Decomposer-plant interactions:

Effects of Collembola on plant performance and competitiveness

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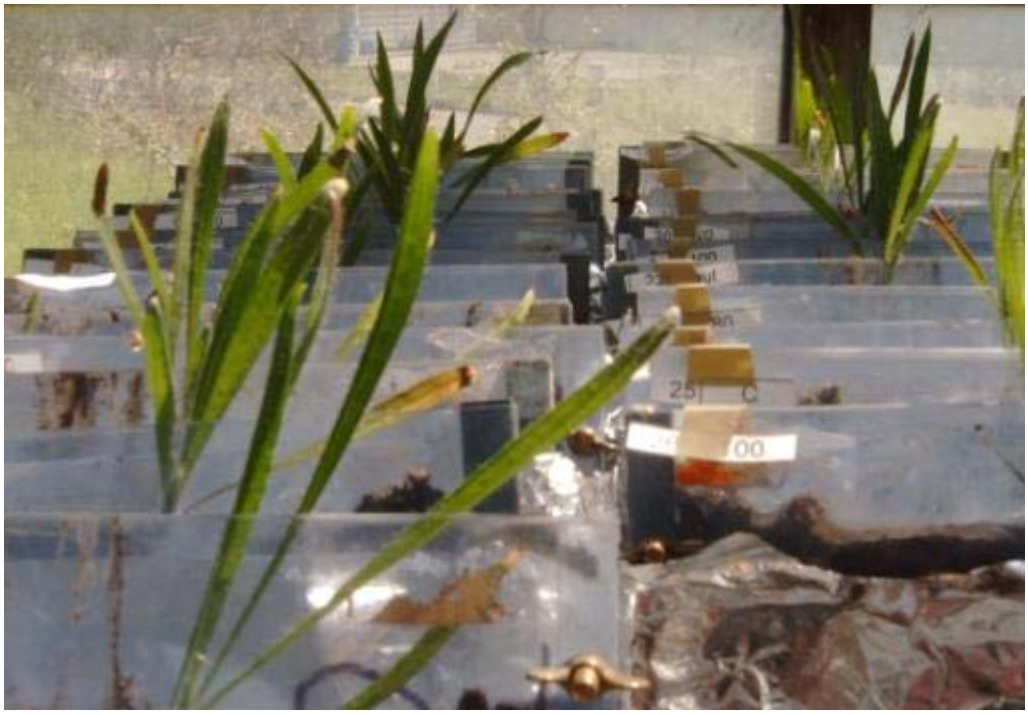

Never does nature say one thing and wisdom another.

Juvenal

LIST OF PUBLICATIONS

Endlweber K, Scheu S (2006) Effects of Collembola on root properties of two competing ruderal plant species. Soil Biology & Biochemistry 38: 2025-2031.
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Endlweber K, Ruess L, Scheu S (2007) Dietary routing in Collembola: Determining Collembola feeding preferences by stable isotope and compound specific fatty acid analysis. (in preparation)
(Chapter 4)

Endlweber K, Schäffner AR, Scheu S (2007) Analysis of decomposer-induced changes of gene expression profiles in *Arabidopsis thaliana*. (in preparation)
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OTHER PUBLICATIONS

Endlweber K, Scheu S (2006) Establishing arbuscular mycorrhiza-free soil: A comparison of six methods and their effects on nutrient mobilization. Applied Soil Ecology 34: 276-279.

Birkhofer K, Endlweber K, Gavish E, Lubin YD, von Berg K, Wise D and Scheu S (2007) Spider functional group diversity does not improve aphid suppression in a winter wheat field in central Germany. (in preparation)

TABLE OF CONTENTS

ZUSAMMENFASSUNG

In Laborexperimenten unterschiedlicher Komplexität wurde der Einfluss von Collembolen auf Pflanzenwachstum und Konkurrenz zwischen Pflanzen untersucht. Das Konkurrenzverhältnis zwischen Pflanzenarten wird durch Unterschiede in der Wurzelmorphologie und Nährstoffaufnahme bestimmt. In einem Experiment wurde überprüft, ob Collembolen das Konkurrenzverhältnis zwischen *Cirsium arvense* und *Epilobium adnatum* durch Veränderungen der Nährstoffverfügbarkeit verschieben. Collembolen beeinflussten weder die Biomasse noch die Nährstoffgehalte beider Pflanzenarten, induzierten jedoch die Bildung längerer, dünnerer Wurzeln und erhöhten die Anzahl an Wurzelspitzen, insbesondere bei *E. adnatum*.

In einem zweiten Experiment wurde überprüft, ob Collembolen über den Fraß an Mykorrhizapilzen das Konkurrenzverhältnis zwischen *Lolium perenne* und *Trifolium repens* verändern. Collembolen reduzierten die Konkurrenzkraft von *L. perenne* gegenüber *T. repens,* diese Reduktion war jedoch unabhängig vom Mykorrhizierungsgrad der Pflanzenwurzeln. Wie im ersten Experiment induzierten Collembolen die Bildung von längeren, dünneren Wurzeln und erhöhten die Anzahl von Wurzelspitzen in beiden Pflanzenarten, wobei die Wirkung bei *L. perenne* stärker ausgeprägt war. Die Veränderungen in der Wurzelmorphologie durch Collembolen beruhten wahrscheinlich auf der Verletzung von Wurzeln beim Fraß der Tiere in der Rhizosphäre und auf der Schaffung nährstoffreicher Bereiche im Boden.

Zur Klärung ob Collembolen direkt an Wurzeln fressen wurde in einem dritten Experiment der Kohlenstoffffluss von Pflanzenwurzeln in Collembolen mit Hilfe stabiler Isotope und komponentenspezifischer Fettsäure-Analyse untersucht. Als Versuchspflanze wurde Mais (C_4-Pflanze) verwendet, dem ^{15}N markierte C_3-Streu zugegeben wurde. Collembolen nahmen bevorzugt Kohlenstoff und Stickstoff aus

Pflanzen auf. Ihr Gehalt an Kohlenstoff und Stickstoff wurde durch die Verfügbarkeit von Pflanzen sowie durch Streu gesteigert, allerdings führte die Kombination beider Ressourcen nicht zu einer weiteren Steigerung. Collembolen nahmen bevorzugt Fettsäuren aus Pflanzenmaterial auf, ihr Fettsäuremuster wurde durch die Verfügbarkeit von Streu nicht beeinflusst.

Durch Verletzung oder Fraß an Pflanzenwurzeln werden sowohl die Abwehr als auch die Bildung von Botenstoffen in Pflanzen induziert. Durch Verwendung von DNA-Micorarrays wurde die Induktion von Pflanzenabwehr und Produktion von Phytohormonen in *Arabidopsis thaliana* durch Collembolen überprüft und diese mit Veränderungen im Rosettenwachstum korreliert. Collembolen reduzierten anfänglich das Wachstum von *A. thaliana,* die Pflanzen kompensierten diese Wachstumsverzögerung jedoch während der weiteren Entwicklung. Die temporäre Wachstumsdepression beruhte vermutlich auf einer Induktion von Abwehrstoffen durch Collembolen. Die Wachstumsverzögerung wurde später jedoch vermutlich durch eine erhöhte Produktion von Auxin ausgeglichen.

Die Ergebnisse der Arbeiten weisen daraufhin, dass Collembolen Pflanzen hauptsächlich über zwei Mechanismen beeinflussen: (i) Durch Beweidung von Mikroorganismen in der Rhizosphäre verändern sie die Verfügbarkeit sowie Verteilung von Nährstoffen im Boden und fördern so die Nährstoffversorgung und das Wachstum von Pflanzen; (ii) durch Weiden in der Rhizosphäre induzieren Collembolen die Abwehr von Pflanzen gegen Schädlinge und erhöhen so den Schutz vor Fraßfeinden. Die dadurch bedingte Wachstumsverzögerung wird durch die von Collembolen induzierte Bildung von Wachstumshormonen ausgeglichen. Das kompensatorische Wachstum wird durch höhere Nährstoffverfügbarkeit und ein expansiv wachsendes Wurzelsystem in Anwesenheit von Collembolen ermöglicht.

ABSTRACT

This study investigated the effect of Collembola on plant growth and competition between plant species. The competitive relationship between plants is determined by differences in root morphology and foraging strategy. In the first experiment the effect of Collembola on the competitive relationship between *Cirsium arvense* and *Epilobium adnatum* was analysed. Collembola neither affected plant biomass nor plant nutrient concentration. However, they induced the production of longer, thinner roots and enhanced the number of root tips with the effect being more pronounced in *E. adnatum.*

The inoculation of plant roots with mycorrhizal fungi is an important factor improving plant nutrition. The second experiment focused on changes in the competitive relationship between *Lolium perenne* and *Trifolium repens* due to Collembola grazing on mycorrhizal fungi. Collembola reduced the competitive superiority of *L. perenne* over *T. repens.* However, the reduction was independent of the rate of mycorrhizal inoculation. Similar to the first experiment Collembola induced the production of longer, thinner roots and increased the number of root tips, particularly in *L. perenne*. The results suggest that Collembola affect root morphology by damaging plant roots while grazing in the rhizosphere and by creating nutrient rich patches in soil.

In a third experiment feeding preferences of Collembola were investigated using analysis of stable isotopes and compound specific analysis of fatty acids. Collembola were introduced to a system consisting of a maize plant (C_4 plant), growing in soil mixed with ^{15}N labelled C_3 litter. Collembola preferentially incorporated plant-born carbon and nitrogen. The concentration of C and N in Collembola tissue increased when either litter or plants were available, whereas the combination of both resources caused no further increase in nutrient concentrations. Collembola

preferentially incorporated fatty acid originating from plant material, whereas the fatty acid composition of Collembola was not affected by the availability of litter.

Damaging or feeding on plant roots induces plant defence as well as the production of plant hormones. In a fourth experiment induction of plant defence and phytohormone production in *Arabidopsis thaliana* by Collembola were investigated employing DNA microarrays. Gene expression patterns were correlated with changes in rosette growth. Collembola initially reduced growth of *A. thaliana* with the deceleration being compensated during further development. The temporary deceleration probably was caused by the induction of plant defence by Collembola thereby reducing plant investment in growth. The enhanced production of the growth promoting hormone auxin presumably facilitated the compensational growth during further development.

The results of this study suggest that Collembola influence plant growth mainly via two mechanisms: (i) Collembola affect nutrient availability and distribution by grazing on microorganisms in rhizosphere and thus enhance plant nutrition and growth; (ii) while grazing in the rhizosphere, Collembola induce plant secondary metabolism and thus increase plant defence against herbivores. The subsequent deceleration in plant growth is compensated by the increased production of growth promoting hormones in presence of Collembola. Further, the compensational growth is facilitated by the increased nutrient availability and the production of a more expansive root system induced by Collembola.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Terrestrial ecosystems

Terrestrial ecosystems consist of two compartments, the above- and below-ground system. Both subsystems are intimately linked by plants, forming the nutritional basis for above- and below-ground biota. Plant growth depends on decomposer communities, which break down organic matter and mobilise nutrients for plant uptake. By increasing the availability of nutrients soil organisms therefore promote plant growth and performance, and subsequently affect their own resource (Wardle 2002).

Soil organisms are assumed to be food limited (Hairston 1989) and thus strong competition and niche differentiation should structure below-ground systems. However, there is limited experimental evidence that competition is the major force structuring soil communities (Scheu and Setälä 2002). Even though many decomposers use similar resources, species tend to switch between alternative food sources. Therefore, below-ground systems are characterised by a low degree of specialisation with most soil organisms being general feeders on a broad spectrum of food sources (Maraun et al. 2003). This low proportion of specialist feeders is due to the absence of coevolution between soil organisms and dead organic matter, the basic resource in soil (Scheu and Setälä 2002). As a consequence ascribing soil organisms to distinct trophic levels in food webs is an “insufficient” approach for structuring soil systems. Alternatively soil organisms are often classified according to body size. Body size restricts the mobility in soil and also determines what resources can be exploited. In this classification system bacteria and fungi (microflora) form the

smallest size class followed by microfauna comprising e.g. nematodes and protozoa (body size less than 0.1 mm). The mesofauna consist of e.g. Collembola, mites and other soil animals with a body width between 0.1 mm and 2 mm. Earthworms, diplopods and chilopods are classified as macrofauna (body width greater than 2 mm).

1.2 Effects of decomposers on plants

Plants and decomposers are linked by a complex system of direct and indirect interactions. Direct effects include antagonistic interactions, such as root feeding and pathogen infection, but also mutualistic interrelationships between plant and root biota such as mycorrhiza. Indirect interactions affect plant performance through several mechanisms, such as modification of physical properties of soil and seed viability. However, the predominant indirect interaction includes the mineralisation and thus the mobilisation of nutrients for plant uptake (Bremer and Vankessel 1992). The microflora (fungi and bacteria) is of fundamental importance for mineralisation processes. Microorganisms break down organic matter, mineralise complex compounds to simpler forms and immobilise the mineralised nutrients by incorporation into microbial biomass (Jonasson et al. 1996). Plants stimulate the mineralisation by allocating up to 20% of the photosynthetically fixed carbon as root exudates to the rhizosphere (Nguyen 2003). The high availability of carbon compounds triggers the increased density and activity of the mainly carbon limited soil organisms in the rhizosphere compared to the bulk soil (Wardle 1992). The increased activity of micro-decomposers and their respective grazers promotes nutrient mineralisation and ultimately increases plant nutrient acquisition (Clarholm 1985).

Depending on soil fertility either a fungal- or a bacterial-based energy channel is facilitated (Wardle et al. 2004). Bacteria based systems in fertile soils support rapid nutrient cycling due to high turnover rates, whereas in fungal based systems the turnover rate is slow and nutrients are immobilised in fungal biomass (Wardle et al. 2004). Microflora activity during such processes is influenced by higher level decomposers e.g. microfauna, meso- and macrofauna (Beare et al. 1992). Microbial grazers affect microbial turnover but also influence competition and composition of the microbial community (Tiunov and Scheu 2005). The soil fauna might shift the fungal to bacterial ratio by influencing the competitive relationship between fungi and bacteria (Hanlon and Anderson 1979) an interaction that consequently affects the rate of nutrient mineralisation (Scheu et al. 1999, Bonkowski et al. 2001). Decomposers also modify the spatial distribution of nutrients in soil, as earthworms mix soil and organic matter (Wurst et al. 2003, Kreuzer et al. 2004). Soil organisms may also create nutrient rich patches by casting (Sjursen and Holmstrup 2004). Particularly earthworm middens are zones of high microbial activity and form microhabitats for other soil organisms (Maraun et al. 1999, Tiunov and Scheu 2000).

