shoots of mycorrhizal and control plants are shown in Chapter 3.7.1.

3.4 Changes in shikimate pathway transcripts due to mycorrhization

During plant response to biotic and abiotic environmental influences, there is often an increased demand for secondary metabolites, which are essential for the formation of signal substances, direct defence mechanisms, changes in cell wall lignification etc. The shikimate pathway is at a central position between primary and secondary metabolism and re-routes carbon to fulfil this demand.

Particularly phenolic compounds derived from the shikimate pathway were influenced by mycorrhizal fungi (Grandmaison *et al.*, 1993). Therefore, the common upstream pathway might also show altered regulation, which has not been shown until now to my knowledge. The regulation of the shikimate pathway during the development of the symbioses was investigated at the transcriptional level.

An enhanced pathogen resistance of mycorrhizal plants was described and defence related genes like PAL have been found to accumulate especially in arbuscule-containing cells (Cordier *et al.*, 1998; Cordier *et al.*, 1996). Therefore changes in shikimate pathway transcripts were not only compared between mycorrhizal (mm, black bar) and non-mycorrhizal (CC, white bar) plants, but also between a mycorrhizal (mC-m, dark grey bar) and a control part (mC-C, light grey bar) of the same root to investigate systemic effects within the same root system (Fig 3-12).

Additionally, the hypothesis should be proved, whether the dual role of the shikimate pathway is ensured via the DAHPS isoforms. On one hand, the pathway provides metabolites for the constitutive demand e.g. amino acids or cell wall components (lignin). On the other hand, aromatic secondary compounds were stress-specifically induced (Fig 4-4).

Roots:

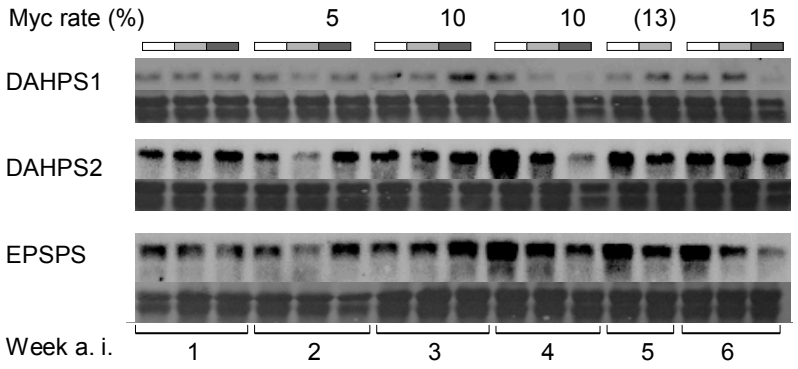

Fig 3-12. Changes in shikimate pathway transcripts in roots during the development of the VAM symbiosis.
The isoforms of the first gene (DAHPS) and the 5th gene (EPSPS) is analysed. The methylene blue stained RNA on the membrane is shown below. The data of the first up to the 6th week after inoculation is shown. The white bar marks the non-mycorrhizal root (CC). The light grey marks the non-mycorrhizal part of the half mycorrhizal root (mC-C); the dark grey bar marks the mycorrhizal part of the half-mycorrhizal root (mC-m). Numbers above indicate the rate of mycorrhization (Myc rate); numbers below indicate the week after inoculation (week a. i.).

In the 2nd and 3rd week after inoculation, the DAHPS2 and EPSPS transcript of mC-m seemed to be enhanced in comparison to mC-C. The isoform DAHPS1 was more or less unaffected. In the following weeks, the transcript levels of control roots were higher (Fig 3-12). In a second experiment, higher mycorrhization rates were reached (Fig 3-13).

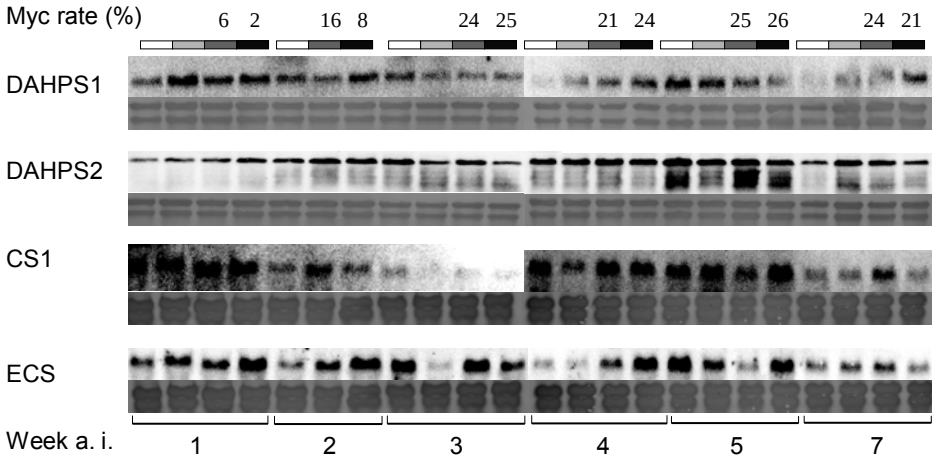

Fig 3-13. Changes in shikimate pathway transcripts in roots during the development of the VAM symbiosis (1st -5th and 7th week).
The isoforms of the first gene (DAHPS) and the 5th gene (EPSPS) of the prechorismate pathway was analysed. The methylene blue stained RNA on the membrane is shown below. The white bar marks the non-mycorrhizal root (CC). The light grey marks the non-mycorrhizal part of the half mycorrhizal root (mC-C); the dark grey bar marks the mycorrhizal part of the half-mycorrhizal root (mC-m). The black bar marks the mycorrhizal root (mm). Numbers above indicate the rate of mycorrhization (Myc rate); numbers below indicate the week after inoculation (week a. i.).

No clear influence of mycorrhization on the prechorismate pathway transcripts was detectable in this experiment, neither for the first gene (DAHPS) nor for the last (CS). The ECS transcript, a gene involved in glutathione biosynthesis, was increased in both fully mycorrhizal root and root parts in the 2nd, 3rd and 4th week after inoculation. No signals were detected after hybridization with PR1a and PR1b probes (not shown), indicating that signalling cascades involving PR protein expression were not induced at the times of harvest.

In a third splitroot experiment conducted in autumn, the mycorrhization rate decreased after reaching 12% in the half mycorrhizal root part in the 3rd week after inoculation (Fig 3-14).

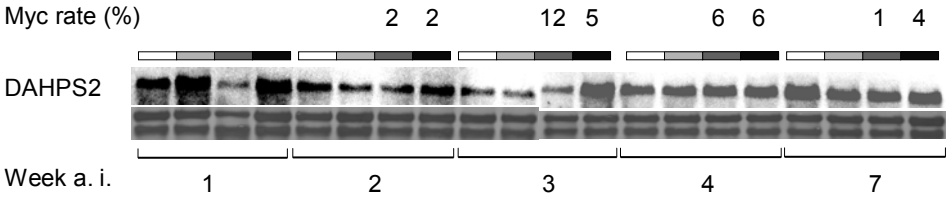

Fig 3-14. Changes in shikimate pathway transcripts in roots during the development of the VAM symbiosis (1st-4th and 7th week).
The methylene blue stained RNA on the membrane is shown below. The white bar marks the non-mycorrhizal root (CC). The light grey marks the non-mycorrhizal part of the half mycorrhizal root (mC-C); the dark grey bar marks the mycorrhizal part of the half-mycorrhizal root (mC-m). The black bar marks the mycorrhizal root (mm). Numbers above indicate the rate of mycorrhization (Myc rate); numbers below indicate the week after inoculation (week a. i.).

There was a slight increase of the DAHPS2 transcript in mycorrhizal roots (mm) compared to controls (CC) at the beginning of the symbiosis in all 3 experiments. The first contact with the fungus releases defence reactions in the plant, which would support the hypothesis that the first prechorismate pathway gene is involved in defence reaction. Additionally, changes in the glutathione pool were investigated (Chap 3.5), especially as the ECS transcript seemed to be increased in mycorrhizal roots (Fig 3-13).

Shoots:

No systemic effect of mycorrhization within the root organ on the prechorismate pathway was detected at the transcriptional level. However there might be systemic effects in the shoot due to mycorrhization (Fig 3-15 to 3-17).

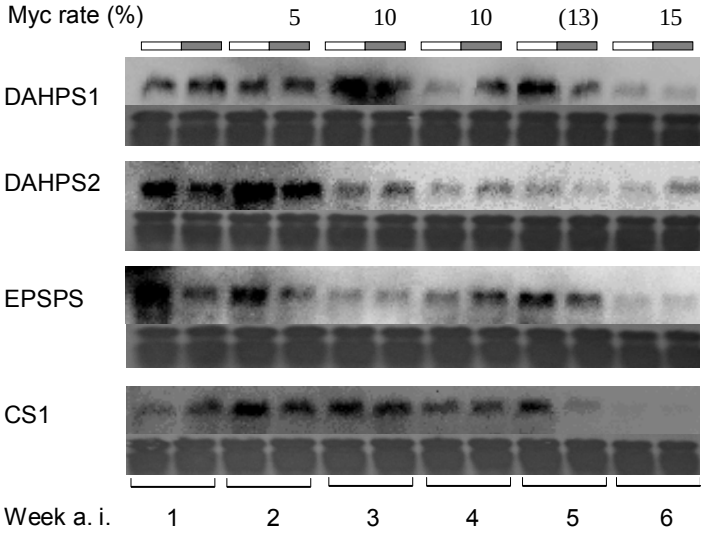

Fig 3-15. Changes in shikimate pathway transcripts in shoots during the development of the VAM symbiosis.
The isoforms of the first gene (DAHPS), the 5th gene (EPSPS) and the last (CS1) of the prechorismate pathway was analysed. The methylene blue stained RNA on the membrane is shown below. The data of the first up to the 6th week after inoculation is shown. The white bar marks the shoots of the non-mycorrhizal root (CC). The grey marks the shoots of the half mycorrhizal plant. Numbers above indicate the rate of mycorrhization (Myc rate); numbers below indicate the week after inoculation (week a. i.).

These results indicate no influence of mycorrhization on the shikimate pathway transcription of the shoot (Fig 3-15). The two isoforms of the DAHP synthase did not behave differently. Only in the first two weeks after inoculation the DAHPS2 and EPSPS transcripts were slightly increased in shoots of control plants.

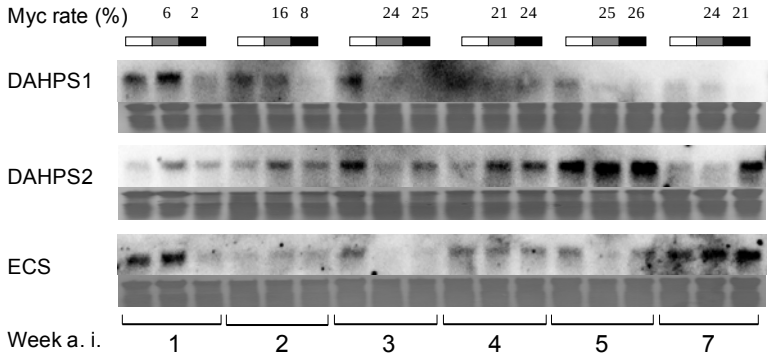

Fig 3-16. Changes in shikimate pathway transcripts in shoots during the development of the VAM symbiosis (1st -5th and 7th week).
The methylene blue stained RNA on the membrane is shown below. The white bar marks the shoots of the non-mycorrhizal root (CC). The grey marks the shoots of the half mycorrhizal root (mC), the black bar marks the shoots of the mycorrhizal root (mm). Numbers above indicate the rate of mycorrhization (Myc rate); numbers below indicate the week after inoculation (week a. i.).

Even at higher mycorrhization rates there was no clear influence of mycorrhization on the shoot DAHP synthase transcription (Fig 3-16). In the 4th, 5th and 7th week after inoculation the

transcript amount of the DAHP synthase was slightly enhanced in shoots of mycorrhizal plants. The ECS, which was induced by mycorrhizal fungi in roots in the 2nd to the 4th week after inoculation (Fig 3-16), seemed to be unaffected in shoots.

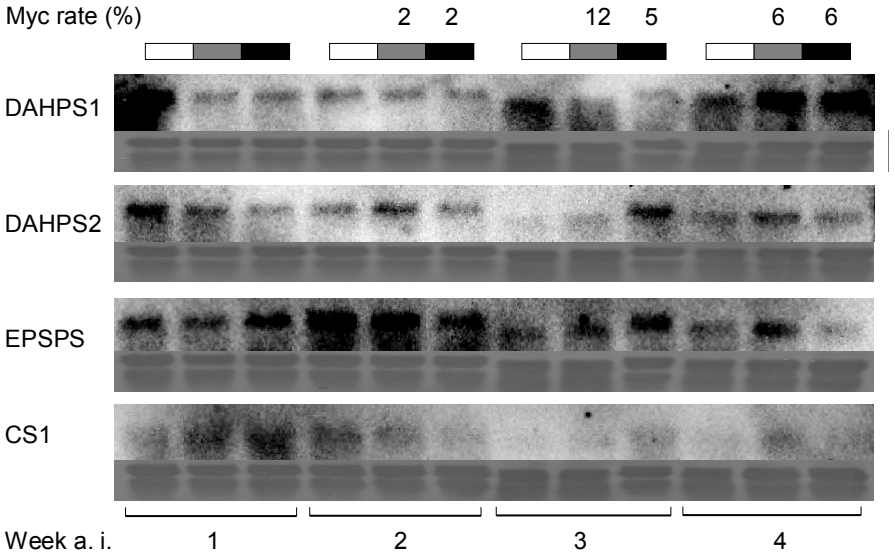

Fig 3-17. Changes in shikimate pathway transcripts in shoots during the development of the VAM symbiosis (1st-4th week).
The methylene blue stained RNA on the membrane is shown below. The white bar marks the non-mycorrhizal root (CC). The grey marks the half mycorrhizal root (mC), the black bar marks the mycorrhizal root (mm). Numbers above indicate the rate of mycorrhization; numbers below indicate the week after inoculation (week a. i.).

