Plant-microbe interactions alter the allocation of carbon in barley (*Hordeum vulgare*)

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Man kann, wenn man will, was man muss (deutscher Volksmund)

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Chapter 3.

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Zusammenfassung

Pflanzen fixierten atmosphärischen Kohlenstoff in ihren oberirdischen Teilen durch Photosynthese und transportieren produzierte Assimilate von aktiven Bereichen (C-Quellen) durch ihr Gefäßsystem zu photsynthetisch inaktiven Pflanzenorganen wie Wurzeln und Früchten mit Kohlenstoffbedarf (C-Senken). Neben dem Kohlenstoffbedarf der Pflanze für Wachstum und ihren Metabolismus stellt pflanzenbürtiger Kohlenstoff die primäre Kohlenstoffquelle für Bodenmikroorganismen da. Diese Dissertation untersucht wie Interaktionen zwischen Pflanzen und Bodenmikroorganismen die Verteilung von Kohlenstoff innerhalb der pflanze beeinflussen.

Im ersten Experiment (Kapitel 2) wurde ein steriles „Split-Root“ System für ^{11}C Messungen mit Gerste (*Hordeum vulgare*) als Modelpflanze etabliert. Experimente mit *gfp* und *DSred* markierten *Pseudomonas fluorescent* Stämmen zeigten, dass sich im etablierte „Split-Root“ System die Präsenz von inokulierte Mikroorganismen über die gesamte Dauer eines Experimentes auf den inokulierten Bereich beschränkte. Pulsmarkierungen von Blättern mit $^{11}CO_2$ demonstrierten, dass mit diesem System die Translokation von Kohlenstoff in die Wurzeln *in vivo* gemessen werden konnte. Die Pflanzen investierten 50 % des aus den Blättern mobilisierten Kohlenstoff in ihr Wurzelsystem. 150 min nach der Markierung war 2-4% des in die Wurzeln exportierten Kohlenstoffes von den Wurzeln veratmet. Wasserlösliche Kohlenhydrate wurden detektiert werden, allerdings war die festgestellte Menge für eine Quantifizierung zu gering.

Im zweiten Experiment (Kapitel 3) wurde im „Split-Root“ Ansatz der Einfluss von extern applizierter Jasmonsäure (JS) auf die Allokation von kurzfristig fixiertem Kohlenstoff untersucht. JS Behandlung einer Wurzelhälfte führte zu einer lokalen Hemmung des

Wurzelwachstums und reduzierte (innerhalb von Minuten) den Kohlenstoff Transport in diese Hälfte, wohingegen die unbehandelte Wurzelhälfte einen verzögerten Anstieg des Kohlenstoffimportes zeigte. Im Gegensatz dazu führte ein durch Kühlung reduzierter Kohlenstoffbedarfs einer Wurzelhälfte nicht zu einem erhöhten Kohlenstofftransportes in die andere Wurzelhälfte. Die unterschiedlichen Effekte von JS und Kühlung lässt einen durch JS spezifische ausgelöste Signaltransduktion von der Wurzeln in den Spross vermuten welche folgend zu einer erhöhte Allokation von Kohlenstoff der nicht wachstumsinhibierten Wurzeln führt.

In dritten Experiment (Kapitel 4) wurde der Einfluss des Wurzelpathogens *Fusarium graminearum* auf die Kohlenstoffallokation in Richtung und innerhalb des Wurzelsystems von Gerste untersucht. Es wurde die Hypothese überprüft, dass die Vorinokulation mit dem pflanzenwachstumsförderden Bodenbakterium *Pseudomonas fluorescens* CHA0 den Effekt vom Pathogenes abschwächen kann. Um zu testen ob die Fähigkeit Sekundärmetabolite zu produzieren involviert ist, wurde ein Stamm (*P. fluorescens* CHA19) genutzt, welcher nicht in der Lage ist Sekundärmetabolite zu produzieren. Behandlung von Gerstenwurzeln mit *F. graminearum* führte zu einer Reduzierung des Kohlenstofftransportes in die infizierte Wurzelhälfte und zu einem Anstieg der Allokation in die unbehandelte. Lokale oder systemische Vorinokulation der Wurzelhälften mit dem Wildtyp *P. fluorescens* führte zu einer Reduktion des *F. graminearum*-Effektes auf die Kohlenstoffallokation, wohin gegen die Mutante CHA19 nicht in der Lage war den Effekt von dem Pathogen abzumildern. Die Ergebnisse legen Nahe, dass die Produktion von Sekundärmetaboliten durch *P. fluorescens* zu einer systemische Resistenz gegen *F. graminearum* in Gerste führt.

Zusammenfassend zeigen die Ergebnisse, dass Pflanzen in der Lage sind die Präsenz von spezifischen Bodenmikroorganismen zu detektieren, um mir einer direkten Änderung ihres Kohlenstofftransportes die Allokation und Investition von Kohlenstoff zu optimieren.

Summary

Plants fix atmospheric carbon via the photosynthetic pathway in above-ground plant parts and translocate the synthesized photoassimilates from places of carbon fixation (source) through the vascular system to organs with carbon demand like roots or fruits (sink). Besides the plants own carbon demand for growth and metabolism plant derived carbon also represents the primary source for microorganisms in soil. This PhD Thesis was performed to investigate how interactions between plants and soil borne microorganisms alter the carbon partitioning within the plant system. Effects of particular signal compounds as well as effects of pathogenic and mutualistic microorganisms on carbon allocation were investigated.

In the first experiment (Chapter 2) a sterile hydroponic split root system allowing ^{11}C measurement was established using barley (*Hordeum vulgare*) as model plant. Experiments with *gfp* and *DSred* labelled *Pseudomonas fluorescens* strains ensured that the established split root system was appropriate to restrict the inoculated bacteria to the inoculated root fraction throughout the experiment. Pulse labelling of plant leaves with $^{11}CO_2$ demonstrated that the system allowed to follow the labelled carbon from the leave into the root system *in vivo*. The plants allocated about 50 % of the mobilized carbon fraction into the root system. From the carbon translocated into roots 2-4% were respired by roots 150 min after labelling. The soluble carbohydrates could be detected, however, the amount of exudates was too low for quantification.

In the second experiment (Chapter 3) the effect of exogenous applied jasmonic acid (JA) to the roots of barley grown in the split root system on the partitioning of recently fixed carbon were investigated. JA applied to one root half inhibited root growth locally and reduced carbon partitioning to the JA-treated tissue within minutes, whereas the

untreated side showed a delayed increase in carbon partitioning. In contrast, the reduction of the carbon sink strength of one root half by cooling did not cause an enhanced carbon partitioning to the other root half. The different effects of JA and cooling suggest that JA triggers a specific signal transduction from root to shoot and further induces an enhanced carbon export to the non-treated root with carbon sink capacity. This was also supported by results of the JA shoot treatment which resulted in a fast increase in carbon partitioning to the root system.

In the third experiment (Chapter 4) the effect of the root pathogen *Fusarium graminearum* on carbon partitioning towards and within the root system of barley was investigated. It was hypothesized that the preincoluation with the plant growth promoing rhizobacterial wild type strain *Pseudomonas fluorescens* CHA0 attenuates the effect of the pathogen on carbon partitioning. To investigate the involvement of bacterial secondary metabolites the *P. fluorescens* mutant CHA19 lacking the ability to produce secondary metabolites was used. The application of *F. graminearum* to barley roots caused a reduction of the carbon allocation towards infected roots and an increase in carbon allocation towards the non-infected root part. Local or systemic preinoculation with the wild type *P. fluorescence* annihilated the effect of *F. graminearum* on carbon allocation, whereas the mutant CHA19 did not repress the pathogen effect. The results suggest that secondary metabolites of *P. fluorescens* induce a systemic resistance against *F. graminearum* in barley plants.

Overall, the results indicate that plants are able to sense the emergence of particular soil microorganisms and to respond to these microorganisms by immediate changes in carbon partitioning to optimize carbon allocation and investment.

1. General Introduction

1.1 Fusarium

The genus *Fusarium* comprises a large group of saprotrophic organisms of the ascomycetes which can act as necrotophic plant pathogens. The taxonomy of the genus *Fusarium* is complex and had been updated several times during the last century (Nelson *et al.*, 1994). The main synapomorphy of *Fusarium* is the production of three different types of spores: the macroconidia, the microconidia and the chlamydospores. The presence or absence of the different spore types, and size and shape of the macronconidia are the classical key characteristics for species determination.

Fusarium species are widespread pathogens of crop species, including all members of Gramnineae and other economical important crop plants including banana (Saravanan *et al.*, 2004), cotton (Assigbetse *et al.*, 1994) and tomato (Mao *et al.*, 1998). All spore types as well as hyphal fragments are infective (Bai & Shaner, 1994), but spores released from soil surface debris are the principal propagules that initiate infection. One of the most common pathogenic species is *Fusarium graminearum* (teleomorph *Gibberella zeae* (Schweinitz) Petch) (Boddu *et al.*, 2006), which infects various plant species including barley (*Hordeum vulgaris*), wheat (*Triticum* spp.), rice (*Oryza sativa*), oats (*Avena*) and maize (*Zea mais*), and a wide range of other species without symptoms.

Fusarium is the causative agent of different diseases in relation to the life cycle of the fungus (Fig. 1), including the *Fusarium* head blight, the stalk rot of corn and the root rot of cereals (McMullen *et al.*, 1997). In fact, the most common cereal disease is the *Fusarium* head blight (FHB) or scap, an infection of the ears and the seeds. Symptoms

of initial infection are similar in all cereals, and consist of water-soaked brownish spots at the base or middle of the ear. FHB is of considerable economic importance. Beside of the direct reduction in crop yield by this disease, the contamination of the seeds with mycotoxins like zearalenone (ZON) or desoxynivalenol (DON) increase the damage since it spoils the seeds for consumption (Velluti *et al.*, 2004). Direct and secondary economic losses due to FHB for all crops in the Northern Great Plains and Central United States were estimated to be $2.7 billion from 1998 to 2000 alone (Nganje, 2002). The control of *Fusarium* diseases like FHB require the application of different disease management strategies, but no one reached until now the required efficiency. A promising attempt to overcome problems with specific pathogen is to breed for resistance cultivars. This, however, requires in depth knowledge on the resistance mechanisms against *Fusarium*. In the case of wheat, a relatively high resistance line, Sumai 3, had been identified (Ban & Suenaga, 2000), but in case of barley no high resistant line could be identified so far (Bai & Shaner, 2004). Even the most resistant cultivar is still infected by *Fusarium* under favorable weather conditions with an infection rate up to 20% and a high accumulation of DON (Legge, 1999). Disease control with fungicide treatments is also often inefficient, the required doses of fungicide being often so high that they become phytotoxic. An interesting phenomenon with great potential for plant protection are so called suppressive soils (Weller *et al.*, 2007). (Baker & Cokk, 1974) defined supressive soils as “soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil.” Studies demonstrated that in most cases the suppressiveness is due to soil microorganisms, since this property can be transferred to other soils, and that soil

sterilization removes the suppressiveness (Scher & Baker, 1980). DAPG (2,4-Diacetylphloroglucinol) producing *Pseudomonads* are key organisms in this process, and a high correlation between the suppressiveness of soils and the concentration of these bacteria has been reported (Weller, 2007), with a minimal threshold for pathogen inhibition at 10^5 CFU g^{-1} root (Raaijmakers & Weller, 1998).

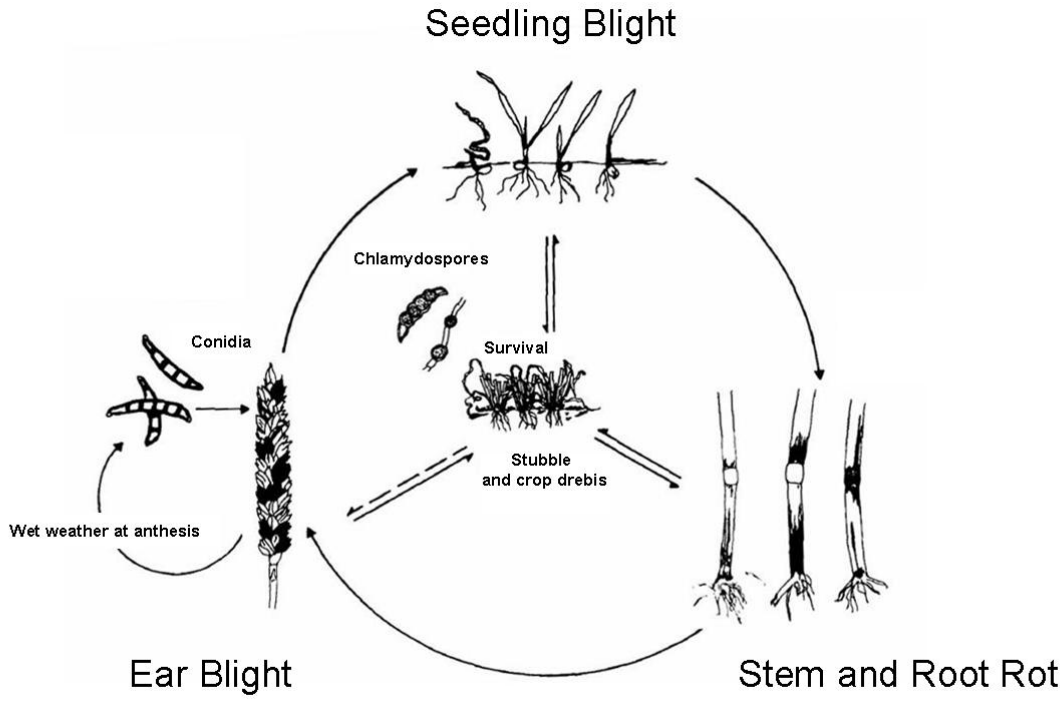

Figure 1 Disease cycle of *Fusarium graminearum*. The vegetative stage of this fungus can live saprotrophically on crop drebis. From there the fungus can spread and contaminate seedlings, stems and roots from healthy plants. On infected ears spores are produced, which can spread the infection to other ears. The figure was adopted from Parry *et al.* (1984).

1.2 Plant growth promoting rhizobacteria

Plant roots are in constant interaction with a highly diverse microbial community. The interaction between plants and microbes can have positive, neutral and negative effects on plant health and growth (Singh *et al.*, 2004). Most interactions between plants and soil-borne micro-organisms take place in the rhizosphere, the soil region close to the roots where microbial activity is strongly stimulated by root exudation and rhizodeposition (Bonkowski, 2004). This zone can be subdivided into endorhizosphere (i.e., the internal root parts), rhizoplane (i.e., the root surface) and ectorhizosphere (the thin layer of soil adhering to the roots; (Lynch & Whipps, 1990; Lugtenberg & Bloemberg, 2004); all are colonized by specific micro-organisms.

Some rhizobacteria have beneficial effects on plant performance, and are designed as plant growth promoting rhizobaceria (PGPR). They can be found in association with many, if not all, plant species (Compant *et al.*, 2005), and influence plant performance by different means. Biofertilizers, such as *Rhizobia* in Legumioses, increase plant growth through nitrogen fixation, phosphate solubilization, production of phytohormones or volatile growth stimulants (Haas & Defago, 2005). Another group of PGPR, called biocontrol bacteria, are antagonists of important plant pathogens, and mainly belong to the genera *Pseudomonas* and *Bacillu*s spp. (Haas & Keel, 2003), but other strains like *Serratia spp.* (Press *et al.*, 1997) or *Burholderia* spp. (Bevivino *et al.*, 1998) are also known to inhibit phytopathogens. The most important mechanisms of pathogen antagonism include competition for a similar ecological niche or a substrate (Chet *et al.*, 1990), the production of toxic compounds that directly inhibit the pathogen (Haas & Keel, 2003), and the induction of plant systemic resistance (Compant *et al.*, 2005).

Pseudomonads are probably the best studied group of biocontrol bacteria, and are thus an ideal model to study biocontrol interactions. In this work, we use *Pseudomonas fluorescens,* a strain which owes its name to the production of pyoverdin, a fluorescent siderophore. It has been shown that under iron limited conditions siderophore production of *P. fluorescens* is involved in the suppression of pathogens like *Fusarium*, probably by limiting the iron availability for the pathogen (Kloepper *et al.*, 1980b; Kloepper *et al.*, 1980a). Beside of the pigment Pyoverdin Pseudomonads are able to produce a wide range of secondary metabolites witch are involved in the repression of other microorganisms. Rhizosphere Pseudomonads often produce toxic exometabolites playing a role in the suppression of root diseases, such as phenanzines, pyoluteorin, pyrrolnitrin, lipopeptides and HCN (Haas & Keel, 2003). Of these metabolites, DAPG is probably the one with the best characterized biocontrol acitivity (Weller *et al.*, 2007; Haas & Keel, 2003).

Several factors influence the production of biocontrol antibiotics by Pseudomonads, almost always under the control of a quorum sensing mechanism. When the population of bacteria is low, there is only a low production of antibiotics but when the population density exceed a certain threshold, the production is strongly increased (Waters & Bassler, 2005). The production of antibiotics in *Pseudomonas* spp. is regulated both at the transcriptional and posttranscriptional level (Haas & Keel, 2003). At the transcriptional level, gene expression is influenced by biotic and abiotic factors. The translation of the produced mRNA then is regulated by the activity of the two components system GacS/GacA (Lapouge *et al.*, 2008). This post-transcriptional pathway responds to unknown signals through the GacS protein, an inner membrane bound histidine kinase which phosphorylates and activates the response regulator

GacA. The activated response regulator activates the transcription of the three regulatory small RNAs rsmX, rsmY and rsmZ, which then trigger the translation inhibitor rsmA, activating ribosomal translation of the mRNAs (Fig. 3). Beside of competition for resources or production of antagonistic compounds, *Pseudomonas fluorescens* strains also promote plant health by inducing resistance systemically and protecting plants without direct interaction with the pathogen (Pieterse *et al.*, 2001). Current knowledge on this topic is summarized in the following chapter of the introduction.

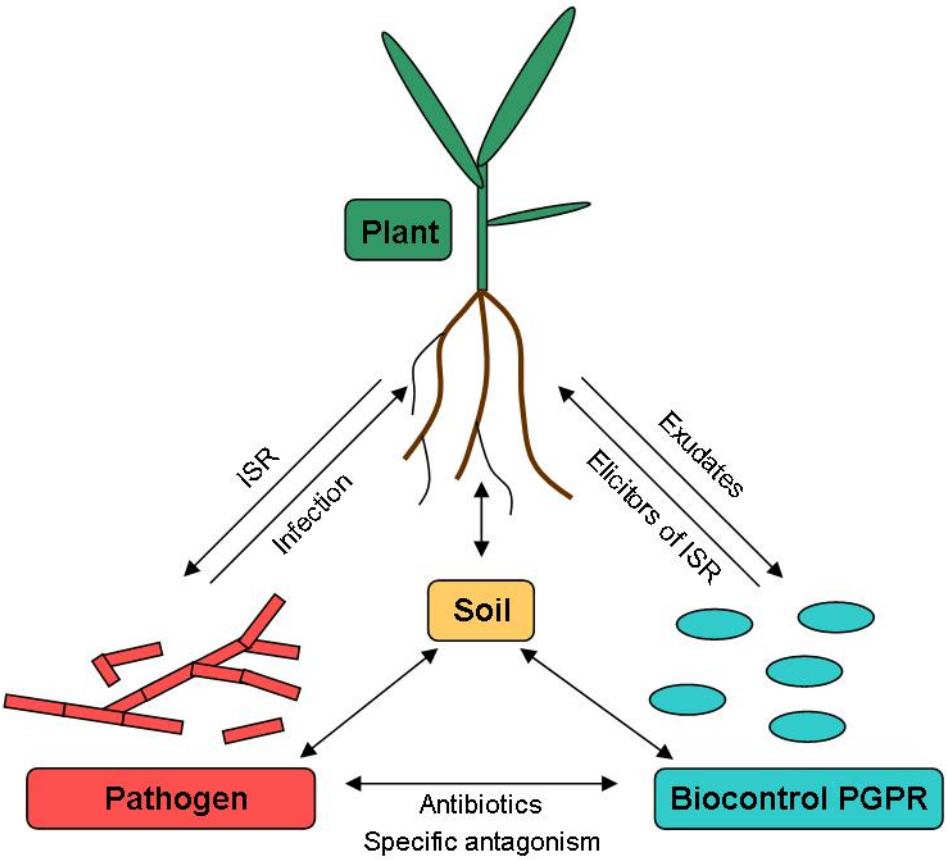

Figure 2 Possible interactions between plants, PGPR, plant pathogens and soil, modified after Haas & Defago (2005).

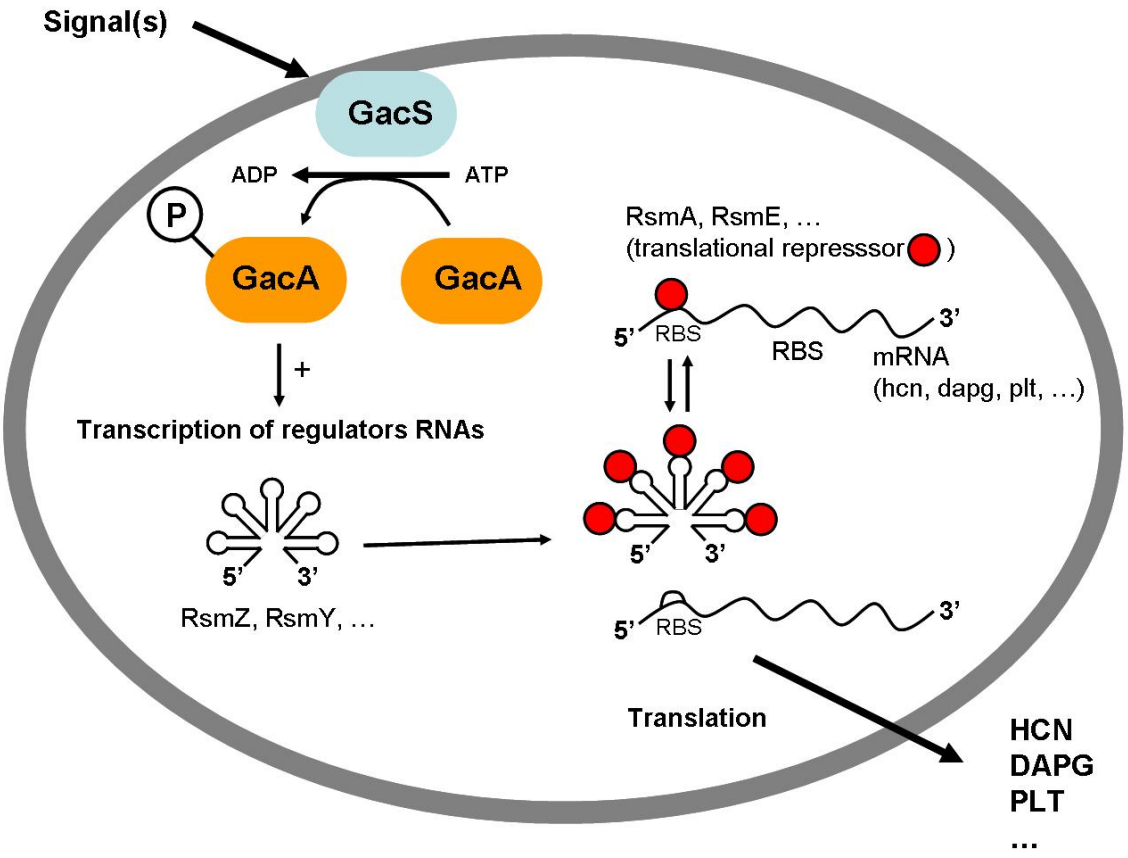

Figure 3 Current model of the signal transduction pathway involving the two-component system GacS/GacA in *Pseudomonas fluorescens* CHA0. Activation of the sensor kinase GacS by unknown signals triggers the phosphorylation of the response regulator GacA. Phosphorylated GacA positively controls the transcription of regulatory RNAs such as RsmZ and RsmY. These small RNAs control translational repressors like RsmA and RsmE, thereby making the ribosome binding site (RBS) of target mRNAs, e.g., those of the antibiotic biosynthesis, accessible for translation. Figure adopted from (Haas & Defago, 2005).