The mineralisation of nutrients und thus the nutrient availability for plants depends on a complex interplay of indirect interactions that structure the soil food web. This system of close interrelationships between plants and soil organisms has not been sufficiently investigated and therefore its functioning is little understood.

1.3 Plant performance and mycorrhiza

Arbuscular mycorrhizal (AM) fungi form symbiosis with about 80% of all terrestrial plant genera (Smith and Read 1997). Their widespread hyphal network extends the absorptive surface of the root system and facilitates plant nutrient acquisition. The symbiosis is associated with the allocation of up to 20% of the photosynthetically

fixed carbon to the root symbiont (Wang et al. 1989). Indeed, photosynthetically inactive plants are not inoculated with mycorrhizal fungi (Lerat et al. 2002). The AM fungus enables the plant to take up relative immobile ions such as phosphorus but also improves water uptake. Recent studies demonstrated a translocation of nitrogen from the AM fungus to the plant root. Mycorrhizal fungi may directly gain nutrients from organic matter and allocate these to the plant (Perez-Moreno and Read 2000). Therefore, plants do not exclusively depend on nitrogen mineralisation by free living soil microorganisms. Nevertheless, the availability of nitrogen is a major factor limiting plant growth and it is still unclear to what extend nitrogen allocation by AM fungi improves plant performance (Johansen 1999).

The symbiosis between plants and AM fungi depends on the availability of nutrients; a high nutrient availability might reduce the effectiveness of the symbiosis (Klironomos et al. 1996). Under such conditions the mycorrhizal association may turn from mutualism into parasitism (Johnson et al. 1997).

Mycorrhizal fungi differ in their ability to supply plants with nutrients with strong consequences for plant growth (Smith et al. 2000, van der Heijden et al. 2003). Their performance optimum is determined by abiotic factors, such as pH and soil moisture content as well as nutrient availability (van Aarle 2002). Species identity of the fungal partner mainly determines plant nutrient acquisition and subsequently plant growth and performance. However, plants also vary in their dependency on nutrients supplied by mycorrhizal symbiosis. The effectiveness of nutrient acquisition determines plant growth and performance and therefore the competitiveness. Thus, plant coexistence as well as competitive superiority might be influenced by mycorrhizal inoculation (van der Heijden et al. 2003).

As soil organisms AM fungi are part of the complex system of interactions below ground, the activity of other soil biota might influence the functioning of mycorrhizal association and consequently affect plant performance. Soil invertebrates could reduce the effectiveness of mycorrhiza by grazing on hyphae and reducing hyphal density (Setälä 1995). Faunal activity might also improve nutrient availability for plants by stimulation of root colonisation and by dispersing mycorrhizal spores (Gange 1993, Klironomos and Kendrick 1996).

1.4 Plant nutrient uptake, plant competition and root morphology

Plant nutrient uptake is mainly governed by the morphology of the root system. The heterogeneous distribution of nutrients in soil forces plant roots to proliferate into nutrient rich patches. Efficient root foraging is probably a trade off between developing and sustaining an extensive root system and precise proliferation into nutrient rich patches (Campbell et al. 1991). The root foraging potential of plants differs between species and therefore has important implications for plant competition (Hutchings et al. 2003). Fast growing plants with an extensive root system might reach and exploit nutrient rich patches quickly and suppress smaller plants in heterogeneous soils (Rajaniemi and Reynolds 2004). Plant root structure and morphology is affected by competition with other plant roots (Maina et al. 2002). Plants increase root production in presence of other roots to gain a competitive advantage in exploiting resources but preferentially proliferate roots in unoccupied areas to avoid root competition (Gersani et al. 2001).

Root morphology and structure can be affected by soil organisms such as root microorganisms or soil invertebrates. The activity of soil invertebrates might affect nutrient availability resulting in an increased root growth (Canellas et al. 2002). The facilitation of nutrient uptake by mycorrhizal fungi enables the plant to reduce the root

system (Wulf et al. 2003). Bonkowski et al. (2001) demonstrated that plants reduce the length of roots as well as the number of root tips when inoculated with mycorrhizal fungi.

1.5 Collembola

Collembola are an ubiquitous arthropod group with 7500 described species worldwide. They colonise a large range of terrestrial ecosystems from arid habitats such as the Antarctica and sand deserts to the humid zones of sea coasts and rivers (Usher and Booth 1986; Andre et al. 1997). The characteristic morphological feature of Collembola is the furca, which enables the animals to cover distances that span many times their own body length. The furca is an effective mechanism that allows Collembola to escape predators. However, several mainly edaphic Collembola species have reduced or lost the furca.

Collembola belong to the mesofauna and are generally of small size. Euedaphic species grow up to 10 mm but most edaphic species reach only a few millimetres. In terrestrial habitats they are particularly abundant in soil and in the litter layer. In soil Collembola reach densities ranging between 50.000/m² in agricultural systems and up to 1 Mio /m² in boreal coniferous forests (Peterson and Luxton 1982). Collembola are mainly regarded as saprophageous or microphytophageous, but more likely are food generalists (Hopkin 1997). They feed on decaying plant material, bacteria, algae, pollen and also on living plant material (Wolters 1985, Chen et al. 1995). A few species feed on nematodes or eggs of other Collembola (Lee and Widden 1996). Analysis of stable isotope signatures indicates that Collembola inhabit a wide range of trophical niches (Chahartaghi et al. 2005). However, feeding habits are influenced by the availability of food resources and therefore depend on both, habitat and season (Anderson and Healey 1972, Wolters 1998).

Edaphic Collembola are particularly active in the rhizosphere of plants. Albers et al. (2006) demonstrated that plant born carbon is rapidly incorporated into Collembola, indicating that their food resources are either closely related to root exudates or to microorganisms utilising root derived carbon, e.g. mycorrhizal fungi. Collembola probably graze on hyphae of mycorrhizal fungi (Moore et al. 1987). The effect of Collembola on the mutualistic relationship between plant and fungus has been demonstrated to be either positive or negative depending on Collembola density (Bakonyi et al. 2002). However, by affecting the mycorrhizal fungus inoculation they might influence plant nutrition and ultimately affect plant growth and performance (Kaiser and Lussenhop 1991, Gange 2000). Changes in plant nutrition and performance might also be due to indirect effects. By grazing on microorganisms in the rhizosphere of plants Collembola affect nutrient cycling by changing the microbial activity and community composition (Bardgett et al. 1993, Chen et al. 1995, Maire et al. 1999, Petersen 2002). They might also increase nutrient availability by excretion and faecal pellet deposition (Petersen 2000, Sjursen and Holmstrup 2004). As a consequence, Collembola also affect nutrient availability to plants and therefore plant growth and nutrient content (Lussenhop 1992, 1996, Filser 2002). Effects of Collembola on plant nutrient content cascade up and ultimately affect above-ground phytophagous insects (Scheu et al. 1999, Wurst and Jones 2003).

The influence of Collembola on plant nutrition probably varies with plant species and functional group. Plant nutrient acquisition is closely related to the root morphology and inoculation with root biota. Resource availability in soil varies with space and time. The reaction of plants to heterogeneous resource distribution depends on their functional group and root morphology. Many plant species increase their nutrient uptake by proliferating roots in nutrient rich patches (Hodge et al. 1998, Hodge 2004). Thus nutrient acquisition depends on plant species and influences the

competition between plant species (Hodge 1999). The patchiness of nutrients as well as the overall availability is probably influenced by Collembola. Hence, Collembola might affect nutrition of plant species or functional groups differently and thus affect plant competition.

1.6 Objectives

This study investigated the effect of Collembola on plant performance and competitiveness. In a first experiment the impact of Collembola on plant performance and competition was determined by comparing morphological and growth related parameters e.g. plant biomass, nutrient content and root morphology (Chapter 2). The experiment tested the hypothesis that Collembola affect the competitive ability of different plant species by altering nutrient availability and individual plant performance. The results of the first experiment were followed up in a second approach focusing on plant nutrient acquisition as influenced by mycorrhizal fungi (Chapter 3). Analysing nutrient contents, root morphology and the inoculation of plant roots with mycorrhizal fungi of *Trifolium repens* and *Lolium perenne,* the hypothesis was tested that Collembola affect competition between plants of different functional groups by influencing the interaction between plants and mycorrhizal fungi.

In a third approach Collembola feeding habits were determined by a combined analysis of Collembola stable isotope signatures of nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) as well as fatty acid composition and compound specific analysis (Chapter 5). Collembola were introduced in pots with Maize plants growing in soil mixed with labelled (^{15}N) litter of *Lolium perenne* L (C_3 plant). Fatty acid and stable isotope analysis provided information about the resources used by Collembola and connected this data with information derived from earlier experiments.

The fourth experiment related the morphological response of *Arabidopsis thaliana* to changes in gene expression. Using a custom made DNA-Microarray the effect of Collembola on plant secondary metabolism and hormone production was investigated (Chapter 4).

CHAPTER 2

EFFECTS OF COLLEMBOLA ON ROOT PROPERTIES OF TWO COMPETING RUDERAL PLANT SPECIES

2.1 Abstract

Plant roots compete for nutrients mineralised by the decomposer community in soil. By affecting microbial biomass and activity Collembola influence the nutrient availability to plants. We investigated the effect of Collembola (*Protaphorura fimata* Gisin) on growth and competition between of two plant species, *Cirsium arvense* L (creeping thistle) and *Epilobium adnatum* Griseb. (square-stemmed willow herb), in a laboratory experiment. Two seedlings of each plant species were planted in rhizotrons either in combination or in monoculture (intra- and interspecific competition). Interspecific competition strongly reduced total biomass of *C. arvense* whereas *E. adnatum* suffered most from intraspecific competition. Collembola neither affected the competitive relationship of the two plant species nor shoot and root biomass. Although Collembola did not affect total root biomass they influenced root morphology of both plant species. Roots grew longer and thinner and had more root tips in presence of Collembola. Root elongation is generally ascribed to the exploitation of nutrient rich patches in soil. We hypothesise that changes in root morphology in presence of Collembola are due to Collembola-mediated changes in nutrient availability and distribution.

2.2 Introduction

Plants depend on the availability of nutrients mineralised by the decomposer community in soil. Biotic interactions in the rhizosphere of plants affect plant growth and competitiveness by a variety of mechanisms. Even though direct effects, such as root feeding, are more apparent, indirect effects of decomposers are vitally important (Setälä 1995, Wurst et al. 2003). Indirect effects of decomposers include mineralisation and distribution of nutrients, changes in activity and composition of microorganisms and modification of soil structure und root environment (Scheu and Setälä 2002, Wardle 2002).

Mineralisation of nutrients in soil is mainly due to bacterial and fungal activity (Beare et al. 1992). Collembola are among the most abundant microarthropods in the rhizosphere of plants (Bardgett et al. 1993). They stimulate or reduce growth and respiration of microorganisms (van der Drift and Jansen 1977, Bakonyi 1989, Kandeler et al. 1999, Cragg and Bardgett 2001) with the direction of the effects being density-dependent (Theenhaus et al. 1999, Cole et al. 2004a). By grazing on fungi and bacteria Collembola mobilise nutrients and therefore affect plant nutrition (Teuben 1991, Lussenhop 1992, Jones 1998, Filser 2002). In fact, it has been shown that Collembola alter the content of nitrogen and phosphorus in plant shoots (Bardgett and Cook 1998, Bardgett and Chan 1999, Lussenhop and BassiRad 2005). Scheu et al. (1999) documented that in presence of Collembola both plant growth and plant shoot N content is increased. In addition, root growth is also modified by the activity of Collembola (Theenhaus et al. 1999, Cole et al. 2004a). Plant roots respond to increased nutrient availability by proliferation or an increased density and elongation of root hairs (Hodge et al. 1999). The response of plant roots depends on plant species and functional group. Theenhaus et al. (1999) demonstrated that in presence of Collembola root biomass of *Trifolium repens* and

Poa annua is decreased but the shoot/root ratio of *P. annua* increased. By changing plant growth and plant nutrition Collembola likely affect the competitive relationship between plant species. However, the effect of Collembola and other soil arthropods on plant competition is not clear. In a laboratory experiment Collembola increased the competitive strength of *T. repens* against *Lolium perenne* (Kreuzer et al. 2004).

Schädler et al. (2004) investigated the effect of herbivorous insects on secondary plant succession of an early set-aside arable field. By applying soil and foliar insecticide, above- and belowground insects were excluded from experimental plots. Applying soil insecticide strongly affected the dominance structure of the plant community. *Cirsium arvense* (creeping thistle) dominated the plots with soil insecticide application, whereas in foliar insecticide treatments and in control plots *Epilobium adnatum* (square-stemmed willow herb) prevailed. The effects caused by insecticide applications were ascribed to herbivore insects being reduced by the insecticides. However, the observed changes in plant community may also have been due to changes in the decomposer community, e.g. in Collembola, since particularly the application of the belowground insecticide strongly reduced the density of Collembola populations and altered the dominance structure of the Collembola community (Endlweber et al. 2006).

In the present study we investigated if Collembola in fact affect the competitive relationship between *E. adnatum* and *C. arvense*; these plant species were taken as model organisms to investigate the effects of Collembola on plant competition. We hypothesized that Collembola alter plant growth, root morphology and plant nutrient contents resulting in changes in the competitive strength of the two plant species.

2.3 Materials and Methods

Rhizotrons (height 20 cm, width 15 cm, thickness 1 cm) were filled with 100 g sieved (< 1 cm) and defaunated (5 days at -21°C) soil taken from the upper 20 cm of a set-aside field near Halle (Saxony-Anhalt, Germany; cf. Schädler et al. 2004). Seedlings of *C. arvense* and *E. adnatum* were grown from seeds in pots filled with defaunated soil in the greenhouse. The seedlings were transplanted into the rhizotrons four weeks after sowing according to a replacement series design: (1) 2 seedlings of *E. adnatum*, (2) 1 seedling of *C. arvense* and 1 seedling of *E. adnatum* and (3) 2 seedlings of *C. arvense.* Two days after transplantation of the plants 60 Collembola of the euedaphic species *Protaphorura fimata* taken from laboratory cultures were added to half of the rhizotrons (Collembola treatments). Each treatment was replicated nine times giving a total of 54 chambers. Rhizotrons were incubated in a greenhouse (16 h light, 18°C) and arranged in a complete randomised block design.