In the experiment shown in Fig 3-17, the fungal arbuscules did not expand and no influence on the shikimate pathway transcripts could be observed.

In summary, no clear influence of VAM fungi on the shikimate pathway transcription in roots and shoots could be detected in the used splitroot system under the described conditions. Only in the first week after inoculation, the DAHPS2 was slightly induced specifically in mycorrhizal roots.
Possibly, the synthesis of aromatic compounds (and emission of MeSA) may have been too cost intensive, as carbon, sugar and nitrogen concentrations were generally very low in these plants compared to optimally supplied ones (Chap 4.1).

3.5 Glutathione

As seen in Fig 3-13, mycorrhization affects the ECS transcription, a gene for glutathione biosynthesis. Glutathione itself plays a role in sulphur metabolism, complexation of heavy metals and in symbiotic interactions, in which an oxidative burst occurs (Hérouart *et al.*, 2002). The influence of mycorrhization on total glutathione (GSH + GSSG) content in shoots and roots is shown in Fig 3-18.

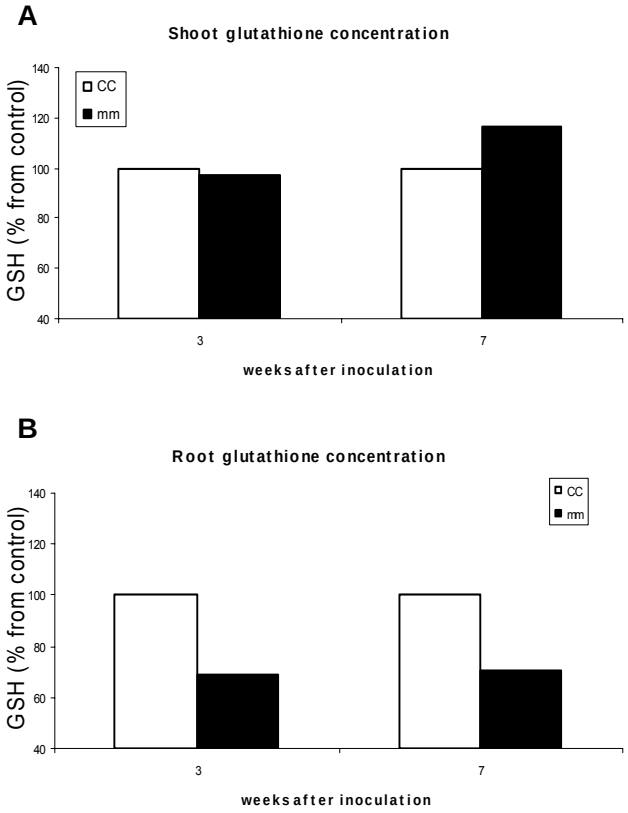

Fig 3-18. Glutathione content (µmol/g fresh weight) of shoots (A) and roots (B) of mycorrhizal (black bars) plants in percent of control plants (white bars) grown in splitroot pots.
In the 3rd and 7th week after inoculation the mycorrhization rate was about 24% in both roots parts. In the figure the mean of two independent measurements is shown. All differences between the treatments were not significant due to high error of measurement.

The induced ECS gene in mycorrhizal roots 3 weeks after inoculation did neither significantly affect the concentration of elemental sulphur (Tab 3-1) nor the total glutathione concentration (Fig 3-18). The content in roots in the 7th week and in the shoot was also not affected by mycorrhization in comparison to control plants. Even in the 2nd week, when a slight induction of the DAHPS2 in roots (Fig 3-13) indicated a weak defence reaction, no differences in the glutathione content were observed (data not shown). In this experiment, the fungus reached maximal mycorrhization rates of over 25% (Fig 3-1).

In another splitroot experiment, the mycorrhization rate reached only 12% and was decreasing after the 3rd week. In the 7th week there were nearly no arbuscules left. A closer look to the glutathione concentrations of shoots and roots in this experiment showed a trend to higher glutathione contents, increased simultaneously in shoots and roots in the 7th week after inoculation compared to controls of the same week (Fig 3-19).

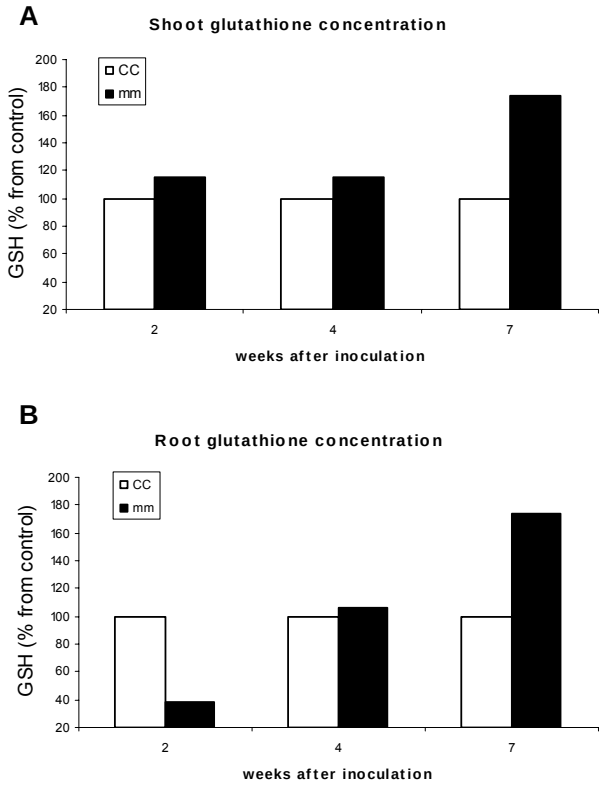

Fig 3-19. Total glutathione (oxidised and reduced form) content of shoots (A) and roots (B) of mycorrhizal (black) and half mycorrhizal (grey) plants in percent of control plants (white) grown in splitroot pots. In the 2nd week the mycorrhization rate was about 2%, in the 4th week 6% and in the 7th week only 1-4%. The GSH concentration (µmol/g FW) relative to the control was calculated in two independent measurements and the mean value is shown. All differences between the treatments were not significant due to high error of measurement.

In half mycorrhizal plants, the shoot glutathione concentration was also increased in the 7th week (data not shown). In this experiment, the DAHPS2 was induced in the 3rd week in mycorrhizal roots (Fig 3-14). The stress-induced isoform could have elicited defence reactions, which could have led to a glutathione-mediated suppression of the fungus in the 7th week after inoculation. These findings indicate that the symbiosis and the development of the fungus within the root might be regulated via the glutathione redox status in the late stage of the symbiosis. In this context, it is interesting to see also how oxidative stress modulates the plants reaction to mycorrhization. Ozone is a common natural occurring, defence reaction releasing shoot stressor, which is well characterised concerning the plant reaction. The

influence of ozone itself on the plant was first characterised (Chap 3.6) before combining it with mycorrhizal fungi (Chap 3.7.2).

3.6 Changes in Shikimate pathway transcripts due to ozone fumigation

A common reaction to ozone is a change in the glutathione pool (Foyer & Rennenberg, 2000; Noctor *et al.*, 2002). GSH reacts chemically with a range of active oxygen species, which results in an increase of the GSH_{red}/GSH_{ox} ratio followed by an increase in total glutathione concentration, reviewed in (Foyer *et al.*, 2005; Smirnoff, 2005). The accumulation of aromatic secondary metabolites is another well-known symptom of the plant response to ozone. Most of these metabolites are synthesised from the three aromatic amino acids. In the following paragraphs, the influence of ozone on the prechorismate pathway transcript levels and on the glutathione pool is described.

Shoot:

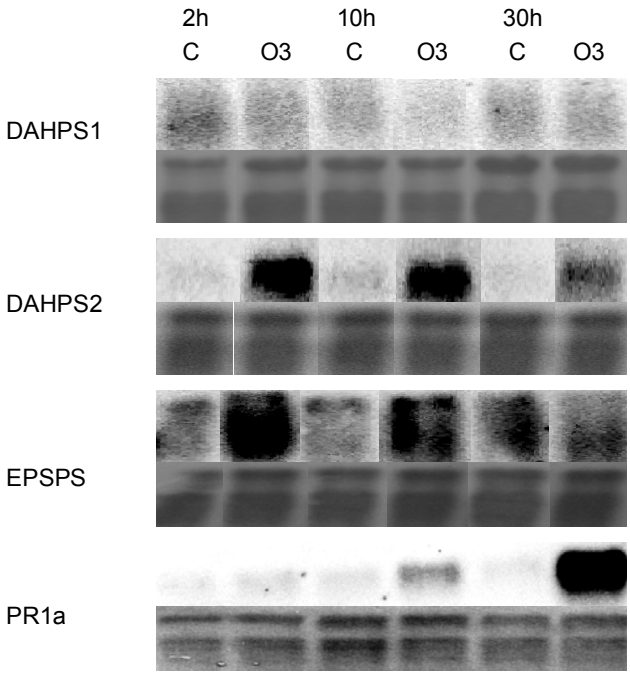

Fig 3-20. Induction of genes in the prechorismate pathway in shoots after ozone fumigation (5 h, 200 ppb).
7 plants were harvested 2 h, 10 h and 30 h after onset of ozone (O3) and the material was pooled. Harvest and transcriptional analysis were done according to Chap 2.5 and 2.9. Signals from 10 µg RNA and the methylene stained membrane are shown.

Ozone, a known inducer of the prechorismate pathway in tobacco (Janzik *et al.*, 2005), did also induce genes of the prechorismate pathway in tomato shoots (Fig 3-20). An isoform specific induction of the DAHP-synthase could be confirmed. The DAHPS1 did not respond while the DAHPS2 was quickly induced 2 h after the onset of the ozone fumigation (200 ppb). The 5th enzyme (EPSPS) was also induced by ozone as well as the PR1a that was included in the experiment as a positive control for induction of defence reactions.

Roots:

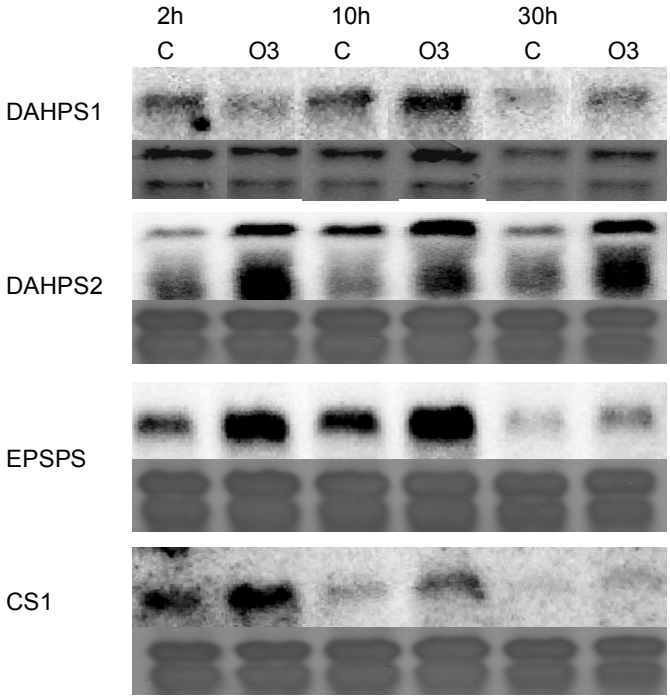

Fig 3-21. Induction of genes in the prechorismate pathway in roots after ozone fumigation (5 h, 200 ppb).
7 plants were harvested 2 h, 10 h and 30 h after onset of ozone (O3) and the material was pooled. Harvest and transcriptional analysis were done according to Chap 2.5 and 2.9. Signals from 10 µg RNA and the methylene stained membrane are shown.

Also in the roots the DAHPS2 as well as other genes of the prechorismate pathway were induced by ozone in this experiment (Fig 3-21). Ozone as a shoot stressor may induce the shikimate pathway systemically also in roots, mediated by an unknown signal. It can not be excluded, however, that the prechorismate pathway induction was due to roots that were growing out of the pot and might have been in direct contact with ozone. But after hybridization with PR1a probes, no signals were detectable in roots (not shown), and thus, such a direct induction in roots is unlikely.