1.3 Induced resistance in plants

Plants, as sessile organisms, need the ability for acclimatisation to changing abiotic conditions and for adaptation when biotic factors are changing. There is always the risk for a plant to be attacked by pathogens or herbivores, and plants developed different mechanisms to rapidly respond to these attacks. Because the defence requires ample resources, plants may invest only in defence when necessary. Otherwise the individual plant would be outcompeted by plants which invest less in defence and have more resources for growth and production of offspring. This resource-based trade-off between growth and defence plays has been demonstrated for a number of plant species (Zangerl, 1997; Heil, 2002; Zavala *et al.*, 2004; Walls *et al.*, 2005). There is a range of defence responses based on morphological and biochemical changes in plants which are induced by pathogens or herbivores. This includes the formation of thorns, trichomes and scleromorphy (Traw & Dawson, 2002; Dalin & Bjorkman, 2003; Hummel *et al.*, 2007), changed allocation pattern of carbon and nutrients to save the resources for regrowth (Schwachtje *et al.*, 2006; Babst *et al.*, 2008), production of toxic defence compounds or synthesis of specific proteins like protease inhibitors or chitinases (Karban & Baldwin, 1997), or the release of volatile compounds to attract predators or parasites of the attacker (De Moraes *et al.*, 1998).

Induced resistance to pathogens can be grouped into two major categories, the systemic acquired resistance (SAR), and the induced systemic resistance (ISR). The results for the plant may be similar, but of the two types of induced resistance are based on different biochemical pathways. SAR is mediated via the phytohormone salicylic acid (SA) and the production of pathogen related proteins (Durrant & Dong, 2004). For example the attack of a pathogen on old leaves induces the resistance of young leaves

against the pathogen. The typical SAR response of a plant is a dramatic increase in SA levels (Klessig & Malamy, 1994), which leads to a local and systemic upregulation of plant defence. However, it is still not known if SA itself is the transmitted signal which moves from the side of attack to the non-affected parts of the plants. Beside of real pathogens, SAR can also be triggered by external application of SA, which is often done in experiments to induce plant defence (Gaffney *et al.*, 1993).

The second type of systemic resistance, the induced systemic resistance (ISR), is based on a different mechanism and is caused by the colonisation of certain non-pathogenic rhizosphere bacteria (van Loon *et al.*, 1998). ISR is mediated by the phytohormone jasmonic acid (JA) which is known to induce the upregulation of plant defensins (Thomma *et al.*, 2002). SAR and ISR are often confusingly used in the literature. For example, SAR mediated resistance was some times called ISR, so that (Tuzun, 2005) suggests to use terms like SAR or ISR only in combination with the induced pathways to avoid misleading interpretation of results. In this study, the term ISR is used to describe a JA dependent induced resistance (JA-ISR), especially in relation to the interaction between plants and rhizobacteria.

Specific *Pseudomonas* strains are known to trigger ISR against soilborne (Siddiqui & Shaukat, 2002) and aboveground pathogens (Maurhofer *et al.*, 1994a; Iavicoli *et al.*, 2003). Experiments with *Arabidopsis thaliana* demonstrated that the biocontrol strain *P. fluorescens* WCS417r induces resistance against *Fusarium* ssp. and the leave pathogen *P. syringae* independently from the SA pathway (Pieterse *et al.*, 1996). In some cases bacterial determinants for ISR could be identified. It has been shown that the presence of bacterial lipopolysaccharides (LPS) induce resistance against fusarium wilt in radish and carnation to the same extent than living bacteria (Vanpeer & Schippers, 1992;

Leeman *et al.*, 1995). Similarly, mutant bacteria altered in their LPS structure were not able to trigger ISR anymore (Duijff *et al.*, 1997). Interestingly, not only plant roots are able to sense the presences of LPS, and leaf treatment with LPS also leads to an ISR response.

A second example for compounds triggering ISR are siderophores. Because only specific siderophores can trigger ISR, whereas others with similar affinities to Fe^{3+} can not, iron limitation in the plant can be excluded as mechanisms for ISR (van Loon *et al.*, 1998). Also antibiotic compounds were considered to be involved as signals triggering ISR. Examples are 2,3-butandiol produced by *Bacillus* spp. (Ryu *et al.*, 2004) and DAPG produced by *P. fluorescens* CHA0 (Iavicoli *et al.*, 2003). In both cases, the isolated antibiotics induced ISR, but not mutants of the bacteria without antibiotic production. However, in several ISR competent strains no specific elicitor could be identified. The ISR effect is further complicated by the fact that the ISR response varies with plant species and even cultivar (Haas & Defago, 2005).

Per definition, ISR has to function without any direct interaction between plant defence inducing bacteria and pathogens, and therefore split-root systems allowing spatial separation have been widely used to study ISR (Zhou & Paulitz, 1994; Leeman *et al.*, 1995; Liu *et al.*, 1995; Siddiqui & Shaukat, 2004). A problem is always to have good separation between defence inducing organisms and pathogens, because a number of bacteria triggering ISR have also direct antagonistic effects against pathogens. In many split root studies it was only assumed, but not verified, that the presence of micro-organisms, or there influence through exudates or volatiles, was restricted over the entire experiment on the inoculated part of the root system. To avoid contamination which could lead to fatal misinterpretations, a specific split-root system was established

for this study, which allows plant cultivation under sterile conditions and restrict inoculated micro-organisms through a gastight separation of compartments to specific parts of the root system.

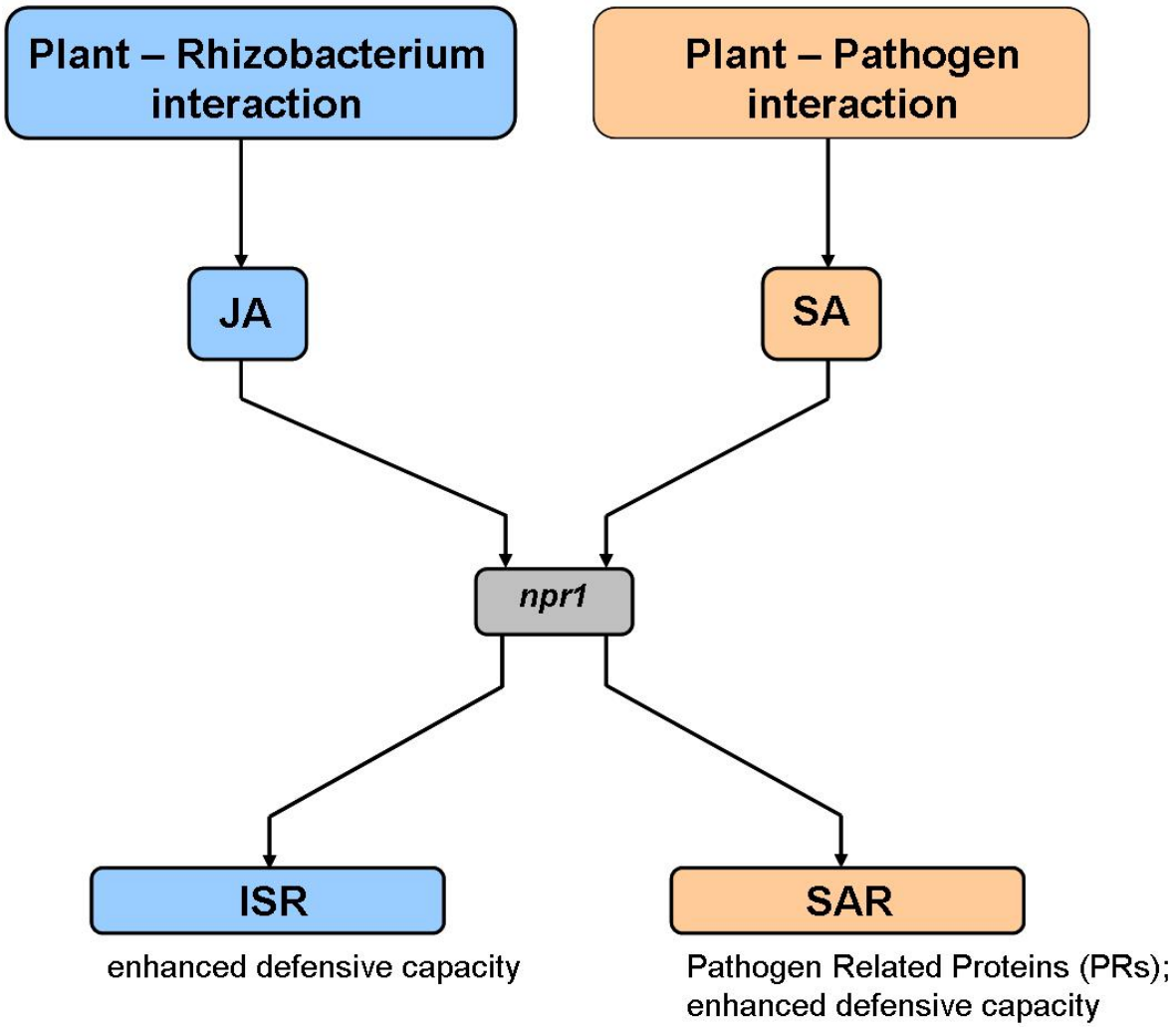

Figure 4 Signal-transduction pathways leading to pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis thaliana*. *npr1*= nonexpressor of PRGENs1.

1.4 Carbon partitioning in plants

Plants, as autotrophic organisms, gain their energy for growth and maintenance metabolism by the fixation of atmospheric CO_2 via photosynthesis. Photosynthesis is a biochemical process, using the energy of light photons to convert CO_2 to glucose. The fixed carbon must be translocated from carbon-fixing parts (C-sources) to parts which are not photosynthetically active, like roots or fruits (C-sinks). The photo-assimilates are transported from the place of fixation (chloroplasts) to the cytosol. The surplus, that is not needed for leaf metabolism, converts to sucrose or amino acids and is transported through the vascular system to organs with sink capacity (Frommer & Sonnewald, 1995). In cereals, as in most plants, the disaccharide sucrose is the main form of carbon which is transported through the phloem from the leaves to the other parts of the plant (Ziegler, 1975). Different kinds of plant tissues differ in their sink strength. Developing flowers, fruits or seeds have the strongest sink strength, and in general the sink strength for carbon is higher in the shoots than in roots (Wardlaw, 1968). The pattern of carbon partitioning is not only influenced by the demand, but also by the supply of carbon from the sources (Wardlaw, 1990).

A major C-sink in young growing cereals is the root system, which receives ~50% of the fixed assimilates (Gregory *et al.*, 1996). When the plant gets older and ears develop, virtually all fixed carbon goes into the ear, allocation to the root is dramatically decreased (Roeb *et al.*, 1986b). Furthermore, a large fraction of the carbon entering roots is released as soluble organic compounds into the rhizosphere thereby inducing high activity of the microbial biomass (Walker *et al.*, 2003; Jones *et al.*, 2004). The fraction of carbon exported from the shoot into the roots is influenced by a number of factors including plant species (Xu & Juma, 1992), develomental stage (Roeb *et al.*,

1986b), light conditions (Minchin & Thorpe, 1996), and nutrient or water status (Palta & Gregory, 1997). But also biotic interactions involving soil micro-organisms can alter the sink strength of roots strongly.

It has been demonstrated that infection by mycorrhizal fungi (Koch & Johnson, 1984), nematodes (Jones, 1976; Poskuta *et al.*, 1986) and nodule inducing bacteria (Pate & Herridge, 1978; Caldwell *et al.*, 1984) increases the carbon demand of roots. In split root systems herbivorous soil nematodes were demonstrated to increase the carbon sink in those parts of the root with high nematode activity (Freckman *et al.*, 1991). More recently, ^{11}C has been used as tracer to investigate how plants alter short-term C-partitioning in response to above-ground biotic interactions. It was shown that plants responded with a fast and strong shift on carbon translocation towards the roots after herbivory (Babst *et al.*, 2008) or the application of phytohormones which mimic herbivory (Babst *et al.*, 2005; Schwachtje *et al.*, 2006; Babst *et al.*, 2008).

1.4.1 In vivo measurement of carbon allocation with 11C

The use of the carbon radio isotope ^{11}C to investigate plant physiological processes has a long history. In 1939 ^{11}C was used as tracer in studies on the photosynthetic metabolism of barley plants (Ruben *et al.*, 1939), but as soon as ^{14}C became available, with its more convenient half-life and ease of measurement, the use of ^{11}C for plant science stopped. For medical purpose ^{11}C was firstly used 1945 as ^{11}CO in the study of blood pools in the human circulatory system (Tobias *et al.*, 1945).

A big advantage of short-lived isotopes like ^{11}C, ^{13}N or ^{18}F is that in vivo measurements are possible, so that the same specimen can be used for a number of experiments. Furthermore, dynamic studies can be readily made. The sensitivity is very high, because of high radiation of the isotopes, such as ^{11}C. The half-life and the associated radiation of tracers is so tiny that the physiology is not affected. The disadvantages are that the experiments must be performed near to a cyclotron, and only short-term phenomena can be studied because measurements can only be continued for about 12-16 half-lives after pulse labelling (Minchin, 1986).

The carbon isotope ^{11}C is decaying in a β+ mode, with a half-live of 20.4 minutes (Fig. 4). During the decay of ^{11}C, beta particles (positrons) are emitted. These energy rich particles loose their energy in collision with electrons. After the beta energy is lowered and the beta particle encounters an electron, both electron and beta are annihilated but two identical ‘annihilation gamma photons’ with specific energy are generated, and travel in opposite directions from this annihilation event. The detection of a ^{11}C-decay event is therefore possible either by measuring the beta particle directly, e.g. with a Geiger-

Muller tube, or by measuring one or both annihilation gammas (Thorpe, 1986; Thompson *et al.*, 1979).

There are different ways of labelling plants with carbon isotopes. It is possible to label the entire shoot (Thorpe *et al.*, 1998b), or only a part of the plant, like one single leaf (Minchin & Mcnaughton, 1984; Roeb *et al.*, 1986b). The latter has the advantage that also the export of tracer carbon from the leaf can be followed. Another aspect is how the labelling itself is done. There a two general ways: pulse labelling, where the plant is exposed for a short time to a varying amount of the isotope, and constant labelling, where the plant is exposed over a long-time period to a constant tracer concentration (Roeb *et al.*, 1986a).

Commonly, the emission of γ-rays is measured during an ^{11}C plant experiment with detectors at specific regions of interest. In this situation good shielding is necessary to avoid incorrect detection of radioactivity, which in other regions from the detector's intended field of view. This 'classical' approach has been commonly used, but recently facilities have been developed which can give a 2D (Kawachi *et al.*, 2006) or even 3D dynamic picture of the distribution of ^{11}C in plants in vivo (Streun *et al.*, 2007), by reconstruction of the exact position of positron decay by coincidence detection of the two emitted γ-rays. Another technique is to directly detect the positrons, before their annihilation, by placing the tissue on a phospho-imaging plate. Because the positron has limited range within plant tissue (about 2 mm in water), a good image of the location of the tracer which is near to the plate can be obtained (Babst *et al.*, 2005; Thorpe *et al.*, 2007).

For this study, one leaf was pulse labelled repeatedly so that in combination with input-output analysis (Minchin & Troughton, 1980), a precise description of the carbon

partitioning between the shoot and root system could be measured through a long time period, as long as labelling continued.

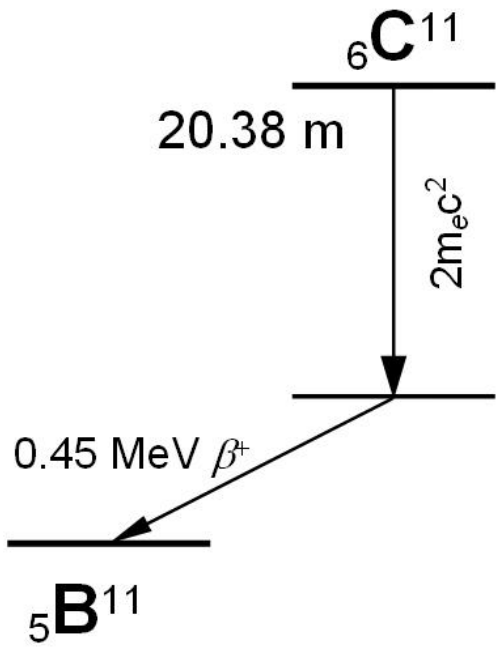

Figure 4 Decay of the carbon isotope ^{11}C. It decays with a half-live of 20.38 min by positron emission to the boron isotope ^{11}B which emits γ-rays with an energie of 0.45 MeV.

1.5 Objectives

This study investigates the effect of plant-microbe interaction on the allocation of recently fixed carbon towards and within the root system. A specific plant growth system was established allowing to investigate the translocation of recently fixed carbon non-invasively in sterile root conditions with the radio isotope ^{11}C, so that time-variation of any response in a plant could be observed for several days. To distinguish between local and systemic plant responses, a split-root setup was designed which was optimized for ^{11}C measurement under sterile conditions. Barley was chosen for this split-root system, because it is an economical important crop plant, and it is already well investigated. Furthermore, barley has a good anatomy for split-root experiments, because photoassimilates are not confined to sectors of the root system but uniformly. In Chapter 2 the developed system is described in detail, and it is tested if the tracer ^{11}C can be used to measure the respiration and exudation of roots in vivo under sterile conditions. Furthermore, it was verified by inoculation with *gfp*-labelled bacteria, if it is possible to restrict colonisation of roots to those in only one compartment of the split-root system.

The second experiment (Chapter 3) deals with the effect of the phytohormone jasmonic acid on the partitioning of recently fixed carbon in barley plants. Recent studies have demonstrated that leaf application of this stress inducible hormone, to mimic herbivorie by animals, alters the partitioning of carbon with in the plant system (Babst *et al.*, 2005; Schwachtje *et al.*, 2006) and in a similar way to a real herbivores (Babst *et al.*, 2008). We hypothesized that also a root application can be induced by this phytohormone, and that exogenouse application of JA leads to fast changes in C-allocation. Because JA is known to have a direct negative effect on root growth, we used the split-root system.

This gives an untreated (control) part of the root in order to show any systemic responses. To investigate the nature of the plant responses that were induced by the compound, we compared the responses to JA with those where metabolism was modified by cooling.

In the third set of experiments (Chapter 4) we investigated how pre-inoculation with PGPR alters the responses in carbon allocation to subsequent infection by another pathogen. First we analysed to what extent and in what chronological sequence does root infection with a hyphal inoculum of the soilborn pathogen *F. graminiearum* change the allocation of carbon within the plant. Furthermore, we hypothesised that the effect of the pathogen on carbon partitioning could be modified by preinoculation with the wild type biocontrol strain *P. fluorescens* CHA0, which is known to repress root pathogens like *Fusarium*. To investigate if the effects depends on a direct interaction, biocontrol agent and pathogen were inoculated either together or separately between the spatially separated roots. To check if the secondary metabolite production of *P. fluorescens* CHA0 is involved in this interaction, we investigated whether the gacS mutant CHA19 (which is unable to produce antibiotics like DAPG) has the same effect on the pathogen. By using ^{11}C as a tracer, non-invasive measurements of effects on carbon partitioning could be followed before changes due to growth occur.

2. Establishing a system to measure root respiration and exudation of soluble compounds using the radio isotope ^{11}C in sterile split root conditions

2.1 Abstract

Plants allocate a large fraction of their fixed carbon below the ground to support their root system. A high proportion of this carbon entering the roots is released, either in the form of soluble organic compounds (exudates) into the surrounding soil or by respiration. The carbon delivered from plants into the belowground system is an important C source fuelling the rhiziosphere food web.

The aim of this study was to establish a system where respired and exuded carbon could be analysed under sterile split-root conditions with the radio-isotope ^{11}C. Therefore, a specific setup was developed which was optimized for sterile root growth and ^{11}C measurement. To verify if the separation of the two root compartments was efficient enough to use this setup in a separate study with soil bacteria, *gfp* and *DSred* labelled *Pseudomonas fluorescens* were inoculated to one root compartment of barley plants, and transfer of the bacteria from one root compartment to the other was checked. Our result show that over a period of 8 days inoculated bacteria were restricted to the root compartment to which they were inoculated. Labelling with ^{11}C indeed allowed to measure root respiration. About 1.5% of the tracer mobilized from the labelled leaf was respired 150 min after labelling. Exudates could also be measured, but were only present in trace amounts.

The study documents that using ^{11}C the developed split root system allows measurement of carbon release under sterile root condition.

2.2 Introduction

A major carbon sink in most growing plants is their root system. Beside its utilization for growth and metabolism, roots release a high proportion of their primary fixed carbon to fuel belowground processes in the vicinity of roots. Depending on plant species and age the release of carbon from the roots may constitute 17-40% from the carbon fixed by photosynthesis (Lynch & Whipps 1990). Field experiments showed that cereals like wheat and barley transfer 20-30% of total assimilated C below the ground (Kuzyakov 2000). In a review covering several plant species Lambers (1987) estimated that 12-29% of carbon translocated to the roots is lost by respiration and 5% by exudation.

The respiration of roots can be viewed as an essential cost for the maintenance of growth and function (Gregory 1991). Root respiration provides the energy (ATP and reducing equivalents) and C skeletons necessary for ion uptake and for the synthesis and maintenance of root biomass (Atkin *et al.* 2000). Root respiration represents a mayor source of CO_2 loss in plants, amounting to 8-52% of the fixed carbon which is returned to the atmosphere (Lambers *et al.* 1998), and is thereby responsible for up to 60% of total soil respiration (Pregitzer *et al.* 1998). It has been shown that the rate of root respiration is related to temperature, the root growth and also depends on the illumination of the shoot (Farrar 1981). A problem in measuring respiration under non-sterile conditions is to distinguish between respiration of roots and micro-organisms (Kuzyakov 2002). For this reason root respiration is often quantified under sterile conditions (Barber & Gunn 1974; McCrady & Andersen 2000). However, the presence of bacteria can induce a strong increase in root respiration (Phillips *et al.* 1999), so a system where changes in vivo could be measured would be an promising tool to investigate such plant - microbe interactions.