The rhizotrons were watered every other day with 5 ml deionised water. Plants were harvested by cutting at soil level after 5 weeks, when plant roots reached the bottom of the chambers. Plant height was recorded, shoots were dried at 60°C for three days and the dry weight of each shoot was determined. Dried shoots were milled in a ball mill (Retsch, Haan, Germany). Total N and C content of the plant material was analysed by an elemental analyser (Carlo Erba, Milan, Italy).

Roots were washed and root length, number of root tips, root diameter and root volume were analysed using WinRHIZO (Regent Instruments Inc., Sainte-Foy, Canada). While washing the roots Collembola floating on the water surface were collected and counted. Soil respiration was measured using an automated respirometer based on electrolytic O_2 microcompensation (Scheu 1992). Oxygen consumption rates at 22°C were measured every 0.5 h. Microbial basal respiration was measured as mean O_2 consumption during hours 10-20 after attachment of the

vessels to the respirometer. Microbial biomass was assessed by measuring the maximum initial respiratory response (MIRR, µg O_2 g^{-1} h^{-1}) to glucose addition (substrate-induced respiration; Anderson and Domsch 1978, Beck et al. 1997). Glucose (4 mg g^{-1} dry wt soil) was added as an aqueous solution adjusting the soil water content to 80-90% (dry wt). MIRR was at a maximum at these glucose concentrations and moisture levels as proven in preliminary experiments. The mean of the eight lowest measurements during the first 10 h after glucose addition was taken as MIRR.

The experiment was set up in a two-factorial design with the factors plant competition and Collembola (with and without). The factor plant competition analysed differences between intra- and interspecific competition for each plant species (*E. adnatum* and *C. arvense*). Individual below- and aboveground plant biomass was taken as dependent factor. Means of the factors above and belowground biomass, root length, number of root tips, root diameter, root volume and C and N content were used in treatments with intraspecific competition. The effect of Collembola on microbial biomass was analysed by single factor analyses of variance (ANOVA). Differences between means were inspected using Tukey's honestly significant difference test. Statistical analyses were performed using the ANOVA procedure in SAS 6.12 (SAS Institute, Cary, N.C.).

2.4 Results

2.4.1 Collembola

Collembola communities developed differently in the competition treatments. In treatments with *E. adnatum* and treatments with both plant species, collembolan numbers did not exceed the initially added 60 individuals. Whereas in treatments with only *C. arvense* collembolan numbers increased to an average of 85 individuals (SD = 24.5). Statistically, however, the two treatments did not differ significantly ($F_{2,24}$=2.99, P=0.0703).

2.4.2 Plant performance

Generally, shoot biomass of *C. arvense* exceeded that of *E. adnatum.* Competition affected the aboveground biomass of *C. arvense* and *E. adnatum* differently. Shoot biomass of *C. arvense* was significantly higher when plants grew with intraspecific competition compared to treatments with interspecific competition with *E. adnatum* (Table 1, Fig. 1a). Overall, root biomass as well as root length and number of root tips in *E. adnatum* exceeded that of *C. arvense*. Interspecific competition significantly reduced root biomass in *C. arvense* (Table 1, Fig. 1b) and caused a decrease in root length and number of root tips (Table 1, Fig. 1c, d). *E. adnatum* responded in the opposite way compared to treatments with interspecific competition shoot biomass decreased significantly when plants grew with intraspecific competition (Table 2, Fig. 1a). Root biomass of *E. adnatum* increased significantly in treatments with interspecific competition (Table 2, Fig. 1b) and this was also true for root length and number of root tips (Table 2, Fig 1c, d).

Table 1. Two-factor ANOVA on the effects of competition and Collembola on shoot biomass, root biomass, root volume, root diameter, root length, number of root tips, root C content, root N content and root C/N content of *Cirsium arvense* (n= 32).

	Shoot biomass		*Root biomass*		*Root volume*		*Root diameter*		*Root length*	
	DF	F	P	F	P	F	P	F	P	F	P
Collembola	1	5.97	0.020	1.54	0.220	3.79	0.050	0	0.988	3.11	0.090
Competition	1	0.25	0.621	12.08	0.001	5.56	0.025	0.28	0.757	10.53	0.003
Comp. x Coll.	1	2.83	0.102	0.49	0.486	0.01	0.907	0.57	0.452	0.06	0.816

	Number of root tips		*Root C content*		*Root N content*		*Root C/N content*	
	DF	F	P	F	P	F	P	F	P
Collembola	1	9.79	0.003	0	0.990	2.09	0.160	1.60	0.210
Competition	1	9.06	0.005	0.30	0.587	1.98	0.165	0.39	0.559
Comp. x Coll.	1	2.21	0.147	0.10	0.752	0.22	0.641	0.94	0.337

Fig. 1

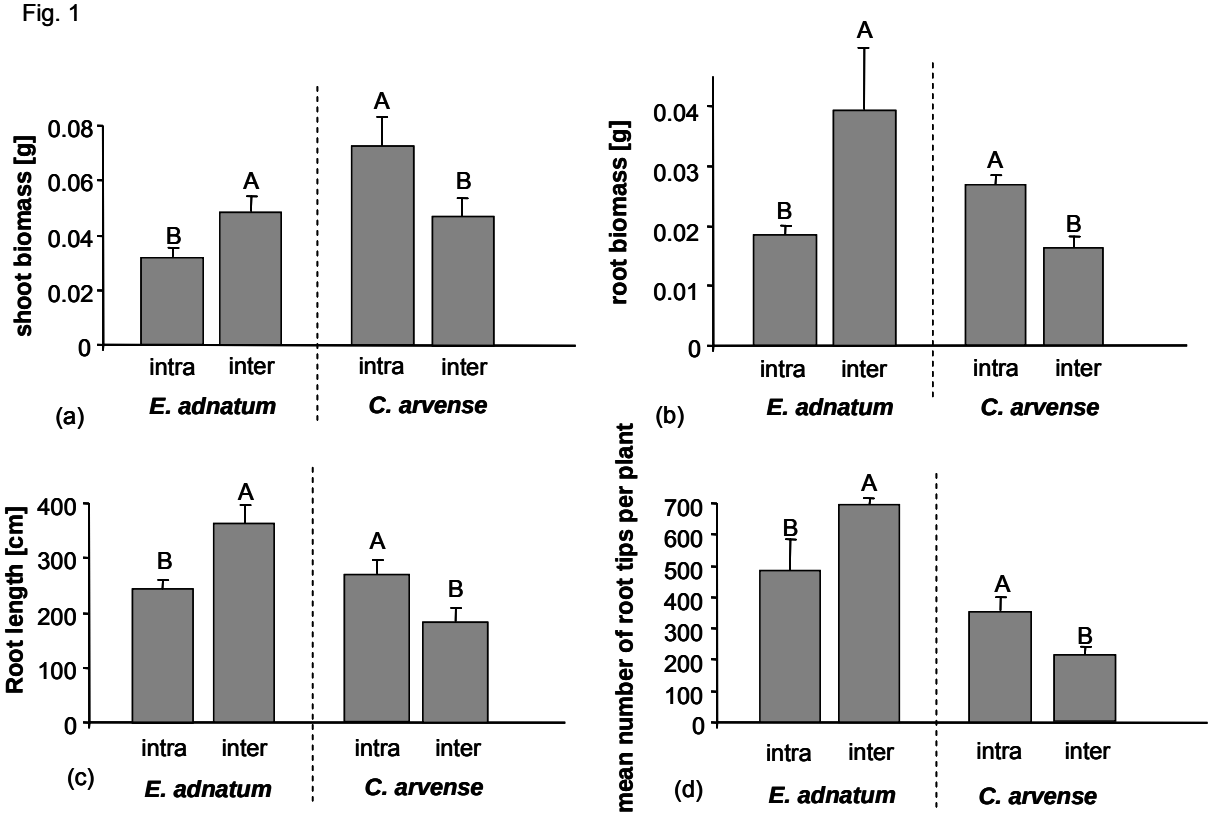


Figure 1. Effect of inter- and intraspecific competition on shoot biomass (a), root biomass (b), root length (c) and number of root tips (d) in *C. arvense* and *E. adnatum.* Error bars are one standard error from the mean. Bars with the same letter are not significantly different (Tukey's honestly significant difference, $P<0.05$)

Collembola generally did not affect shoot and root biomass of *C. arvense* (Table 1) and *E. adnatum* (Table 2). However, they strongly affected the structure of the root system of both plant species. Overall, Collembola reduced the diameter and volume of roots in *E. adnatum* (Table 2, Fig. 2a, b). In contrast, root length was significantly increased (Table 2, Fig. 3). They also increased the number of root tips (Table 2, Fig. 4). In contrast to *E. adnatum*, Collembola neither affected root length nor root volume of *C. arvense.* However, similar to *E. adnatum* presence of Collembola reduced the diameter of roots in *C. arvense* (Table 1, Fig. 2a) and increased the number of root tips (Table 1, Fig. 4). There was generally no significant interaction between Collembola and plant competition on any of the plant response variables studied (Table 1, 2).

Table 2. Two-factor ANOVA on the effects of competition and Collembola on shoot biomass, root biomass, root volume, root diameter, root length, number of root tips, root C content, root N content and root C/N content of *Epilobium adnatum* (n= 32).

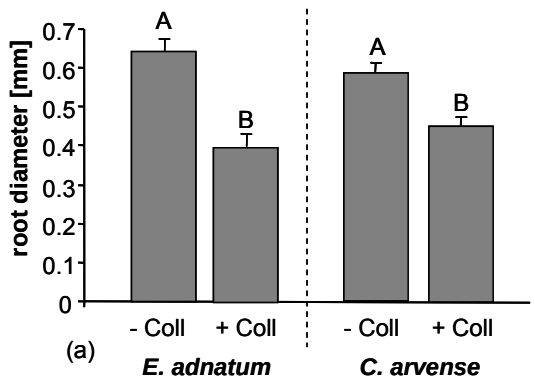
	Shoot biomass		*Root biomass*		*Root volume*		*Root diameter*		*Root length*	
	DF	F	P	F	P	F	P	F	P	F	P
Collembola	1	7.75	0.009	1.30	0.260	21.30	<0.0001	28.41	<0.0001	5.42	0.026
Competition	1	0.44	0.511	4.70	0.035	3.30	0.079	0.03	0.857	8.47	0.007
Comp. x Coll.	1	0.74	0.395	1.67	0.208	1.24	0.274	2.30	0.136	3.24	0.081

	Number of root tips		*Root C content*		*Root N content*		*Root C/N content*	
	DF	F	P	F	P	F	P	F	P
Collembola	1	9.36	0.005	0.02	0.878	0.65	0.410	0.95	0.335
Competition	1	4.23	0.048	7.15	0.010	0.13	0.722	4.52	0.039
Comp. x Coll.	1	1.08	0.308	1.42	0.239	0.10	0.658	3.90	0.060

Presence of Collembola did not cause any changes in microbial basal respiration (overall mean 2.36 µg O_2 h^{-1} g^{-1} dry weight) nor in microbial biomass (overall mean 249.3 µg C_{mic} g^{-1} dry weight). Although Collembola and plant competition affected plant biomass and root performance plant tissue nitrogen and carbon concentration were not affected except for root C content of *E. adnatum* which was significantly

increased when grown together with *C. arvense* (Table 2). The C content of *E. adnatum* was increased from 34.68% when grown as single species to 36.74% in interspecific competition treatments; leading to an increased C/N ratio in *E. adnatum* roots when grown in competition with *C. arvense* (from 27.59% to 29.59%; Table 2).

Fig. 2



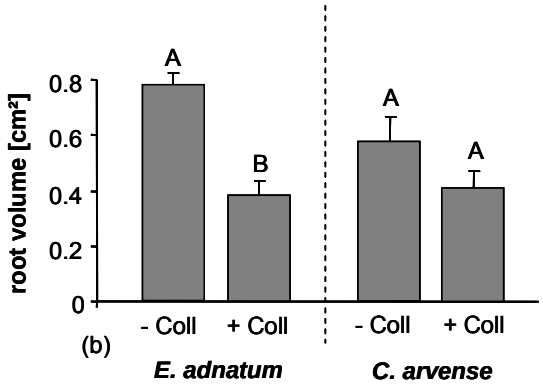


Figure 2. Effect of Collembola on root diameter (a) and root volume (b) in *C. arvense* and *E. adnatum.* Error bars are one standard error from the mean. Bars with the same letter are not significantly different (Tukey's honestly significant difference, P<0.05)

Fig. 3

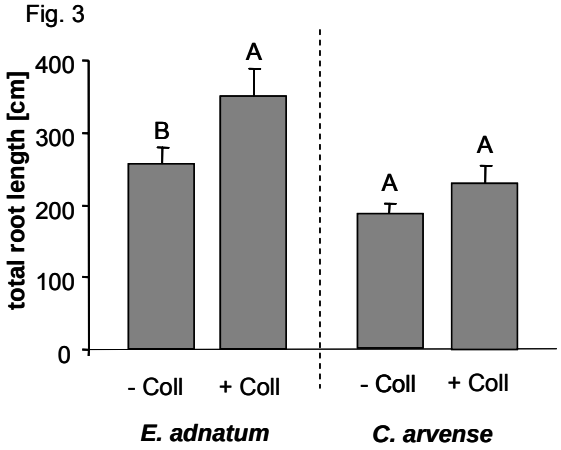


Figure 3. Effect of Collembola on total root length in *C. arvense* and *E. adnatum.* Error bars are one standard error from the mean. Bars with the same letter are not significantly different (Tukey's honestly significant difference, P<0.05)

Fig. 4

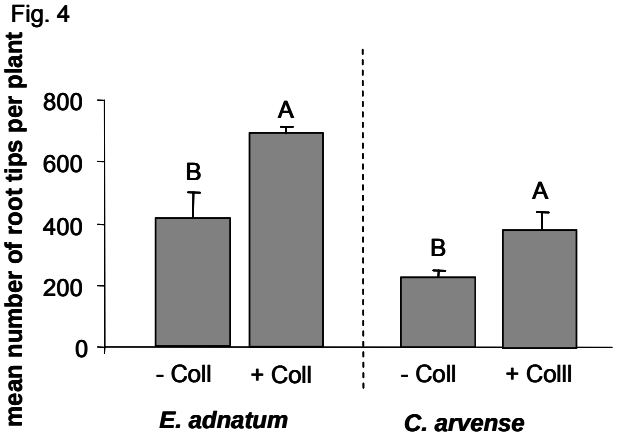


Figure 4. Effect of Collembola on number of root tips in *C. arvense* and *E. adnatum.* Error bars are one standard error from the mean. Bars with the same letter are not significantly different (Tukey's honestly significant difference, P<0.05)

2.5 Discussion

Competition between plants for nutrients in soil is an important factor forming plant communities (Aerts 1999). Intra- and interspecific competition of plant roots influences root proliferation and structure of root systems (Gersani et al. 1998). Gersani et al. (2001) and Maina et al. (2002) documented that plants react to inter- and intraspecific competition by the production of more roots and less yield. However, the response of plants to inter- and intraspecific competition may vary with plant species. Schädler et al. (2004) suggested that *C. arvense* and *E. adnatum*, two common plant species on arable land and fallow fields, compete for resources in early successional stages after cessation of cultivation. In the present study the effect of inter- and intraspecific competition on plant growth differed between the two plant species. Intraspecific competition decreased root and shoot biomass in *E. adnatum* whereas *C. arvense* suffered most from interspecific competition suggesting that *E. adnatum* is the stronger interspecific competitor (Ang et al. 1995). Besides competition for space and light plants compete mainly for nutrients in soil. Belowground competition influences the root structure of the plants (Volis and Shani 2000) but the extent depends on root architecture of the competing plant species (Rubio et al. 2001).