In roots and shoots, 30 h after the onset of ozone, when the PR1a was strongly induced in shoots, the induction of the prechorismate pathway genes was already declining. This

expression pattern reminds of the weak induction of the DAHPS2 in mycorrhizal roots in the 3rd week, which was followed by an accumulation of glutathione in the 4th and 7th week (Fig 3-14, -19). Both PR1a transcript and glutathione were induced after ozone stress. However, in this experiment the total glutathione content was not affected by ozone in neither shoots nor roots (data not shown).

If a shoot stressor influences root gene expression, as was observed in the experiment shown in Figure 3-21, it could also work vice versa. However, first results did not indicate an effect of mycorrhization on general shoot shikimate pathway gene expression (Chap 3.4).

3.7 Systemic changes in the plant response to ozone, affected by mycorrhization

The shikimate pathway is an interface between primary and secondary metabolism and the biosynthetic pathway for tryptophan, tyrosine and phenylalanine. The three aromatic amino acids are precursors for many aromatic metabolites, e.g. MeSA, which accumulate during, or play a role in, stress responses (e.g. ozone fumigation). In this chapter, altered reactions to ozone treatment on shikimate pathway transcription, carbohydrate content, glutathione concentration, and VOC emission were investigated, especially in shoots.

3.7.1 Changes in VOC emissions due to mycorrhization

VAM is known to induce plant defence reactions and increased resistance against root pathogens (reviewed by Harrison, 2005; Gianinazzi-Pearson, 1996). Little is known about the influence of mycorrhization on the shoot. In this study, mycorrhization did affect the shoot growth (Fig 3-2) and the shoot N concentration (Fig 3-8). Changes in the carbon-nitrogen-balance in turn could lead to altered secondary compounds, which accumulate in response to stress.

To characterise how mycorrhization affects emission of secondary compounds, a broad spectrum of different compounds was analysed, including monoterpenes, LOX products and products derived from the shikimate pathway, which are known to be involved in defence reactions. LOX products occur as a general response of plants to stress, e.g. the increase of LOX products was observed in tomato after an ozone pulse (Heiden *et al.*, 2003). Here it was investigated if mycorrhization affects the ozone induced LOX emission (Fig 3-22 B).

Beauchamp *et al.* (2005) found that the VOC emission is related to ozone flux density into the plant for short ozone pulses. To control this parameter, the stomatal conductance (Fig 3-22 A) was taken into consideration in the interpretation of the obtained VOC emissions.

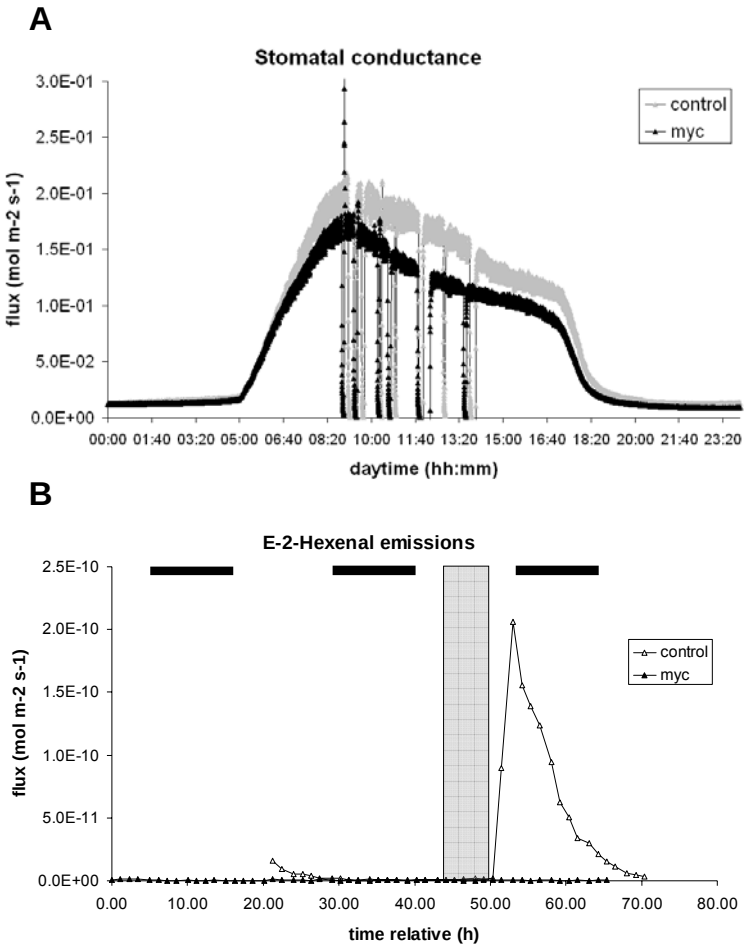

Fig 3-22. Stomatal conductance (A) and LOX-product emissions (B) of mycorrhizal and control plants. The fluxes resulted from 6 shoots measured simultaneously. The black bars in B symbolise darkness, the grey box symbolises the period of ozone fumigation. The data were obtained from a single experiment.

Fig 3-22 A shows the diurnal changes of the stomatal conductance. At dawn, the plants were well watered and the stomata opened. Hence, ozone fluxes into the plants could be considered as nearly equal in both mycorrhizal and non-mycorrhizal plants. However, the decline in conductance towards the dusk was faster in mycorrhizal plants.

Although the stomatal conductance of mycorrhizal plants nearly reached the values of control plants, mycorrhizal plants emitted negligible amounts of E-2-hexenal after ozone fumigation. E-2-hexenal is considered as an example for the whole LOX pathway induction (Heiden *et al.*, 2003) and is a typical marker for stress response.

The changes in terpene emissions of mycorrhizal and control plants after ozone fumigation (5 h, 200 ppb) are shown in Fig 3-23.

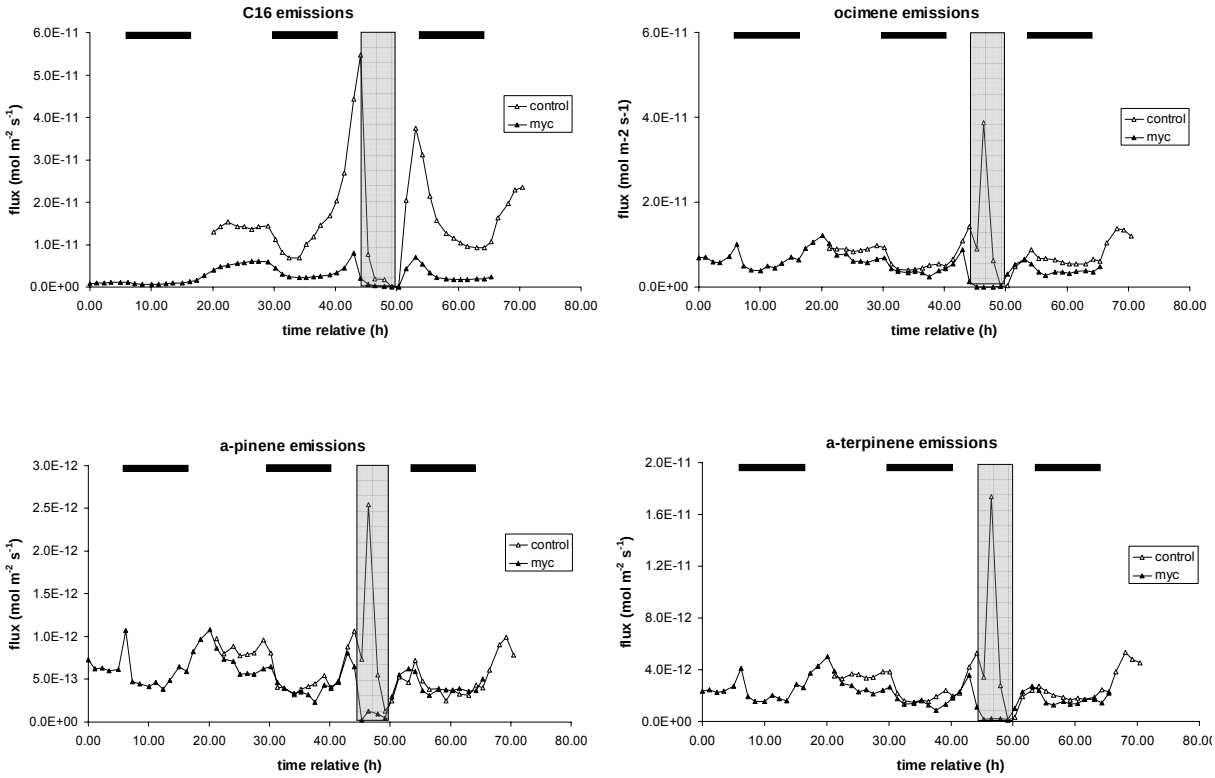

Fig 3-23. Terpene emissions of mycorrhizal and control plants.
The black bars symbolise darkness, the grey box symbolises the period of ozone fumigation. The fluxes resulted from 6 shoots measured simultaneously. Ocimene, pinene and terpinene are monoterpenes. TMTT (Trimethyl-tridecatetraene, C16) is a diterpene, with C_4 lost. The data were obtained from a single experiment.

The basal emission rates of mycorrhizal plants were slightly lower, which might be a result of the lower stomatal conductance (Fig 3-22 A). Interestingly, all monoterpenes were induced during ozone exposure in non-mycorrhizal plants but not in mycorrhizal plants. In contrast, the diterpene Trimethyl-tridecatetraene (TMTT) was already elevated before ozone treatment and was decreased by ozone, again only in non-mycorrhizal plants. Jasmonic acid (JA), proposed to be produced via the octadecanoid pathway (Vick & Zimmermann, 1984), is able to trigger terpene emissions (Farag & Paré, 2002), which might indicate a certain pre-stress, perhaps manifested by the pre-induction of TMTT.

Like JA, phenolic compounds play a role in plant defence reactions. Benzoic acid and methylsalicylate (MeSA) are phenolic secondary compounds derived from the shikimate pathway. Salicylic acid (SA) is reported to have several functions in plants and has an

important signalling role in plant defence against pathogens, reviewed in Raskin (1992). SA is a phenolic compound which can directly be formed via chorismate, the end product of the shikimate pathway, or via the phenylpropanoid pathway (Wildermuth *et al.*, 2001; Nugroho *et al.*, 2001) with benzoic acid as intermediate. In Fig 3-24, the emission rates of these compounds are shown before and after ozone fumigation.

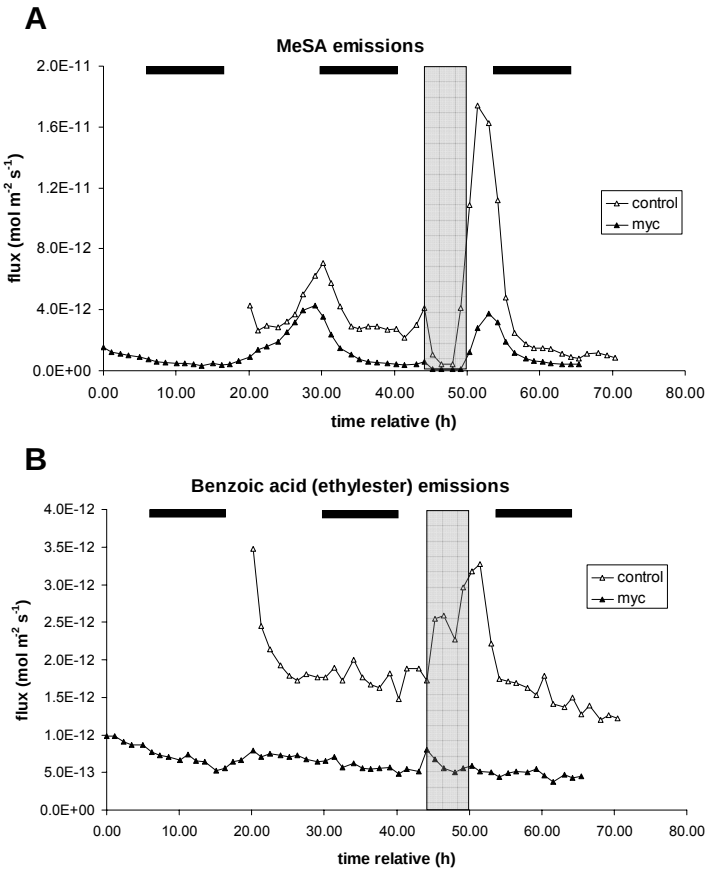

Fig 3-24. Benzoic acid and methylsalicylate (MeSA) emissions of mycorrhizal and control plants. The black bars symbolise darkness, the grey box symbolises the period of ozone fumigation. The fluxes resulted from 6 simultaneously measured shoots. The data were obtained from a single experiment.

Again, emissions from mycorrhizal plants were generally lower. Both mycorrhizal and non-mycorrhizal plants showed a circadian rhythm of MeSA emission, but only control plants exhibited ozone-induced MeSA emission.

The stomatal conductance was slightly higher in control plants (Fig 3-22 A). Therefore the obtained results should be carefully examined because the differences between mycorrhizal plants and control plants could be due to mycorrhization or lower ozone fluxes caused by lower stomatal conductance. These preliminary results need to be confirmed in further experiments.