Beside respiration, roots release a high proportion of their fixed carbon into the surrounding soil in form of soluble exudates like polysaccharides (mucilage), sugars, organic acids, amino acids, secondary metabolites and proteins (Bais *et al.* 2006). In plants grown in soil, approximately 5-10% of the net fixed C is exuded by roots (Jones *et al.* 2004), however, in hydroponic systems exudation is significantly lower (typically between 0.5-1.5%: (Farrar *et al.* 2003). Jones et al. (2004) suggest that methodical bias is responsible for this big difference between exudation in soil and in hydroponic systems, with exudation in soil being overestimated whereas in hydroponic systems being underestimated. They estimated the amount of C exuded under natural condition to be 2-4% of the net fixed carbon. It has been suggested (Meharg 1994) that exudation measurements using isotopes in soil may result in an overestimation of exudation rates, and that the method of labelling affects the measured fraction of fixed carbon exuded by roots.

Minchin & Mcnaughton (1996) demonstrated with non-sterile barley plants that the process of exudation is fast enough to quantify the released soluble carbon compounds using ^{11}C tracer technology. They showed that the amount of labelled exudates was increased after antibiotic treatment and concluded that micro-organisms metabolized the exudates. However, the plants in these experiments were not grown in sterile conditions and therefore the influence of micro-organisms on exudation could not be quantified. Micro-organisms care known to increase root exudation by the production of hormones and other chemicals (Barber & Lynch 1977).

The aim of this study is to establish a system to measure the release of both CO_2 and soluble organic compounds (exudates) by using the radioisotope ^{11}C in sterile hydroponic root systems. To enable discrimination between local and systemic

responses in future studies, the setup was designed as a split-root system. The advantage of using ^{11}C as tracer in plant systems is that it can be measured in vivo and with a high temporal resolution allowing to analyse the kinetics of carbon allocation patterns. On the other hand, due to the short half-live of the isotope, ^{11}C studies are restricted to investigating the fate of recently fixed carbon only. The developed split-root system was tested to ensure that there is no migration of micro-organisms from one half of the split root system to the other. This is the prerequisite for studying systemic effects of rhizosphere micro-organisms on plant carbon allocation patterns.

2.3 Material and Methods

2.3.1 Split-Root Rhyzotrons

For the experiments planting pots with special features were needed. They need to be sterilized easyly to, allow root growth without contaminations, and split the root system into two parts with separate rhizospheres. To avoid problems with anoxia, each root half was provided with sterile filtered air. A further requirement was to improve the geometry for non-invasive radio isotope measurements. A thin, almost planar, shape was chosen with the advantage that all the radioactive tracer in roots will decay within a relatively constant distance to the detector and so the sensitivity is similar in different parts of the root system. After testing different materials, polypropylene and polycarbonate for the main body were selected because this material can stand the temperature during autoclaving. Treatment of PVC-Rhizotrons with 70% EtOH proved to be insufficient for sterilization. The final version of the split root rhizotrons is shown in Fig. 1 and Fig. 2 in detail. This rhizotron was used for all ^{11}C experiments. To ensure complete separation the Y-gap where the roots were split was sealed with sterile silicon grease (Baysilone, Bayer, Germany).

2.3.2 Plants

Seeds of barley (*Hordeum vulgare* L. cv. “Barcke”, Irnich Inc., Frechen, Germany) were dehusked by incubation in 50% H_2SO_4 for 60 min under agitation, and washed three times with distilled water to remove the acid. Seeds were sterilized with a freshly prepared 2% $AgNO_3$ solution for 20 min on a shaker at 200 rpm, washed with a sterile 1% NaCl solution, with distilled water, again with NaCl, and 5 times with distilled water to completely remove $AgNO_3$.

The seeds were germinated in darkness at 20°C on a diluted nutrient agar (agar 8 g l^{-1}, nutrient broth 0.8 g l^{-1} in Neff's modified amoeba saline; Page 1976). After 4 days, seedlings were checked visually for contaminations with microorganisms. Sterile plants were transferred into silicon closed cell foam rubber stoppers with a longitudinal slit (VWR, Darmstadt, Germany) which sealed the roots into glass tubes (length 135 mm, diameter 25 mm) containing 50 ml sterile 50% Hoagland solution. The plants were grown at 60% relative humidity (RH) with 16-h day (100 µE m^{-2} s^{-1}, 25°C) and 8-h night (20°C). After 7 days, each plant was transferred into a two-chamber split-root rhizotron. The roots were separated in two equal parts in the two chambers of the rhizotron, and each chamber was sealed with silicon grease (Baysilone, Bayer, Germany). Each rhizotron chamber was supplied with 300 ml 50% Hoagland solution containing 5 mM MES buffer (pH 5.8) and plants were allowed to grow for an additional 5-7 days until radiotracer experiments were started.

2.3.3 Pseudomonas fluorescens

For this study the rhizosphere bacteria *Pseudomonas fluorescens* CHA0 was used, tagged with Gfp or DsRed using a mini tn7 delivery system as described in *(*Jousse*t et al.* 2008). Tagged strains were routinely kept on nutrient agar supplemented with 25 µg ml^{-1} Kanamycine or 8 µg ml^{-1} gentamycine, respectively (Jousset *et al.* 2006). Prior to inoculation, bacteria were grown in NYB medium (nutrient broth 25 g l^{-1}, yeast extract 5 g l^{-1}) at 30°C with agitation. Late exponential-phase bacteria were harvested by centrifugation (13,000 rpm for 2 min) and washed three times in Neff's modified amoeba saline (NMAS). The concentration of bacteria was determined by measuring optical

density at 600 nm (OD600) and checked under a Zeiss Axioscope 2 epifluorescence microscope at 400x magnification.

2.3.4 Testing for Cross Contaminations between Split-Root Compartments

To verify that the two root halves in the split-root rhizotrons were completely separated and micro-organisms introduced on one side were not able to pass to the other side, a set of 4 plants were inoculated with different labelled bacteria on each root half. One root compartment was inoculated with 1:5 ml (OD_{600}=0.71) Gfp tagged *P. fluorescens* CHA0, the other root compartment in the same way with DsRed tagged *P. fluorescens* CHA0. On day 1, 2, 3, 4, 5 and 8 after inoculation, 10 ml from the hydroponic solution was sampled with a sterile syringe by removing the filter from the support tube. The removed volume was replaced with sterile 50% Hoagland solution. The bacteria were fixed by adding formaldehyde to give a final concentration of 1% v/v. The OD_{600} of each sample was measured with a photometer to estimate the population size. An aliquot 100 µl of resuspended bacteria were filtered on a 0.2µm polycarbonate membrane, and counted under a Zeiss Axioscop 2 plus epifluorescence microscope at 400x magnification. The filter settings for Gfp and DsRed counts were, respectively, an excitation filter of 470 and 546 nm, a dichroic filter of 493 and 580 nm, and an emission filter of 505-530 and 590 nm.

2.3.5 ^{11}C labelling

Two to three days prior to an ^{11}C labelling experiment, a plant was transferred to the climate chamber for acclimation. It was connected to the ^{11}C-labelling system at least 16 h before the start of the measurement, to ensure that it had fully recovered from

mechanical disturbance before it was labelled. The second leaf was sealed with 2-pot silicone rubber (Xantopren VL, Heraeus Kulzer, Hanau, Germany) in a cylindrical Plexiglas™ chamber (length 70 mm, diameter 18 mm) and labelled three times with about 100 MBq $^{11}CO_2$ in air at 5, 7.5 and 10.5 h into the light phase. Plants were treated during the second application of ^{11}C when tracer activity in the root detectors was at a maximum (i.e. equal rates of decay and arrival), ~60-70 min after the start of labelling. The $^{11}CO_2$ was produced with a Baby cyclotron in the Research Centre Jülich

2.3.6 Measurement of Respired $^{11}CO_2$

For measuring the respired $^{11}CO_2$, barley plants were grown hydroponically using one root compartment of the split root rhizotrons. The hydroponic solution was aerated with a high flow rate of sterile filtered air to obtain a good gas exchange from the solution. Because the solubility of CO_2 in water depends on pH, the pH of the hydroponic solution was adjusted with a MES buffer (2-(*N*-morpholino)-ethane sulphonic acid) to a relative low value of 5.5 to get fast exchange of soluble CO_2 into the gas phase. The used pH is well within the physiological potential of barely and other crop plants (Islam *et al.* 1980). The outflow of root compartments was directed into a CO_2 trap, which was a 10 ml syringe filled with Carbosorb. Plants were measured over two following days, labellings with 3 pulses of $^{11}CO_2$ (~100 MBq) per day.

2.3.7 Measurement of Water Soluble ^{11}C Exudates

To get information on the exudation of recently fixed carbon, we measured the fraction of water soluble compounds. For the calibration of detectors in the exudate setup, an equal amount of solved $^{11}CO_2$ was placed into the Rhizotron, into the washing bottle and in the position of the CO_2 trap (Fig. 3). After some minutes, when enough measurements

were done for the sensitivies, the syringe with the activity was removed from the CO_2 trap. Than the aeration of the solution bottle was started to wash the solved CO_2 out of the hydroponic solution.

2.3.8 Data Analyses

For analysing ^{11}C data two different analyses were used, an input-output analysis (Minchin & Troughton 1980), and a more crude method where the percentage distribution of activity was calculated at specific time points for the compartment of interest.

The input-output method estimates the transfer function for movement of the tracer through a pathway into the plant (Minchin & Thorpe 1989; Minchin & Thorpe 2003), and by accounting for radioisotope decay the analysis quantifies the transport of 'recently assimilated carbon'. In the analysis, the total mobilised tracer (the sum of the three plant parts) was taken as input, and the tracer entering a specific sink (either or both root portions) was taken as output. The steady-state gain of the transfer function is the fraction of the mobilised photosynthate reaching the sink - that is, the partitioning into that sink. Because processes like exudation and respiration had a long time constant, it was not possible to analyse these data with the input-output analyses. For the respiration experiment, the data were corrected for background, detector dead-time and sensitivity. The relative fraction of the shoot (except load leaf), root, and CO_2 trap, were calculated with the data from 150 min after each labelling by dividing the different compartments by the total mobilized fraction (everything what was transported from the load leaf).

2.4 Results

2.4.1 Seed sterilisation

The seed sterilisation protocol for barley efficiently sterilized the seeds. After 7 days 86% of the sterilized seeds were free of micro-organisms (Table 1). Germination rate of the seeds was not affected by the sterilisation procedure. Almost all germinated seedlings developed normally into shoots and roots. Because of the high rate of germination of sterile seeds, it was possible to select seedling for experiments from agar plates which remained sterile, thereby minimizing the risk of contamination.

Table 1. Number of germinated and sterile remained barley seeds 7 days after sterilisation with 2% AgNO3 in three independent Experiments.

Exp.	Number of seeds:	Germinated seeds:	Sterile seeds:
1	45	36 (80%)	39 (87%)
2	44	40 (91%)	38 (86%)
3	42	38 (91%)	36 (86%)
∑	**131**	**114 (87%)**	**113 (86%)**

2.4.2 Migration of Pseudomonas fluorescens

In all four inoculated plants *dsRed* or *gfp* tagged *P. fluorescens* CHA0 cells could be recovered after 1, 2, 3, 4 and 8 days from the root solution of the inoculated root compartment (data not shown.), but in no case could labelled bacteria be detected in the root half which had not been inoculated.

2.4.3. Carbon partitioning towards the Root System

It was possible to follow the export of recently fixed carbon from the load leaf through the rest of the shoot into the root system in the established system. After ^{11}C pulse labelling, the activity of the load zone directly increased, followed by the shoot, root, solution and CO_2 trap, with increasing time delay (Fig. 5a), which was caused by the transport. The half-live corrected data (Fig. 5b) suggest, that the $^{11}CO_2$ activity in the closed gas loop which supplied the load leaf peaked after 8 min, and then started to drop, while the activity in the load-leaf continued to increase.

The installed lead-shielding was efficient, problems with radiation see-through from other parts of the plants with a similar activity as the shoot was negligible (Fig. 5a). An exception was the solution detector, where very little tracer could be detected and so see-through was inevitably a higher fraction of the observed tracer.

A total of 59% of the mobilized activity was recovered in the root zone 150 min after labelling, whereas an input-output modelling of the same data lead to a slightly higher root fraction of 61% at this time point (Fig. 6). In comparison to the calculated fraction at each time point the modelled output data gave a more constant root fraction over the entire measurement period. The plant allocated a relatively high fraction of carbon to the root system, as compared to another set of 6 barley plants, in which 150 min after the last labelling at the first day on average only 44% of the mobilized tracer could be recovered in the root system. On the second day, at this time point the fraction was increased to 51%. However, this increase of the root fraction was statistically not significant.

2.4.4 Root 11C Respiration

About 150 min after labelling the average fraction of released ^{11}C in form of CO_2 was 1.65%, 1.65%, 1.32% at the first day, and 1.74%, 1.62% and 1.28% at the second of the entire mobilized fraction, respectively (Fig. 7). There was no statistically significant diurnal change in respiration, and no significant difference between the values of the first and the second sampling day.

3.4.5 Measurement of Soluble ^{11}C Compounds

The calibration measurements of the exudation setup (Fig. 4) demonstrated that the radioactive CO_2 solution injected into the root compartment was transported into the solution bottle when the solution pump was started, and from there washed out into the CO_2 trap. Whereas the exchange between solution bottle and trap was efficient, the export of radioactivity from the root compartment was incomplete; about 15 % of the initially applied ^{11}C activity remained fixed in the sterile root compartment. This calibration procedure allowed measurement of exuded and respired ^{11}C. However, measurements with real plants in the same setup showed only a relatively low activity of labelled compounds in the bathing the solution. When the circulation pump was stopped (to avoid import of new solved CO_2 from the roots) only a really small activity remains in the bathing solution (Figs 5a,b), indicating that most activity seen by the solution detector was solved respired $^{11}CO_2$ passing through the system and not soluble exudates. The activity in the solution was too low for allowing quantification.

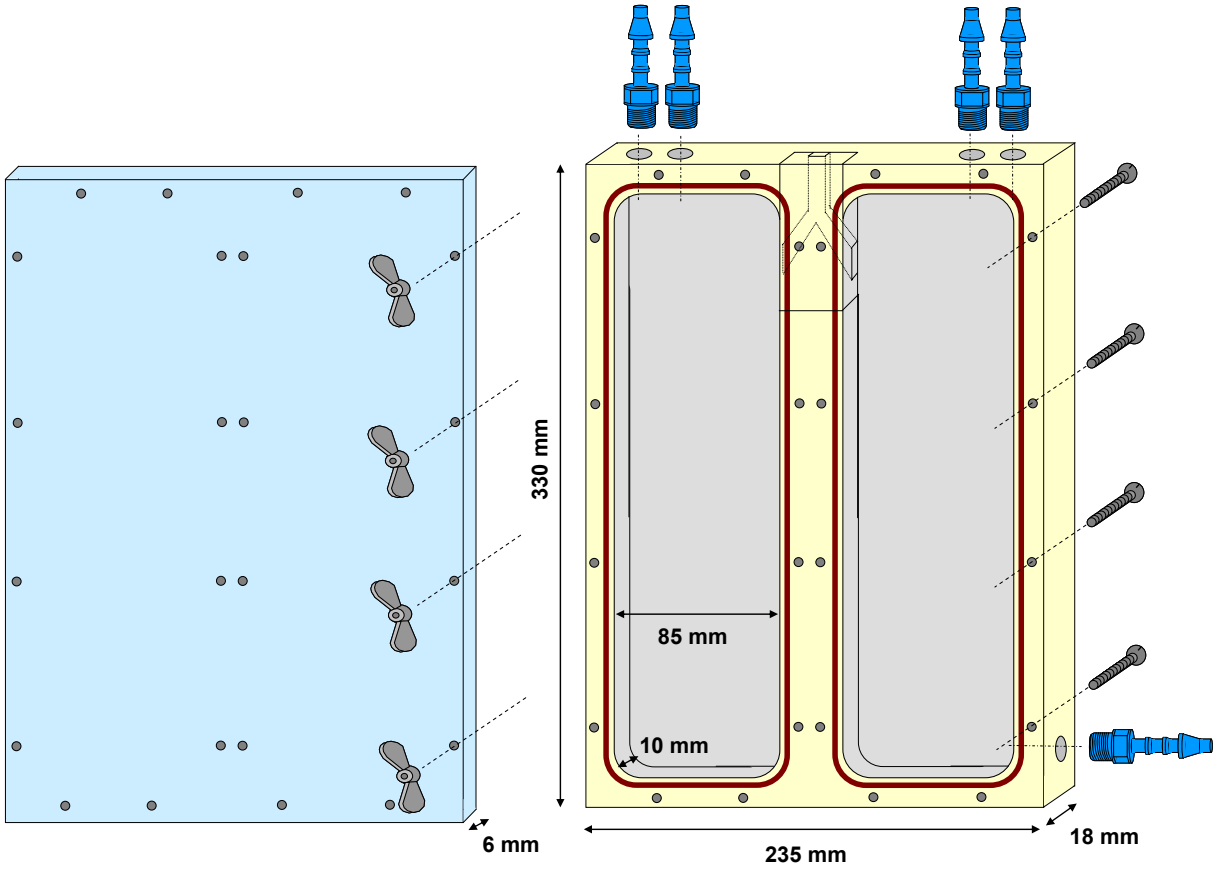

Figure 1. The developed split-root rhizotron. The cover plate is made of transparent polycarbonate (PC), the main body out of polypropylene (PP) allowing autoclaving. The tubes are silicon, and connectors are made out of polyamide (PA) and are connected with a 1/8'' NPT thread to the rhizotron.

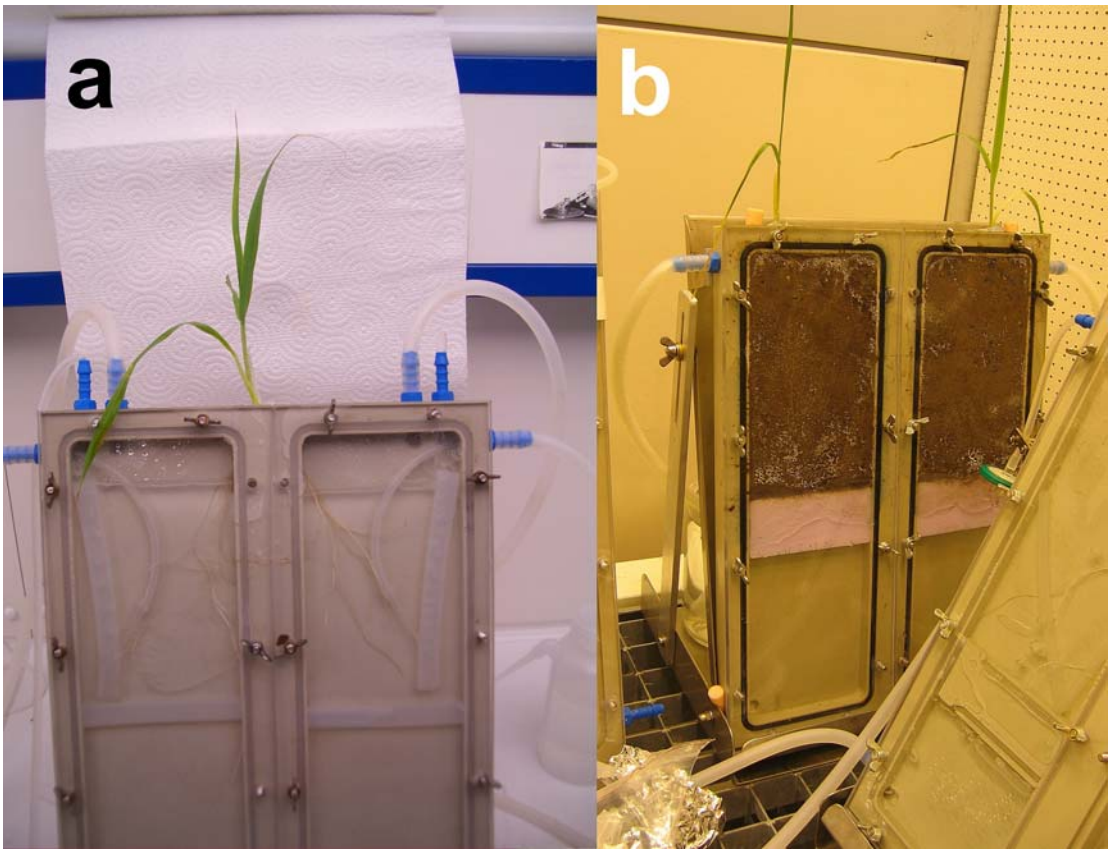

Figure 2. (a) Hydroponically grown barley plant in the developed split-root system. Aeration, via a tube which ensured mixing of the solution but restricted air bubbles to a narrow part of the root chamber, was stopped for taking the photograph. (b) Two non-sterile grown barley plants in the rhizotron with soil as substrate. The soil is watered automatically through glas fiber packing to maintain constant humidity.