In contrast to our hypothesis that Collembola affect the competitive relationship between *E. adnatum* and *C. arvense*, results of the present experiment suggest that the competitive relationship between the two plant species is independent of the presence of Collembola. Collembola neither affected shoot nor root biomass in the two plant species which is in contrast to previous studies reporting an increase in plant biomass in presence of Collembola (Scheu et al. 1999, Kreuzer et al. 2004, Lussenhop and BassiriRad 2005). These contrasting results might be due to variable responses of plant species and plant developmental stages to the presence of

Collembola. The effect of Collembola on plant growth varies with plant functional group (Partsch et al. 2006). Presence of different Collembola species with different functional characteristics might be more important than Collembola density (Cragg and Bardgett 2001, Cole et al. 2004a).

Previous experiments documenting changes in root and shoot biomass in presence of Collembola lasted for 8-12 weeks (Scheu et al. 1999, Kreuzer et al. 2004, Gormsen et al. 2004, Lussenhop and BassiriRad 2005, Partsch et al. 2006), whereas experiments which did not find any changes in plant growth due to the presence of Collembola were running for about 3-5 weeks (Larsen and Jakobsen 1996). In the present study though Collembola did not affect total root biomass they affected root morphology of both plant species. Roots generally were longer and thinner and developed more root tips in presence of Collembola. This was more pronounced in *E. adnatum* than in *C. arvense.* A major determinant of the structure of the root system of plants is the availability and distribution of nutrients in soil. Roots respond to nutrient rich patches by proliferation and growth towards these patches. Proliferation is primarily triggered by the availability of nitrogen and includes a variety of responses comprising increased production of root tissue and production of longer roots (van Vuuren et al. 1996, Hodge et al. 1999, Hodge et al. 2000). The ability of roots to grow to nutrient rich patches differs between species and is influenced by competition between plants for resources in soil (Hutchings et al. 2003).

Collembola may affect root structure by influencing the nutrient availability and spatial heterogeneity of nutrients in soil (McGonigle 1995). Collembola form discrete nutrient rich patches through excretion and faecal pellet deposition (Petersen 2000, Sjursen and Holmstrup 2004). As indicated by increased root elongation and number of root tips in *E. adnatum* and *C. arvense* plant roots exploited these nutrient rich patches by growing longer. Root elongation and root branching are typical reactions to patchily

distributed nutrient rich sources (Linkohr et al. 2002, Mantelin and Touraine 2004). Furthermore, Collembola affect the availability of nutrients by modification of microbial biomass and activity. It has been demonstrated that mineralisation of nitrogen in presence of Collembola and other soil microarthropods is increased (Bardgett and Chan 1999). Plants benefit from the increased availability of nitrogen resulting in increased plant growth and tissue nitrogen concentration (Kreuzer et al. 2004, Lussenhop and BassiRad 2005). In the present study no effects of Collembola on microbial biomass and activity could be detected which is in contrast to previous experiments (Hanlon and Anderson 1979, Bakonyi 1989, Teuben 1991, Kandeler et al. 1999). It is known that the effect of Collembola on microorganisms depends on microarthropod density and on species composition (Cragg and Bardgett 2001, Cole et al. 2004a, Cole et al. 2004b). Microbial biomass may have remained unaffected since Collembola density was low compared to other studies (Scheu et al. 1999, Kreuzer et al. 2004, Partsch et al. 2006).

Root elongation and an increase in the number of root tips suggest increased availability of nitrate (Zhang and Forde 2000, Mantelin and Touraine 2004). In fact, Cragg and Bardgett (2001) found enhanced leaching of nitrate in presence of Collembola and attributed this to an increased activity of nitrifying bacteria. Further studies are necessary combining the analysis of the structure of the plant root system and the composition of microbial communities in the rhizosphere as affected by the presence of Collembola.

2.6 Conclusions

Overall, results of the present study documented that despite plant biomass production and plant competition remain unaffected, Collembola altered the structure of the root system of plants and root resource exploitation. Considering that the effect of Collembola varies with plant species and soil conditions these are likely to alter plant growth and plant competition in the field. Therefore, experiments using insecticides to exclude herbivore insects for investigating their effect on plant communities have to consider that the observed effects in fact at least in part might be due to changes in the decomposer community.

In the insecticide treatments of the studied oldfield community decomposers, such as collembolans, might have contributed to the observed changes in dominance between *C. arvense* and *E. adnatum*.

CHAPTER 3

INTERACTIONS BETWEEN MYCORRHIZAL FUNGI AND COLLEMBOLA: EFFECTS ON ROOT STRUCTURE OF COMPETING PLANT SPECIES

3.1 Abstract

Mycorrhizal fungi influence plant nutrition and therefore likely modify competition between plants. By affecting mycorrhiza formation and nutrient availability of plants, Collembola may influence competitive interactions of plant roots. We investigated the effect of Collembola (*Protaphorura fimata* Gisin), a mycorrhizal fungus (*Glomus intraradices* Schenck and Smith), and their interaction on plant growth and root structure of two plant species, *Lolium perenne* L (perennial ryegrass) and *Trifolium repens* L (white clover). In a laboratory experiment two individuals of each plant species were grown either in monoculture or in competition to the respective other plant species.

Overall, *L. perenne* built up more biomass than *T. repens*. The clover competed poorly with grass, whereas the *L. perenne* grew less in presence of conspecifics. In particular, presence of conspecifics in the grass and presence of grass in clover reduced shoot and root biomass, root length, number of root tips and root volume. Collembola reduced shoot biomass in *L. perenne,* enhanced root length and number of root tips, but reduced root diameter and volume. Effects of Collembola on *T. repens* were less pronounced but Collembola enhanced root length and number of root tips. In contrast to our hypothesis, changes in plant biomass and root structure in the presence of Collembola were not associated with a reduction in mycorrhizal formation. Presumably, Collembola affected root structure via changes in the amount of nutrients available and their spatial distribution.

3.2 Introduction

One of the most important systems affecting plant nutrition is the symbiosis of plant roots with mycorrhizal fungi (Smith and Read 1997). Mycorrhizal fungi facilitate plant nutrient uptake, in particular that of phosphate, but also that of other nutrients, such as zinc and copper. Additionally, the symbiosis can provide the plant with inorganic nitrogen (Javelle et al. 1999, Hawkins et al. 2000, Hawkins and George 2001). The functioning of mycorrhizas, however, is affected by other biota. Mycorrhizal fungi are imbedded in a complex food web of decomposer invertebrates including soil arthropods, such as Collembola. Collembola graze on hyphae and spores of arbuscular mycorrhizal fungi (Moore et al. 1987, Bakonyi et al. 2002) and this may significantly affect plant growth (Kaiser and Lussenhop 1991, Gange 2000, Kreuzer et al. 2004).

The effect of Collembola on mycorrhizal functioning has been shown to be density dependent, with high Collembola densities hampering but low densities increasing mycorrhizal nutrient transfer to plants (Ek et al. 1994). Reduced mycorrhizal functioning might be due to lower infection of roots with mycorrhizal fungi (Lussenhop 1996) caused by feeding on spores and hyphae (Klironomos and Ursic 1998, Bakonyi et al. 2002). The increase in mycorrhizal functioning at low densities of Collembola is likely due to a stimulation of hyphal growth and functioning (Kandeler et al. 1999, Gange 2000, Cragg and Bardgett 2001). In addition, Collembola might also beneficially affect ectomycorrhizal fungi and increase root infection by transporting spores. It has been shown that spores of more than 100 fungal species adhere to the body surface of *Onychiurus subtenuis* (Visser et al. 1987).

Most Collembola species preferentially feed on saprotrophic rather than mycorrhizal fungi (Klironomos and Ursic 1998, Schreiner and Bethlenfalvay 2003). Selective grazing of Collembola on fungi may result in changes in the structure of fungal

communities, e.g. by decreasing the competitive strength of saprotrophic fungi and increasing that of mycorrhizal fungi (Sabatini and Innocenti 2000, Tiunov and Scheu 2005). Changes in the structure of the soil fungal community likely result in changes in plant nutrition and therefore affect plant growth. Furthermore, Collembola affect plant growth by increasing nutrient availability by feeding on bacteria and fungi, and mobilising microbial nutrient pools (Lussenhop 1992, Theenhaus et al. 1999, Filser 2002, Cole et al. 2004a). Plant roots respond to modified nutrient availability by proliferation and elongation even though shoot and root biomass may remain unaffected (Hodge et al. 1999, Endlweber and Scheu 2006).

By changing plant nutrition, root growth and root structure, Collembola not only affect plant growth but likely also plant competition. In the laboratory Collembola indeed increased the competitive strength of *Trifolium repens* L against *Lolium perenne* L (Kreuzer et al. 2004). The present experiment builds on these results by investigating if these changes are mediated by mycorrhiza. We hypothesise that changes in plant biomass and root structure are due to a reduction in mycorrhizal formation by Collembola. Therefore, we investigated effects of Collembola on arbuscular mycorrhizal fungus infection, plant nutrient uptake and root morphology of *L. perenne* and *T. repens* growing in monoculture and in combination of both plant species.

3.3 Materials and Methods

The experiment was conducted in a temperature controlled greenhouse (16 h light, 18°C). Rhizotrons (height 35 cm, width 15 cm, thickness 1 cm) were filled with 280 g soil taken from the upper 20 cm of a set-aside field (early successional stage) in Bad Lauchstädt near Halle (Saxony-Anhalt, Germany). The soil is a Chernosem with an average pH of 7.14 and an average content of 10.19 µg PO^-_4-P/g soil. Carbonate-

extractable phosphate was extracted as reported by Olsen and Sommers (1982). Average ammonium and nitrate contents were 0.43 µg NH_4^+/ g soil and 0.96 µg NO_3^- /g soil respectively. Mineral N was extracted from sub-samples and was determined as reported by Keeney and Nelson (1982).

The soil was sieved (1 cm mesh) and autoclaved at 120°C for 2 h for defaunation and elimination of mycorrhizal fungi. It was stored for 3 days at 15°C after autoclaving and then filled into the experimental containers. Fresh soil (172g dry weight) was suspended in 200 ml distilled water to re-inoculate the soil with microorganisms. The suspension (150 ml) was filtered through 25 µm gauze to exclude mycorrhizal fungus spores and made up to 750 ml with distilled water. Soil suspension (12 ml) was evenly distributed over the soil of each rhizotron.

Seven-day-old seedlings of *L. perenne* and *T. repens* were transplanted into the rhizotrons to establish the following treatments in a replacement series design: (1) 2 seedlings of *L. perenne,* (2) 1 seedling of *L. perenne* and 1 seedling of *T. repens,* (3) 2 seedlings of *T. repens.* Each treatment was inoculated with or without mycorrhizal fungi, and with and without Collembola. Each treatment was replicated five times giving a total of 60 rhizotrons. Mycorrhizal fungus (14 g) as spores and hyphae of *Glomus intraradices* Schenck and Smith (Dr. C. Grotkass, Institut für Pflanzenkultur, Schnega, Germany), was evenly spread over the soil in each rhizotron. Non-mycorrhizal treatments received the same amount of inoculum which had been autoclaved (120°C, 2 h). In order to control for potential effects caused by microorganisms other than mycorrhizal fungi in the inoculum 12 ml of a filtrate of the inoculum were added to each experimental container. The filtrate was prepared by suspending 100 g of the inoculum in 800 ml distilled water and filtered through 25 µm gauze. Half of the rhizotrons received 100 collembolans of the euedaphic species

Protaphorura fimata Gisin. The rhizotrons were watered with 15 ml distilled water every other day through the experiment.

Plants were harvested after eight weeks. Shoots were dried at 60°C for three days and weighed. Dried shoots were milled in a ball mill (Retsch, Haan, Germany) and the shoot C and N contents were analysed by an elemental analyser (Carlo Erba, Milan, Italy). Plant roots were washed and scanned. Images were analysed in terms of root length, number of root tips, root diameter and root volume using WinRHIZO (Regent Instruments Inc., Sainte-Foy, Canada). Collembola floating on the water surface during root washing were collected and counted. The roots were weighed and a subsample was bleached by boiling in 1 N KOH. Then, the roots were dyed in 10 ml 1 N HCl mixed with two drops ink (Quink, Parker Permanent Blue, Germany) and bleached in a mixture of 10 ml lactic acid and 10 ml distilled water. Colonisation of roots by mycorrhizal fungi was analysed using the gridline intersection method (Giovannetti and Mosse 1980). To determine root biomass, roots were dried at 60°C for three days and weighed.

The experiment was set up in a complete factorial design with three factors: plant combination (con- and heterospecifics), Collembola (with and without) and mycorrhiza (with and without). Effects of these factors on above and belowground biomass, root length, number of root tips, root diameter, root volume and C and N content were analysed by three factorial ANOVA. In treatments with conspecific competitors (monocultures) means of the dependent variables of the two plant individuals per rhizotron were used for the analyses. Collembola density and mycorrhizal fungus inoculation were analysed by two factor ANOVA with mycorrhiza and plant competition as independent variables. Differences between means were inspected using Tukey's honestly significant difference test. Statistical analyses were performed using the ANOVA procedure in SAS 6.12 (SAS Institute, Cary, N.C.).