The finding that MeSA emissions were induced by ozone only in shoots of non-mycorrhizal plants could mean that the shoot defence system is less sensitive to ozone due to VAM fungi. Enhanced MeSA emissions could be a result of shikimate pathway induction, which is the upstream pathway for aromatic secondary compounds. Furthermore, these secondary metabolites could be a quenching route for the excess carbon in control roots (Fig 3-6). Although the shikimate pathway was not regulated in roots 4 weeks after inoculation (Chapter 3.4), when VOC emissions were measured, it was regulated by ozone and this regulation is likely influenced by mycorrhization, because the fungus provides a C-sink in roots. Consequently the carbon might be missing to build defence compounds in shoots after ozone exposure. Furthermore, a regulation of this pathway by the plant seems to be likely, because it provides the metabolites for all 3 aromatic amino acids and other important organic compounds, like chlorogenic acid, which are synthesised from intermediates.

In the following chapter, the regulation of the shikimate pathway by ozone and mycorrhization is investigated at the transcriptional level in roots and shoots of tomato plants.

3.7.2 Shikimate pathway transcripts

Previous results indicated a clear influence of ozone and no significant influence of mycorrhization on the shikimate pathway transcription. In this chapter the influence of mycorrhization on the prechorismate pathway induction by ozone is investigated. The experiment was focussed on systemic effects of mycorrhization in the root on transcriptional changes in the shoot. Additionally, the influence of ozone fumigation on carbohydrates and glutathione is elucidated in mycorrhizal tomato plant (Chap 3.7.3/4).

In Fig 3-25, the influence of mycorrhization and ozone on the prechorismate pathway transcription is shown. 4 weeks after inoculation (mycorrhization rate of about 10%) the plants were fumigated with ozone (5 h, 200 ppb) and harvested 10 h after the onset of ozone.

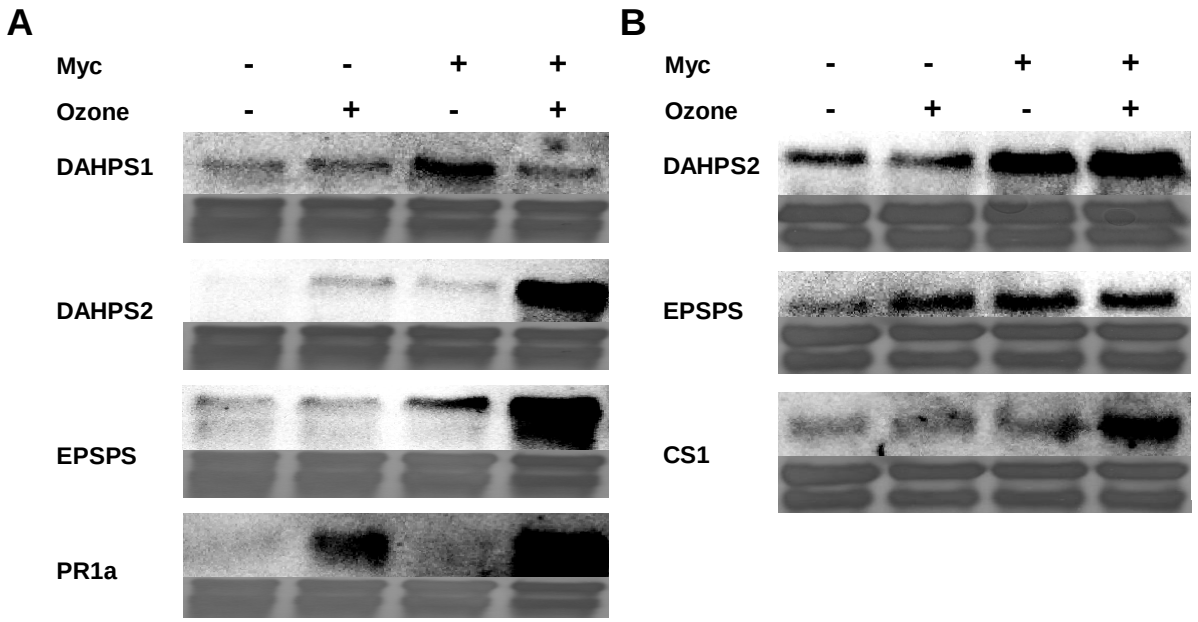

Fig 3-25. Induction of genes in the prechorismate pathway by ozone fumigation in shoots (A) and roots (B) of mycorrhizal plants.
4 weeks after inoculation the plants were fumigated with ozone (5 h, 200 ppb) and harvested 10 h after the onset of ozone. The mycorrhization rate was about 10%. Harvest and transcriptional analysis were done according to Chap 2.5 and 2.9. Signals from 10 µg RNA and the methylene blue stained membrane are shown.

In shoots, the induction of the PR1a and the DAHPS2 genes by ozone could be confirmed (first and 2nd lane), although the intensity of induction in this experiment was much lower than in the experiment shown in Fig 3-20 A. The DAHPS1 and EPSPS seemed to be induced in the shoot by mycorrhization (first and 3rd lane). For DAHPS2, EPSPS and PR1a the induction by ozone was additionally enhanced by mycorrhization. In roots, the DAHPS2 and EPSPS transcripts were increased by mycorrhization whereas the CS1 transcript was increased by ozone fumigation (Fig 3-25 B). An additive effect of mycorrhization and ozone could not be detected.

Mycorrhization led to a systemic induction of genes in the shikimate pathway in shoots and did even enhance the ozone-induced response. The question as to how mycorrhization and ozone influence the gene transcription was addressed in a further experiment using plants that showed a mycorrhization rate of 3% in the 4th week (Fig 3-26). In this experiment, ozone did not induce the shikimate pathway in roots (Fig 3-27), corresponding to overall very weak induction of DAHPS2 by ozone also in shoots (Fig 3-26).

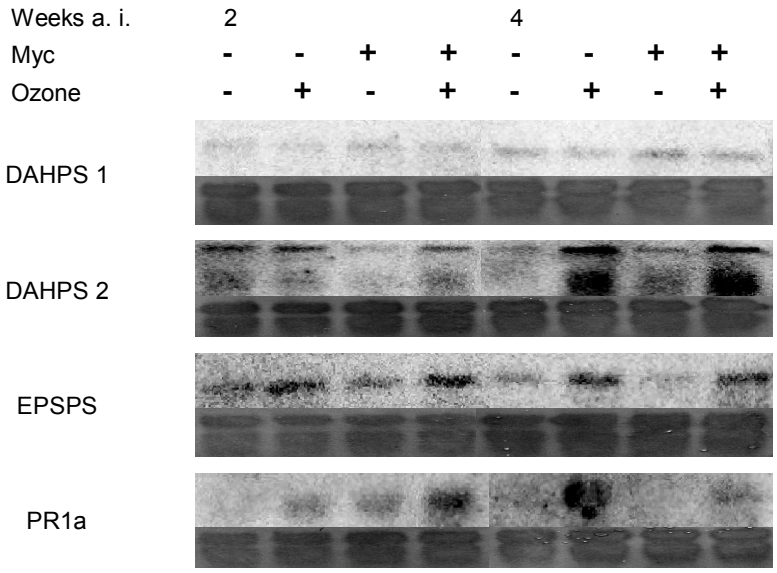

Fig 3-26. Influence of ozone exposure and mycorrhization on gene expression in the prechorismate pathway in shoots.
The plants were fumigated with ozone (5 h, 200 ppb) 4 weeks after inoculation (weeks a.i.) and harvested 10 h after onset of ozone. The mycorrhization rate reached only about 4% in the 4th week. Harvest and transcriptional analysis were done according to Chapter 2.5 and 2.9. Signals from 10 µg RNA and the methylene blue stained membrane are shown.

Again, in the 4th week after inoculation, the prechorismate pathway genes (DAHPS2, EPSPS) were induced by ozone in the shoot. At the transcriptional level, there were no differences in ozone induction of the shikimate pathway between mycorrhizal and control plants, although shikimate pathway derived MeSA was induced only in control plants in the same experiment. The observed discrepancy could be explained by the fact that the shikimate pathway branches into many sideways and controls the carbon flow into the whole aromatic metabolism. Hence, a strong correlation between shikimate pathway induction and the level of one aromatic metabolite is not necessarily expected. Rather the data indicate that MeSA emission could be regulated independently from the overall flow in the aromatic metabolism.

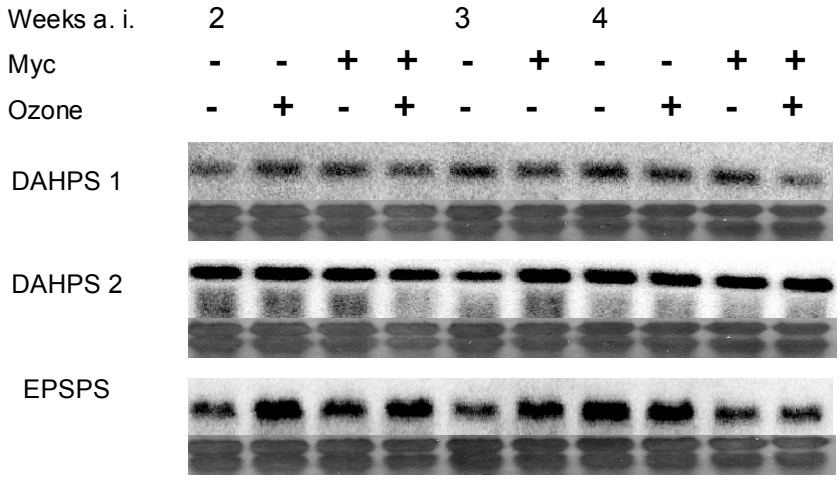

Fig 3-27. Influence of ozone exposure and mycorrhization on gene expression in the prechorismate pathway in roots.
The plants were fumigated with ozone (5 h, 200 ppb) 4 weeks after inoculation (weeks a.i.) and harvested 10 h after the onset of ozone. The mycorrhization rate reached only about 4% in the 4th week. Harvest and transcriptional analysis were done according to Chapter 2.5 and 2.9. Signals from 10 µg RNA and the methylene blue stained membrane are shown.

In roots the DAHPS2 and EPSPS were induced by mycorrhization in the 3rd week although the roots were colonised at a very low level (Fig 3-27). Mycorrhization induced the same DAHPS isoform in roots as ozone did in shoots. This supports the hypothesis that the dual role of the shikimate pathway involves differential regulation of the DAHPS isoforms (Chap 4.2), as the pathway has to fulfil the constitutive demand on aromatic amino acids on the one hand and is stress-induced on the other hand.
In the 2nd week, the EPSPS was induced by ozone in mycorrhizal and non-mycorrhizal roots. This may imply that the root shikimate pathway was systemically induced in response to a shoot stress. No additive effect of mycorrhization and ozone was detected in roots.

Summarising, the results from these single pot experiments showed that the shikimate pathway was influenced at the transcriptional level by ozone and mycorrhization. An additive effect of mycorrhization on the ozone induction of the prechorismate pathway was found, albeit it was not confirmed in a second experiment, presumably due to the very low mycorrhization rate (3-4%).

Regulation of the secondary metabolism, and thus the shikimate pathway, is discussed in the context of carbon metabolism. On the other hand, the shikimate pathway provides aromatic metabolites for defence mechanisms. In the following section, the influence of mycorrhization and ozone on carbohydrates (Tab 3-2) and glutathione (Fig 3-28) was investigated. The assays were done with the same plant material as for the transcriptional analysis (Fig 3-25)

in order to relate changes in shikimate pathway transcription with changes in primary carbohydrate metabolism (sugar/starch) or defence reactions (glutathione, VOC emission).

3.7.3 Carbohydrates

Tab 3-2. Changes in sugar and starch concentration in shoots and roots of mycorrhizal (+ myc) and non-mycorrhizal (- myc) plants after ozone fumigation
(+ ozone, 5 h at 200 ppb, harvest 10 h after onset of ozone). The carbohydrate concentrations were measured from one set of pooled plant material. The plants were harvested 4 weeks after inoculation at a mycorrhization rate of about 10%.

			soluble sugars (µmol/g FW)	starch (µmol glucose units/g FW)
- myc	- ozone	shoot	3.1389	334.8928
		root	16.7445	4.2752
	+ ozone	shoot	3.9342	336.3165
		root	21.1774	5.9961
+ myc	- ozone	shoot	4.6803	379.8283
		root	4.4879	1.9664
	+ ozone	shoot	2.1645	183.7372
		root	3.4334	2.2046

The sugar and starch concentration was reduced in mycorrhizal roots, while there was no influence on the shoot. This is in line with the results obtained from splitroot experiments described in Fig 3-10.

Ozone alone had no significant influence on sugar and starch concentrations in shoots and roots. Therefore, induction of the DAHPS2 in shoots by ozone (Fig 3-25, -26) was probably not caused by higher sugar concentrations. However, first results from *Arabidopsis* leaves indicated a higher sugar turnover in leaves incubated in sugar solutions in the dark for 8 h, which was also found to be accompanied by DAHP-synthase inductions (Martin, 2006). A combination of mycorrhization and ozone seems to result in a decrease in shoot sugar and starch, which might indicate a higher turnover in sugar metabolism.