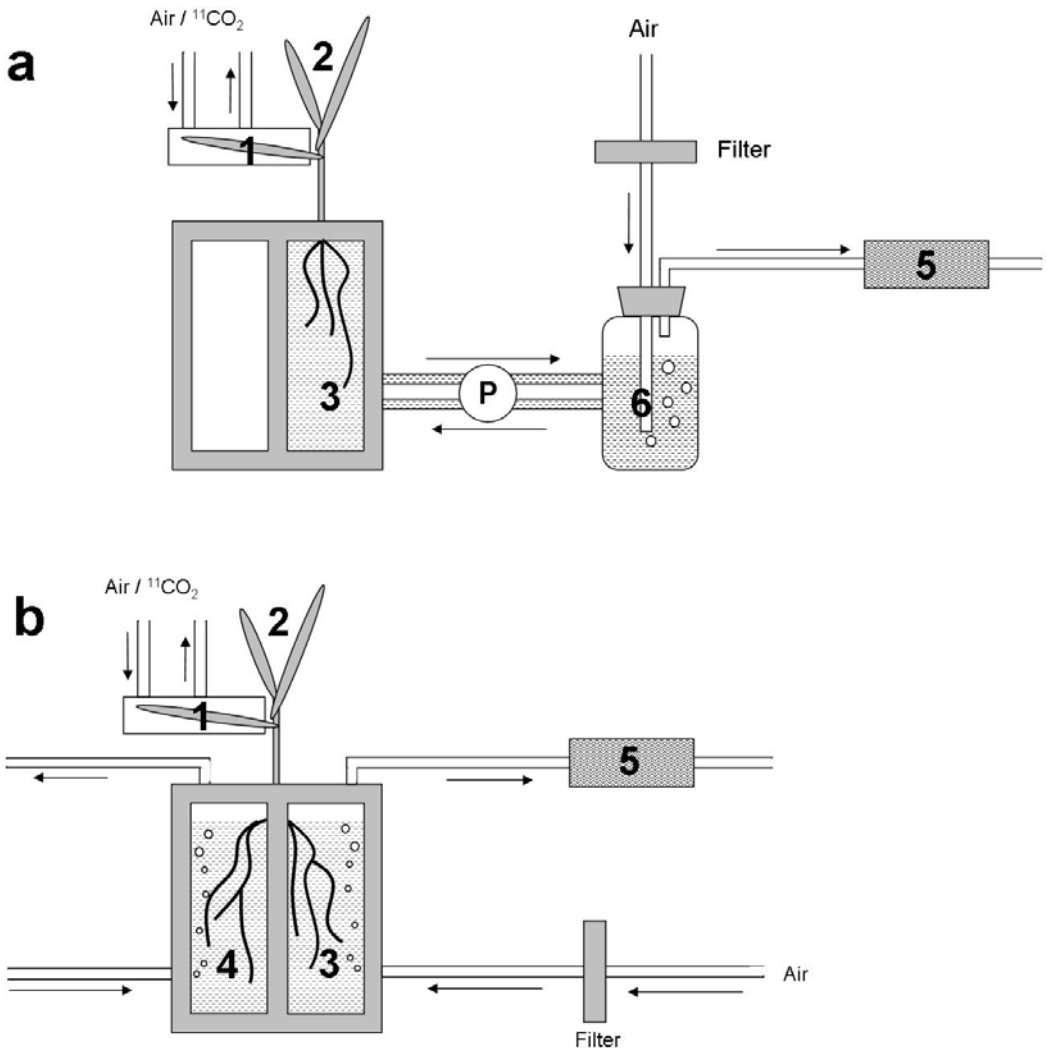

Figure 3. Experimental setup for investigating root exudation and respiration or root respiration in split-root conditions following labelling of a single leaf with $^{11}CO_2$. **(a) A single b**arley plant was placed with the roots in one compartment of a split-root rhizotron containing 50% Hoagland solution circulated by peristaltic pump (P) through a root bathing reservoir. Air is bubbled through the bathing solution and passed through a CO_2 trap containing Carbosorb. (b) The barley plant is grown in split-root conditions, where each root half is aerated separately. Radiation detectors are positioned to be sensitive to tracer within specific regions (radiation shielding not shown); (1) load-leave, (2) shoot, (3) root 1, (4) root 2, (5) CO_2 trap, (6) bathing solution.

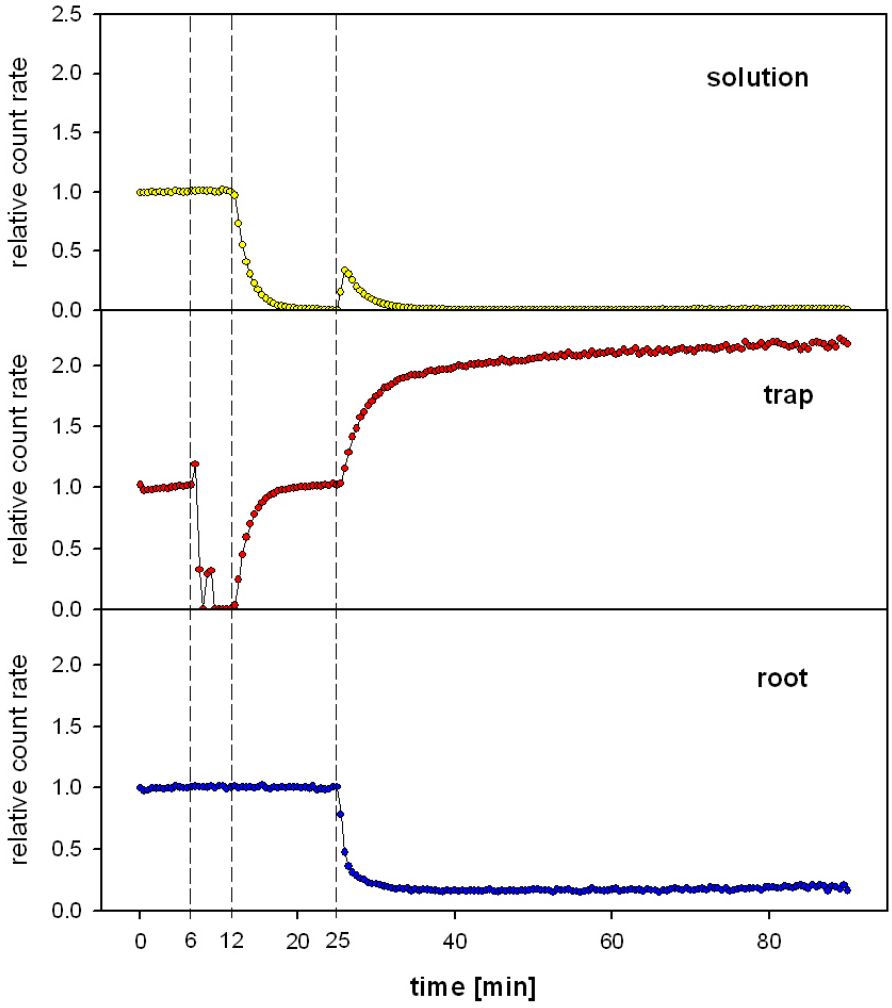

Figure 4. Calibration of the exudation setup (Fig. 3a), showing ^{11}C activity in the root chamber (**root**), the root solution (**solution**) and the CO_2 trap (**trap**). The counts were corrected for background and decay. At time 0, the same activity of dissolved $^{11}CO_2$ was added into the root chamber, the solution bottle and the CO_2 trap. At 6 min the CO_2 trap was replaced, and after 12 min aeration of the solution was started thus washing the activity from the solution into the CO_2 trap. At minute 25, the solution pump was started mixing the activity uniformly between root and solution bottles and transporting the CO_2 at the end in the CO_2 trap.

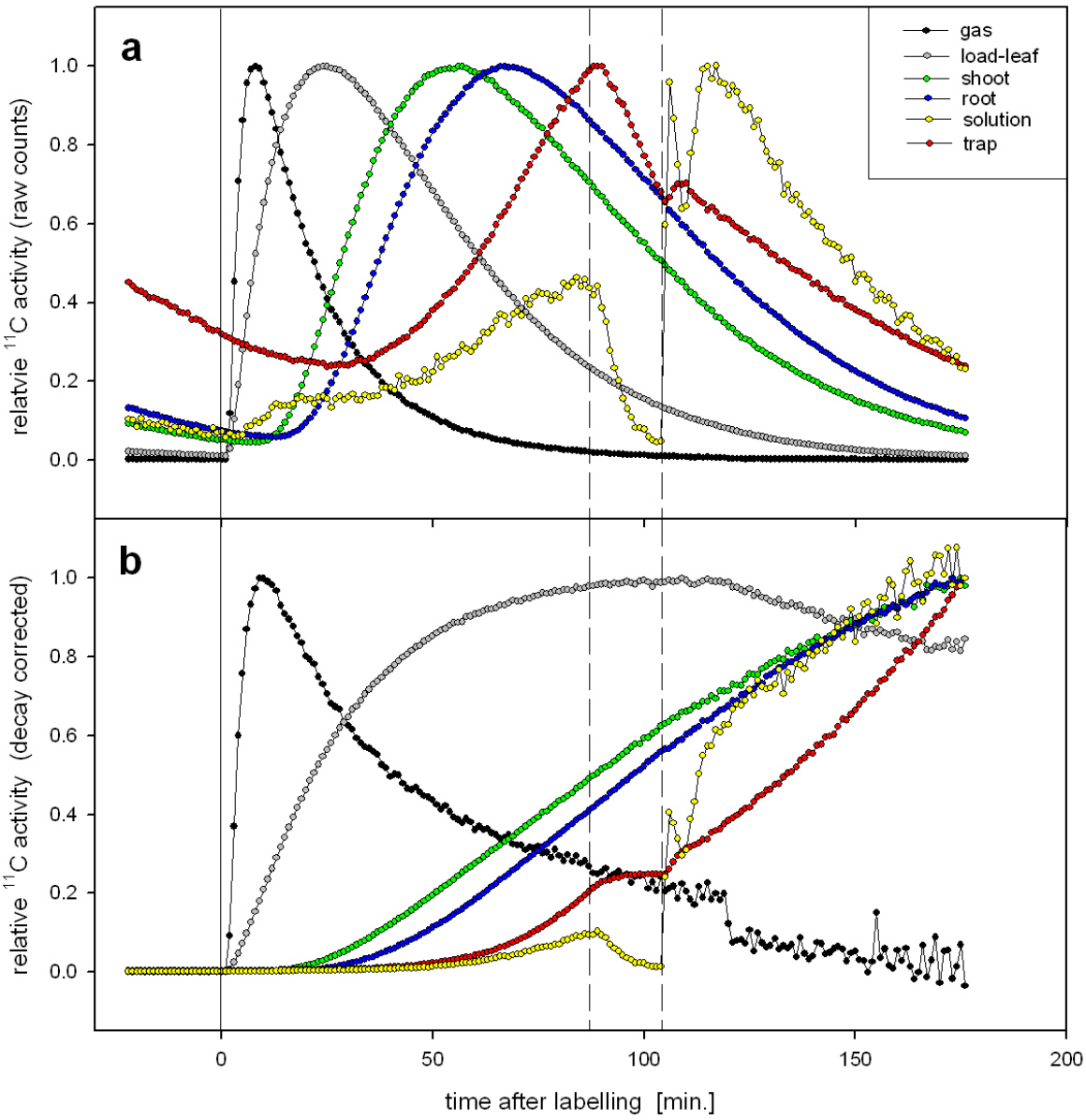

Figure 5. Count of decays at different compartments during a typical $^{11}CO2$ pulse labelling experiment. A new pulse of $^{11}CO_2$ was applied to the load leaf at 0 min; (a) data without decay correction (b) the same data corrected for decay. The counts are not corrected for different sensitivities. Each plot was normalized with the data from the maximum=1. After 98 min the pump which transport the solution between root compartment and provsioning bottle was stopped for 17 min.

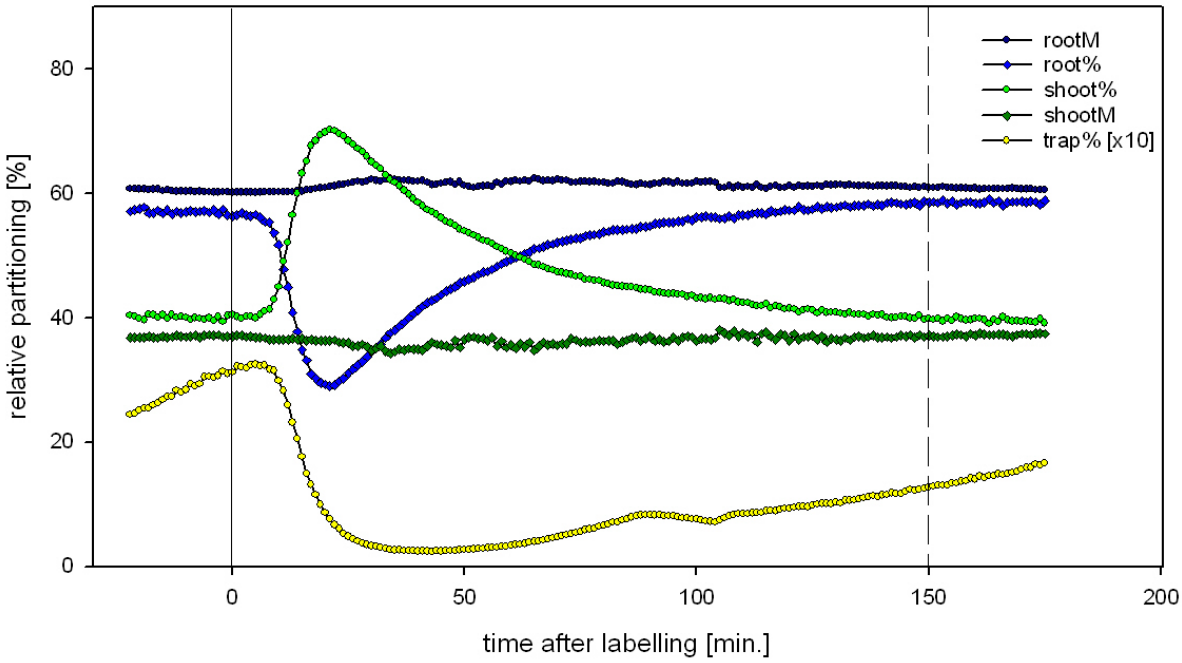

Figure 6 Partitioning of ^{11}C tracer in a plant at different compartments analysed with two different methods over a time period of 180min after labelling. The plots “shoot%”, “root%” and “trap%” show the percentage of mobilized activity in the shoot (without load-leave), the root compartment and the CO2-trap, respectively (total 100% at each time point). The plots shootM and rootM show time-variation of partitioning as calculated by input-output analysis. The respiration data are too slow to permit analysis (see text). The data from the CO_2-Trap are multiplied by the factor 10. There is activity before the analysed pulse which remains from a previous pulse of ^{11}C.

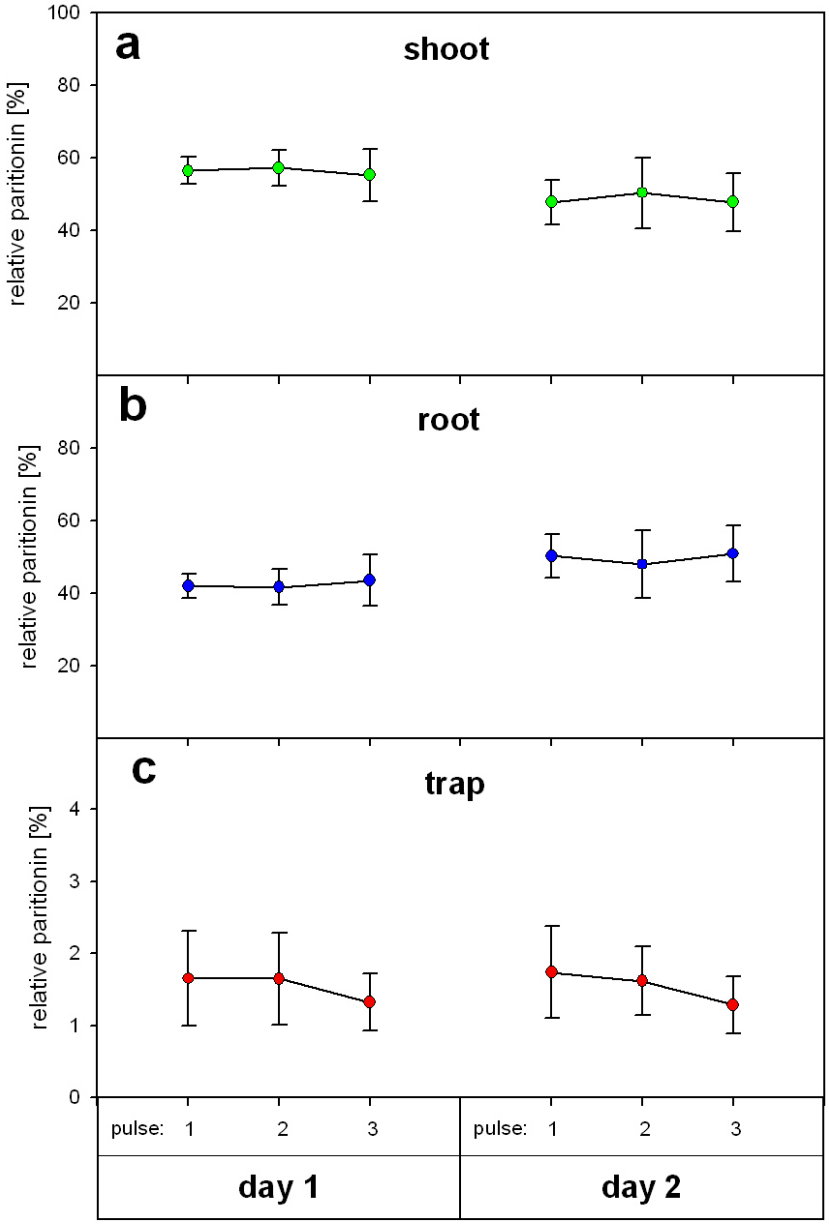

Figure 7. Partitioning of ^{11}C tracer after its mobilization from the load leaf in barley at three different regions after pulse labelling. (a) shoot fraction, (b) root fraction, (c) root respired fraction 150 min after labelling over two days (means±SD, n=5 for shoot and root, n=6 for respirated CO_2).

2.5 Discussion

2.5.1 Split-Root Rhizotron

The developed split-root rhizotron allowed the measurement of root growth under controlled conditions. Further, the experiment with DsRed and Gfp labelled Pseudomonas strains verified that the separation between both root compartments of the Rhizotrons was efficient and avoided transfer of micro-organisms from one compartment to the other over a period of 8 days. Prevention of cross contaminations with micro-organisms allow to investigate local and systemic effects in future studies. In other split-root studies the separation between two root halves was not proven and therefore immigration of bacteria into sterile root compartments cannot be excluded (cit). This is critical as even low numbers of bacteria can be sensed by a plant and induce changes in rate and composition of exudates or other changes in plant physiology (Walker et al. 2003). Further, certain soil bacteria like Bacillus spp. are known to interact with plants without direct contact through the release of volatile compounds such as 2-3-butandiol (Ping & Boland 2004). Because the tested system is gas tight even volatile compounds can be excluded; volatile compounds released by plant roots are unlikely to induce untreated roots or the shoot. However, such a response has been demonstrated for tobacco (Heil et al. 2007).

2.5.2 ^{11}C Measurements and Analyses

Results of this study demonstrated that the developed rhizotrons were suitable for conducting ^{11}C tracer experiments. The flat geometry of the rhizotrons in relation to the distance of the detectors lead to uniform sensitivity through the entire root compartment, much better than the commonly used circular planting pots. The shielding gave an efficient separation of the different compartments of interest so that see-through was negligible, and it was possible to analyse data with Minchin's input-output analysis. The

root partitioning estimated from the input-output analysis allowed to delineate the fraction of recently fixed carbon that is being delivered to roots at each time. Without this procedure it only the fraction of activity in each region at each time point can be analysed, but these results are of limited value as they vary at different times after tracer-labelling, and this variation is not a variation in the plant's behaviour.

We measured a slight daily increase in the fraction of carbon allocated to roots. This was likely due to the emerging new source leaves in the upper stem axis, so that the loaded leaf was providing roots with an increasing fraction of carbon. However, this daily increase of root partitioning was not statistically significant.

2.5.3 Respiration

In previous studies ^{11}C has been used to investigate root respiration by calculating the respired fraction of tracer at particular time points (Thorpe *et al.* 1998b). Also in the present study the respired fraction was calculated 150 min. after labelling because Input-output analysis is not suitable to analyse respiration because the process is too slow in comparison with tracer decay. A further problem of the developed setup is that the used polypropylene material of the rhizotron absorbs CO_2, as clearly shown in the calibration measurement of the exudation experiment (Fig. 4). The activity of tracer remaining in the rhizotron underestimates the actual respired fraction, however, only slightly. This may be overcome by using a more inert material, like glass or teflon, which does not absorb CO_2, or simply by correcting the calculations.

2.5.4 Exudates

Minchin and McNaughton (1984) demonstrated that ^{11}C can be used to analyse exudation in cereals; 200 min after pulse labelling about 10% of the carbon allocated to

barley roots was released by the roots. We also detected the tracer in the rhizosphere solution 150 min after labelling, but the signal was not high enough to allow separation from the background level and therefore could not be quantified. This difference in exudation rates between our data and those of Minchin and McNaughton (1984) might be explained by the fact that we measured earlier and that we used sterile plants. Indeed, it has been demonstrated that exudation rates under sterile conditions are much lower compared to non-sterile conditions (Baber & Martin 1976; Farrar *et al.* 2003). Furthermore, Barber and Gunn (1974) demonstrated that sterile grown cereals exuded less carbohydrates and amino acids when they were grown hydroponically without any solid substrate or presence of micro-organisms. The low exudation rate of ^{11}C labelled compounds therefore partly can be explained by the missing biotic and abiotic root stimuli. In addition, the solution volume of the entire system was high resulting in a strong dilution of the exudates thereby reducing the detection level.

The use of the developed split-root rhizotron is not restricted to ^{11}C experiments, the systems might be used e.g. to analyse the consequence of plant-microbe interactions for other parameters like gene expression and biosynthesis of specific compounds. The split-root setup is especially suitable to investigate if induced plant responses are local or systemical, providing information about the mechanisms involved. The transparent cover plate of the rhizotron also allows root growth to be measured under hydroponic conditions (Henkes *et al.* 2008). An interesting application would be to combine measurement of root growth and ^{11}C to study how root growth and allocation of carbon to the root are related.

Overall, the developed split-root system allowed successful analysis of the allocation of recently fixed carbon towards the root system in sterile split-root conditions *in vivo*.

Further, carbon released from the roots could be followed. However, only the respired fraction could be quantified; soluble exudates were present in trace amounts only. Separation of the two root halves allowed to confine micro-organisms to one root half over a period of several days.

3. Jasmonic acid treatment to part of the root system is consistent with simulated leaf herbivory, diverting recently-assimilated carbon towards untreated roots within an hour

3.1 Abstract

It is known that shoot application of jasmonic acid (JA) leads to an increased carbon export from leaves to stem and roots, and that root treatment with JA inhibits root growth. Using the radioisotope ^{11}C we measured JA effects on carbon partitioning in sterile, split-root, barley plants. JA applied to one root half reduced carbon partitioning to the JA-treated tissue within minutes, whereas the untreated side showed a corresponding—but slower—increase. This response was not observed when instead of applying JA, the sink strength of one root half was reduced by cooling it: there was no enhanced partitioning to the untreated roots. The slower response in the JA-untreated roots, and the difference between the effect of JA and temperature, suggest that root JA treatment caused transduction of a signal from the treated roots to the shoot, leading to an increase in carbon allocation from the leaves to the untreated root tissue, as was indeed observed 10 min after shoot-application of JA. This supports the hypothesis that the response of some plant species to both leaf and root herbivores may be the diversion of resources to safer locations.