3.4 Results

3.4.1 Plant combination

The shoot yield of *L. perenne* was significantly higher when grown in combination with *T. repens* compared to when grown in monoculture (Table 1, Fig. 1a). Increased shoot biomass was not associated with changes in root biomass. However, roots of *L. perenne* were significantly longer (Table 1, Fig. 2a) and the number of root tips was enhanced (Table 1, Fig. 3a) when grown with *T. repens* compared to monoculture. Although root biomass and the diameter of roots (Table1, Fig. 4) did not differ between the treatments, root volume of *L. perenne* was significantly increased when grown with *T. repens* (Table 1).

Fig. 1

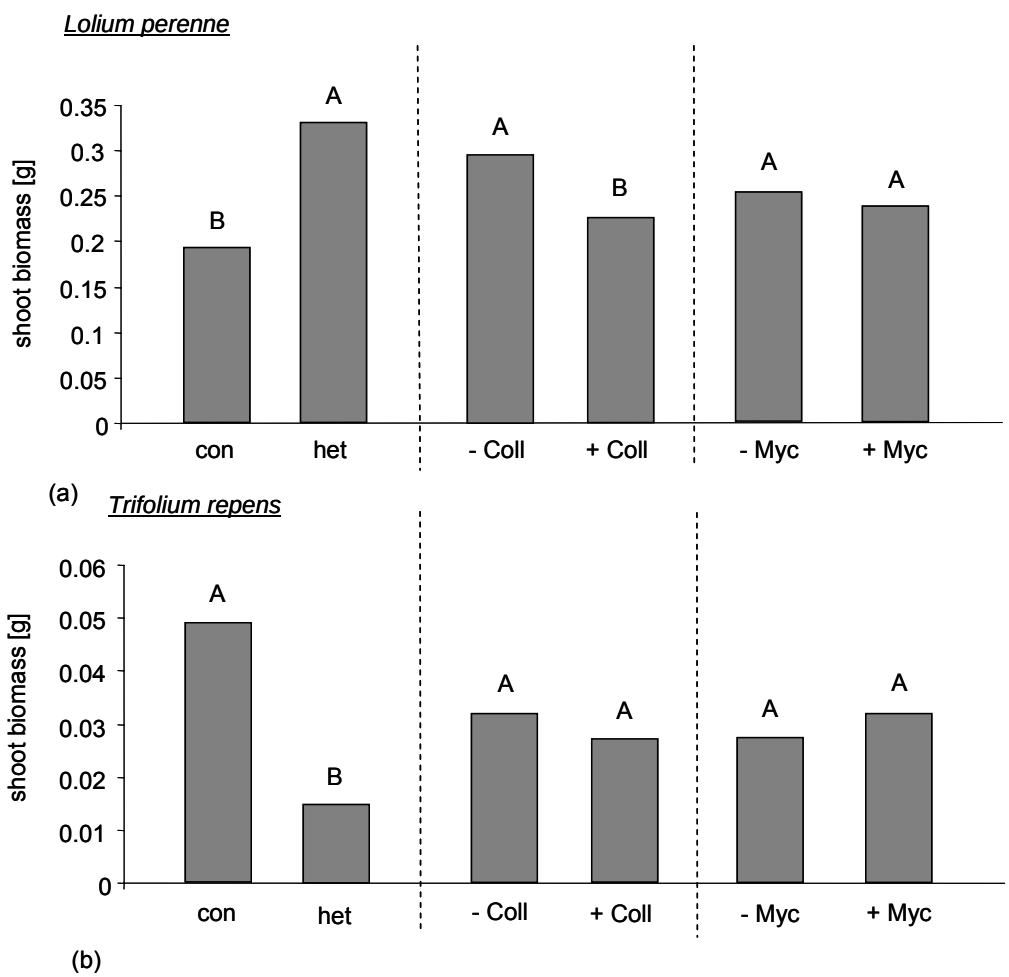


Figure 1. Effect of presence of con- and heterospecific competitors, Collembola and mycorrhization on shoot biomass of *Lolium perenne* and *Trifolium repens* respectively. Bars sharing the same letter are not significantly different (Tukey's honestly significant difference, $P<0.05$)

The shoot biomass of *T. repens* grown with conspecifics was significantly higher than when grown with *L. perenne* (Table 2, Fig. 1b). When grown with *L. perenne* root biomass (Table 2) and the length of roots (Table 2, Fig. 2b) of *T. repens* were significantly reduced. However, root diameter (Table 2, Fig. 4), root volume and number of root tips remained unaffected (Table 2).

Shoot C/N ratio in *T. repens* and *L. perenne* were not significantly affected by plant combination (average: *T. repens*: 15.389, *L. perenne*: 9.831).

Fig. 2

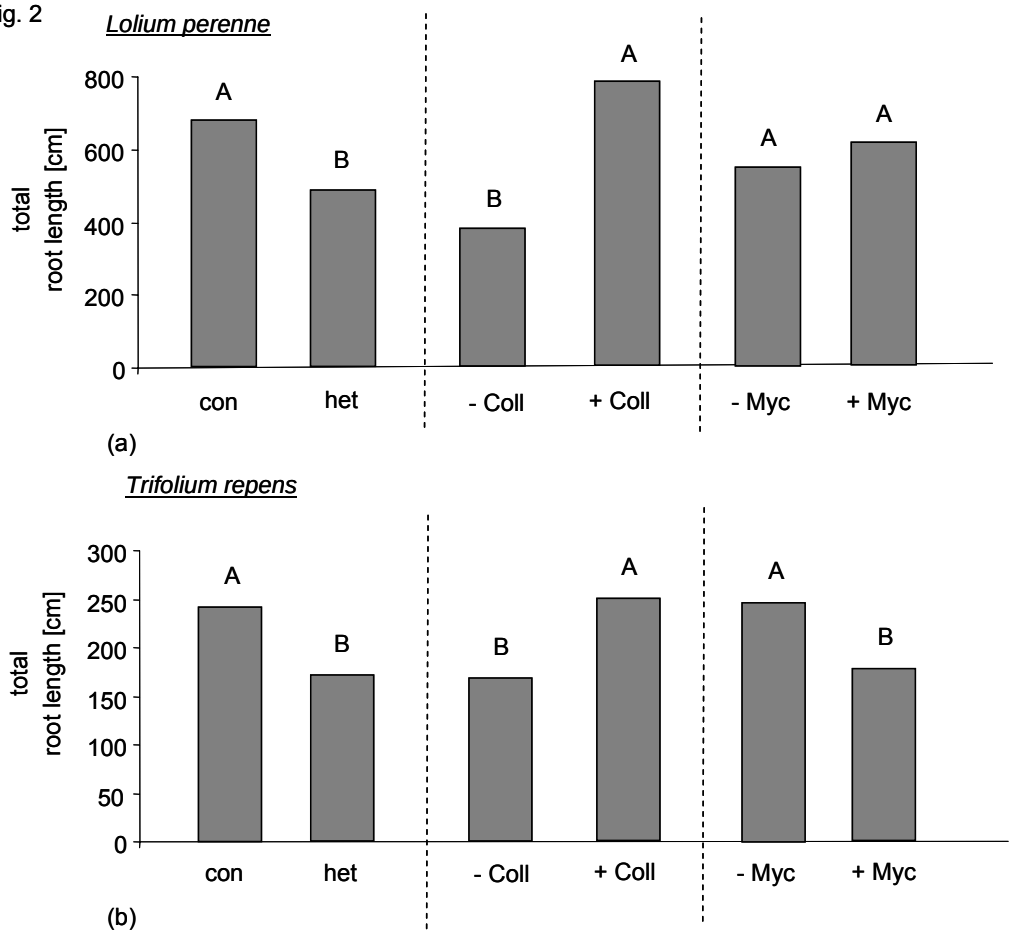


Figure 2. Effect of presence of con- and heterospecific competitors, Collembola and mycorrhization on root length of *Lolium perenne* and *Trifolium repens* respectively. Bars sharing the same letter are not significantly different (Tukey's honestly significant difference, $P<0.05$)

Table 1. F-values of a three-factor ANOVA on the effects of Collembola, presence of con-/heterospecific competitors and mycorrhiza on shoot biomass, root biomass, root volume, root diameter, root length and number of root tips of *Lolium perenne* (n= 5).

		Shoot biomass		Root biomass		Root volume		Root diameter		Root length		Root tips	
	df	F	P	F	P	F	P	F	P	F	P	F	P
Collembola (Coll.)	1	5.72	0.0228	3.22	0.0823	131.86	<0.0001	42.18	<0.0001	104.60	<0.0001	5.72	0.0228
Plant combination (Com.)	1	22.59	<0.0001	0.05	0.8201	23.70	<0.0001	0.14	0.7117	23.93	<0.0001	22.59	<0.0001
Mycorrhiza (Myc.)	1	0.19	0.6629	0.02	0.8804	0.98	0.3298	0.12	0.7317	2.77	0.1061	0.19	0.6629
Coll. x Com.	1	2.80	0.1043	1.94	0.1728	32.36	<0.0001	9.69	0.0039	20.49	<0.0001	2.80	0.1043
Coll. x Myc.	1	0.85	0.3635	0.05	0.8201	0.44	0.5114	0.14	0.7090	0.04	0.8417	0.85	0.3635
Com. x Myc.	1	0.29	0.5945	0.21	0.6522	0.24	0.6266	0.35	0.5597	1.45	0.2379	0.29	0.5945
Coll. x Com. x Myc.	1	0.05	0.8164	0.18	0.6708	2.63	0.1146	1.95	0.1724	4.17	0.0494	0.05	0.8164

df = degrees of freedom

Table 2. F-values of a three-factor ANOVA on the effects of Collembola, presence of con-/heterospecific competitors and mycorrhiza on shoot biomass, root biomass, root volume, root diameter, root length and number of root tips of Trifolium repens (n= 5).

		Shoot biomass		Root biomass		Root volume		Root diameter		Root length		Root tips	
	df	F	P	F	P	F	P	F	P	F	P	F	P
Collembola (Coll.)	1	0.55	0.4625	0.31	0.5795	2.31	0.1396	0.79	0.3801	6.98	0.0131	4.39	0.0450
Plant combination (Com.)	1	24.93	<0.0001	4.85	0.0347	0.47	0.4966	0.08	0.7755	4.96	0.0338	2.01	0.1672
Mycorrhiza (Myc.)	1	0.51	0.4784	1.31	0.2601	3.45	0.0733	5.30	0.0287	5.45	0.0267	7.13	0.0123
Coll. x Com.	1	1.45	0.2381	0.06	0.8051	1.88	0.1809	0.73	0.3990	3.28	0.0806	0.41	0.5249
Coll. x Myc.	1	1.05	0.3131	0.00	1.0000	0.70	0.4099	1.30	0.2644	0.30	0.5905	0.01	0.9124
Com. x Myc.	1	2.07	0.1599	1.15	0.2919	0.40	0.5306	0.06	0.8112	0.15	0.6975	0.73	0.3985
Coll. x Com. x Myc.	1	0.10	0.7527	1.14	0.2925	4.45	0.0436	2.29	0.1412	8.78	0.0060	6.61	0.0155

df = degrees of freedom

3.4.2 Effects of mycorrhiza

Inoculation with *G. intraradices* mycorrhiza affected neither below- nor aboveground biomass of *L. perenne* (Table 1, Fig. 1a). Furthermore, inoculation of plant roots with mycorrhizal fungi did not affect root length, root volume and root diameter or the number of root tips (Fig. 2a, 3a, 4a).

Fig. 3

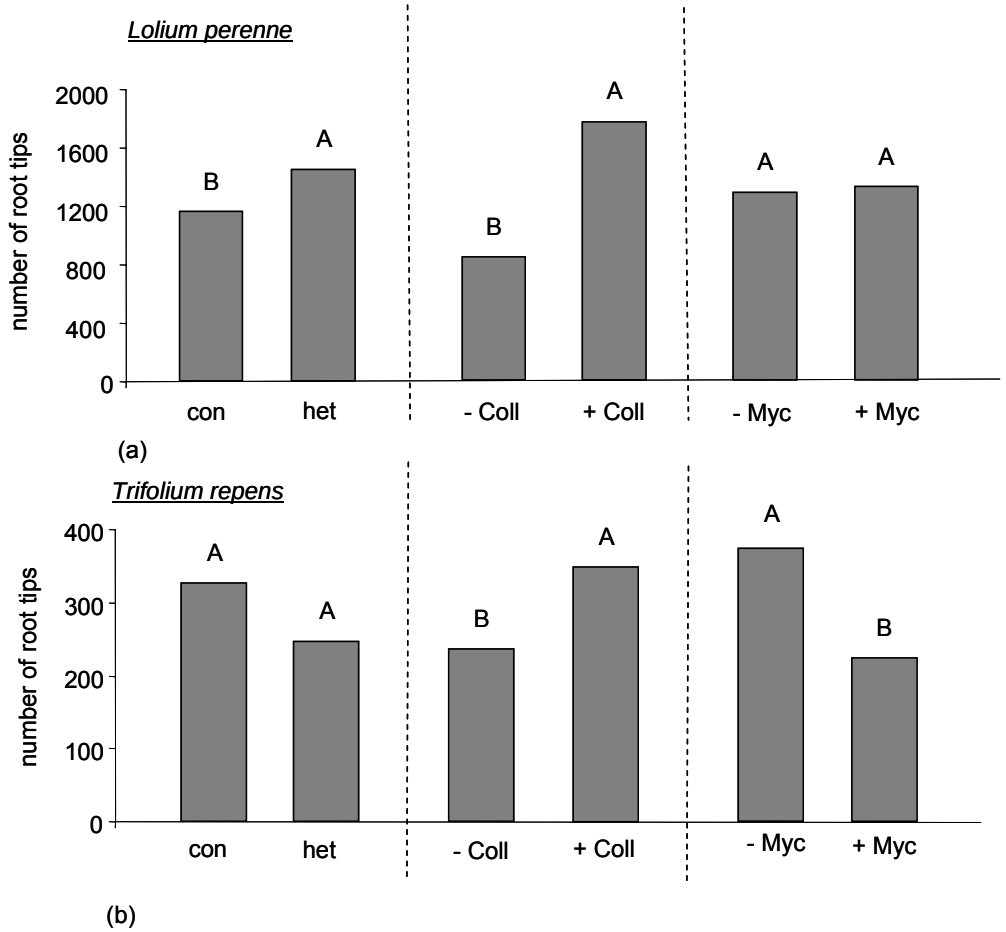


Figure 3. Effect of presence of con- and heterospecific competitors, Collembola and mycorrhization on root diameter of *Lolium perenne* and *Trifolium repens* respectively. Bars sharing the same letter are not significantly different (Tukey's honestly significant difference, $P<0.05$)

As in *L. perenne,* inoculation with the mycorrhizal fungus did not affect shoot or root biomass of *T. repens,* but significantly reduced root length (Table 1, 2, Fig. 2b), root diameter and the number of root tips (Table 2, Fig. 3b, 4b). Furthermore, the extent of mycorrhizal inoculation of roots of *T. repens* grown with *L. perenne* significantly exceeded that when grown with conspecifics ($F_{5,24}$=4.74, P=0.0410).