3.7.4 Glutathione

Total glutathione measurements did not show a clear trend concerning the influence of ozone and mycorrhization of root. Fig 3-28 summarises three independent shoot glutathione measurements from plants with a mycorrhization rate of 10%. Two measurements were done independently with plants (four weeks after inoculation) from the experiment shown in Fig 3-

25 and Tab 3-2. The 3rd measurement was done with plants of a second experiment, in which the plants reached 10% mycorrhization rate already 2 weeks after inoculation.

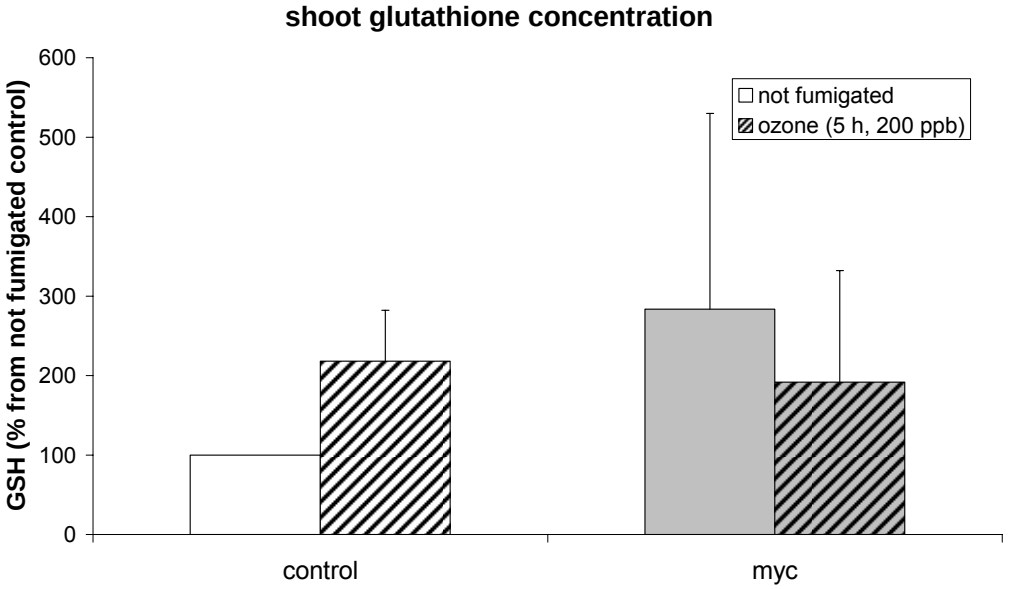

Fig 3-28. Glutathione content in shoots of mycorrhizal (grey bars) and control plants (white bars). The glutathione concentration (µmol/g FW) relative to the control (white bar) was calculated in 3 independent measurements (see text above) and the mean value is shown. In each measurement the extract was measured twice and the mean was taken.

These results show the same trend as the MeSA induction by ozone, which was observed only in non-mycorrhizal shoots (Fig 3-24 A). Again, there might be a systemic influence of mycorrhization on ozone induced defence reactions, like for VOC emissions (Chap 3.7.1). The glutathione concentration in shoots seemed to be slightly induced by ozone in control plants but not in mycorrhizal plants at 10% mycorrhization rate (Fig 3-28). Mycorrhization alone may induce a weak increase in shoot glutathione concentration. The shoot defence system seemed to be less sensitive to ozone in mycorrhizal plants, but further research needs to be done on this topic.

4 Discussion

The interactions between plant and fungi during mycorrhization are very complex. One important part is the regulation of defence reactions in the plant, which is different in the different stages of the symbiosis. Phenols, synthesised via the shikimate pathway, are one important kind of compounds relevant in plant defence processes and are accumulated at the site of infection. The regulation of shikimate pathway transcription at the different stages of the symbiosis locally as well as systemically in the non-mycorrhizal root part and the shoots of mycorrhizal plants have been investigated in this study in splitroot pots and will be discussed in Chapter 4.2. The splitroot system did not only allow the comparison of mycorrhizal to non-mycorrhizal plants, but the half mycorrhizal system helped to understand systemic effects on non-mycorrhizal roots of mycorrhizal plants. As both root parts were separated from each other, changes in the non-mycorrhizal part must be shoot-mediated, what underlines the role of indirect changes in mycorrhizal roots by physiological changes in shoots of mycorrhizal plants.

Furthermore it has been shown that mycorrhization has an impact on shikimate pathway regulation and stress reactions in the shoot response to ozone. A hypothesis is developed on possible signalling cascades and mechanisms leading to these mycorrhiza-dependent changes in the shoot stress response to ozone.

One major impact on metabolic processes in mycorrhizal plants is the change in carbon and nutrient flow between primary and secondary metabolism on one hand and between shoots and roots on the other hand. Therefore, local and systemic variations in carbon and nutrient concentrations of roots and shoots will be discussed first (Chap 4.1). Fluctuations in the pool of carbohydrates and nutrients indicate changes in source-sink relations and could therefore be correlated with changes in the secondary metabolism in the whole plant and especially with the shikimate pathway.

4.1 Local and systemic changes in physiology of the tomato - *Glomus intraradices* symbiosis

The results obtained from this study provide evidence that mycorrhization of tomato plants with *Glomus intraradices* has deep impacts on the carbon-nutrient balance and on the source-sink balance. Many interactions and complex regulation mechanisms exist between nitrogen- and carbon-metabolism in order to regulate the C/N ratio under a large range of growth conditions (Krapp *et al.*, 2005; Lerdau, 2002; Koricheva, 2002; Palenchar *et al.*, 2004; Grace & Logan, 2000). An ample nutrient availability decreases the C/N ratio, favours

growth, lower levels of N free secondary compounds and increased synthesis of nitrogen-containing secondary compounds such as alkaloids and cyanogenic glycosides. In comparison, a low nutrient availability result in a higher C/N ratio, low growth rate and an increased synthesis of carbon based metabolites such as phenolics and terpenes (Hamilton *et al.*, 2001; Amâncio & Stulen, 2004). It is generally accepted that it is the balance between C and N metabolism rather than the plant´s N status alone that is likely to be under regulatory control (Walch-Liu *et al.*, 2005). Nitrate is proposed to act as a signal to initiate coordinated changes in carbon and nitrogen metabolism (Scheible *et al.*, 1997).

4.1.1 Growth depression in mycorrhizal plants

In this study, tomato growth was reduced by inoculation with *Glomus intraradices* (*G.i.*) in a splitroot system. The biomasses of shoots and roots were lower in mycorrhizal plants from the 4th week after inoculation (Fig 3-2). This is in agreement with results on tomato and *G.intraradices* from Pozo *et al.* (2002), although the growth depression was mainly established at the shoot level in their experiment. Their plants were much better fertilised (at low phosphorus levels), which could have caused the differences in growth depression as well as the two times higher mycorrhization rate (about 56%). The growth depression was already observed two weeks after inoculation in their study, possibly due to the higher mycorrhization rate and thus greater sink strength of the fungus. Pozo *et al.* (2002) observed a slightly higher biomass in the mycorrhizal part of the half mycorrhizal root, what is different from our results (Fig 3-3). We observed no differences between the two halves except for week 3, where the biomass of the non-mycorrhizal half was higher. In this week, when the arbuscle rate reached its maximum, modifications in the half mycorrhizal roots took place, which was also reflected in the carbon and nitrogen tissue concentrations, discussed in Chapter 4.1.2. The shortly reduced biomass of the mycorrhizal half indicates that the nutrient status was very low in our system and that the fungus had only negative effects at the beginning of the experiment.

It is known that mycorrhization with *G. intraradices* does not necessarily result in increased **growth**. In fact, similar growth depressions have previously been shown for *G.intraradices* and for many other AM fungal species, listed by Lerat *et al.* (2003). Johnson *et al.* (1997) reviewed the plant responses along the mutualism-parasitism continuum of VAM symbiosis and stated: “Carbon allocated to a fungus is only a cost if it could otherwise have been allocated to increase plant fitness, and resources gained through the activities of a fungal symbiont are only beneficial if those resources are in limiting supply.” Thus, if the root growth

is reduced by mycorrhization, the costs due to C flow to the fungus might be less than the cost resulting from additional root formation. Moreover, not only the involvement in interspecific interactions other than herbivory makes demand on resources and thus could lead to lower biomasses, but also defence and protection from abiotic stresses like ozone (Hamilton *et al.*, 2001).

Fig 4-1 underlines the link between colonisation and sink strength causing growth reduction.

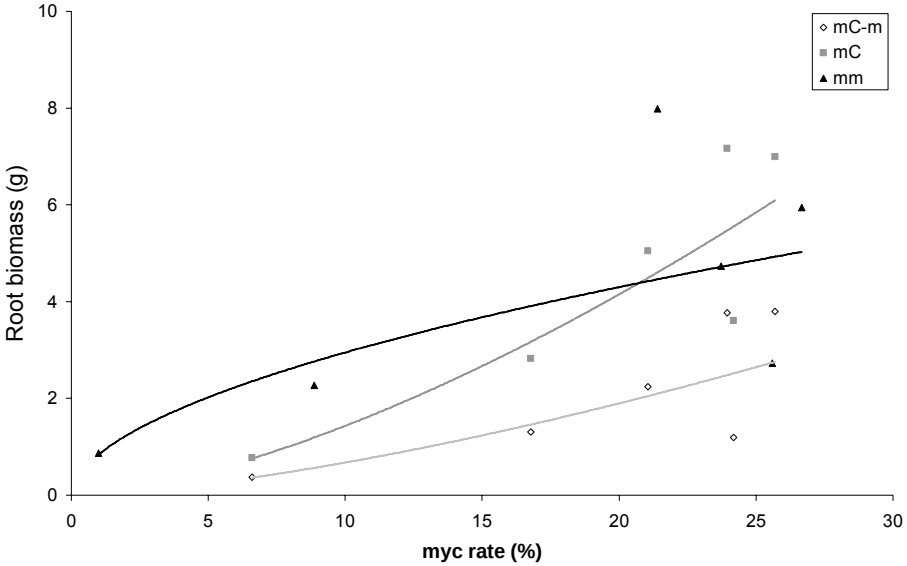

Fig 4-1. Increase in root biomass (fresh weight) of fully mycorrhizal roots (mm), half mycorrhizal roots (mC) and the mycorrhizal part of half mycorrhizal roots (mC-m) in relation to the mycorrhization rate (arbuscules). The data was extracted from Fig 3-1,-2.

Increases in mycorrhization rate and biomasses are correlated with the plant development and growth. With increasing mycorrhization rate, the increase in biomass of the fully mycorrhizal root (mm) declines, whereas in the total half mycorrhizal root (mC) and especially the mycorrhizal part of the half mycorrhizal root (mC-m) the increase in biomass still becomes larger. The more reduced growth of total mycorrhizal roots compared to only half mycorrhizal roots supports the great sink strength of the fungus. Because the total amount of arbuscules and vesicles in mC-roots is only half of those in mm-roots at the same mycorrhization rates, indicating a lower C sink in mC and thus more carbon, which potentially is invested into growth or other processes. This is in agreement with the higher C concentration in half mycorrhizal roots (Fig 3-6, -7).

Despite the fact that in the mycorrhizal plants growth was reduced, for the shoot-root ratio no differences between the mycorrhizal, half mycorrhizal and non-mycorrhizal plants were

detected in this study, except for the 3rd week (Fig 3-4). In contrast, several works are published with mycorrhizal plants typically having lower root-shoot ratios than their non-mycorrhizal controls (Douds jr *et al.*, 2000). A possible explanation is that since the mycorrhizal root system is more efficient per unit root length in the uptake of nutrients, a given mass of mycorrhizal root can supply a larger mass of shoot relative to that of non-mycorrhizal plants.

Together, the nutrient availability seemed to be quite low, respectively leading to high amounts of carbon based metabolites. The differences in nutrient and carbohydrate concentrations of mycorrhizal and non-mycorrhizal plants are discussed in Chapter 4.1.2.

4.1.2 Better nutrient acquisition due to VAM fungi

In this study, the concentration of nitrogen, phosphorus and sulphur was enhanced rather in shoots than in roots of mycorrhizal plants (Tab 3-1), although the absolute nutrient concentrations were low in comparison to other investigations (see below). The higher tissue concentrations in mycorrhizal roots compared to control roots are probably caused by increased mineral nutrition und enhanced uptake of elements. The external hyphae of VAM can deliver up to 80% of plant P and 25% of plant N (Marschner & Dell, 1994). The improved plant mineral nutrition, reviewed in Clark & Zeto (2000), can be a direct effect of mycorrhizal fungi or indirectly mediated by modification of root morphology and physiology (George, 2000). Because of the lower root biomass of mycorrhizal plants, improved element uptake by VAM is more likely the reason for increased N and P concentrations rather than root morphology.