3.2 Introduction

Plants can allocate recently fixed carbon (C) to, for example, reproduction, growth, storage or the synthesis of defence compounds. When recently fixed carbon is allocated to growth, it can be invested into new photosynthetic or reproductive tissue, or allocated to root growth that occurs particularly in the elongation zone of the root tips to increase uptake of nutrients and water (Pritchard *et al.*, 2004). However, roots may also store

photoassimilates or may synthesize defence compounds. It has been suggested that, in response to herbivory on leaves, plants may very rapidly alter carbon partitioning patterns in favour of the lower stem or into roots for storage to be used for later regrowth or reproduction (Strauss & Agrawal, 1999; Babst *et al.*, 2005; Schwachtje *et al.*, 2006). The diverted resources may also protect the existing tissue, through lignification or thickening of the stem (Hudgins *et al.*, 2004; Babst *et al.*, 2005). While resistance traits like the synthesis of defence compounds prevent plants from being consumed, or reduce the extent of feeding by herbivores, tolerance traits reduce the fitness-impact of herbivores on the infested plant. Tolerance behaviour in response to herbivory is thought to be associated with a change in carbon partitioning (Babst *et al.*, 2005; Schwachtje *et al.*, 2006). In response to herbivory, signalling cascades are activated in the plant that lead to the formation of jasmonic acid, a signalling compound involved in a multitude of plant responses. Therefore, jasmonic acid is frequently applied to plants in an attempt to mimic herbivory, as are caterpillar oral secretions (Thaler *et al.*, 1996; Baldwin, 1996; Rose & Tumlinson, 2005). Both treatments have been shown to mimic the induction of plant resistance by herbivores when applied to the leaves (McCloud & Baldwin, 1997; Halitschke *et al.*, 2001; Roda *et al.*, 2004). When leaves of the woody perennial *Populus tremuloides* (Babst *et al.*, 2005) were treated with jasmonic acid, and leaves of the annual *Nicotiana attenuata* (Schwachtje *et al.*, 2006) were treated with caterpillar oral secretions, the distribution of photoassimilates changed immediately in favour of the roots. However, it appears that methyl jasmonate, the methyl ester of jasmonic acid, is not responsible for the changes in carbon partitioning in *Nicotiana attenuata*. An antisense genotype with a silenced β-subunit of SnRK1 protein kinase was shown to be

involved in the regulation of assimilate transport to the roots and was not regulated by methyl jasmonate (Schwachtje *et al.* 2006).

Given that localised treatment of roots with high concentrations of 1-10 µM jasmonic acid is known to inhibit root growth, for example in tomato root cultures (Tung *et al.*, 1996), *Arabidopsis* (Staswick *et al.*, 1992), potato (Ravnikar *et al.*, 1992) and in spruce (Regvar & Gogala, 1996), whereas much lower concentrations of 10^{-3} µM can promote the frequency of lateral root initiation and elongation (Tung *et al.*, 1996), we expect changes in sink strength that may also affect carbon partitioning. However, depending on the developmental stage of the plant, the concentration of jasmonic acid applied and the duration of the experiment, the effect of jasmonic acid on root biomass may vary (van Dam *et al.*, 2004).

Our aim was to investigate the effect of shoot application of jasmonic acid on shoot/root partitioning of recently fixed carbon compared with the effect of root application. Therefore, we compared the carbon partitioning response to jasmonic acid treatment of shoots with that of partial roots. We also examined the effect of lowered root temperature, hypothesizing that cooling parts of the root system would reduce root metabolism and thus the sink strength of the root: this would provide a reference for the effect of jasmonic acid treatment that may include a signalling function throughout the plant. Changes in partitioning of recent photoassimilates were determined by applying the radioisotope ^{11}C as $^{11}CO_2$ to source leaves. The transport of the ^{11}C in the plant can be measured *in vivo* by scintillation detectors because of the high energy of its decay products. Repeated experiments with a single plant at high time-resolution were possible because there is no build-up of tracer with repeated applications due to the short half-life

of 20 min for ^{11}C. To allow separation of a sterile root system in two fractions, we developed a tightly sealed split-root chamber design in combination with an ^{11}C detection system to determine short-term changes in carbon distribution within split-root barley plants.3.3 Material and Methods

3.3.1 Plant Material

Barley (*Hordeum vulgare* L.) plants of the variety 'Barcke' (Irnich Inc., Frechen, Germany) were grown from seeds harvested in 2003. To avoid microbial contamination that may affect carbon partitioning within the plant, seeds were sterilized prior to planting: Approximately 200 seeds were incubated in 50 ml 50% H_2SO_4 for one hour, shaken to remove the glumes, and then rinsed three times with bi-distilled water to remove the sulphuric acid. Seeds were subsequently sterilized with a freshly prepared 2% $AgNO_3$ solution for 20 min on a shaker at 200 rpm. Then in a sterile laminar-flow cabinet, the seeds were washed once with sterile 1% NaCl solution, once with $Aqua_{bidest,}$ again with NaCl, and 5 times with $Aqua_{bidest}$ to remove the AgNO3 completely.

To verify sterility, the seed were germinated on agar plates (20 on each) with 50 ml 1/10 NB-NMAS (Page, 1976) 0.8% agar in darkness for 4 days at 20°C. The germinated seed were checked visually for contamination with microorganisms: only sterile seedlings were transferred singly into individual autoclaved glass tubes (length 135 mm, 25 mm diameter) containing 50 ml sterile 50% Hoagland solution. A silicon closed-cell foam rubber stopper (VWR, Darmstadt, Germany; with a longitudinal slit for the plant stem) sealed roots, seed and shoot-base into the glass tube to exclude microorganisms from the roots. The plants were grown at 60% RH with a 16-h day (100 µE m^{-2} s^{-1}, 25°C) and 8-h night (20°C).

After 7 days, each plant was transferred into its autoclaved two-chamber rhizotron. The roots were divided in two roughly equal parts and inserted into the Y-junction leading to the two chambers of the rhizotron (Fig. 1). Sterile silicon grease (Baysilone, Bayer, Germany) was used to isolate both sides. The rhizotrons were filled on each side with 300 ml 50% Hoagland solution, buffered with 5 mM MES adjusted to pH 5.8 with KOH. Fresh Hoagland solution was added as necessary through a sterilizing filter. Plants were taken for radiotracer experiments after 5-7 days when there were 3 mature leaves. Light intensity was 350 µE m^{-2} s^{-1} at the load leaf and 300 µE m^{-2} s^{-1} at the rest of the shoot.

3.3.2 Split-Root Rhizotrons

To allow plant cultivation under sterile conditions, the Split-Root Rhizotrons were constructed from autoclavable materials and autoclaved for 20 min at 120°C before use. The main body (330 mm height, 235 mm with, 18 mm depth) was built from polypropylene, the transparent cover plate out of 6 mm thick polycarbonate (Fig. 1). To avoid anoxia during the experiments, both root halves were aerated with sterile filtered air.

3.3.3 ^{11}C Labelling

Two to three days prior to a ^{11}C labelling experiment, a plant was transferred to the climatic chamber for adaptation. It was connected to the ^{11}C-labelling system at least 16 hours before the start of the measurement, to ensure that it had fully recovered from mechanical disturbance before it was labelled. The second leaf was sealed with 2-pot silicone rubber (Xantopren VL, Heraeus Kulzer, Hanau, Germany) in a cylindrical Plexiglas™ chamber (70 mm length, 18 mm diameter) and labelled three times with about 100 MBq $^{11}CO_2$ in air at 5 h, 7.5 h and 10.5 h into the light phase. Plants were

treated during the second application of ^{11}C when tracer activity in the root detectors was maximal (i.e. equal rates of decay and arrival), ~60-70 minutes after the start of labelling, giving a good measure of transport changes. The $^{11}CO_2$ was produced with a Baby cyclotron in the Research Centre Jülich.

3.3.4 ^{11}C detection and analysis

Scintillation detectors were positioned within radiation shielding to be uniformly sensitive to well-defined parts of the plant. The counts were corrected for background, dead-time and their different sensitivities to equal amounts of tracer. The measured plant parts were the shoot excluding the load leaf, and both root portions. Strips of clear 4 mm-thick Plexiglas™ were placed around the shoot of the plant to ensure that ß+ radiation escaping from the plants was annihilated near its source (Minchin *et al.*, 2002).

The data were analysed by the 'input-output' method for the analysis of carbon-11 tracer profiles (Minchin & Troughton, 1980). The method estimates the transfer function for movement of tracer through a pathway in the plant (Minchin & Thorpe, 1989: Minchin & Thorpe, 2003), and by accounting for radioisotope decay the analysis quantifies the transport of 'recently assimilated carbon'. In the analysis, the input was the total mobilised tracer (the sum of the three plant parts), and the output was the tracer entering a specific sink (either or both root portions). The steady-state gain of the transfer function is the fraction of the mobilised photosynthate reaching the sink that is partitioning into that sink. To allow comparison between plants, treatment responses were normalised to the value observed at the time of treatment.

3.3.5 Jasmonic acid treatment

To investigate how external application of Jasmonic acid (JA) affects the C partitioning between shoot and root, and within the root system, we treated barley plants with this phytohormone either on the shoots or on the roots. For the root treatment, 5 ml of a JA stock solution containing 3 mM JA (Sigma, Steinheim, Germany) and 0.6% EtOH (v:v) was added to the hydroponic solution in one half of the rhizotron through a sterile filter to give a final concentration of 50 µM JA. One root half was exposed to this solution throughout the subsequent measurement time. Both root halves were aerated with sterile-filtered air taken from outside the plant cabinet and vented outside. Completely untreated plants were used as control plants. To account for possible effects of EtOH or Triton-X 100 on C-partitioning, both compounds were applied in the same concentration and manner to a second set of barley plants as controls. For the JA shoot experiments, 200 µl of a solution containing 1 mM JA in 1% EtOH and 0.125% (v:v) Triton-X 100 were applied with a pipette to form small droplets on the leaf surface (similar to (Arnold & Schultz, 2002).

3.3.6 Root cooling

For root-cooling experiments, the hydroponic solution of one root chamber was cooled with a stainless steel tube (350 mm length, 6 mm dia) as a heat-exchanger receiving water from a temperature-regulated waterbath (Typ: F32-MC, Julabo, Seelbach, Germany) set at 14°C. With the pump set to maximum speed, a chamber temperature of 15°C was reached within 20 min (Fig. 4). The non-cooled, chamber maintained a constant temperature of 26°C, because of the low thermal conductance of the plastic walls of the rhizotrons; even after 120 min of cooling, no temperature change in the

solution of the non-cooled root chamber was detectable. Plants where both roots were not cooled were used as controls.

3.3.7 Root elongation

An image of the split root system was taken through the transparent front plate of the rhizotron every 5 min using a CCD camera (Sony XC-ST75; Sony, Köln, Germany) with a chip-resolution of 700 x 480 pixels. JA root treatment was administered to one root half as described above. The root elongation measurements were performed for 48 hours with three independent plants.

3.3.8 Statistical analysis

Normalized partitioning measurements were analysed using repeated measurement analysis of variance (rm-ANOVA) with time as the repeated factor. The normalized partitioning to individual root portions was analysed independently, by comparing each root half separately with the mean of control root halves. The data of the control root halves were tested for homogeneity of variance (Levene Test). Statistical analyses were carried out using SAS 9.1 (Cary, Florida, USA).

3.4 RESULTS

3.4.1 Effect of JA on root growth

Application of 50 µM JA to one root chamber completely stopped the elongation of the treated barley roots (Fig. 1). No increase in root length of primary or lateral roots was detectable within 48 hours after JA application. In contrast, the untreated roots continued to grow.

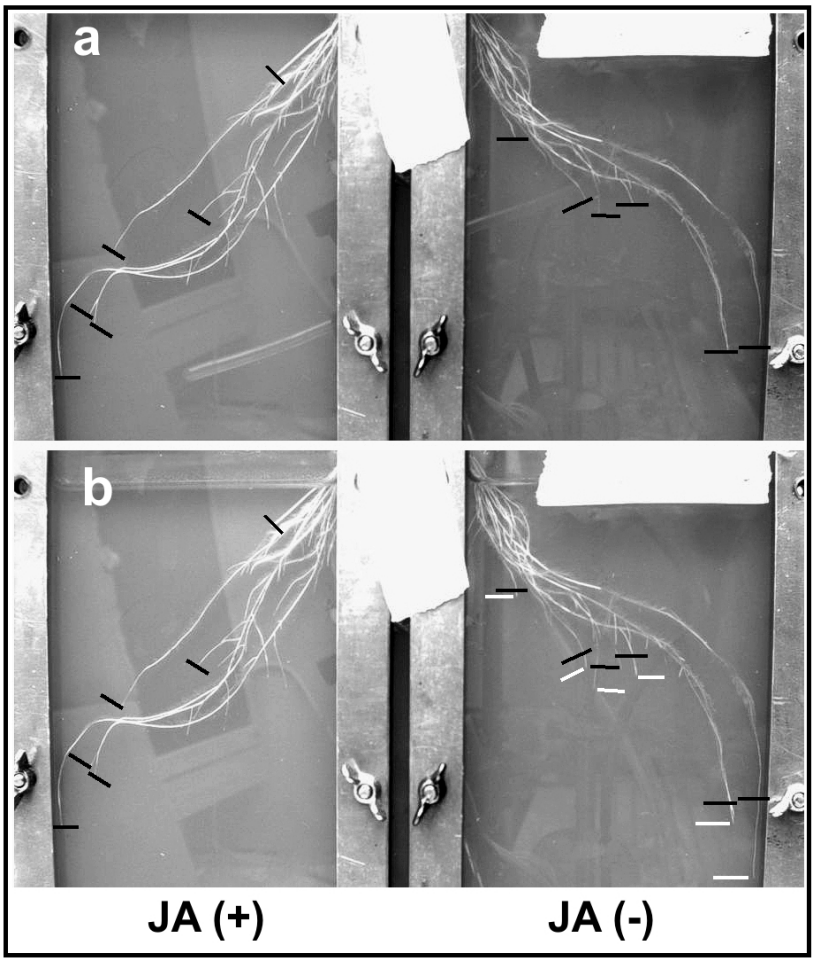

Figure 1. Effect of application of 50 µM jasmonic acid (JA) to part of a barley root system on root elongation. The root system is shown in a rhizotron (a) before treatment and (b) 48 hours after treatment. Root chambers: JA(+) treated, JA(-) untreated. Black lines mark the positions of some root tips at the time of treatment; white bars the position

of growing tips after 48 hours. Aeration was interrupted briefly while taking the photographs.

3.4.2 Effect of JA root treatment on C-partitioning

Our barley plants invested on average 51% of the ^{11}C mobilized from the load leaf into their root system (data not shown), which is comparable to other ^{11}C tracer studies with barley seedlings (Minchin *et al.*, 1994). There was a slight reduction in C-partitioning over the time of measurements (Fig. 2), with both root portions of untreated control plants showing a similar reduction. Because there were no statistically detectable differences between the two portions, they were combined for all controls. Treatment of one root portion with 50 µM JA led to a rapid reduction of its ^{11}C partitioning compared with the root portion in control plants (Fig. 2; Table 1); partitioning to the untreated portion of the same plants increased but with a longer delay. The magnitudes of decrease and increase were similar: 120 min after treatment, the treated roots received 11% less than in control plants ($F_{1,17}$ =13.07, P = 0.002), and the untreated roots received 11% more ($F_{1,17}$ =12.67, P = 0.0024). However, first significant differences were detected for the treated roots (compared with controls) after a delay of 30 min, whereas first significant differences for untreated roots were only detected after 50 min (Fig. 2). Because the magnitudes of increase and decrease of root partitioning were indistinguishable, we expected no change in ^{11}C partitioning to the entire root. Fig. 5 confirms there was no change in the fraction of mobilised ^{11}C partitioned to the entire root system after JA treatment of one root half, compared with control plants. This lack of response occurred even though the treated fraction of the root system varied from 38 to 62%.

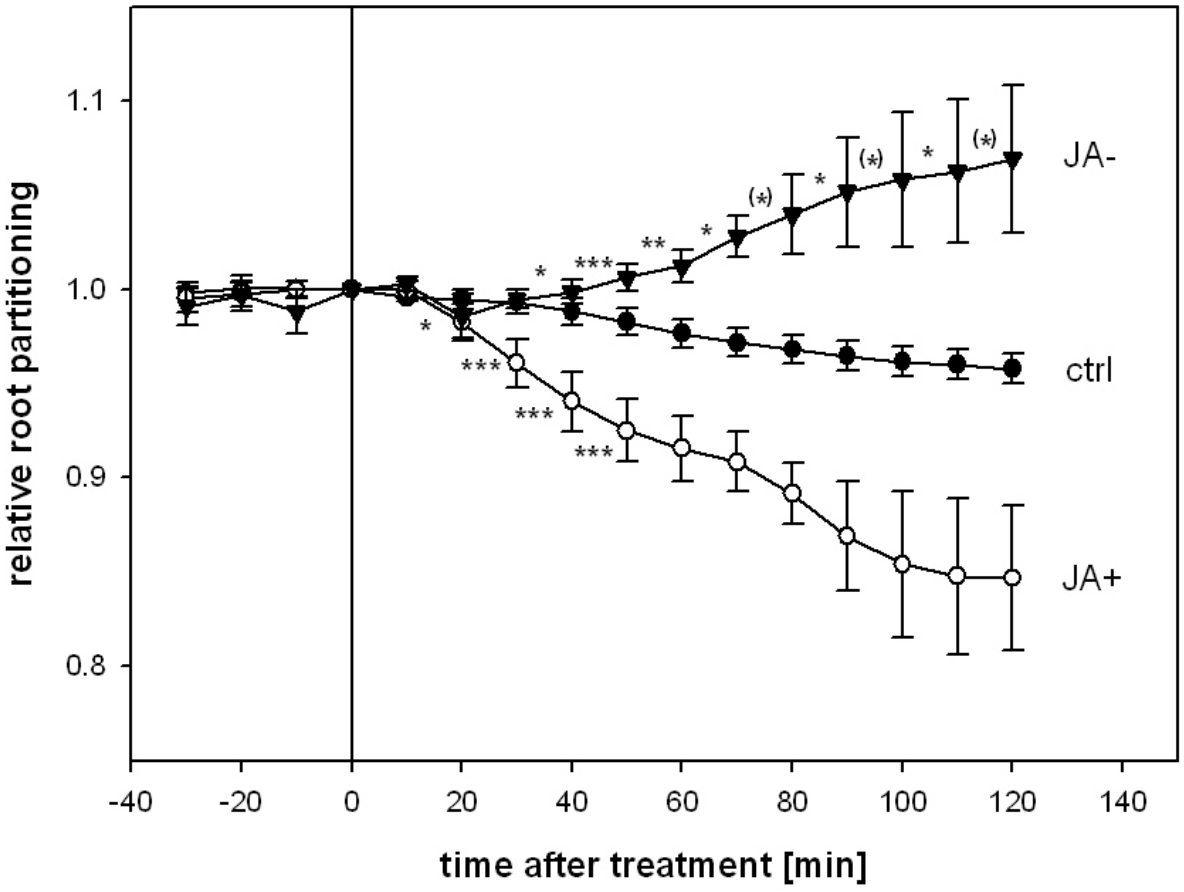

Figure 2. Effect of application of 50 µM jasmonic acid (JA) to one part of the barley split root system on carbon partitioning between both root halves, showing the normalised fraction of recent photoassimilate (mobilized from the $^{11}CO_2$ labelled leaf) that was delivered to each root portion. The data were normalised by setting the relative root partitioning to 1 at the time of treatment and adjusting all values by dividing each measurement by its value at the time of treatment t=0. Ctrl shows the mean of the two root portions of untreated control plants. The two portions of the split-root plants are designated JA(+), JA(-) for the JA-treated plants. (Mean±SE, n=12 for control plants, n=7 for JA treated plants). Asterisks indicate significant changes in time compared to the control (i.e. different slopes). ($^{(*)}P$<0.1, *P<0.05, **P<0.01, ***P<0.001).

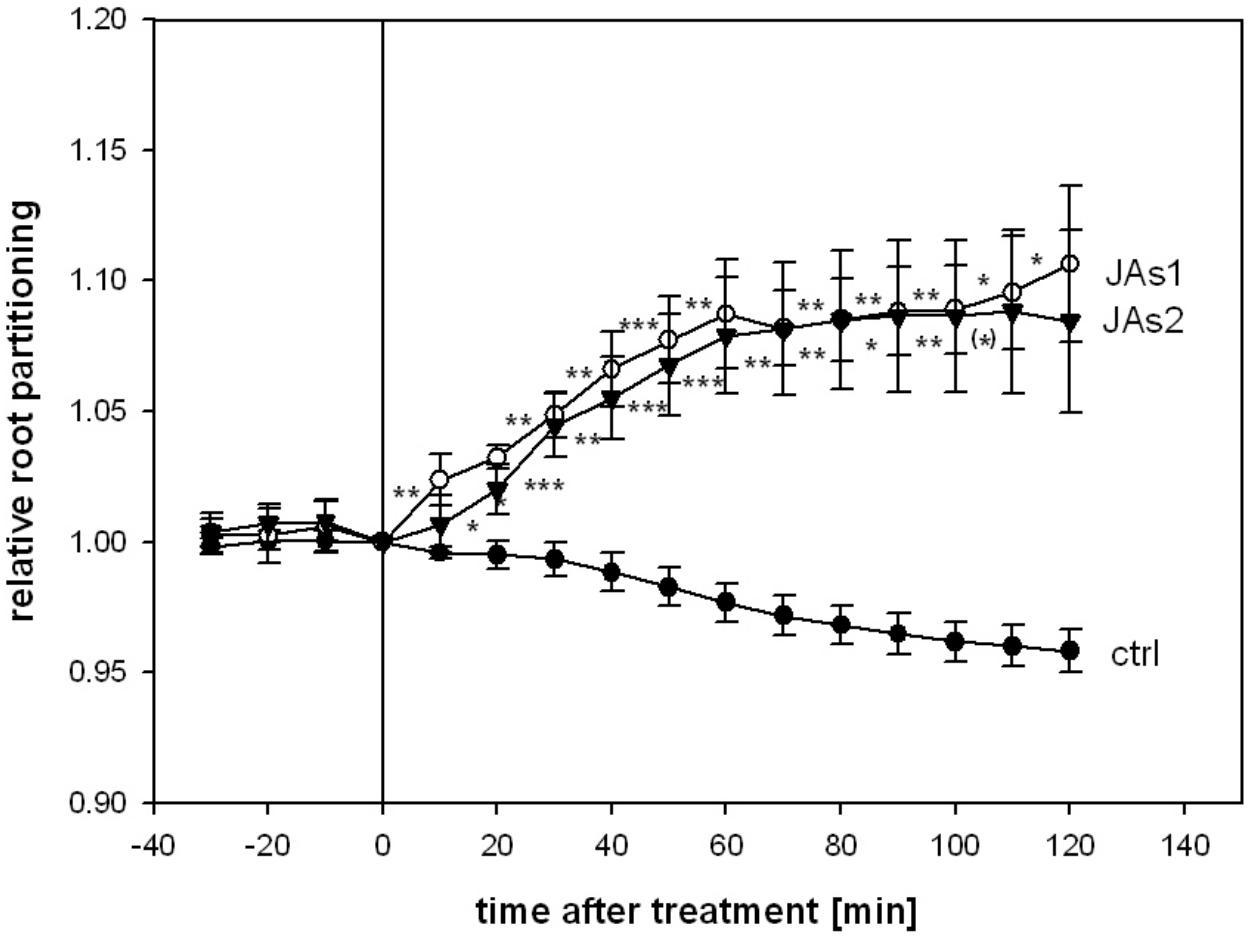

Figure 3. Effect of 1 mM jasmonic acid (JA) application to the shoot on the fraction of mobilized ^{11}C-tracer that was transported to roots in split-root barley plants. The data were normalised at time of treatment. Ctrl shows the mean of the two root portions of untreated control plants. The two root portions of the split-root plants are designated JAs1 and JAs2 for the JA-shoot-treated plants. (Mean±SE, n=12 for control plants, n=6 for JA treated plants). Asterisks indicate significant changes in time compared to the control (i.e. different slopes). ($^{(*)}P$<0.1, *P<0.05, **P<0.01, ***P<0.001).