The average colonisation of roots of *L. perenne* by *G. intraradices* (86.66% of root length) generally exceeded that of *T. repens* (67.97%). Inoculation with mycorrhizal fungi affected neither C/N ratio of *T. repens* nor that of *L. perenne* (average: *T. repens*: 15.336, *L. perenne*: 9.809).

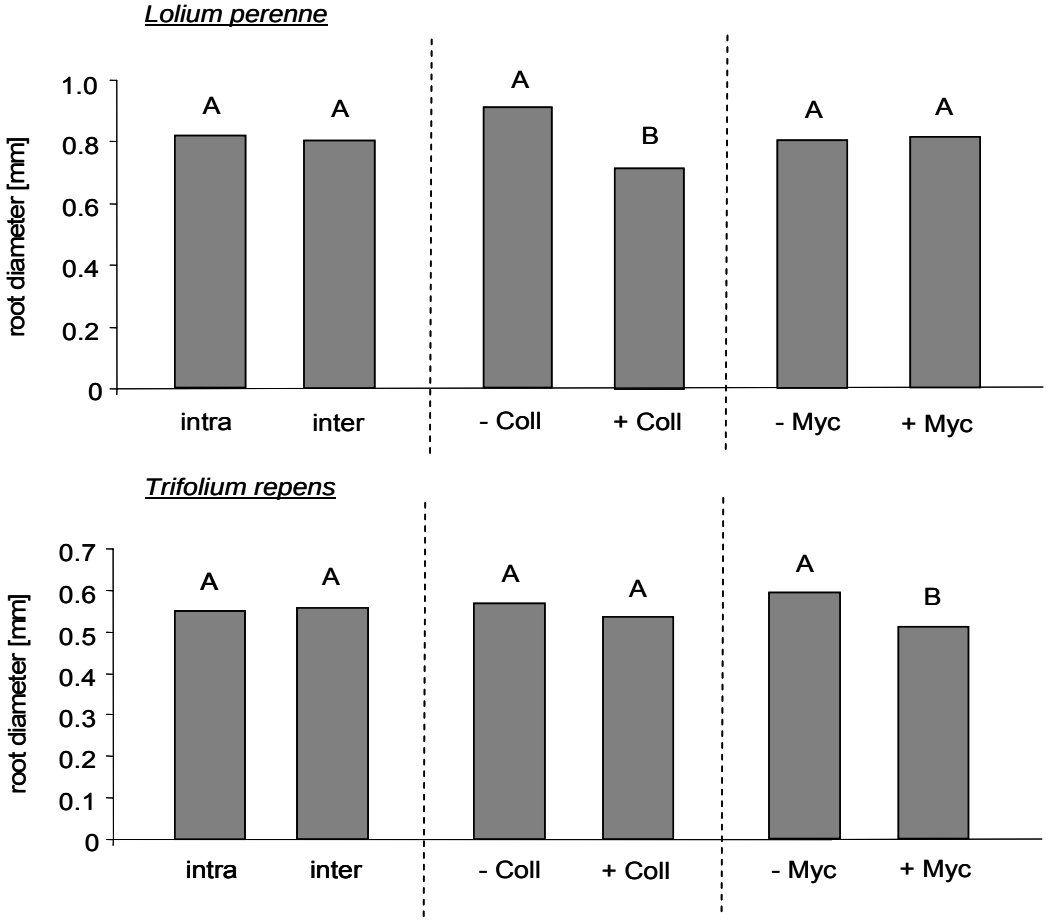


Figure 4. Effect of presence of con- and heterospecific competitors, Collembola and mycorrhization on root number of root tips *Lolium perenne* and *Trifolium repens* respectively. Bars sharing the same letter are not significantly different (Tukey's honestly significant difference, P<0.05)

3.4.3 Effects of Collembola

Collembola significantly reduced shoot but not root biomass of *L. perenne* (Table 1, Fig. 1a). However, Collembola significantly increased root length (Table 1, Fig. 2a) and the number of root tips (Table 1, Fig. 3a), but decreased root volume and root diameter (Table 1, Fig. 4a).

Collembola did not affect above- and belowground biomass, root volume and root diameter of *T. repens*. However, they did enhance the length of roots of *T. repens,* but reduced the number of root tips (Table 2, Fig. 1a, 2a, 3a, and 4a). In contrast, Collembola significantly reduced the colonisation of roots of *T. repens* by mycorrhizal fungi by about 20% ($F_{3,26}$=5.23 P=0.0327), but did not affect mycorrhizal infection of roots of *L. perenne*. Collembola generally did not affect the C/N ratio of the two plant species (average: *T. repens*: 15.338, *L. perenne*: 9.809).

Inoculation of plant roots with *G. intraradices* generally did not affect Collembola density, but the number of Collembola significantly differed between the competition treatments ($F_{5,24}$=13.79, P=0.0006), with an average of 162 individuals per rhizotron in *T. repens* monocultures and an approximately three times higher density in *L. perenne* monocultures and in combinations of *T. repens* and *L. perenne* (average of 403 and 435 individuals per rhizotron, respectively).

3.5 Discussion

3.5.1 Plant combination

Competition is one of the most important factors structuring plant communities. Plant responses may differ between intra- and interspecific competition depending on plant species and plant functional group (Gersani et al. 2001, Maina et al. 2002). Plant competitiveness varies with plant root structure and root foraging strategy (Lodge 2000, Rajaniemi and Reynolds 2004). Hence, plant competitiveness likely increases with the growth rate of roots and the extension of the root system (Aerts 1999). In the present experiment *L. perenne* grew faster and built up significantly more biomass than *T. repens*. The presence of *L. perenne* was associated with a reduced biomass of *T. repens* compared to monoculture treatments. This is consistent with findings of other studies demonstrating a competitive superiority of ryegrass over clover (Munoz and Weaver 1999, Lucero et al. 1999). Strong competitiveness of *L. perenne* likely is due to the ramified root system which is characteristic for grasses and allows effective uptake of nutrients (Stone et al. 1998). Furthermore, in soils with high N availability the competitive advantage of nitrogen-fixing legumes is abrogated. Autoclaving soil, as done in the present experiment to eliminate mycorrhiza, mobilises nutrients, especially N, and may have contributed to the low competitiveness of *T. repens* in our experiment. This is supported by the low number of nodules observed for *T. repens*.

The different responses of the two plant species to monoculture and the combined treatment are probably due to the different growth rates of both plant species. The grass had a higher growth rate than the clover. Therefore, competition between conspecifics of *L. perenne* was higher in the limited space of the rhizotrons compared to monocultures of *T. repens*. Furthermore the grass outcompeted clover in the combined treatments.

In addition to shoot biomass, presence of con- and heterospecific competitors also affected root structure; similar results have been documented (Gersani 1998). Intra- and interspecific competition often results in an increase in root biomass (Maina et al. 2002, O'Brien et al. 2005). Generally, competition between plant roots is more intense among plants with similar root morphology (Rubio et al. 2001). In the present experiment, *L. perenne* produced longer roots, more root tips and an enhanced root volume when competing with *T. repens* as compared to when grown with conspecifics. In contrast, *T. repens* produced longer roots when grown in monoculture as compared to when grown in competition with *L. perenne*. Root proliferation is strongly affected by the availability of nutrients and allows plants to exploit resources to the disadvantage of the competitor (Hodge et al. 1999, Gersani et al. 2001). There is evidence that plant species are able to differentiate conspecific and heterospecific roots and adjust the response of the root system accordingly (Huber-Sannwald et al. 1996).

3.5.2 Mycorrhiza

The response of plants to inoculation with AM fungi species varies with the mycorrhizal fungus (Joner and Leyval 2001, Rogers et al. 2001, Klironomos 2003). In the present experiment root colonisation with mycorrhizal fungi in *L. perenne* exceeded that in *T. repens*. Nevertheless, plant growth and root morphology in *L. perenne* were little affected by mycorrhiza whereas in *T. repens* mycorrhiza reduced root length, number of root tips and root diameter.

Mycorrhizal fungi allow plants to reduce investment into roots since mycorrhizal hyphae compensate for reduced extension of the root system (Smith and Read 1997, Harrison 1997, van der Heijden 2004). Colonisation of roots by mycorrhizal fungi

therefore likely affects competition of plant species (van der Heijden et al. 2003, Smith et al. 1999). Indeed, in previous experiments mycorrhizal fungi increased the competitive strength of clover against ryegrass (Hamel et al. 1992, Joner and Leyval 2001). This might have been due to higher colonisation of roots by mycorrhizal fungi of the legume compared to the grass. However, in the present experiment mycorrhizal fungi did not increase the competitiveness of clover. This might have been due to the higher colonisation by mycorrhizal fungi of *L. perenne* roots compared to roots of *T. repens*. Colonisation by mycorrhizal fungi is probably correlated with the plant nutrient status. Blanke et al. (2005) demonstrated a negative correlation between root colonisation of *Artemisia vulgaris* by AMF and tissue N concentration. Therefore, differences in colonisation by mycorrhizal fungi are possibly due to different nutrient contents.

3.5.3 Collembola

Collembola may stimulate or reduce plant nutrition and growth (Harris and Boerner 1990, Bardgett and Chan 1999, Scheu et al. 1999, Lussenhop and BassiriRad 2005). In addition, Collembola may significantly affect root growth without affecting shoot growth (Scheu et al. 1999, Endlweber and Scheu 2006). In the present experiment, Collembola increased root length and number of root tips in both plant species but reduced shoot biomass, root volume and root diameter in *L. perenne*. The reduction in shoot biomass of *L. perenne* was more pronounced when grown with *T. repens* suggesting that Collembola reduced the competitive superiority of *L. perenne* over *T. repens*. These findings and previous experiments (Kreuzer et al. 2004, Partsch et al. 2006) suggest that Collembola generally increase the competitive strength of legumes against grasses. The increase in the competitive strength of legumes may

be caused by increased nodule occupancy in legumes in the presence of Collembola (Lussenhop 1993).

Increased root elongation and number of root tips likely reflect an increase in the availability of nitrate (Zhang and Forde 2000, Mantelin and Touraine 2004). Collembola may increase N availability in the rhizosphere via enhancing microbial N mineralisation and by forming nutrient rich patches through excretion (Bardgett and Chan 1999, Petersen 2000, Sjursen and Holmstrup 2004). Presumably, in the present experiment mineral N made available by Collembola at microsites in soil, such as droppings of excreta, was nitrified quickly and this stimulated root elongation and branching. However, the potentially increased availability of N by Collembola was not reflected by shoot N concentration. In fact, the reduction of shoot biomass by Collembola contradicts the assumption that Collembola enhanced nutrient availability to plants. This reduction might have been due to a decline in nutrients provided by mycorrhizas. Collembola have been shown to alter mycorrhization of plant roots with the effect being density dependent (Ek et al. 1994, Lussenhop 1996, Bakonyi et al. 2002). In the present experiment Collembola reduced the colonisation of roots by mycorrhiza of *T. repens* which likely was caused by grazing on mycorrhizal hyphae. Surprisingly, however, Collembola did not significantly affect root colonisation by mycorrhiza in *L. perenne* although mycorrhizal colonisation of roots in *L. perenne* exceeded that in *T. repens*.

Collembola density was significantly higher in treatments with ryegrass suggesting that they benefited from high root biomass and associated high root exudates and increased biomass of saprophytic fungi (cf. Salamon et al. 2004, Sung et al. 2006). Milcu et al. (2006) found Collembola density to be reduced in the presence of legumes, whereas it was increased in the presence of grasses.

3.6 Conclusions

Presence of Collembola alters root structure with longer and thinner roots and therefore likely affects plant resource exploitation. Although plant C and N content remained unaffected presence of Collembola declined shoot biomass. Overall, results of the present experiment and previous studies suggest that the effect of Collembola depends on plant species with grasses being more vulnerable than legumes. Therefore, Collembola likely reduce the competitive superiority of grasses over legumes. The Collembola-mediated reduction in shoot biomass and changes in root structure were not related to changes in mycorrhizal fungus colonisation of roots. Hence, the effect of Collembola on root morphology and shoot biomass presumably is not caused by affecting plant-mycorrhiza interrelationships, but possibly by direct grazing on roots and on saprotrophic fungi.

CHAPTER 4

DIETARY ROUTING IN COLLEMBOLA: DETERMINING COLLEMBOLA FEEDING PREFERENCES BY STABLE ISOTOPE AND COMPOUND SPECIFIC FATTY ACID ANALYSIS

4.1 Abstract

Collembola are abundant and ubiquitous soil decomposers, being particularly active in the rhizosphere of plants where they are assumed to be attracted by high microbial activity and biomass. While feeding on root associated microorganisms or organic matter they may damage and ingest plant roots e.g. particularly root hairs and fine roots. Employing stable isotope analysis and compound specific ^{13}C analysis of fatty acids we investigated Collembola feeding preferences and types of ingested resources. We offered Collembola two resources with distinct isotope signature: a C_4 plant (*Zea mays* L.) planted in soil with ^{15}N labelled litter of *Lolium perenne* L. (C_3 plant). We hypothesised that Collembola obtain their nutrients (C and N) from different resources, with their carbon being mainly derived from resources that are closely associated to the plant root e.g. root exudates causing enrichment in ^{13}C in Collembola tissue, while the incorporated nitrogen originates from litter resources.