Nitrogen concentrations ranged from about 1.0-1.8% in shoots and 1.4-1.8% in roots in the splitroot and in single pot experiments (Fig 3-8, -9; Tab 3-1). In comparison, concentrations of 3.85-5.5% are usual in mature leaves (Sanders *et al.*, 1981; Marschner, 1995), which is still slightly higher than in the optimal supplied control plant (Tab 3-1). Although the concentrations are generally very low and indicate malnutrition, the contents between the treatments are partly significantly different.
The higher nitrogen concentration in fully mycorrhizal roots in comparison to controls is also reflected by higher nitrogen concentration in the mycorrhizal part of the half mycorrhizal roots at least 7 weeks after inoculation (Fig 3-9) and in 2 single pot experiments (Tab 3-1). For roots it is not clear, whether the nitrogen is part of the plant or the fungus. The lower N concentration in fully and half mycorrhizal plants in the 3rd week was possibly due to

intensive fungal development and thus synthesis of new N-rich cell wall material (chitin) (Hawkins & George, 1999). In the 2nd and 3rd week the arbuscules developed faster in the half mycorrhizal root, but possibly also the extraradical mycelium proliferates and thus provides a sink for N. The fungus does not only absorb and translocate NH_4^+ and NO_3^-, but may also assimilate amino acids and re-transport it into the extraradical mycelium (Govindarajulu *et al.*, 2005), which might play an important role in the turnover of inorganic N (Johansen *et al.*, 1996). Thus, the symbiosis might contain overall a higher concentration of nitrogen also in the 2nd and 3rd week, but the extraradical mycelium is not harvested and therefore not measured in the element analysis.

There is no systemic beneficial effect of mycorrhization on the nitrogen concentration of the control part of the half mycorrhizal roots (Fig 3-9). In the control half, nitrogen is locally depleted respectively not supplied indirectly by the mycorrhizal half (mC-m). Possibly the shoot, which regulates the signalling for N uptake (Forde, 2002), prefers the “better supplier”, which is the mycorrhizal half due to the fungal uptake. Higher tissue element concentrations and thus better supply may indicate higher uptake rates. In tomato, rapid nitrogen absorption and translocation to the aerial parts of the plant occurs, which has been demonstrated by MacVicar & Burris (1948), using ammonium sulphate with labelled N^{15}. The uptake systems in turn are regulated by demand from the shoot (Forde, 2002) and the whole plant N status influences root growth and transport activity (Parson & Sunley, 2001). In tomato plants, elevated transcript levels of a putative high-affinity nitrate transporter gene due to mycorrhization with *G.i.* was detected in roots (Hildebrandt, 2000). As already mentioned, the applied method does not distinguish between nitrogen of the plant and the fungus. So it is still possible that the higher nitrogen content in mycorrhizal roots is just due to the existence of the fungus.

In the shoot, however, the differences between the treatments are the same as in the root, indicating an influence of mycorrhization on the whole plant nitrogen status and a beneficial N supply by the fungus. Because of the generally low N acquisition, the shoot possibly stored the N and did not additionally supply the control part of the half mycorrhizal root in this study. It is not clear, whether the hyphal N uptake is driven by the demand of the plant (Forde, 2002), so that mycorrhization is an adaptation to low soil N supply (Hawkins & George, 1999).

In contrast to the analysed mycorrhizal plants (C/N ratio 25:1), the C/N ratio of optimally supplied control plants (Tab 3-1) correspond to the ratio commonly described for tomato plants of 12:1 (www.oregonbd.org/Class/CtoN.htm). So, at least in the case of nitrogen, the growth and fertilisation conditions for mycorrhizal plants in splitroot and single pot experiments in this study were probably too restrictive, which could be another reason for the

low growth rates. This is in agreement with the complex interactions between carbon- and nitrogen metabolism, which predicts low growth rates under conditions of low nutrient availability (see above).

The **phosphorus** concentrations of shoots and roots are in the range of 0.4-0.65% (Tab 3-1), what is proposed by Marschner (1995) for a mature leaf. In all experiments, the phosphorus concentration is slightly higher in mycorrhizal roots and shoots of mycorrhizal plants, indicating a mycorrhiza-supported uptake, in which specific uptake systems could be involved. Indeed, for the symbiotic uptake of phosphorus mycorrhiza-specific transporters are found to be induced in tomato (Requena, 2005).
P is a key element in photosynthesis and high foliar concentrations enable the translocation of C compounds towards other organs (Herold, 1980). Thus, changes in the nutritional (P) status of the plant could result in changes in the C budget. Increased P acquisition due to VAM fungi might result in higher photosynthesis rates and potentially in higher C assimilation. However, nitrogen nutrition also influences photosynthesis, e.g. by competing with CO_2 for assimilatory power and reduction equivalents (Khamis *et al.*, 1990). Research has indicated that N acquisition is enhanced in mycorrhizal plants even when the P supply is adequate, reviewed by Clark & Zeto (2000). Phosphate does not only influence C assimilation, but also the transfer of P from *G. intraradices* to transformed carrot roots was stimulated by increased carbohydrate availability to roots (Bücking & Shachar-Hill, 2005), indicating complex interaction between carbon-, nitrogen- and phosphate metabolism. The status of carbohydrate pools itself (Chap 4.1.3) may be considered as an indicator of the source-sink balance.

As discussed above, in this study the biomasses were slightly higher in non-mycorrhizal shoots and roots and the tissue phosphate concentration was as high as in optimally supplied plants, possibly indicating at least sufficient soil phosphate. Rather sufficient than low phosphorus levels are also supported by the following connections, summarised by Marschner (1995, Fig 4-2).

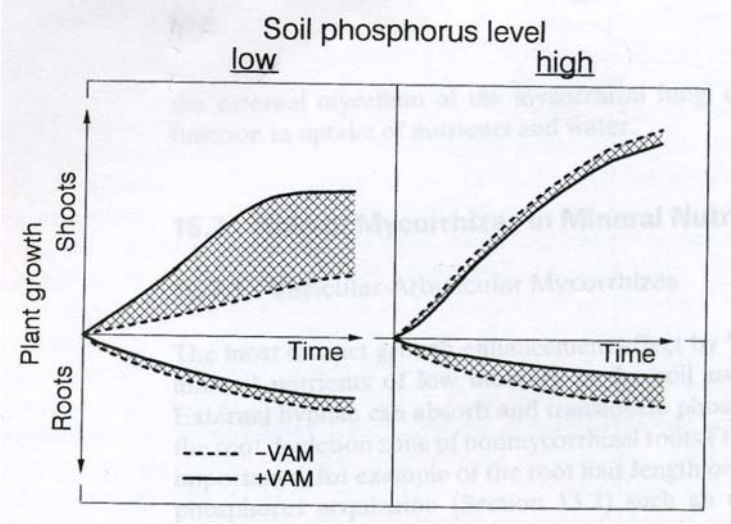

Fig 4-2. Growth of shoots and roots of mycorrhizal (+ VAM) and non-mycorrhizal (- VAM) plants at low and high soil phosphorus levels (Marschner, 1995).

The differences in growth between mycorrhizal and non-mycorrhizal plants, shown in Fig 3-2, reflect the situation under high soil phosphorus levels (Fig 4-2), indicating that there is a critical colonisation level (influenced by the phosphorus level) above which the plants receive no benefits from the fungus and might even be depressed in growth (see also Douds *et al.*, 1988). In potato, raising abiotic P supply indirectly increased the root resistance to VAM infection (McArthur & Knowles, 1992). However, *Glomus intraradices* is one of the "aggressive" fungi, which can also effectively colonise at high P (Douds jr *et al.*, 2000).

Only little is known about the influence of mycorrhization on the **sulphur** (S) metabolism and uptake of plants. The concentrations in this study vary between 0.1-0.9% in shoots and 0.2-0.5% in roots. In comparison, Sanders *et al.* (1981) reported tomato foliage concentrations of 0.76%. Generally, shoots but also roots of mycorrhizal plants contain higher concentrations of sulphur than controls in this study, although the absolute values and the differences between shoots and roots differ a lot among the different experiments (Tab 3-1). It is also remarkable that the optimal supplied plants contain lower amounts of S than the plants of exp. 1&2 (Tab 3-1), but again, the concentrations are higher in the shoot. A possible reason for the generally higher concentrations in shoots could be the sulphate assimilation, which mainly takes place in the chloroplasts. One important transport form of sulphur with many other functions is glutathione, which is discussed below in its role as antioxidant and inducer of phenylpropanoid biosynthetic enzymes (Wingate *et al.*, 1988).
Together, we found on one hand reduced growth in mycorrhizal plants, but on the other hand higher concentrations of nitrogen and phosphate. This indicates that the nutrients are not used for growth, but for other physiological functions, like a generally increase in defence

compounds. Additionally, there might be other (not measured) elements, which were in deficiency and inhibited growth, e.g. potassium (3.0-6.0%; Marschner, 1995), which occur in higher amounts in tomato leaf tissue. For example, enhanced leaf K acquisition was the only nutrient that was related to differences in growth (Clark & Zeto, 2000). But also calcium (3.0-4.0%) and magnesium (0.35-0.8%) are important elements for growth (Marschner, 1995). Perhaps these nutrients could not be taken up more efficiently by mycorrhization and occurred in too low concentrations in the soil substrate.

Also other physiological changes in VAM plants may affect plant growth and thus increased nutrient supply. For example, mycorrhizal plants may differ in their abscisic acid concentration in the xylem sap, which influences stomatal closure with consequences for mass flow and nutrient acquisition. It is possible that mycorrhization leads to a better nutrient acquisition, but that not enough carbon is available for growth or that increased disease susceptibility affects the ability of the root to take up nutrients (George, 2000), what is discussed later (Chap 4.2).

4.1.3 Changes in carbon and carbohydrates concentrations due to VAM fungi

All plants contained only about 1/10 of soluble sugars and starch compared to flowering tomato plants grown in a hydroponic system (Gary *et al.*, 2003). The shoot carbohydrate concentration was about 4-12 µmol/g FW (sugar) and 300-500 µmol/g FW (starch). Gary *et al.* reported 100-200 µmol/g FW (sugar) and 600-1200 µmol/g FW (starch). The root concentration was about 10-40 µmol/g FW (sugar) and 2-15 µmol/g FW (starch). Gary *et al.* reported 100-150 µmol/g FW (sugar) and about 20 µmol/g FW (starch), indicating that carbon fixation was very much affected by low nutrition.

But mycorrhization additionally alters carbon partitioning, because the fungus receives most if not all carbon from his host (Jennings, 1995). 4-20% of the plant photoassimilates are transferred to the fungus, reviewed by Bago *et al.* (2000). Thus, 7 weeks after inoculation the carbon and carbohydrate concentration of mycorrhizal roots is even lower than control roots (Fig 3-10), confirmed by single pot experiments (Tab 3-2), so that only minimal concentrations of starch could be stored. The fact that the shoot concentrations are not different between the treatments is probably due to the missing reserves, indicating that the shoot regulates the transport of sugar to the roots and to the symbionts.

Although the sugar concentration is unaffected, also in the half mycorrhizal plant the starch concentration is significantly lower in the mycorrhizal part (Fig 3-11), indicating the sink strength of *Glomus intraradices* probably due to formation of large quantities of vesicles. Additionally, respiration is enhanced in mycorrhizal roots (Berta *et al.*, 2000). With ^{14}C

labelling experiments, Lerat *et al.* (2003) could show that the degree of colonisation, the sink strength and the carbon supply to the roots is correlated.
Much of the carbon additionally delivered to mycorrhizal roots is taken up via arbuscules as hexose. In this study, carbohydrates - delivered to the fungus or being lost by respiration make up 80% of the "missing" elemental carbon in fully mycorrhizal roots in the 7th week after inoculation (calculations in Chap 3.3). In other words, the resting 20% more carbon might be routed into metabolites other than carbohydrates in mycorrhizal roots, possibly root exudates or emitted compounds (Chap 4.2.3).
This reflects the increased metabolic demand and necessity to supply the carbon needs of the fungus. Indeed, pulse/chase experiments with $^{14}CO_2$ show that a greater percentage of labelled photosynthates are transported out of the leaves of mycorrhizal plants during the chase period than for non-mycorrhizal plants, grown in a splitroot system (Douds *et al.*, 1988). The percentage of $^{14}CO_2$ in mycorrhizal roots was higher than in one or neither half root systems colonised with *G. i.*. In the half mycorrhizal root system the percentage was slightly higher in the mycorrhizal part, indicating the enhanced demand.

Taken together, the nitrogen concentration was higher and the carbohydrate concentration lower in mycorrhizal plants 7 weeks after inoculation, indicating nitrogen supply to be supported by the fungus, but carbohydrate assimilation to be even more reduced in this system. Nitrogen nutrition influences photosynthesis and thus carbohydrate assimilation in several ways, reviewed by Khamis *et al.* (1990). First, NO_2^- and CO_2 reduction potentially compete for reducing equivalents, produced by the electron transport chain in the chloroplast (Bloom, 1997). If more nitrogen is available, quantity, structure, and composition of the photosynthetic apparatus is affected since this accounts for most of the leaf nitrogen (Khamis *et al.*, 1990; Krapp *et al.*, 2005). The nitrogen nutrition – like all other nutrients - was not optimal in this study in comparison with normal element concentrations (see above), so a largely reduced photosynthetic capacity would be expected and explain the low levels of carbon. Even the better supply of mycorrhizal plants with nitrogen and sulphure could not compensate for even higher consumption of carbon by the fungus. This may also directly affect defence reactions, as many aromatic, stress-induced compounds contain amino groups and these metabolites might be increased in response to the high nitrogen content (Fritz, 2006).