3.4.3 Effect of JA shoot treatment on C-partitioning

Application of 1 mM JA to the shoot induced a very rapid increase in ^{11}C allocation to the roots with no difference in allocation between the two root portions (Fig. 3, Table 1). Within 10 min after JA application, root partitioning had significantly increased compared with that in untreated plants (P=0.007): after 120 min the C-partitioning was increased by 14%. Control experiments with application of Triton-X and EtOH (no JA) to the shoot showed no significant effect on ^{11}C partitioning.

3.4.4 Effect of root cooling on C-partitioning

The cooling treatment reduced the temperature of the cooled roots by 10ºC within 10 min, while the temperature of the untreated roots remained unaffected (Fig. 4a, Table 1). Correlating with the decline in temperature, partitioning of ^{11}C to the cooled roots declined quickly (Fig. 4b) and was significantly reduced (P≤0.001) within the first 10 min after onset of cooling. After 120 min, the ^{11}C partitioning to cooled roots was reduced by 19% compared with that in control plants. Despite the reduction in ^{11}C partitioning to the cooled roots, no change in ^{11}C partitioning to the untreated roots was detectable compared with roots of control plants and therefore the total partitioning to the roots was significantly reduced (Fig. 5).

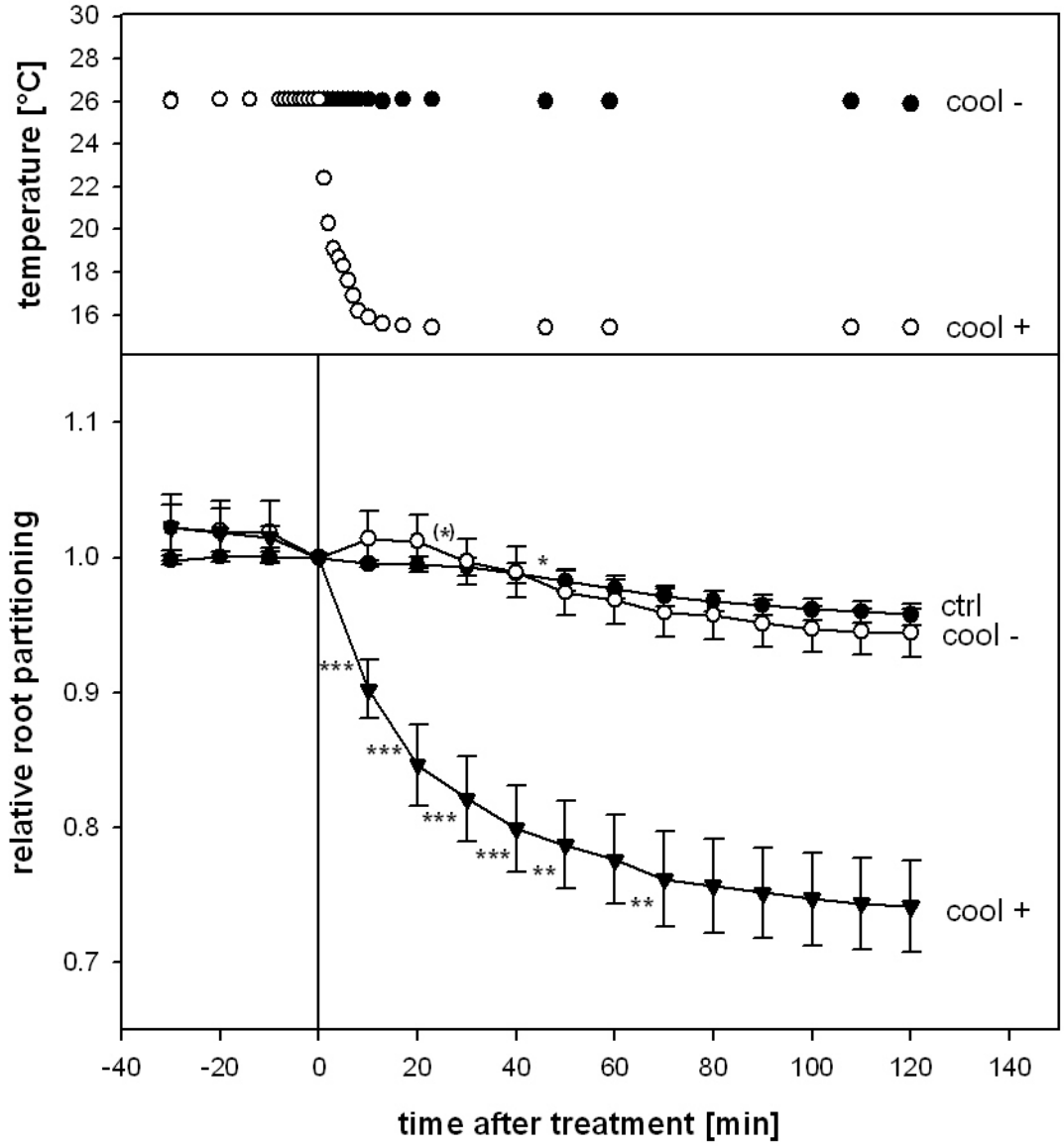

Figure 4. Effect of cooling part of the root system of split-root grown barley plants on the fraction of ^{11}C-tracer that was transported to each root portion. (**a)** Temperature of the two hydroponic solutions. (**b)** Relative ^{11}C partitioning of each root portion. The data were normalised at time of treatment. Ctrl shows the mean of the two root portions of untreated control plants. The two root portions of the split-root plants are designated cool+, cool-, for the treated plants. (Mean±SE, n=12 for control plants, n=7 for root cooled plants). Asterisks indicate significant changes in time compared to the control (i.e. different slopes). ($^{(*)}P$<0.1, *P<0.05, **P<0.01, ***P<0.001).

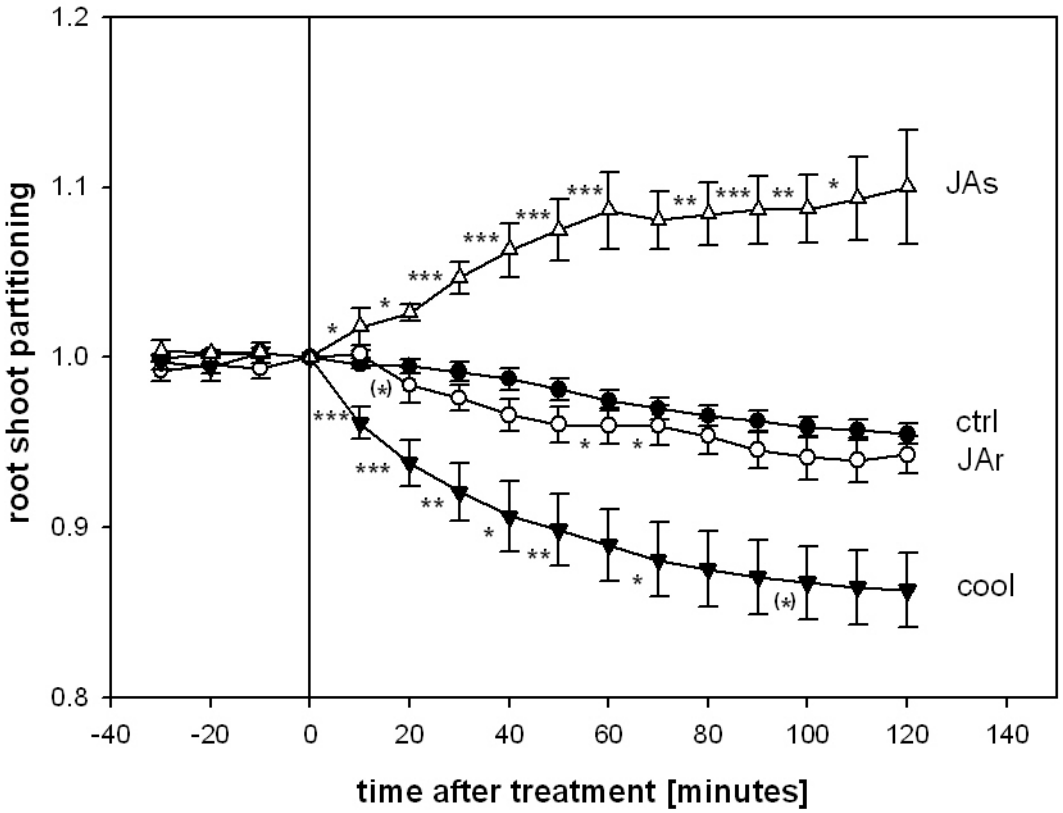

Figure 5. A comparison of shoot treatment by jasmonic acid (JA) with part-root treatment (by cooling or by JA) on the fraction of ^{11}C-tracer transported to the entire root system of split-root barley plants**.** The data were normalised at time of treatment. ctrl: control plants; JAr: 50µM JA treatment of one root half; JAs: JA treatment of the shoot; cool: plants where one root portion was cooled. (Mean±SE, n=12 for control plants, n=7 for JA root, n=6 for JA shoot; n=7 for root cooled plants). Asterisks indicate significant changes in time compared to the control (i.e. different slopes). ($^{(*)}P$<0.1, *P<0.05, **P<0.01, ***P<0.001).

3.5 DISCUSSION

Our aim was to compare the effect of exogenously applied JA to leaves or roots on the partitioning of recently fixed carbon. It has been shown for leaf-treatment with JA that carbon export is enhanced, for example in poplar and tobacco (Babst *et al.*, 2005; Thorpe *et al.*, 2007), and partitioning of the exported carbon changes in favour of roots (Babst *et al.*, 2005; Schwachtje *et al.*, 2006). To our knowledge there is no corresponding information on carbon partitioning in response to root treatment, even though local treatment of roots inhibits root growth, so that 'inhibition of root growth became the most prominent assay for screening of mutants affected in respect of JA signalling' (Wasternack, 2007). Further, it has recently been reported that in very young *Nicotiana attenuata* seedlings, shoot application of JA or caterpillar oral secretions is followed rapidly by a significant but transient reduction in root elongation rate (Hummel and colleagues, unpublished) and, within a day, an increase in defence compounds such as nicotine (Hummel *et al.*, 2007). In addition, amino acid composition and sugar content are affected by JA application within 7 days (van Dam & Oomen 2008). Our method employing the short-lived isotope ^{11}C quantifies the fate of "mobilised tracer" (the tracer carbon that has exited a labelled leaf), giving the fraction of that mobilised tracer that eventually enters each root portion (the balance of the mobilised tracer remains within the shoot) (Minchin & Thorpe, 1989; Minchin & Thorpe, 2003). The analysis accounts for isotope decay but, because the isotope has a half-life of only 20 min, the tracer reflects only recent photoassimilate and not all the carbon that is moving. Our measurements thus give 'partitioning of recent photoassimilate' that mainly consists of sucrose. Nevertheless, in a split root barley plant, it can be assumed that *changes* in partitioning of recent assimilate in treated roots, relative to untreated roots, reflect

changes in the partitioning of *total carbon*, since we can expect both old and new carbon arriving from the shoot into the crown of graminaceous plants to be distributed in the same ratio between all parts of the root system (Williams *et al.*, 1991), unlike many dicotyledons where the vascular architecture is sectorial (Orians, 2005). This combined use of short-lived isotopes, which allow repeated and non-invasive monitoring of plant function, with the facility to maintain sterile hydroponic split root systems, is expected to form a valuable platform for the study of biotic interactions.

In our experiment root elongation was inhibited locally by 50 µM JA, which is in accordance with observations on the entire root systems of *Arabidopsis* and tomato plant roots treated with JA at similarly high concentrations (Staswick *et al.*, 1992; Tung *et al.*, 1996; Creelman & Mullet, 1997). Associated with this inhibition of root elongation, there was a highly significant reduction in carbon partitioning to the JA-treated part of a root system, within the first 30 min of the treatment. After a further time-delay of 30 min, partitioning to the untreated roots increased significantly: the consequence of this compensatory interaction between the two root systems was no significant overall change in root/shoot partitioning. Such short-term changes in carbon distribution, where one sink benefits at the expense of another, have been reported frequently (Pickard *et al.*, 1979; Thorpe & Lang, 1983), and are commonly utilized in horticulture. However, (Minchin *et al.*, 1994) reported that cooling one part of a barley split-root system caused *no* compensatory increase in carbon partitioning into the untreated roots, despite a strong reduction of carbon partitioning to the cooled roots. Because plant growth conditions can affect source-sink interactions by altering carbon status by altering the relative priority of sinks (Minchin & Thorpe, 1996), and by affecting the cellular pathway for radial exchange with phloem (Hayes *et al.*, 1987), we investigated whether this

difference in response was related to the nature of the treatment, or to the physiological state of our barley plants that were grown with sterile roots and under about 50% lower irradiance compared with those of Minchin *et al.* (1994). Because our plants showed no compensatory response between root portions when one portion was cooled (Fig. 4). the differences between JA and cooling responses cannot be ascribed to growing conditions, rather to the nature of the treatment.

The lack of a compensating response in untreated roots after cooling one root portion of split-root barley was explained by a model of osmotically-driven Münch phloem transport through water-impermeable tubes with saturable carbohydrate (and water) unloading at sinks (V_{max}) (Minchin *et al.*, 1993). In their split-root plant model, with sinks near saturation, and with cooling merely reducing V_{max}, no compensating increase in partitioning is expected in the non-cooled root until some time later when, for example, gene expression responds to sugar levels (Chiou & Bush, 1998). In this model, carbon partitioning responds to mass action and fluid dynamics, without the need for a chemical signal.

Because this compensatory response of sinks does occur after partial JA root treatment, but not in response to cooling, we conclude that JA caused more widespread physiological changes than mere cooling. We suggest that the JA or a related signal, moves to the shoot in response to root application where it promotes both, export and root partitioning, so root partitioning can be expected to change in response to leaf export alone, as has been seen in response to shading (Minchin *et al.* 1993); however, inhibition of local root elongation by the treatment makes for a lower carbon demand. Several facts are relevant. First, the response-time after JA treatment was much slower in the non-treated root, suggesting a delay in signal transport to the shoot, and for the

subsequent partitioning response. The additional time needed for a response (30 min) corresponded roughly to the response time after shoot treatment, suggesting that increased root partitioning was induced by a signal in the shoot. Second, it is likely that some JA or a conjugate will move to the shoot in the xylem (Babst *et al.*, 2005; Schwachtje *et al.*, 2006; Thorpe *et al.*, 2007; Babst *et al.*, 2008). Finally, leaf-treatment with jasmonate or caterpillar oral secretion increases the export of recent photoassimilate to the roots (Babst *et al.*, 2005; Schwachtje *et al.*, 2006; Babst *et al.*, 2008), and jasmonates or caterpillar oral secretion promote partitioning of photosynthate towards stem and roots (Babst *et al.*, 2005; Schwachtje *et al.*, 2006; Babst *et al.*, 2008). We attribute the lower carbon partitioning to JA-treated roots to the total inhibition of their elongation rate. Incidentally, we noted that the treated roots quickly took on a yellow coloration, quite unlike senescent roots, suggesting the presence of phenolic compounds like flavenoids, as we observed in response to biotic stress (Lanoue *et al.*, unpublished) which may correspond to the synthesis of defence compounds in response to shoot treatments, as observed in *Nicotiana* (Hummel *et al.* 2007). Assuming that JA treatment partially mimics both herbivore attack (Baldwin, 1998; Walls *et al.*, 2005; Hare & Walling, 2006) and also root attack (van Dam *et al.*, 2004; van Dam & Raaijmakers, 2006;) it seems possible that root treatment may simulate an attack by root herbivores, giving rise to a tolerance response in which resources are diverted to other roots that have escaped attack, a response analogous to that after leaf herbivory, where resources are diverted to a less vulnerable location than the shoot (Babst *et al.*, 2005; Schwachtje *et al.*, 2006). Mimicked herbivory on leaves led to an upregulation of invertase activity in the roots within 5 hours, suggesting that an increase in sugar-cleaving activity leads to an increased sink strength of roots (Schwachtje *et al*. 2006). Recent results

demonstrated that the allocation of recently fixed carbon in response to partial root pathogen infection in barley also led to an enhanced allocation of carbon to untreated roots without changes in shoot/root partitioning (Henkes *et al*., unpublished), suggesting that barley plants can adjust carbon partitioning within the root system in response to biotic stress.

We have demonstrated the benefit of a combined use of short-lived isotopes, which allow for a non-invasive, repeated monitoring of plant function with high time-resolution in combination with the facility to maintain sterile hydroponic split root systems for the study of biotic and abiotic stresses and sundry signalling compounds. In a split root system, JA treatment of one root portion resulted in a shift in carbon partitioning in favour of the other roots, this effect was not able to be achieved with cooling. Despite complete cessation of elongation of treated roots, their carbon import continued (at a reduced rate). We suggest that the diversion of resources to root systems is a tolerance behaviour exhibited following simulation of both herbivory and root attack.

4. Modification of carbon delivery to roots by *Fusarium graminearum* and its systemic repression by *Pseudomonas fluorescens* in barley

4.1 Summary

We investigated how infection of barley roots with the pathogen *Fusarium graminearum* affects allocation of recently fixed carbon from the shoot to infested and uninfested roots. We tested the hypothesis that detrimental effects of *F. graminearum* on carbon allocation will be attenuated by the colonization of roots with the biocontrol bacterium *Pseudomonas fluorescens*.

We established a hydroponic split-root system of barley and inoculated one root portion with *F. graminearum* in combination with the *P. fluorescens* strain CHA0 or the mutant CHA19 defective in the production of secondary metabolites. Bacteria were inoculated either together or in separate halves of the root system to separate local and systemic effects. Partitioning of recently fixed carbon was analysed using $^{11}CO_2$.

Plants reduced carbon allocation towards infected roots for the benefit of the non-infected ones. Local or systemic preinoculation with CHA0 annihilated the effect of *F. graminearum* on carbon allocation, whereas CHA19 did not repress the pathogen effect. Both local and systemic effects were of similar intensity.

Our results show that roots can divert carbon from infected towards uninfected roots, suggesting rapid first line defence against pathogens. *P. fluorescens* CHA0 can rapidly repress the effect of *F. gramineraum* on carbon partitioning locally and systemically, whereas the non-antibiotic producing CHA19 lacks this ability.

4.2 Introduction

The necrotrophic fungus *Fusarium graminearum* (Schwabe) is a devastating pathogen of barley and other cereals, causing significant yield losses by diseases known as “Fusarium head blight” and “root rot” (Parry *et al.*, 1995; McMullen *et al.*, 1997). In addition to a direct reduction of plant fitness by reduced seed number and grain quality through destruction of starch and protein (Sutton, 1982) as well as necrosis, the contamination of harvested grains with fungal toxins decrease the economic value of grains. Deoxynivalenol of the group of trichothecenes, a sesquiterpene produced by the fungus and Zearalenon are the most prominent fungal toxins (Bagi *et al.*, 2000; Mesterhazy, 2002). *Fusarium graminearum* is a saprophyte which grows on plant residues and does not require a living host. From these sources of contamination the fungal pathogen spreads and infects aboveground parts of plants like stems and ears but also roots of living plants, affecting the growth and functioning of the roots and their demand for carbon.

Plants are partially protected from microbial pathogens by constitutive as well as inducible defences. For example, lignification of the cell wall in wheat increases strongly in response to infection by *F. culmorum* (Kang & Buchenauer, 2000). However, to date no effective resistance to *F. graminareum* has been identified. In transgenic AtNPR1-expressing wheat the increased resistance against Fusarium head blight is associated with faster activation of defense responses when challenged by the fungus. In fungus-challenged spikes of AtNPR1-expressing wheat, PR1 expression is induced rapidly to high level. Furthermore, benzothiadiazole, a functional analog of salicylic acid, induced PR1 expression faster and to a higher level in AtNPR1-expressing wheat than in nontransgenic plants (Makandar, 2006).

Because resistant cultivars are currently not available, disease suppression by microbes as is known from suppressive soils play an important role (Haas & Defago, 2005). Rhizobacteria with beneficial effects on plants, i.e. plant growth promoting rhizobacteria (PGPR), can either directly provide nutrients or growth factors, or indirectly suppress plant diseases caused by microbial pathogens (Davison, 1988; Persello-Cartieaux *et al.*, 2003; Ping & Boland, 2004; Barea *et al.*, 2005). A group of well studied antagonists of soil-borne pathogens are fluorescent pseudomonads (Haas & Defago, 2005; Weller, 2007) which produce a wide range of secondary metabolites such as 2,4-diacetylphloroglucinol (DAPG), phenanzine, pyoluteorin and pyrrolnitrin (Haas & Keel, 2003). In particular DAPG plays a crucial role in suppressing plant pathogens due to its direct inhibition of pathogenic fungi (De Souza *et al.*, 2003). Besides their direct effect on pathogens, PGPR are known to trigger induced systemic resistance (ISR) (Bloemberg & Lugtenberg, 2001; Bakker *et al.*, 2003). One of these bacteria strains, *Pseudomonas fluorescens* CHA0, has been shown to induce systemic resistance in *Nicotiana* and *Arabidopsis*, resulting in the protection of plants against both leaf (Maurhofer *et al.*, 1994a; Iavicoli *et al.*, 2003) and root pathogens (Siddiqui & Shaukat, 2002).

Growth of *Fusarium*, like most soil microorganisms, is mainly limited by carbon. Therefore, we investigated how infection of barley roots with *F. graminearum* affects allocation of recently fixed carbon from the shoot to the infested and uninfested roots. By dividing the roots in a split-root system, the infection by microorganisms was confined to half of the root system, while the other half remained uninfected. Because pseudomonads are known to inhibit fungal pathogens of plants, we hypothesized that changes in carbon allocation induced by *Fusarium* are attenuated by preinoculation of roots with the antagonistic biocontrol bacterium *P. fluorescens* CHA0. We compared the

local and systemic effects of the antibiotic-producing *P. fluorescens* strain CHA0 to that of the isogenic GacS mutant CHA19 that lacks production of antibiotics (Blumer *et al.*, 1999). Rapid changes in carbon partitioning were investigated by application of $^{11}CO_2$ to a leaf and precisely monitoring C-allocation of the tracer in the plant in a non-invasive manner. The short half-live of the ^{11}C tracer of 20.4 min permits repeated labelling of each plant resulting in a high resolution of the plant response in time.