In contrast to our hypothesis, bulk stable isotope analysis and compound specific analysis of fatty acids suggest that Collembola derived the majority of incorporated C and N from plant roots. Fatty acid $\delta^{13}C$ and tissue $\delta^{13}C$ and $\delta^{15}N$ ratios of Collembola resembled those of maize plants. Furthermore of ^{13}C signatures of fatty acids in Collembola corresponded to fatty acids in maize rather than to fatty acids from soil microorganisms. The results indicate that Collembola in the rhizosphere of plants, being assumed to be mainly decomposers, predominately live on plant resources,

presumably fine roots or root hairs i.e. are herbivorous rather than decomposers or fungivorous.

4.2 Introduction

Collembola are among the most abundant soil arthropods, reaching particularly high densities in the rhizosphere of plants. Collembola affect plant growth and nutrition as well as plant performance in a variety of ways (Theenhaus et al. 1999, Endlweber and Scheu 2006). Changes in plant growth in presence of Collembola often are ascribed to increased nutrient mineralization and an associated increased nutrient uptake by plants (Bardgett and Cook 1998, Bardgett and Chan 1999, Lussenhop and BassiRad 1995). In fact, Collembola increase plant nutrient contents but the effect varies between plant species and functional groups (Scheu et al. 1999, Kreuzer et al. 2004, Partsch et al. 2006).

Collembola mobilise nutrients by grazing on fungi and bacteria and by the formation of nutrient rich patches, i.e. by depositing faecal pellets (Teuben 1991, Lussenhop 1992, Jones 1998, Filser 2002). The formation of nutrient rich patches and increased nutrient mineralization therein likely induces root proliferation toward these “hotspots” (van Vuuren et al. 1996, Hodge et al. 1999, Hodge et al. 2000). In fact, Collembola affect root performance and induce the production of longer and thinner roots (Endlweber and Scheu 2006, 2007). However, the changes in root performance are not necessarily associated with an increase in tissue nutrient concentration or plant biomass. The mechanisms responsible for the changes in root morphology in presence of Collembola therefore remain little understood. In particular, it need to be resolved if Collembola feed on root tissue or accidentally damage roots when feeding on fungal hyphae and organic matter in the vicinity of roots. Root hairs may be

ingested or at least damaged by Collembola, and damaged plant roots may induce changes in plant metabolism resulting in altered root performance. Detailed knowledge on the feeding behaviour of Collembola in situ is needed to disentangle Collembola-plant interrelationships.

Collembola are generalist feeders, ingesting a wide variety of resources. They probably obtain nutrients such as N and C from different resources with the ratio of incorporated nutrients depending on food quality (Scheu and Folger 2004). The analysis of stable isotope ratios in combination with fatty acid analysis offers the opportunity for investigating the contribution of different food resources to the diet of Collembola and therefore may allow uncovering trophic relationships between Collembola, rhizosphere fungi and plants.

Stable isotope ratios of animal tissue, in particular ^{13}C signatures, reflect the stable isotope ratio of the resources ingested (Peterson and Frey 1987, Post et al. 2002). By offering two resources with different stable isotope signatures the proportion of incorporated C and N derived from both resources can be determined, and this has been used to analyse dietary preferences of Collembola in the laboratory (Scheu and Folger 2002, Chamberlain et al. 2006). Food resources with different $^{13}C/^{12}C$ ratios can easily be obtained by using plant materials originating from C3 or C4 plants due to the discrimination of ^{13}C by Rubisco in the photosynthetic pathway of C3 plants.

Another recently introduced tool to analyse trophic links between Collembola and their food resources is the evaluation of the composition and ^{13}C signature of fatty acids in the diet and the consumer (Ruess et al. 2005, Chamberlain et al. 2006). Phospholipid fatty acids (PLFAs) as components of cell membranes differ in particular between bacteria and fungi but also between bacterial phyla. In consumers neutral fatty acids (NLFAs), i.e. storage lipids, are linked to the animal's diet. Fatty acids of the diet in part are incorporated into animal tissue without or with slight

modifications. The evaluation of NLFA patterns combined with compound specific ^{13}C analysis of fatty acids provides a unique and powerful tool to trace food resources of Collembola (Ruess et al. 2004, Ruess et al. 2005).

In the present study we evaluated the diet of Collembola by offering resources with distinct isotope signature. We established a laboratory system consisting of a C4 plant (*Zea mays* L.) planted in soil mixed with ^{15}N labelled litter of *Lolium perenne* L. (C3 plant). We hypothesized that (i) the diet of Collembola is based in large on carbon resources entering the soil via roots, such as root exudates, therefore Collembola in our laboratory system with maize will be enriched in ^{13}C, and (ii) a large fraction of the nitrogen in Collembola tissue originates from litter resources, i.e. Collembola obtain N and C for tissue formation from different dietary resources.

4.3 Materials and Methods

The experiment was conducted in microcosms (diameter 10 cm, height 25 cm), sealed with a 45 µm mesh at the bottom to allow drainage. Microcosms were filled with 1 kg soil taken from an arable field (Jena, Thuringia, Germany). Prior to adding to the microcosms the soil was sieved (4 mm mesh) and frozen at -20°C for defaunation. The experiment was set up in a two factorial design with the factors Litter (with and without) and Plant (Maize; with and without). Litter (1.25 g dry weight) consisting of thoroughly mixed 250 mg labelled ($\delta^{15}N$ = 11084) and 1000 mg unlabelled *Lolium perenne* L. shoots was added to half of the microcosms. The litter was reduced to small pieces (<0.5mm) and homogeneously mixed into the soil. Plant treatments received one Maize seed (*Zea mays* L) per microcosm. The treatments were replicated 10 times and watered every other day with 15 ml deionised water. The microcosms were incubated in a temperature controlled greenhouse equipped

with lamps for increasing radiation and setting day/night cycles (16 h light, 18°C) and arranged in a complete randomised design. After germination of the maize seeds each treatment received 100 individuals of the Collembola species *Protaphorura fimata* Gisin taken from laboratory cultures.

Microcosms were harvested after 8 weeks. Plant roots were washed, weighed and divided into two subsamples. Half of the subsamples were frozen at -20°C until further analysis. The other half was dried at 60°C for three days. Soil samples were taken prior to washing the roots. Half of the soil samples were stored at -20°C the other half dried at 60°C for three days. During the washing procedure, Collembola floating on the water surface were collected and frozen at -20°C. Collembola taken from treatments with plants were divided into two subsamples which were dried at 60°C or stored at -20°C, respectively. All other Collembola were dried at 60°C for stable isotope analysis.

The dried soil and plant materials as well as the litter samples were milled in a ball mill (Retsch, Haan, Germany). Collembola and subsamples of the milled materials were weighed into tin capsules for stable isotope analysis. Stable isotope ratios of the samples were analysed in a system consisting of an elemental analyser (NA 1500, Carlo Erba, Milan) coupled with a mass spectrometer (MAT 251, Finnigan; Reineking et al. 1993). Acetanilide (C_8H_9NO; Merck, Darmstadt) was used for internal calibration. The ratio between ^{13}C and ^{12}C was expressed relative to that in Pee Dee Belemnite (marine limestone). For ^{15}N atmospheric nitrogen served as primary standard. Ratios [‰] were calculated according to the following formula: $\delta X = (R_{sample} - R_{standard})/(R_{standard}) \times 1000$ (Peterson and Fry 1987), with X representing the heavier isotope (^{15}N or ^{13}C), and R the ratio between the heavy and the light isotope ($^{15}N/^{14}N$ respectively $^{13}C/^{12}C$). The proportion of N incorporated in Collembola tissue

derived from litter was calculated by a two-source mixing model (Newman Gearing 1991): $F=(\delta^{15}N_{Collembola\ +litter} - \delta^{15}N_{litter})/ (\delta^{15}N_{maize} - \delta^{15}N_{litter})*100$

Three samples from each treatment were chosen for fatty acid analysis of Collembola, soil microorganisms and root material. Lipids of Collembola and of microorganisms in soil were extracted by shaking in a single phase extraction solvent consisting of chloroform, methanol and citrate buffer in ratios of 1.0:2.0:0.8 (Bligh and Dyer 1959). After addition of distilled water and $CHCl_3$ and centrifugation the chloroform fraction of each sample was transferred to a silicic acid column. The lipids were eluted by consecutively adding chloroform (neutral lipids), acetone (glycolipids) and methanol (phospholipids). For soil microorganisms phospholipids were used for further analysis, whereas neutral lipids were used for analyses of the fatty acid composition of Collembola.

Plant material and samples of Collembola and soil microorganisms were saponified and methylated (procedure given for the Sherlock Microbial Identification System; MIDI, Newark, USA). The samples were stored at -20°C until further analysis. Samples were analysed by gas chromatography (Clarus 500 GC, PerkinElmer Inc., Waltham, Massachusetts, USA).

Compound specific $\delta^{13}C$ analysis of fatty acids was conducted in a gas-chromatography-combustion-isotope-ratio-monitoring-mass spectrometer system (GC-C-IRM-MS) consisting of a gas chromatograph (6890 Series, Agilent Technology, USA) coupled via a Conflow III interface (ThermoFinnigan, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Germany). A select FAME polar capillary column (50 m, 0.25 mm i.d., film thickness 0.25 mm) was used for the separation of the fatty acid methyl esters.

4.4.1 Statistical analysis:

Stable isotope signatures ($\delta^{13}C$ and $\delta^{15}N$) and differences in total N and C concentrations in Collembola tissue were analysed by two factorial ANOVA with the factors Litter and Plant. Differences between means were inspected using Tukey's honestly significant difference test. Statistical analyses were performed using the ANOVA procedure in SAS 6.12 (SAS Institute, Cary, USA).

4.5 Results

4.5.1 Stable Isotopes

4.5.1.1 Plants

The addition of labelled litter (^{15}N) significantly increased plant shoot biomass ($F_{1,16}$=5.88, P=0.020) but did not affect root biomass. Total C and N concentration in plant tissue was not affected by the addition of litter, but added litter increased the $\delta^{15}N$ signature of plant roots from 2.73‰ to 385.20‰ ($F_{1,16}$=14.74, P< 0.001; Fig. 1). In contrast, plant shoots were significantly depleted in ^{13}C in presence of litter with the ratio dropping from -13.73‰ to -14.60‰ ($F_{1,16}$=19.87, P< 0.001; Fig. 1).

4.5.1.2 Collembola

Total Collembola biomass was increased by about 40% in treatments with litter and with both litter and plants compared to the control without litter and plants. Presence of plants increased Collembola biomass by approximately 25%. Further, the availability of litter significantly increased tissue N concentration in Collembola ($F_{3,36}$=13.53, P=0.003), whereas the presence of maize had no significant effect. Also, the addition of labelled litter significantly enhanced the $\delta^{15}N$ ratio of Collembola from 9.10‰ to 1803‰ ($F_{3,36}$=96.57, P<0.0001; Fig. 1).

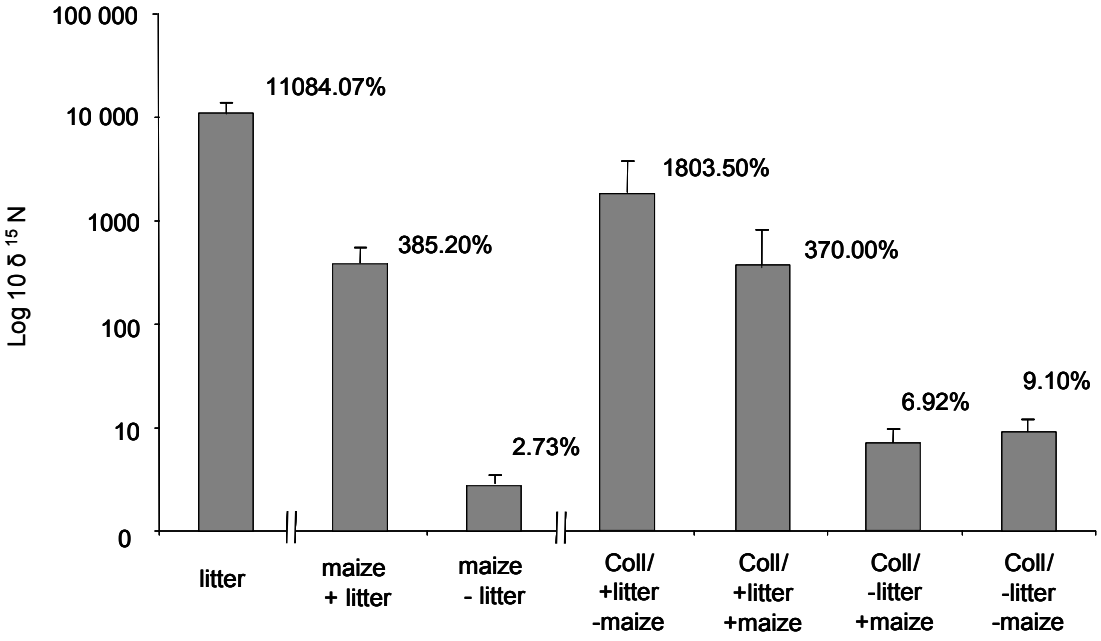


Figure 1. $\delta^{15}N$ ratios of Collembola (Coll) in treatments with ^{15}N labelled litter (+ litter - maize), plants (- litter + maize), and both litter and plants (+ litter + maize). $\delta^{15}N$ ratios of the ^{15}N labelled litter, and maize grown with (maize + litter) and without litter (maize - litter) are given as control; note log-scale. (log-transformed data).

Although total N concentration remained unaffected, the presence of plants decreased the $\delta^{15}N$ ratio of Collembola (from 1803‰ to 370‰; $F_{3,36}$=39.10, P<0.0001; Fig. 1); it was at a minimum in plant treatments without litter addition (6.92‰). The proportion of ^{15}N obtained from litter decreased in presence of plants from 16.22% in treatments without plants to 3.31% in plant treatments.

The $\delta^{13}C$ ratio of Collembola was marginally higher in treatments without plants and without litter (-24.13‰) compared to treatments with litter (–23.41‰). In treatments with maize plants Collembola were significantly enriched in ^{13}C by approximately 10‰ compared to Collembola in treatments without plants, with the signature increasing from -14.05‰ with litter addition to -12.88‰ without litter ($F_{3,36}$=141.61, P<0.001; Fig. 2).