4.2 Defence and stress responses are affected by mycorrhization

Plants have developed a broad range of defence responses against pathogen infection, wounding or oxidative stress including deposition of lignin-like polymers and structural proteins, formation of low-molecular-weight antimicrobial phytoalexines, an oxidative burst and accumulation of pathogenesis-related (PR) proteins (Somssich & Hahlbrock, 1998). Once activated, the defence systems maintain an enhanced defence capacity for a certain period. This stimulated enhanced defensive capacity has been termed systemic acquired resistance (SAR) or induced systemic resistance (ISR). SAR induction is strongly correlated with the coordinated expression of a set of PR genes and requires the signal molecules salicylic acid (SA) (Métraux *et al.*, 1990) and/or (methyl)-jasmonate (JA) (Schaller *et al.*, 2005; Sasaki-Sekimoto *et al.*, 2005). Typically, SA is the signalling molecule in the pathogen-induced pathway, whereas JA acts as signalling molecule in the pathway induced by wounding or necrosis-inducing pathogens, e.g. herbivory. For an overview, see Fig. 4-3. The third type of induced resistance is called rhizobacteria-induced systemic resistance (RISR) and is phenotypically similar to the two other pathways, as it results in systemic resistance to plant diseases, reviewed by Harman *et al.* (2004).

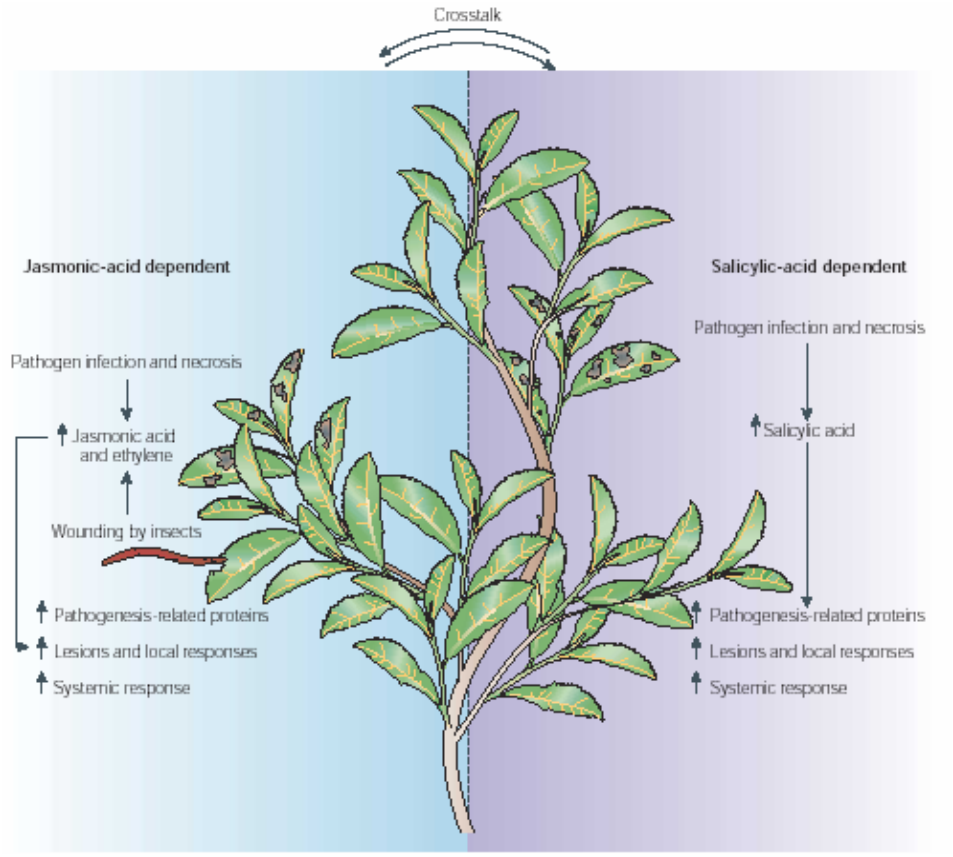

Fig 4-3. Jasmonic- and salicylic acid-dependent defence reactions (Harman *et al.*, 2004).

4.2.1 Defence reactions and signalling in mycorrhizal symbiosis

Mycorrhiza is a typically mutualistic symbiosis and modulated balance between defence reactions of the plants and their suppression as well as carbon supply of the plant to the fungus regulates the symbiosis. On one hand, plant defence reactions are ensured via the phenylpropanoid pathway, phenolic compounds and PAL, but on the other hand, ROS and PR proteins are involved in resistance as well.

The **shikimate pathway** is an upstream pathway for aromatic metabolites involved in defence reaction and is therefore likely also regulated during mycorrhization, especially at the sites of fungal infection. However, this study shows no clear regulation of the shikimate pathway by mycorrhization (Chap 3.4).
The DAHP-synthase was found to be induced by fungal elicitors in cell cultures of tobacco, tomato and parsley (Suzuki *et al.*, 1995; Görlach *et al.*, 1995), indicating that fungal elicitor *per se* is able to induce defence reactions, among them DAHPS induction. The non-induction observed in our study can be explained by different reasons: First by the different time scales (in cell cultures induction after 3 h, harvest in this study weekly) and that the elicitor was not mycorrhiza but pathogen derived. Additionally, the cells and their metabolism probably react differently, depending on whether they grow in cell cultures or if organs and whole plants were treated. Another possibility for the non-regulation of the shikimate pathway by mycorhization in roots might be the constitutive high transcript levels (Görlach *et al.*, 1994), together with relatively low mycorrhization rates, so that the possible induction is simply overlaid by the signal of non-mycorrhizal roots for constitutive supply of aromatic compounds.

Often in the **early stages** of the mycorrhiza, we have observed an induction of the DAHPS2 in mycorrhizal roots and a reduction in shoots of these control plants (Chap 3.4). In the early stages during the first contact between fungus and roots, the defence reaction in roots has a weak and transient character, involving chalcone synthase, PAL, an oxidative burst, catalase, peroxidase, and SA accumulation, reviewed by Garcìa-Garrido & Ocampo (2002). This early induction of the DAHPS could indicate an oxidative burst-specific reaction of DAHP-synthase with ROS as primary signal (Desikan *et al.*, 2005), as suggested by Janzik *et al.* (2005) for the ozone induced DAHPS up-regulation in tobacco. For *G. i*. an oxidative burst is involved in the early responses to the fungus (Garcìa-Garrido & Ocampo, 2002). In the first week after inoculation, the mycorrhization was estimated to be 1% in mm roots (Fig 3-1), because only a few arbuscules are observed and thus could be considered as early stage.

In **established symbiosis** during the intraradical growth of the fungus and arbuscule formation, the defence becomes stronger, but mostly located at the site of fungal structures. In this reaction, the phenylpropanoid pathway, flavonoids and ROS are involved. At the transcriptional and enzymatic level, PAL, CHS, catalase, peroxidase, hydrolase, chitinase and β 1,3-glucanase are involved (Garcìa-Garrido & Ocampo, 2002). Some authors propose a regulation of defence gene expression by a sensor system, based on the flux of carbohydrates (Blee & Anderson, 2000). The shikimate pathway was not regulated at the transcriptional level at this stage in our system (Chap 3.4), indicating that the defence response is suppressed to a basal level by the fungus. Another explanation could be the lack of carbon in our system, as discussed above, especially at the sites of plant fungus interaction and this strongly might influence shikimate pathway regulation.
A 3rd type of reaction was observed, when the **plant suppressed the spreading of the fungus** in the later symbiosis stage resulting in a decline in mycorrhization rates, the shikimate pathway is again slightly induced (Fig 3-14). These results indicate a fine tuned balance of acceptance and defence reactions influenced by carbon and nutrient status to regulate the symbiosis, indicating that pathogenesis and symbiosis are only variations of a common theme (Baron & Zambryski, 1995).

Besides the local induction of the shikimate pathway in roots also a systemic induction could be possible, influencing the systemic defence system of the plant. However, a clear induction of the shikimate pathway in the splitroot experiments was also absent **systemically** in the control part of mycorrhizal roots and in shoots. In several experiments, induced systemic resistance (ISR) against other pathogens within the root organ in mycorrhizal plants was observed. In splitroot systems, root colonisation of barley with *Glomus mosseae* suppressed colonisation on the second side, when colonisation levels increased on the first side (Vierheilig, 2004), indicating a defence not only against pathogens. In further experiments, mycorrhization with *G. intraradices* and *G. m*osseae induced local and systemic resistance against *Phytophthora* in tomato. In our system we could not decide, whether the non-induction of the shikimate pathway was correlated with a non-induction of systemic resistance or not. However, we found indications that SA-dependent defence reactions were absent in our system (as discussed below).

Overall, the DAHPS1 is mycorrhiza-related generally weaker expressed and regulated than the DAHPS2, what is in agreement with Görlach *et al.* (1994) for roots. And the induction of the root DAHPS by mycorrhization (see above) in the first week was only observed for the 2nd isoform (DAHPS2). This might indicate a realisation of the dual role of the shikimate pathway via the isoforms of the entry enzyme, as discussed in Chapter 4.2.2. In the context of

accumulation of reactive oxygen species in VAM roots (Salzer *et al.*, 1999; Fester & Hause, 2005), the isoform-specific mycorrhiza-related induction of the superoxide dismutase, which is involved in protection against oxidative stress, is known. Also other plant defence-related enzymes showed a local induction of mycorrhiza-related new isoforms of hydrolytic enzymes (Pozo *et al.*, 2002).

Signalling cascades

SA, temporarily correlated with PR induction, is also involved in arbuscule regulation in the mycorrhizal symbiosis (Ludwig-Müller, 2006) and the SA content of transgenic tobacco plants affects the root colonisation (Medina *et al.*, 2003). However, in this study, the SA-induced **PR1a** transcript was not induced in mycorrhizal shoots and roots (not shown). This is in agreement with other splitroot experiments, in which PR1a was only induced by *Phytophthora* infection, not by mycorrhization (Cordier *et al.*, 1998; Pozo *et al.*, 2002).

SA and JA have an antagonistic effect on the expression of PR proteins (Niki *et al.*, 1998), indicating a stronger influence of JA rather than SA in our system. However, also the tomato PR1b transcript, normally induced by JA (Tornero *et al.*, 1997), showed no detectable signals in the splitroot experiments (not shown). JA is a signalling molecule for oxidative stress, but its role in VAM symbiosis is not clear (Ludwig-Müller, 2000). JA accumulation is, together with SA accumulation, observed after ozone fumigation and leads to glutathione induction, summarised by Sasaki-Sekimoto *et al.* (2005). Neither in the third week nor in the 7th week after inoculation accumulation of total **glutathione** was observed in shoots and roots of mycorrhizal plants grown in splitroot pots (Chap 3.5). This shows that also SA does not seem to be a key player during the established mycorrhiza. Probably, JA-signalling and a glutathione accumulation are more likely involved in the early stages of the symbiosis and not three weeks after inoculation, when maximal mycorrhization rate was reached. This is supported by the finding that a positive role in the establishment of the legume-*Rhizobium* symbiosis can be proposed with glutathione as a key intermediate for gene expression via the modification of the redox balance (Hérouart *et al.*, 2002). Furthermore reduced glutathione stimulated transcription of phenylpropanoid biosynthetic enzymes in cultured bean cells (Wingate *et al.*, 1988), indicating that glutathione-mediated signalling cascades influence aromatic metabolism.

4.2.2 Influence of ozone on shikimate pathway transcription

To test whether the shikimate pathway is at all inducible under our restrictive nutrient conditions, we fumigated tomato plants with ozone, which is a potent inducer of the shikimate pathway. In this study, genes of the prechorismate pathway were found to be rapidly induced in shoots compared to controls after 2 h ozone exposure (Chap 3.6). 10 h after onset of ozone, the levels of the first and 5th enzyme of the pathway were still high and decreased after 30 h, when the PR1a-transcript reached its maximum. This is in agreement with Janzik *et al.* (2005), who found this induction pattern for the first time in tobacco plants.

This co-regulation of many shikimate pathway enzymes has also been investigated after fungal elicitor treatment by Görlach *et al.* (1995) in tomato cell culture. Görlach *et al.* observed an induction of SK and EPSPS as well as an isoform specific induction of DAHPS and CS. DAHPS and SK were induced in parallel with or after PAL, while EPSPS and CS was induced in parallel with ethylene accumulation.

In this study, tomato “Roma” showed even 30 h after ozone exposure no lesions, although it reacted sensitively in another study (Wohlgemuth, 2002), supporting an involvement of the shikimate pathway in ROS resistance mediation due to ozone. Furthermore it shows that the reaction of the tomato plants in our system does not resemble typical defence reactions as they are induced by ozone or elicitors of fungal pathogens.

In tomato, **PAL** and shikimate dehydrogenase were induced 24 h after the end of ozone exposition (3 h, 150 ppb) only in a resistant line (Guidi *et al.*, 2005), confirming an involvement of phenylpropanoids in defence reactions induced by ozone. In bean cultivars, PAL was already induced 1 h after beginning of the O_3 treatment (4 h, 120 ppb) exclusively in the sensitive cultivar (Paolacci *et al.*, 2001). In tobacco, the maximal induction of PAL was later than the maximal induction of DAHPS (Janzik *et al.*, 2005). Together this might indicate that induction of the prechorismate pathway, namely the first enzyme, is necessary to provide supply for synthesis of secondary metabolites, accumulated in response to ozone.