4.3 Material and Methods

4.3.1 Plant Material

Seeds of barley (*Hordeum vulgare* L. cv. “Barcke”, Irnich Inc., Frechen, Germany) were dehusked by incubation in 50% H_2SO_4 for 60 min under agitation, and washed three times with distilled water to remove the acid. Seeds were sterilized with a freshly prepared 2% $AgNO_3$ solution for 20 min on a shaker at 200 rpm, washed with a sterile 1% NaCl solution, with distilled water, again with NaCl, and 5 times with distilled water to completely remove remaining $AgNO_3$.

The seeds were germinated in darkness at 20°C on a diluted nutrient agar (agar 8 g l^{-1}, nutrient broth 0.8 g l^{-1} in Neff’s modified amoeba saline (NMAS; (Page, 1976). After 4 days seedlings were checked visually for contaminations with microorganisms. Sterile plants were transferred into silicon closed-cell foam rubber stoppers with a longitudinal slit (VWR, Darmstadt, Germany) which then sealed the roots into glass tubes (length 135 mm, 25 mm diameter) containing 50 ml sterile 50% Hoagland solution. The plants were grown at 60% relative humidity with 16 h day (100 µE m^{-2} s^{-1}, 25°C) and 8 h night (20°C). After 7 days each plant was transferred into a two-chamber split-root rhizotron.

The roots were separated in two roughly equal parts in the two chambers of the rhizotron, and each chamber was sealed with silicon grease (Baysilone, Bayer, Germany). The rhizotron chambers were each supplied with 300 ml 50% Hoagland solution containing 5 mM MES buffer (pH 5.8) and plants were allowed to grow for an additional 5-7 days until radiotracer experiments were started.

4.3.2 Fusarium inoculum

The pathogenic fungus *F. graminearum* (Schwabe) strain DSM 1095, isolated from *Zea mays* roots, was obtained from 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' (DSMZ; Braunschweig, Germany). The strain was kept on Luria-Bartani (LB) agar plates at 20°C. Prior to inoculation, a piece of hyphae material of the *F. graminearum* culture was taken from the agar plate and grown in liquid LB medium for 2 days at 20°C under agitation of 200 rpm, and adjusted to an OD_{600} of 0.45. The inoculum consisted exclusively of fungal hyphae, with no detectable macrospores. Depending on the treatment, 5 ml of this inoculum was introduced to one or both sides of the split-root system as described below.

4.3.3 Pseudomonas strains

Pseudomonas fluorescens CHA0 and its isogenic GacS deficient mutant CHA19, carrying a mini Tn7 chromosomal GFP insert (Jousset *et al.*, 2006), were kept routinely on nutrient agar (Blood agar base 40g l^{-1}, yeast extract 5 g l^{-1}) supplemented with 25 μg ml^{-1} kanamycin (Sigma, Germany). Prior to inoculation, bacteria were grown in NYB medium (nutrient broth 25 g l^{-1}, yeast extract 5 g l^{-1}) at 30°C with agitation of 200 rpm.

Late exponential phase bacteria were harvested by centrifugation (5300 g for 2 min), washed in saline solution (NaCl 9 g l^{-1}) and resuspended to an OD_{600} of 0.2 in NMAS.

4.3.4 Split-Root Rhyzotrons

Split-root rhizotrons (Fig. 1) were built from a single block of polypropylene (330 mm height, 235 mm width, 18 mm depth) with a polycarbonate transparent cover. Prior to each experiment, the rhizotrons were autoclaved for 20 min at 120°C and 1.1 bar. Each side of the rhizotron was filled with 300 ml of a sterile hydroponic solution containing 50% Hoagland, buffered with 5 mM MES adjusted to pH 5.8 with KOH. To avoid anoxia during the experiments, both root halves were aerated with sterile filtered air.

4.3.5 ^{11}C Labelling

Two to three days prior to the ^{11}C labelling experiments the plants were transferred to the climatic-chamber for acclimation. Fifteen hours prior to labelling the second leaf was sealed with 2-component silicone rubber (Xantopren VL, Heraeus Kulzer, Hanau, Germany) into a cylindrical Plexiglas™ chamber (70 mm length, 18 mm diameter), and allowed to recover from possible mechanical disturbances. The leaf was then labelled three times per day on two subsequent days with about 100 MBq $^{11}CO_2$ 5, 7.5 and 10.5 h after switching to the light phase. Light intensity was 350 µE m^{-2} s^{-1} at the load leaf and 300 µE m^{-2} s^{-1} at the rest of the shoot. Fresh Hoagland solution was added as necessary through a 0.22 µm filter. The $^{11}CO_2$ was produced with a Baby cyclotron in the Research Centre Jülich.

4.3.6 ^{11}C detection and analysis

Scintillation detectors (Bicron NaI(Tl) detectors, Saint-Gobain Crystals, Houston, Texas) were positioned within radiation shielding to be uniformly sensitive to well-defined parts of the plant. The counts were corrected for background, dead-time and their different sensitivities to equal amounts of tracer. Activity in the following plant parts were measured independently: (1) shoot, the complete shoot except the load leaf, (2) left root and (3) right root. Strips of clear 4 mm-thick Perspex™ were placed around the shoot of the plant, to ensure that ß+ radiation escaping from the tissue was annihilated near its source (Minchin *et al.*, 2002).

To analyze the ^{11}C tracer time-series, the 'input-output' method was applied (Minchin & Troughton, 1980). The method estimates the transfer function for movement of tracer through a pathway in the plant (Minchin & Thorpe, 1989; Minchin & Thorpe, 2003). By accounting for radioisotope decay the analysis quantifies the transport of 'recently assimilated carbon'. An input and an output flow of tracer define each pathway. We considered the mobilised carbon from the leaf as input, and tracer entering either of the root-portions (or their sum) as output. The steady-state gain of the transfer function is equal to the partitioning of the mobilised photoassimilate into the respective sink tissue. Partitioning values were normalised to facilitate comparison of treatment responses between plants, assuming that a response was proportional, by dividing each data of measurement by its value at the first measurement.

4.3.7 Treatments

Two to three-week-old barley plants were inoculated during the second pulse of ^{11}C labelling when tracer activity in the root detectors peaked (i.e. equal rates of decay and

arrival), 60-70 min after the start of labelling, giving a good measure of any immediate change in transport.

4.3.8 Fusarium graminearum infection

To investigate the effect of *F. graminearum* on the carbon partitioning between the two root halves, one side of the root system was inoculated with 5 ml of fresh prepared *F. graminearum* inoculum in LB medium in each experiment (Fus+). The control side (Fus-) received an equal quantity of sterile LB medium. This experiment was replicated eight times, and was denoted (Fus) when the ratio of both root halves was plotted to compare total shoot/root ratio (Fig. 2a, Fig. 3). In a second set of three plants the entire root system was inoculated with 10 ml of *Fusarium* inoculum (denoted Fus/Fus).

4.3.9 Pseudomonas fluorescens strains CHA0 and CHA19

To investigate if *P. fluorescens* induced a shift in the plant carbon allocation, 3 ml of a suspension of CHA0 or CHA19 (OD_{600} = 0.2) in NMAS medium were added during the second ^{11}C labelling to one side of the root system. The control side received 3 ml of sterile NMAS. This experiment was repeated nine times with the strain CHA0, and ten times with the GacS mutant CHA19.

4.3.10 Interaction between Fusarium graminearum and Pseudomonas fluorescens

To test for a local and systemic effect of *P. fluorescens* CHA0 upon subsequent infection by *F. graminearum*, one side of the root system was inoculated with one of the

pseudomonas strains as described above, and left for 48 h to allow the bacteria to colonize the roots. Then the plants were placed into the ^{11}C measuring system and *F. graminearum* was inoculated as described above, with the following combinations: (1) CHA0 and *F. graminearum* on the same root half to test the direct effect of *P. fluorescens* on subsequent infection by *F. graminearum,* (Fus&CHA0; n=5); (2) CHA19 and *F. graminearum* on the same root half as a control with a non-toxin producing mutant (Fus&CHA19; n=10); and (3) CHA0 and *F. graminearum* on different sides of the root system to test for a systemic effect of *P. fluorescens* on subsequent infection by *F. graminearum* (Fus/CHA0; n=5).

4.3.11 Statistical analyses

The individual values of the partitioning of mobilised carbon for each side of the root system, and the ratio between partitioning to the two sides of the root system, were analysed using repeated measurement one-way ANOVA, with the treatment as categorical predictor and time as repeated factor. Data for each time point were then analysed with an independent one-way ANOVA followed by a Tuckey's HSD test. Statistical analyses were carried out using STATISTICA 6.0 (Statsoft, Tulsa, USA).

4.4 Results

4.4.1 Effect of Fusarium graminearum on C partitioning

In all cases inoculation with *F. graminearum* led to a successful infection of the roots, as was visible by brown necrotic spots on the treated roots (Fig. 1). On the non-treated roots (Fus-) of partially root-infected plants no fungal infection was visible and no hyphae were detected in the hydroponic solution. Inoculation of one (Fus+) or both root halves

(Fus/Fus) had no effect on root/shoot partitioning of ^{11}C tracer within 28 h compared to control plants (Fig. 2a). However, ^{11}C partitioning to individual root-sides did change when only one root half was infected (Fig. 2b). ^{11}C partitioning to the untreated roots (Fus-) of infected plants was significantly increased by 33%, 35%, and 46% within 23.5 h, 24.5 h and 28 h after infection, respectively, compared to root halves of control plants. In contrast, partitioning to the infected root portion (Fus+) was reduced by 28% within 28 h after infection compared to control plants. The ratio (r) between partitioning to each root half of infected compared to uninfected roots [r = (^{11}C partitioning to Fus+) / (^{11}C partitioning to Fus-)] was also significantly affected (Fig. 3; Table 2). While the ratio between both root halves of control plants remained stable over time, the ratio of treated to untreated root halves was strongly decreased in the plants partially treated with *F. graminearum*.

4.4.2 Direct effects of Pseudomonas fluorescens CHA0 and CHA19 on C partitioning

Root inoculation with either the wild type strain CHA0 or the gacS-mutant CHA19 had no significant effect on C partitioning over 30 h. Neither the ^{11}C allocation from the shoot to the entire root system, nor partitioning between treated and untreated root halves differed from control plants (data not shown).

4.4.3 Interaction between Pseudomonas fluorescens and Fusarium graminearum

In all tested combinations *F. graminearum* successfully colonized the inoculated root halves in the presence of *P. fluorescens* visible by brown necrotic spots on the infected

roots. However, it was not possible to quantify the infection rate of the fungi during the ^{11}C measurement. Comparable to the single treatments with either *F. graminearum* or the *Pseudomonas* strains CHA0 and CHA19, C allocation from the shoot to the entire root system was unaffected in all the combined treatments of P. *fluorescens* and *F. graminearum*. However, preinoculation with *Pseudomonas* strains CHA0 strongly influenced the effect of *F. graminearum* on carbon partitioning. Preinoculation of a root half with the wild-type strain CHA0 2 d prior to inoculation of those roots by *F. graminearum* (Fus & CHA0) completely suppressed the negative effect of *F. graminearum* on carbon partitioning and no differences in the ^{11}C partitioning between both root portions compared to control plants were observed over 28.5 h (Fig. 3; Table 1, 2). This annihilation of the negative effect of *F. graminearum* on carbon partitioning not only occurred when the *Pseudomonas* strains CHA0 and *F. graminearum* inoculation were localized on the same root half but also occurred systemically, *i.e.* when one root half was preinoculated with CHA0 but inoculation with *F. graminearum* was conducted to the other root half (CHA0/Fus; Fig.3). This systemic effect was as strong as the direct local effect of CHA0 on suppression of the negative effect of *F. graminearum* on carbon partitioning (Tables 1, 2).

Unlike preinoculation with CHA0, a local preinoculation of roots with the GacS mutant CHA19, which produces no secondary metabolites (CHA19 & Fus), did not prevent changes in ^{11}C partitioning in response to infestation with *F. graminearum* (Fig. 3). Local preinoculation with CHA19 reduced the effect of *F. graminearum* on carbon partitioning only slightly by 18% after 28.5 h and the reduction in C partitioning remained between the values observed for the C partitioning in response to Fus and Fus&CHA0 treatments

(Fig. 3). The difference in the C partitioning between the treatments CHA19&Fus and CHA0&Fus increased with time (Table 1; Fig. 3).

Result Figures & Tables

Table 1. F- and p- table reporting the effects of treatments on the relative root carbon allocation over the entire experiment (Repeated measurement ANOVA). Significant effects ($p<0.05$) are highlighted in bold. Ctrl, no treatment to either root portion; Fus, *F. graminearum* on one root portion; Fus&CHA0, *F.graminearum* and CHA0 on the same root portion; Fus&CHA19 *F.graminearum* and CHA19 on the same root portion; Fus/CHA0, F. graminearum an CHA0 on different root portions.

factors	d.f.	*F*	*p*
Treatment	4, 27	7.15	**<0.001**
Time	4, 27	3.15	**0.017**
Treatment x time	16, 108	3.60	**<0.001**
contrasts			
Ctrl vs. Fus	1, 27	15.49	**<0.001**
Fus&CHA0 vs. Ctrl	1, 27	0.01	0.910
Fus&CHA0 vs. Fus&CHA19	1, 27	6.22	**0.019**
Fus&CHA0 vs. Fus/CHA0	1, 27	0.38	0.539

Tabel 2. Ratio between the partitioning to each roots portion at the different time points. Values for one time point that are followed by the same letter do not differ according to the Tukey HSD test (α=0.05). Ctrl, no treatment to either root portion; Fus, *F. graminearum* on one root portion; Fus&CHA0, *F.graminearum* and CHA0 on the same root portion; Fus&CHA19 *F.graminearum* and CHA19 on the same root portion; Fus/CHA0, F. graminearum an CHA0 on different root portions

time point	**1.5 h**		**4.5 h**		**23 h**		**25.5 h**		**28.5 h**	
Ctrl	1.017	a	1.015	a	1.048	a	1.017	a	0.980	ab
Fus	0.959	a	0.813	a	0.628	b	0.560	c	0.470	c
Fus&CHA0	0.967	a	0.931	a	1.081	a	0.989	ab	0.931	ab
Fus&CHA19	0.860	a	0.858	a	0.773	ab	0.675	bc	0.694	bc
Fus/CHA0	1.010	a	0.919	a	1.106	a	1.062	a	1.073	a

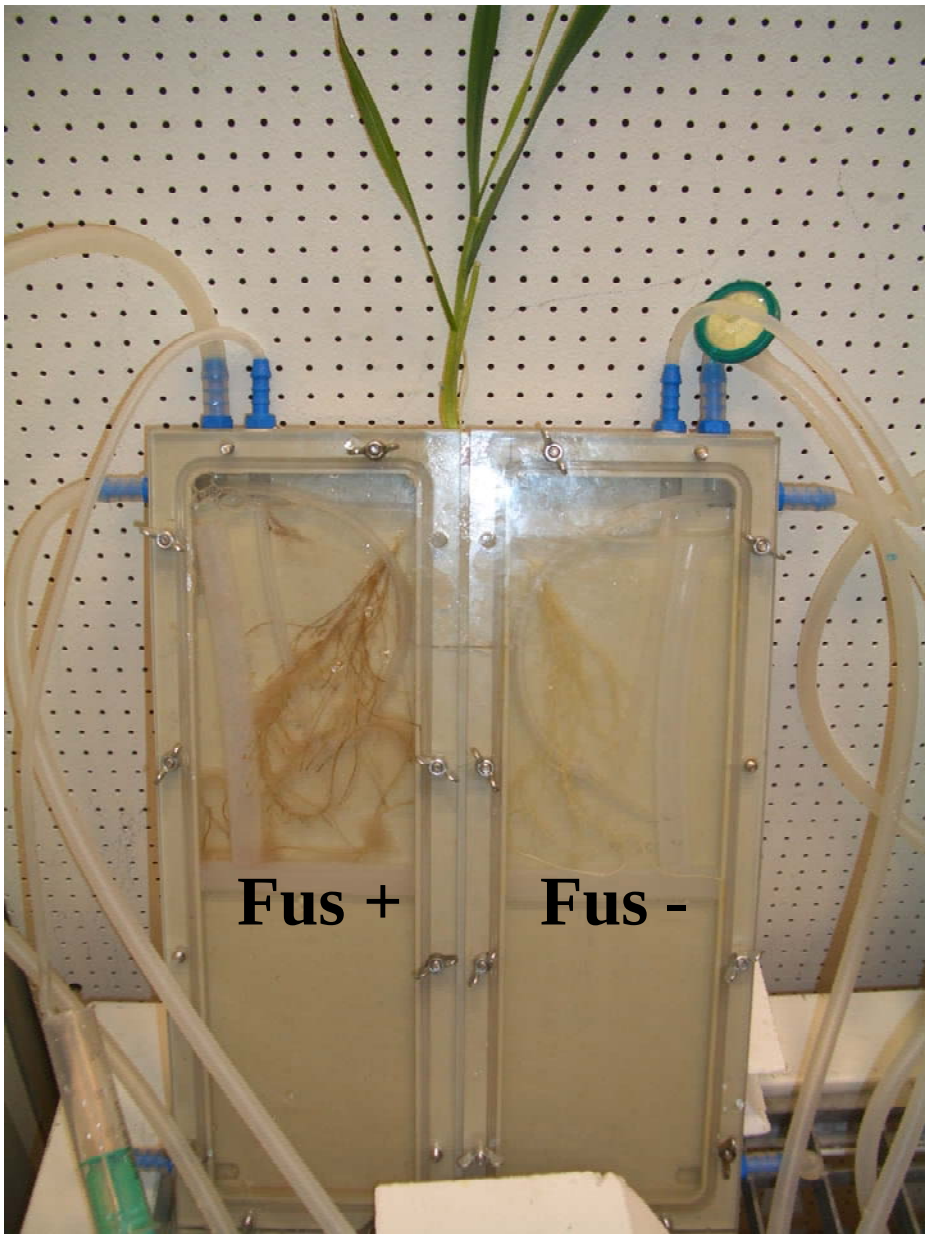

Figure 1. Split-root rhizotron that allows infecting half of the barley root system with *Fusarium graminearum* (Fus+) while the other half is kept sterile (Fus-). Barley plant 4 d after infection by *F. graminearum*. Aeration was stopped while taking the photograph.

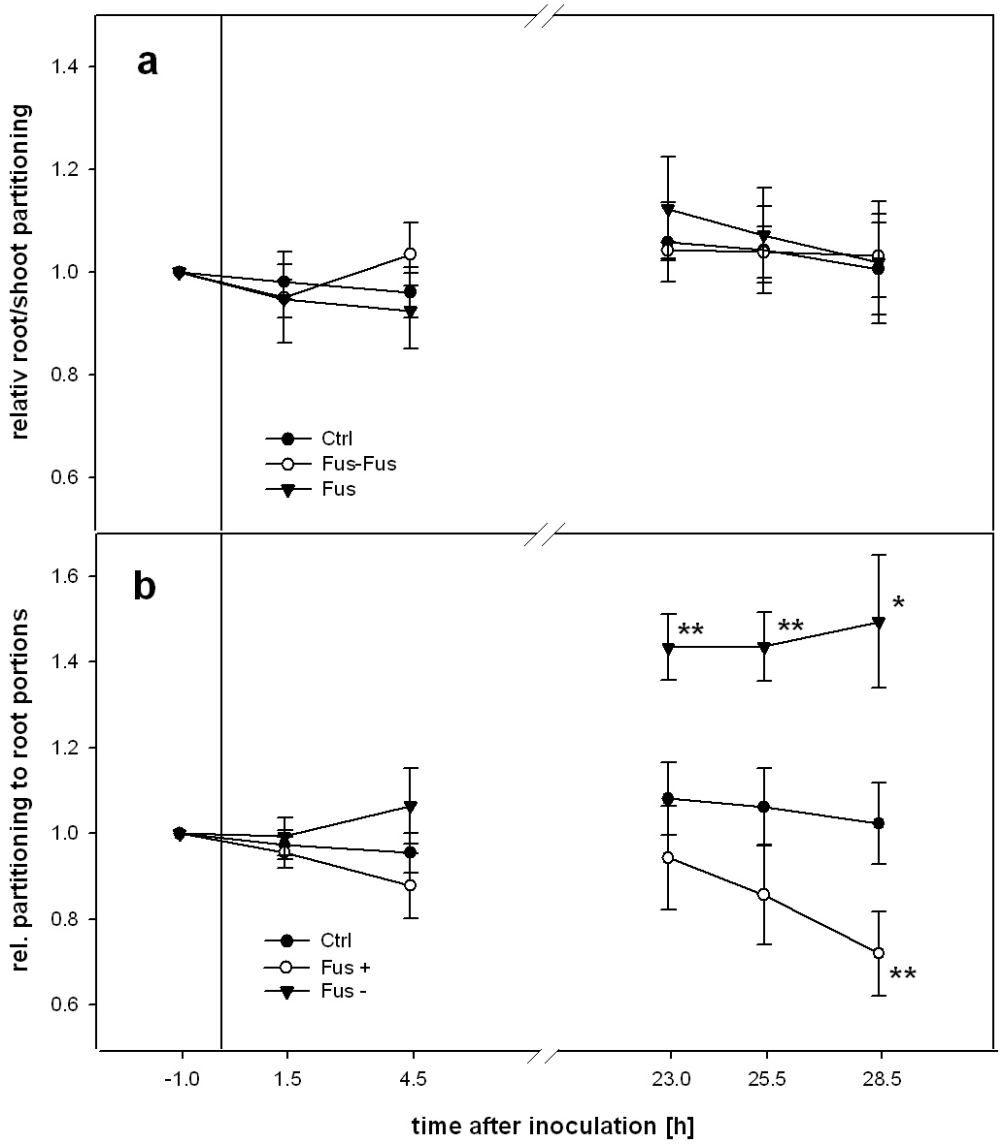

Figure 2. Effect of *Fusarium graminearum* infection of roots in a barley split root system on carbon partitioning to (a) the entire root system and (b) each half of the root system. Carbon partitioning was measured as the fraction of recent photoassimilate mobilized from $^{11}CO_2$ labelled leaf to each root half. Data were normalized at time of treatment. Ctrl, no infection on either root half; Fus, *F. graminearum* infection on one root half (Fus+, treated root half, Fus-, non-treated root half); Fus-Fus, *F. graminearum* infection on both root halves. Means ±SE, n=9 for Ctrl, n=8 for FUS, n=3 for FUS-FUS; significant difference between treatment and control were determined by Tukey's HSD test; *, $P<0.05$, **, $P<0.01$.)

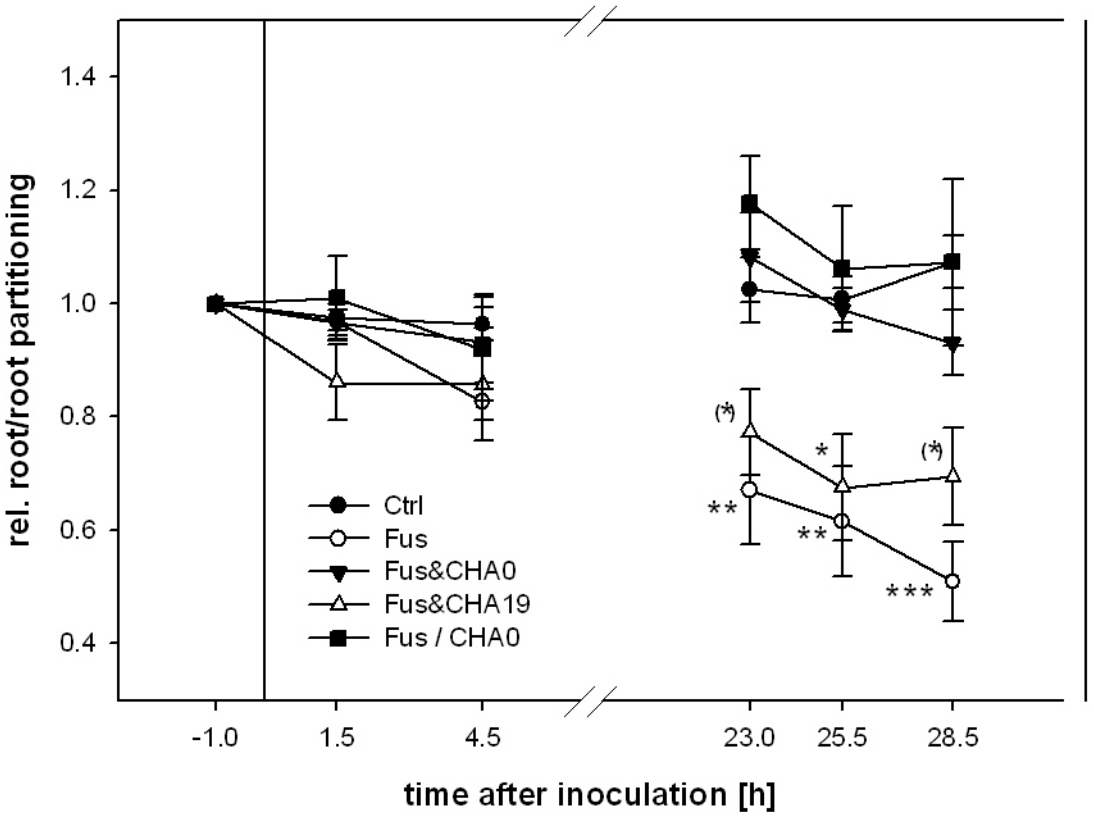

Figure 3. Changes in carbon allocation to barley roots by infection of *Fusarium graminearum* and its modification by preinoculation (2 days) with the *Pseudomonas fluorescens* strains CHA0 or CHA19. Carbon partitioning was measured as the fraction of recent photoassimilate mobilized from a $^{11}CO_2$ labelled leaf to each root half. Data were normalized at time of treatment. Ctrl, no treatment to either root half; Fus, *F. graminearum* treated root half; Fus&CHA0, after priming with CHA0 for 2 d (*F. graminearum* added to the same root half); Fus&CHA19, after priming with CHA19 for 2d (*F. graminearum* added to the same root half); Fus/ CHA0, after priming with CHA0 for 2 d (*F. graminearum* added to different root halfs to avoid direct contact between microorganisms). Means ±SE, n= 8 for Ctrl, n= 8 for Fus, n=5 for Fus&CHA0, n=6 for Fus&CHA19, n=5 for Fus/CHA0; significant difference between treatment and control were determined by Tukey's HSD test; $^{(*)}$, P<0.1 *, P<0.05, **, P<0.01.)

4.5 Discussion

4.5.1 Barley - Fusarium interaction

The ^{11}C tracer technique proved to be an ideal tool to study the dynamics and rapid changes in allocation of recently fixed carbon in barley plants challenged by the fungal pathogen *F. graminearum.* The method is non-invasive and suitable for repeated measurements of the same plant. Being both quantitative and highly sensitive, it allows detecting shifts in plant C allocation long before changes in plant biomass or morphology are becoming apparent. Our split-root system allowed confining the infection by microorganisms to only half of the root system, while the other half remained uninfected. The results showed for the first time that infection of one root portion with *F. graminearum* leads to a rapid shift in C partitioning within the root system towards uninfected roots, which is visible already 4.5 h past inoculation with *F. graminearum*. However, the total shoot/root allocation of carbon in response to inoculation with *F. graminearum* was not affected.

Previous experiments with cooling part of the root system showed no compensatory allocation of carbon to the non-cooled root portion (Minchin *et al.*, 1994; Henkes *et al.*, 2008), whereas application of jasmonic acid resulted in a redirection of carbon to the untreated root portions (Henkes *et al.*, 2008), comparable to the effect we observed in response to *F. graminearum* infestation. This suggests more physiological changes in response to *F. graminearum* infestation or Jasmonic acid treatment whereas cooling part of the root system will only reduce the sink capacity in the treated roots. Our results suggest fast recognition of the pathogen before or during infection that affects the entire plant. Microarray data demonstrate that inoculation with *F. graminearum* changes gene

expression within hours: defence-related genes are up-regulated during early fungal stress (Boddu *et al.*, 2006; Bernardo *et al.*, 2007) and PR-proteins are systemically expressed (Pritsch *et al.*, 2001), so plant-wide adjustments in carbon distribution can be expected as a consequence.

For biotrophic pathogens it is known, that they are able to stimulate movement of photoassimilates towards the infected sites (Hancock & Huisman, 1981). For necrotrophs such behaviour has not been reported. In split-root experiments with barley it was shown that two AM species increased the sink capacity for carbon of the infected root half (Lerat *et al.*, 2003). In contrast, infection of barley seedlings with powdery mildew had little effect on assimilate distribution and the percentage of carbon translocated from the shoot to the roots was only slightly reduced (Walters & Ayres, 1982). We showed allocation of carbon away from the infected barley roots to the uninfested roots, suggesting that resources are diverted to a less vulnerable part the root system indicating a defence strategy of the plant to belowground pathogen attack.

4.5.2 Pseudomonas - Fusarium interaction

It is well established that non-pathogenic root colonizing microorganisms may antagonistically affect plant pathogens, such as *Fusarium*. For example, it has been shown that the presence of non-pathogenic *Fusarium oxysporum* strains reduce the symptoms of pathogenic *Fusarium* infection (Fravel *et al.*, 2003). Split root studies, where pathogenic and non-pathogenic strains had no direct contact, demonstrate that Induced Systemic Resistance is important in this interaction. In a hydroponic tomato system, prior inoculation of one root compartment with the non-pathogenic *Fusarium* strain Fo47 reduced the extend of infection of the other root compartment by the

pathogenic *Fusarium* strain Fol8 from 54 % to 37 % (Fuchs *et al.*, 1997). No fungal infection of Fo47 could be detected in the non-treated root half or in the stem. Similar results were obtained from split-root experiments with water melons (Biles & Martyn, 1989), indicating that direct contact between non-pathogenic and pathogenic strain is not necessary for disease suppression.

Preinocculation with non-pathogenic bacterial strains showed similar effects on subsequent infection by pathogenic fungi. The *P. fluorescens* strain CHA0 is a well known effective biocontrol agent against a wide range of pathogens. The bacteria colonize root surfaces, and it has been shown to reach internal parts of tomato roots without detriment to the plant's health (Troxler *et al.*, 1997). *Pseudomonas fluorescens* induces systemic resistance by activating the jasmonic acid pathway (Pieterse *et al.*, 2002). In particular, production of diacyl-phloroglucinol (DAPG) strongly impacts various plant parameters such as root growth (De Leij *et al.*, 2002) and plant defences (Iavicoli *et al.*, 2003; Rezzonico *et al.*, 2007). Results of the present study demonstrated that *P. fluorescens* CHA0 inoculation represses carbon partitioning to *F. graminearum*.

Our results are in agreement with other studies which showed that PGPRs can induce systemic resistance against soilborne pathogens. In a split root system with cucumber, the inoculation of one side of the root system with pseudomonads impaired the growth of *Pythium aphanidermatum* on the non-treated roots (Chen *et al.*, 1998). In addition, two PGPR strains have been shown to induce resistance against the pathogenic fungus *F. oxysporum* in the same plant system (Liu *et al.*, 1995). Our results indicate that plants are able to rapidly adapt to complex and changing multipartite biotic interactions. Interestingly, we observed that the GacS mutant CHA19 of *P. fluorescens* lacking production of antibiotics (Blumer *et al.*, 1999) did not reverse the effect of *F.*

graminearum as much as the antibiotic producing CHA0 strain. Compared to the wild type, strain CHA19 is lacking ability to produce the secondary metabolite DAPG, which is suspected to induce systemic resistance in plants (Iavicoli *et al.*, 2003). However, the mutant strain CHA19 is also inhibited in the production of other GacS regulated compounds like phenanzines, pyoluteorin and HCN (Haas & Keel, 2003) that may be involved in the interaction. Therefore, the specific mechanisms involved in this plant - bacteria interaction need further investigation.

In conclusion, the use of ^{11}C tracer is a promising technique to investigate plant - microbe interactions. A strong advantage of this method is that it is non-invasive and that changes in plant carbon allocation pattern can be detected before changes in biomass become apparent. Our results show, that infection of barley roots with the pathogen *F. graminearum* did not affect the total shoot/root allocation of carbon. However, infection of one root half with *F. graminearum* lead to a rapid shift in C partitioning within the root system towards the uninfected half. We further demonstrated that *P. fluorescens* CHA0 can repress fungal effects on carbon transport in plants locally and systemically, whereas the *Pseudomonas* strain CHA19 that does not produce antibiotics elicits only marginal local (direct) effects.

5. General discussion

In the present work the effect of plant - microbe interactions on the partitioning of recently fixed carbon towards and within roots of barley (*Hordeum vulgare*) was investigated under hydroponic conditions. The allocation of carbon was analysed *in vivo* with repeated ^{11}C pulse labelling allowing to detect small changes in carbon partitioning with a high temporal resolution. To avoid contaminations a split-root rhizotron was established which ensured sterile conditions in the root compartments during the experiment and enabled a restricted inoculation of micro-organisms to specific parts of the root system. Using the split-root system the effect of an exogenous application of the stress-inducible phytohormone jasmonic acid (JA) to the shoot and root on the short-term partitioning of recently fixed carbon was investigated. In further experiments barley roots were challenged with the necrotrophic root pathogen *Fusarium graminearum* as well as with the bacterial biocontrol strain *Pseudomonas fluorescens* to elucidate effects of detrimental and beneficial microorganisms on carbon partitioning.

5.1 Carbon partitioning in plants

Plants allocate atmospheric CO_2 via the photosynthetic pathway into organic compounds. Higher plants rely on a transport system which permits the translocation of assimilates from places of carbon fixation like mature leaves (sources) to plant tissue with carbon demand (sinks) like roots or fruits (Wardlaw 1990; Lalonde, Wipf & Frommer 2004). In most plants carbohydrates are mainly transported through long distances as disaccharide sucrose (Milthorpe & Moorby 1969) via the vascular system (phloem) and need a fine tuned coordination to support all different organs efficiently. Carbon partitioning in plants can be influenced by a range of factors and the question

whether a particular organ or tissue is adequately supplied with assimilates and can fully express its growth potential (source or sink limitation) is not easy to answer (Wardlaw 1990). Carbon partitioning is strongly affected by the level of illumination of the plant because light intensity is directly linked to the source strength of leaves. During darkness also mature leaves temporally turn into carbon sinks which have to satisfy its own energy demand by the consumption of transitory starch (Zeeman, Smith & Smith 2007). During the increase of the light level leaves switch back to function as carbon sources, but prior to export carbon to other plant parts leaves have to refill their starch memory. These diurnal changes of carbon partitioning in plants was demonstrated for many plant species. In case of barley, which was used in this study, it was demonstrated that young plants need several hours of adaptation to light conditions to reach an equilibrium in carbon partitioning between shoot and root (Williams, Minchin & Farrar 1991). Also, the developmental stage of a plant has a strong influence on carbon partitioning. Whereas young cereals invest around 50% of the fixed carbon into their root system (Williams *et al.* 1991) this fraction is reduced in mature plants producing seeds (Roeb, Wieneke & Führ 1986). Since most physiological processes in plants are highly temperature depended, changes in temperature also strongly affect the source sink relationship in plants (Wardlaw 1990; Minchin, Farrar & Thorpe 1994;). This fact was also used in this study to manipulate the sink strength of barley roots by cooling the hydroponic solution.

Effects of developmental stage or abiotic factors (like temperature or light conditions) on carbon partitioning of recently fixed carbon has to be taken in account to avoid misleading results and suggestions when the effect of specific treatments on carbon partitioning are analysed in experiments. Therefore, plants in the same developmental

stage were grown under controlled conditions and treatments were performed at the same time points of the light-phase (4, 6.5 and 9.5 h after light switch) to receive reliable results.

In addition to abiotic conditions plants also respond to the presence of belowground biotic interactions with a change in carbon partitioning. This might be positive for a plant like colonization by mycorrhiza fungi (Wright, Scholes & Read 1998; Lerat *et al.* 2003;) and rhizobia (Thorpe, Walsh & Minchin 1998) or negative like the attack of herbivores (van Dam, Witjes & Svatos 2004; Schwachtje *et al.* 2006; Babst *et al.* 2008) and pathogens (Johnstone *et al.* 2005) or competition with neighbouring plants (Casper & Jackson 1997). Surprisingly, only few studies have been performed so far focusing on the short-term effects of biotic interactions in the rhizosphere of plants on carbon partitioning. The elucidation of effects of pathogens and biocontrol bacteria on carbon partitioning in plants is necessary to understand how plants respond to changes in environmental conditions and how this determines plant growth.

5.2 Establishment of ^{11}C analysis

We successfully established an experimental split-root system with barley as model plant which allows to investigate effects of plant - microbe interaction on the allocation of recently fixed carbon measured *in vivo* via the ^{11}C tracer technique. Barley was chosen as plant since it is an economically important crop and since it was used in previous studies on carbon partitioning. In addition, barley roots have a suitable anatomy for split-root experiments. Because of using polypropylene as material for the established rhizotron the sytem was easy to sterilise by autoclaving and ensured that the used rhizobacteria or pathogenic fungi inoculated to one root compartment were restricted to the inoculated side.

Our method employing the short-lived isotope ^{11}C quantifies the fate of "mobilised tracer" (the tracer carbon that has exited a labelled leaf), giving the fraction of that mobilised tracer that enters each root portion (Minchin & Thorpe 1989; Minchin & Thorpe 2003). Root respiration (Farrar *et al.* 2003) and exudation (Minchin & Mcnaughton 1984) had been measuerd before using ^{11}C, however, the experimental setup established in this study for the first time allowed to investigate carbon release by roots under sterile rhizosphere conditions.

5.3 Effect of jasmonic acid on carbon partitioning

The phytohorme jasmonic acid (JA) plays a central role as herbivore inducible signal molecule and is essential for the development of defence mechanisms in plants. The production of JA is strongly enhanced by the attack of chewing herbivores (Gatehouse 2002) and thus the application of JA or its methylester methyljasmonate (MeJA) on leaves has been routinely used to mimic aboveground herbivory (Baldwin 1996; Schwachtje *et al.* 2006) However, not only herbivores are able to induce the JA defence pathway. It has been shown, that also Plant Growth Promoting Rhizobacteria (PGPRs) like *Pseudomonas fluorescens* trigger the up-regulation of JA in *Arabidopsis thaliana*. Further, the application of high JA concentrations on plant roots was shown to inhibit root growth (Wasternack 2007). The results obtained in this study demonstrate the local inhibition of root elongation by root treatment with 50 µM JA, which supports similar results from previous studies (Ravnikar, Vilhar & Gogala 1992; Staswick, Su & Howell 1992; Tung *et al.* 1996; Creelman & Mullet 1997). The ^{11}C measurements clearly show that the JA induced growth inhibition of the treated root halves of split-root barley plants was linked to an reduced sink strength of the treated root part, which indicates that less carbon is required for root growth (Farrar & Jones 2000). In contrast to the treated root

part, the import of labelled carbohydrates to the non-treated root half increased about 30 minutes past JA application. These results contradict observations by Minchin *et al.* (1984) showing that the reduction of the sink capacity by cooling of one root half of split-root grown barley plants did not enhance the carbon export into the other root part. Plant growth conditions are known to affect source-sink interactions by either changing the carbon status and thus the priority of sinks (Minchin & Thorpe 1996) or by affecting the cellular pathway for radial exchange with the phloem (Hayes, Patrick & Offler 1987). Thus, we investigated whether the JA treatment or the physiological state of the used barley plants, with their roots grown under sterile conditions and with the shoots exposed to about 50% lower irradiance compared to that in the study of Minchin *et al.* (1994), was responsible for the observed import of carbohydrates into the non-treated root half. As described by Minchin *et al.* (1984) the performance of a cold treatment of one root half did not cause an increased carbon import to the other root part. This indicates that the compensatory response induced by the JA treatment is actively triggered by shoot mediated processes. Further, we investigated the effect of JA shoot treatment and observed a strong and immediate increase of carbon export into the roots. These results support other ^{11}C tracer studies showing that a foliar application of jasmonates result in enhanced carbon allocation towards the root system in tobacco and poplar (Babst *et al.* 2005; Schwachtje *et al.* 2006; Thorpe *et al.* 2007). Assuming that JA treatment partially mimics both herbivore attack of shoots (Baldwin 1998; Hare & Walling 2006; Hummel *et al.* 2007) and roots (van Dam *et al.* 2004; van Dam & Raaijmakers 2006), the JA root treatment presumably simulates the attack by root herbivores. Thus, our results provide evidence for tolerance responses analogous to plant responses to

herbivory, where resources are diverted to less vulnerable plant compartments (Babst *et al.* 2005; Schwachtje *et al.* 2006; Babst *et al.* 2008).

5.4 Effect of Fusarium *and* Pseudomonas *on carbon partitioning*

Infection with plant pathogenic *Fusarium* strains cause tremendous crop losses in cereals and other important crop plants world wide (Parry, Jenkinson & Mcleod 1995; McMullen, Jones & Gallenberg 1997). In this study we investigated effects of *F. graminearum* on partitioning of recently assimilated carbon in barley plants grown in a split root system by using the ^{11}C tracer technique. The results demonstrate that root infection by a necrothrophic plant pathogen results in a rapid reallocation of carbon within the root system towards uninfected roots, which already starts 4.5 h after inoculation with *F. graminearum*. The observed immediate response in carbon partitioning suggests recognition of the pathogen by the plant prior to or during plant infection. Interestingly, in case of an inoculation of the entire root system with *Fusarium* the root shoot partitioning of carbon was not affected. This indicates that the observed enhanced allocation of carbon to uninfected roots is an active plant mediated process which triggers the redirection of resources to uninfected root parts.

A number of studies have demonstrated that root colonizing microorganisms such as PGPRs may antagonistically affect the performance of plant pathogens (Bai & Shaner 1994; Fravel, Olivain & Alabouvette 2003). Results of the present study show that the preinoculation with the biocontrol wild type strain *P. fluorescens* CHA0 alters carbon partitioning in plants. PGPRs are known to inhibit pathogens by effectively competing for resources like nutrients or space or by synthesizing toxic compounds (Kloepper *et al.* 1980; Chet *et al.* 1990; Haas & Keel 2003). However, studies using split-root systems in which pathogens and PGPR strains had no direct contact demonstrated that also an

induced systemic resistance (ISR) plays an essential role in pathogen suppression (Zhou & Paulitz 1994; Leeman *et al.* 1995; Liu, Kloepper & Tuzun 1995; Siddiqui & Shaukat 2004). The present study supports that the direct contact between the biocontrol strain CHA0 and *F. graminearum* is not crucial to annihilate the effect of the pathogen on carbon partitioning.

The synthesis of secondary metabolites by *P. fluorescens* such as DAPG is known to be associated with the ability to repress plant pathogens (Haas & Defago 2005; Weller *et al.* 2007). Recently, it has been demonstrated that purified DAPG may trigger ISR in *Arapidosis* (Iavicoli *et al.* 2003). The used *Pseudomonas* wild type strain CHA0 produces a diverse mixture of secondary metabolites, with the production being controlled by the quorum sensing gacS/gacA sytem (Heeb & Haas 2001; Haas & Keel 2003; Lapouge *et al.* 2008).

To investigate whether bacterial secondary metabolites are responsible for the observed annihilation of *Fusarium* induced carbon reallocation by *Pseudomonas* the gasS deficient mutant CHA19 was used which lacks the ability to produce secondary metabolites (Jousset *et al.* 2006). The preinoculation with CHA19 did not repress the *Fusarium* effect on carbon partitioning in barley roots indicating that the bacterial synthesized secondary metabolites are involved in the inhibition of *Fusarium* effects. This supports previous results which describe that the production of bacterial secondary metabolites is involved in plant pathogen repression (Maurhofer *et al.* 1994). Particularly, DAPG has been suggested to be the responsible elicitor for plant pathogen inhibition. However, the used mutant CHA19 also lacks the production of other gacS related compounds like phenanzines, pyoluteorin and HCN (Haas & Keel 2003) which may also be involved in this interaction. Thus, for identifying the specific elicitor responsible for the

annihilation of *Fusarium* effects on carbon partitioning in barley plants further studies are necessary.

5.5 Conclusion:

The used ^{11}C tracer technique proved to be an indispensible tool to study the dynamics and rapid changes in the allocation of recently fixed carbon in barley plants challenged by varied biotic stress induced by JA and infection with root pathogens. The ^{11}C method is non-invasive and suitable for repeated measurements of the same plant due to the short half-live of the tracer. Being a quantitative and highly sensitive method it allows to detect shifts in plant C allocation before changes in plant biomass or morphology become visible. The used split-root system allowed confining the added microorganisms to one half of the root system, with the other half remaining sterile which enabled to distinguish between local and systemic effects. The results indicate that plants adapt and respond immediately to changes in biotic rhizosphere conditions by a fine tuned reallocation of assimilates. This enables the plant to optimize the investment of resources into growth and reproduction and thus decrease the negative effects of pathogens and herbivores.

This study for the first time combined a well controlled split-root system with the ^{11}C tracer technique to investigate plant-microbe interactions. The established methods and experimental systems are a promising tool to link ^{11}C measurements with other methods like high resolution root growth analyses in further experiments. This may allow to clarify whether changes in growth rate are controlling carbon import into roots or if the transport is affected by the plant which than leads to changed growth rate. Further, the established split-root system may be used to investigate other changes due to plant - microbe interactions on parameters like gene expression or production of secondary

metabolites. For such experiments, the temporally high resolution data from ^{11}C experiments could give precise information for identifying promising sampling dates.

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Curriculum vitae

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Eidestattliche Erklärung

Hiermit versichere ich an Eides statt, dass die vorliegende Dissertation ohne fremde Hilfe angefertigt und mich keiner anderen als die von mir angegebenen Schriften und Hilfsmittel bedient habe. Die benutzen Bakterien aus Kapitel 2 und 4 wurde von Alexandre Jousset transformiert.

Ich habe noch keinen weiteren Promotionsversuch unternommen.

……. (Gunnar Henkes)

Darmstadt, den 14. Mai 2008