Energy dissipation and radical scavenging via the phenylpropanoid pathway could be another reason for the fast induction of the DAHP-synthase in tomato after ozone fumigation. In addition, Grace & Logan (2000) suppose that high levels of phenolics and carbon flow through the phenylpropanoid pathway is inducible by an accumulation of excess carbohydrates and high levels of soluble sugars in leaves. The carbohydrate levels were generally very low in this study (Fig 3-10), but the turnover may be enhanced. This might be significant to increase the flux through the shikimate pathway together with activating stimuli under ozone treatment.

The first enzyme, the DAHP-synthase, was **isoform** specifically induced by ozone, what confirmed the results obtained from tobacco (Janzik *et al.*, 2005). Only the DAHPS2 which has highest homology to DAHPS1 of tobacco (Fig 4-4) was up regulated by ozone while the other isoform was unaffected.

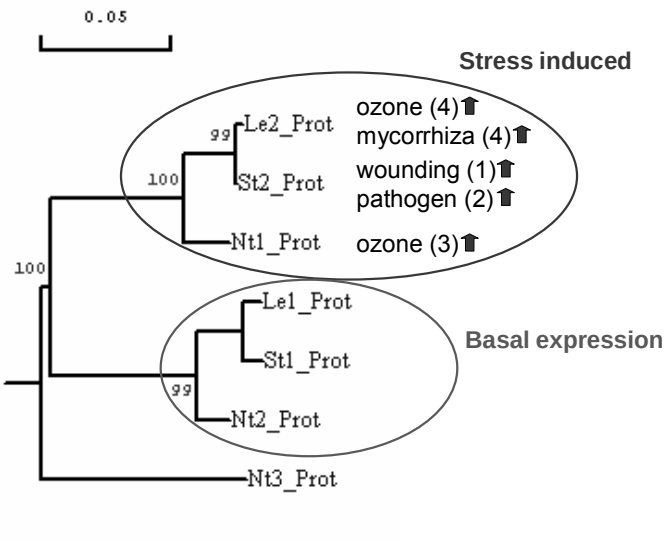

Fig 4-4. Phylogenetic tree of the DAHP synthases of solanacaeous species (Janzik, personal communication). Le = Lycopersicon esculentum, Nt = Nicotiana tabacum, St = Solanum tuberosum. ⇧ = induced. (1) (Dyer *et al.*, 1989). (2) (Nakane *et al.*, 2003). (3) (Janzik *et al.*, 2005). (4) this work.

In tomato, the same DAHPS isoform was induced by ozone and by fungal elicitor (Görlach *et al.*, 1995). This would indicate a stress specific induction of this enzyme, having in mind that ozone induces responses known from plant-pathogen interactions. DAHPS1 could provide a constant flux into the pathway for constitutive demand, while DAHPS2 might be responsible for additional demand to build secondary metabolites and for the supply of side branches, as proposed by Schmid & Amrhein (1999). EPSPS (in shoot & root) and CS1 (in the root, Fig 3-20, -21) were induced as fast as DAHPS, which is in contrast to Janzik *et al.* (2005). Both enzymes could be responsible for biosynthesis of chorismate and secondary compounds after abiotic stress.

In at least two independent experiments, the prechorismate pathway was not only induced in shoots, but also in **roots after ozone exposure** (Fig 3-20, -21). To my knowledge, this has not been shown until now and has to be further investigated, as this requires a fast signal transfer from shoots to roots. Again, already 2 h after onset of ozone, transcript levels are enhanced. In the second experiment, the induction was observed 10 h after starting of ozone exposure. An unknown signalling substance might be transported to the roots or acts as volatile compound to transduce the systemic signal. Ozone stress affects source control of allocation instead of sink control like mycorrhiza, since O_3 does not penetrate the soil and

thus can not directly influence the roots. Perhaps the same substances that are responsible for induction in shoots induce the prechorismate pathway also systemically in roots. Changes in root-shoot interactions due to ozone fumigation are discussed in Chapter 4.2.3.

Possible **signalling** substances which play a role in gene induction due to ozone are ethylene and ROS, which have the first peak already 1-2 h after beginning of pulse treatment (5 h, 150 ppb) in sensitive and resistant tobacco cultivars (Schraudner *et al.*, 1996) and *Arabidopsis* (Rao *et al.*, 2002). As the DAHPS2 is already induced 2 h after onset of ozone, these might be possible signalling substances. The plant response to ozone is discussed to be typical for plant-pathogen-interactions and is associated with hypersensitive response (HR) and programmed cell death, overview in Sandermann *et al.* (1998). Accordingly, the signalling molecules seem to share some common routes.

The **PR1a** transcript was strongly induced only in shoots by ozone (Fig 3-20), neither by mycorrhization in shoots or roots (see above) nor by ozone in roots (Fig 3-21). In shoots not exposed to ozone, no induction was observed. This is in agreement with Niki *et al.* (1998), who proposed an accumulation of SA due to ozone as prerequisite for PR1a induction. In tobacco, expression of all tested acidic PR1 genes was induced by SA and was inhibited by MeJA (Niki *et al.*, 1998). These data show that at the site of ozone action in the shoots, SA not JA is the dominant signal.

Furthermore, **glutathione** concentrations were unaffected by ozone in this study (Chap 3.6), although levels seemed to be slightly enhanced in a second experiment (Fig 3-28). This is in agreement with the obtained results for PR1a, because PR1a is known to be induced by SA, which is an antagonist to JA. For glutathione it is discussed to be induced not by the SA but by the JA signalling cascade at the transcriptional level (Sasaki-Sekimoto *et al.*, 2005).

PR1a is induced only in shoots, but not in roots, what is in contrast to the DAHPS induction, which occurs in both, roots and shoots and much earlier. Taken together, this shows that SA is not very likely the inducing signal for the shikimate pathway under ozone stress.

4.2.3 Mycorrhization affects stress responses induced by ozone

The influence of mycorrhization on the above described plant responses to ozone will be discussed in this chapter, having in mind that it is difficult to value the influence on plant fitness, because tomato “Roma” did not show any lesions due to ozone fumigation. The influence on reproductivity and fruit development was not observed.

As discussed in Chapter 4.2.1, mycorrhization does not have influence on the shikimate pathway in the shoot, while ozone induces the shikimate pathway not only in shoots but also in roots. However, the combination of both environmental factors resulted in additional shikimate pathway shoot induction in one experiment with 10% mycorrhization rate. In another experiment, with only 3-4% mycorrhization rate, this effect was not detected (Chap 3.7.2). Only the ozone induction of shikimate pathway genes was observed in the later experiment. In both experiments the shikimate pathway was slightly induced in roots by mycorrhization (2nd experiment: week 3). Systemic increase of induction of the shikimate pathway in shoots of mycorrhizal plants or roots of ozone fumigated plants was not uniform and probably dependent on the mycorrhization rate. It is very likely that the acute ozone stress is a stronger signal than longer-lasting mycorrhization and releases SA-dependent defence reactions, indicated by the PR1a induction. Four weeks after inoculation, the fungus does probably not induce strong, constitutive defence reactions, but is a strong sink of carbon and energy, which is missing in the shoot. Together with the malnutrition, mycorrhizal plants are probably more affected by ozone, which causes a massive induction of aromatic defence genes, although even in mycorrhizal plants no lesions were observed 30 h after onset of ozone. The observed higher induction of the DAHPS2 in ozone exposed leaf of strong mycorrhizal plants could possibly be correlated with a higher turnover in carbohydrates in these plants. No export stop in response to ozone could be observed, indicated by the lack of increase in sugar concentration in ozone exposed leaves (Tab 3-2). Possibly the turn-over of sugars in the mycorrhizal plants under ozone stress is additionally increased, what might positively regulate DAHPS expression, as supported by data from the diploma work of Martin (2006), who could show a clear correlation of sugar turnover and DAHPS induction.

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Whereas soilborne diseases are often reduced in mycorrhizal plants, those caused by foliar pathogens generally increased (Dehne, 1982). In an experiment with tobacco plants, leaves of mycorrhizal plants infected with a leaf pathogen showed higher incidence and severity of necrotic lesions than those of non-mycorrhizal controls (Shaul *et al.*, 1999).

Indeed, in preliminary emission measurement experiments defence-related **VOC** emissions and glutathione concentrations were only induced by ozone in shoots of non-mycorrhizal plants. E-2-hexenal and MeSA emissions were induced by ozone only in non-mycorrhizal plants (Fig 3-22, -24). During ozone fumigation, the reduction of Trimethyl-tridecatetraene (TMTT) and the induction of the three observed monoterpenes was also only detected in shoots of non-mycorrhizal plants (Fig 3-23). The lack of reaction in shoots of mycorrhizal plants could mean a lack of metabolic substrates or is the consequence of a mycorrhiza-induced systemic signal. It has recently been shown that the C_6-tomato-volatile (E)-2-hexenal

itself induces local and systemic terpene emissions, when exogenously applied, while MeJA (JA is LOX pathway derived) triggered only local emissions (Farag & Paré, 2002). This means that already the non-induction of E-2-hexenal might be sufficient to suppress also following terpene emissions. Anyhow, changes in terpene emissions during ozone fumigation have to be handled carefully, because it can not be excluded that the applied detection method produces artefacts during ozone exposure. All results were obtained only once and have to be confirmed in further experiments.

Shikimate pathway induction as well as MeSA emission are constitutively low in control plants and increased after an ozone pulse. The mycorrhization rate in this experiment was only about 3-4%, indicating weaker sink strength of the fungus and thus less consumption of carbohydrates. This is in agreement with the hypothesis that only in well established mycorrhizal plants an additive induction of DAHPS in response to ozone might occur due to an increased sugar turnover in these plants. In this experiment with low mycorrhization rates and thus less affected carbon nutrient balance no difference in reaction to ozone for the DAHPS induction could be observed. Under these conditions, an ozone induced induction of LOX and MeSA in both mycorrhizal and non-mycorrhizal plants could have been expected, especially as the shikimate pathway was equally induced by ozone in both treatments (Fig 3-26). However this was not the case. LOX products and MeSA emissions in response to ozone were completely suppressed in mycorrhizal plants. These findings indicate that volatile emissions and shikimate pathway induction are triggered by different signals in response to ozone. Neither early JA or hexenal induction nor later MeSA emissions seem to be relevant in the regulation of DAHPS in response to ozone.

5 Outlook

In this study, strong hints were found that the shikimate pathway is involved in defence reactions of the plant in early stages of the mycorrhizal symbiosis. Especially the early stage of the symbiosis, when a shikimate pathway induction was found, would be interesting to study at a higher temporal and spatial resolution concerning regulation of shikimate pathway transcription.

Experiments with a mini-mycorrhizotron are useful to study gene expression, especially during the first contact of both partners. This system allows natural growth and permits continuous non-invasive observation of the development of plant and fungus under the microscope (Bonanomi *et al.*, 2001). Another advantage of the mini-mycorrhizotrons is that fertilisation could better be fine-tuned to optimise and control growth and colonisation conditions.

These experiments could be completed by flux experiments (e.g. with radioactive labelled nitrogen), what would give an idea about the kinetics of the local and systemic differences in element and carbohydrate concentrations investigated in the splitroot system, as strong interactions occur between carbon and nitrogen metabolism and the regulation of the DAHPS by sugar-turnover was proposed.

The preliminary results concerning changes in VOC emissions and shikimate pathway induction in response to ozone, dependent on the mycorrhization rate, have to be confirmed in further experiments. The hypothesis was formulated that neither early JA or hexenal induction nor later MeSA emissions are relevant for the regulation of DAHPS in response to ozone, indicating that volatile emissions and shikimate pathway induction are triggered by different signals in response to ozone. To study the systemic signal, leading to shikimate pathway induction in roots after ozone exposure and to changed shoot reaction to ozone dependent on the mycorrhization rate, experiments with mutants deficient in the synthesis of possible signalling substances or receptors (like nitrate or glutamate receptor), is a useful tool. Another experimental setup to identify mechanisms, which mediate the systemic shikimate pathway induction in roots after ozone exposure of the shoot, is the determination of phloem sap metabolites at protein, amino acid, nitrate and carbohydrate levels.

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Danksagung

Ich danke JuProf Dr. Ingar Janzik für die Übernahme der Berichterstattung, die Aufnahme in die Arbeitsgruppe und die kompetente, engagierte Betreuung.

Prof. Dr. Ulrich Schurr danke ich für die Übernahme des Ko-Referates und die Aufnahme in das Institut.

Dem ZCH, insbesondere M. Michulitz, danke ich für die zuverlässig durchgeführten Elementanalysen.

Mein besonderer Dank gilt Ingar, Sabine, Andrea, Kate, Anika, Imke, Sven, Patric und den Azubis für die innerhalb und außerhalb des Labors verbrachte gemeinsame Zeit in toller kollegialer Atmosphäre. Ohne diese Gruppe wäre ich an den vielen Splitroot-Töpfen gescheitert und am Mikroskopieren verzweifelt.

Danke an Andreas, Beate, Annelie, Maja, Kerstin und das gesamte ICG-III für die gute Zusammenarbeit.

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 20.07.2006

(Katharina Klug)