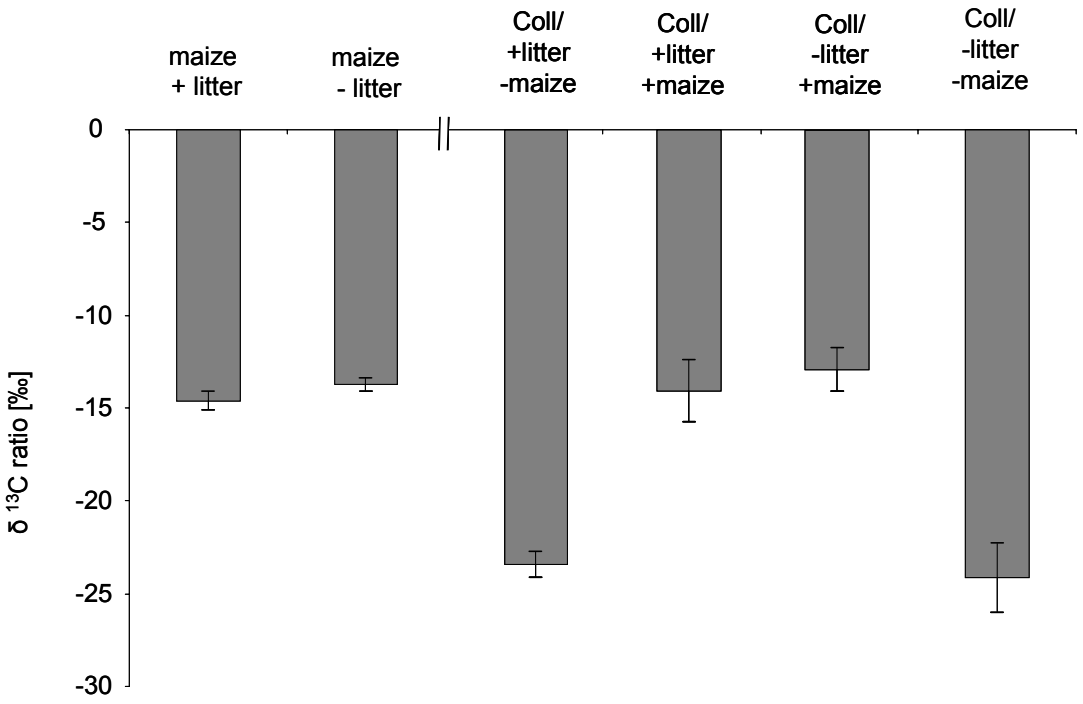


Figure 2. $\delta^{13}C$ [‰] Collembola (Coll) in treatments with ^{15}N labelled litter (+ litter - maize), plants (- litter + maize), and both litter and plants (+ litter + maize). $\delta^{15}N$ ratios of maize grown with (maize + litter) and without litter (maize - litter) are given as control.

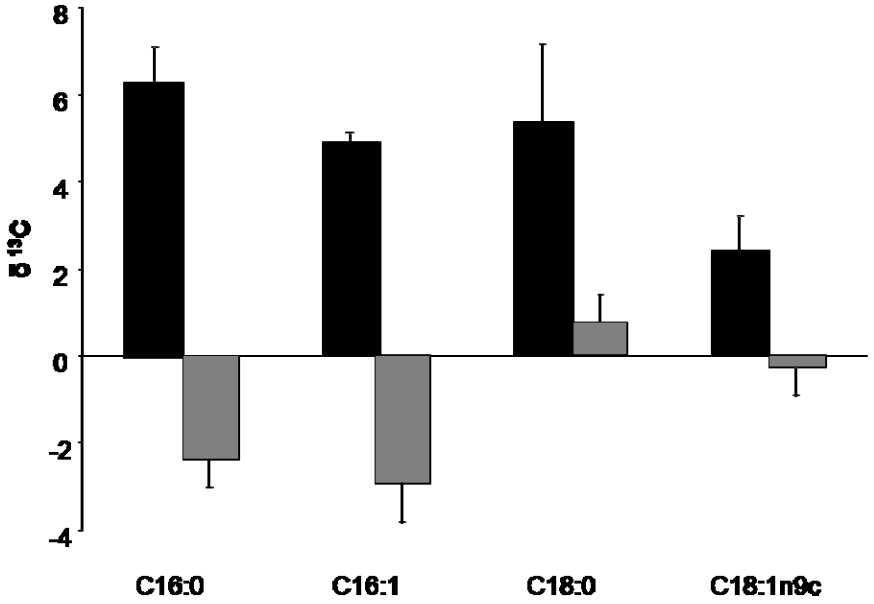
4.5.2 Fatty acids

Soil and litter. The predominant fatty acids obtained from soil microorganisms were myristoleic (14:1), palmitic (16:0), palmitoleic (16:1) and linoleic acid (18:2ω6). Linoleic acid on average accounted for 44% of total fatty acids in treatments with litter addition and 14% in treatments without litter. Except for linoleic acid the pattern as well as the proportion of PLFAs from plant/litter treatments was not significantly different from plant treatments. The $\delta^{13}C$ values of fatty acids in soil corresponded to stable isotope analysis of bulk soil, but were slightly depleted in ^{13}C. The $\delta^{13}C$ values ranged between -27.22‰ and -31.78‰ and remained unaffected by litter addition.

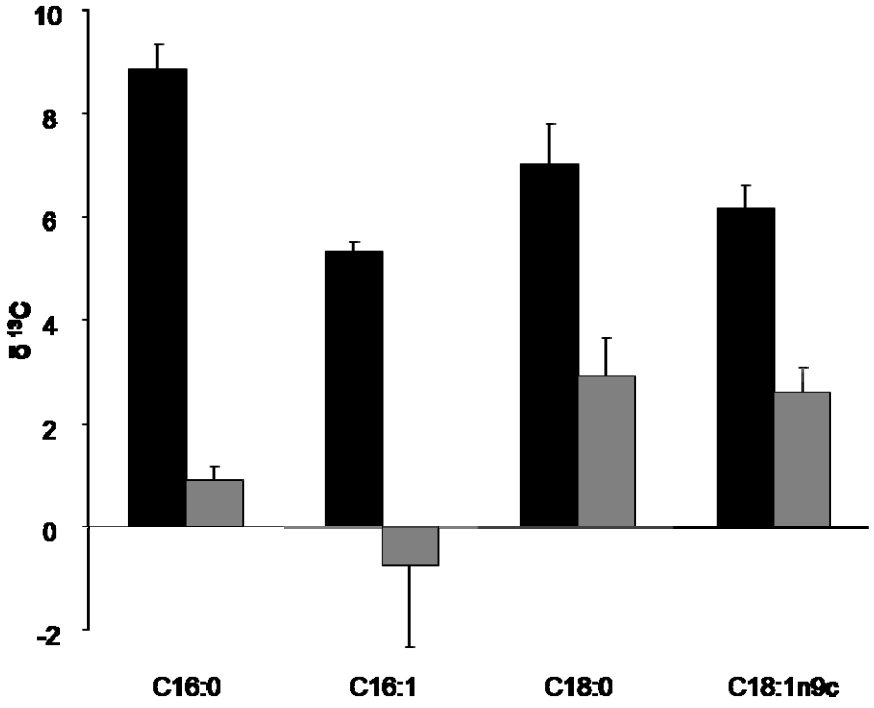
The predominant fatty acids in root material were palmitic (16:0) and linolelaidic (18:2ω6t) acid with 18:2ω6t accounting on average for 50% of the total fatty acids in plant roots. The fatty acid pattern and relative amounts of different fatty acids were not significantly different between treatments with and without litter. The $\delta^{13}C$ values of plants were depleted by 4-10‰ compared to bulk tissues; they ranged between -18.44‰ and -25.38‰.

Collembola. Fatty acid profiles of Collembola were not affected by the availability of litter; the proportion of fatty acids and the fatty acid composition were not significantly different in plant/litter and plant treatments. The predominant fatty acids in both treatments were palmitic (16:0), stearic (18:0) and oleic acid (18:1ω9c). Palmitic acid accounted for 59.87% and stearic acid for 26.35% of total fatty acids. Compared to bulk tissue the $\delta^{13}C$ values of Collembola fatty acids were depleted in ^{13}C by approximately 10‰, with $\delta^{13}C$ ranging between -20.25‰ and -27.04‰.

Generally, Collembola fatty acids reflected the $\delta^{13}C$ signal of fatty acids of maize plants (Fig. 3 a,b). Isotope signatures in Collembola was slightly different in treatments with and without litter addition. The fatty acids 16:0, 16:1, 18:0 and 18:1ω9c of Collembola taken from plant/litter treatments were enriched in ^{13}C by 3.86‰ up to 9.85‰ compared to soil microorganisms, whereas differences in isotope ratios ranged between -1.34‰ and 2.92‰ compared to the corresponding fatty acids in plant material, with the difference being most pronounced in 18:0 and 18:1ω9c (Fig 3a). In contrast, the differences in isotope signature between plant treatments with litter and without litter was highest in 16:0 and 16:1 compared to fatty acids obtained from plant roots (Fig. 3b). Generally, Collembola fatty acids in treatments with plants were enriched in ^{13}C by 2.42‰ to 6.29‰ compared to soil microorganisms and by -2.94‰ to 0.75‰ compared to root material.



(a)



(b)

Figure 3. Differences in carbon stable isotopes ($\delta^{13}C$ [‰]) in the fatty acids 16:0, 16:1, 18:0 and 18:1ω9c between Collembola and preferential resources, i.e. microorganisms (black bars) and plants (grey bars) in plant treatments (a) and plant/litter treatments (b).

4.6 Discussion

Food choice studies and gut content analyses suggest that Collembola are generalist feeders (Bardgett et al. 1993, Scheu and Simmerling 2004). They ingest a wide variety of food sources, e.g. dead organic matter and algae, but are assumed to be mainly fungal feeders. Food choice experiments offering different fungal taxa simultaneously indicated that Collembola preferentially feed on specific fungi species (Maraun 2003, Scheu and Simmerling 2004). Collembola probably ingest resources that are easily available rather than food sources which require an intensive search. However, as indicated by stable isotope analyses the micro-niches exploited by different species of Collembola consist of very different food resources (Chahartaghi et al. 2005). Feeding on a variety of resources, i.e. ingesting mixed diets has been shown to increase Collembola reproduction and fitness even if resources of low food quality are mixed with high quality food (Scheu and Folger 2004). Therefore, access to different food resources presumably enhances Collembola fitness and reproduction.

In the present experiment, Collembola generally benefited from the availability of litter and plants. While starving in control treatments, as indicated by the enrichment in ^{15}N (Haubert et al. 2005), Collembola density as well as tissue nitrogen and carbon concentration increased in presence of both litter and plants. However, the fraction of tissue carbon and nitrogen originating from litter and plants varied between both treatments. In litter treatments the fraction of tissue carbon originating from litter was high (61.8 %), whereas incorporation of litter-born nitrogen was low (16%). Consistent with our initial hypothesis the data indicate that Collembola derived C and N from different resources in litter treatments. Although litter derived nitrogen contributed only a minor part to the diet, Collembola benefited from the availability of litter as alternative food source as indicated by the high increase in tissue N

concentration. The presence of plants caused a similar increase in the concentration of nitrogen and carbon in Collembola tissue, indicating that plant and litter material was of similar food quality. However, in contrast to litter treatments, Collembola derived the majority of incorporated nutrients from plant roots, with the fractions being 97.8% for carbon and 96.8% for nitrogen. The results suggest a close association of Collembola with plant roots.

We predicted that Collembola resemble $\delta^{13}C$ ratios of maize plants and $\delta^{15}N$ of litter in combined treatments. In contrast to our expections, Collembola tissue $\delta^{15}N$ and $\delta^{13}C$ values in combined treatments were almost identical to those of maize plants, indicating that Collembola almost exclusively incorporated C and N originating from plants, whereas litter contributed little to their diet. Obviously, Collembola rely much more on plant resources than previously assumed. The capture of resources originating from plant roots may be via feeding on microorganisms in the rhizosphere, e.g. rhizoplane bacteria or mycorrhizal fungi, or by directly consuming plant roots. Plants release large amounts of resources which are assimilated by rhizosphere microorganisms, in particular by bacteria, resulting in increased microbial biomass. Further, large amounts of plant resources are allocated to mycorrhizal fungi. Both bacteria and mycorrhizal fungi may serve as food for Collembola. However, despite in our experiment $\delta^{13}C$ values of bacteria and mycorrhizal fungi may have been similar to those of plant roots, $\delta^{15}N$ signatures likely differed from those of plant roots. Bacteria and mycorrhizal fungi likely incorporated larger amounts of nitrogen from the added litter labelled with ^{15}N than plants. The virtually identical signatures of both $\delta^{13}C$ and $\delta^{15}N$ of plant roots and Collembola tissue therefore suggest that Collembola directly fed and digested plant roots and root hairs.

Overall, the results contradict our initial hypothesis that Collembola obtain C and N from different resources and particularly benefit from a mixed diet. Presumably, litter

is a less attractive food source due to slow decomposition and high fibre content compared to root hairs and other root derived resources.

In contrast to the plant and the plant and litter treatment, $\delta^{13}C$ ratios of Collembola of the control and litter treatments did not resemble those of soil and litter, i.e. those of their expected diet. Rather, Collembola compared to soil and litter were enriched in $\delta^{13}C$ by approximately 4‰. In contrast to nitrogen, $\delta^{13}C$ signatures differ little between resources and consumers (Post 2002).

Overall, the comparable fatty acid compositions and proportions of Collembola in treatments with and without litter addition indicate that the animals consumed similar diets in both treatments (Chamberlain et al. 2005, Haubert et al. 2006, Ruess et al. 2002, 2005). Corresponding to bulk stable isotope analysis, the compound specific analysis of fatty acids suggests that Collembola preferentially incorporated fatty acids originating from plant roots. However, signatures of fatty acids in Collembola indicate that fatty acids were not incorporated directly from diet into storage fat. Despite the availability of specific fatty acids in food sources, Collembola may synthesise fatty acids de novo (Chamberlain et al. 2005). The de novo synthesis of fatty acids results in a depletion in $\delta^{13}C$ compared to the diet, due to the enzymatic discrimination of the heavier isotope in lipid biosynthesis (DeNiro and Epstein 1977). Ruess et al. (2005) demonstrated the depletion of fatty acids in Collembola to vary between -2.6 and 1.4‰ from the incorporated diet. However, the isotopic discrimination is not constant and probably depends on the food source (Ruess et al. 2005). The generally lower $\delta^{13}C$ ratios of fatty acids in plants and Collembola compared to the stable isotope ratio of the respective bulk tissues are probably also caused by discrimination of ^{13}C in fatty acid biosynthesis in plants. Compared to bulk stable isotope ratios fatty acid ^{13}C signatures may be depleted by 5 - 9‰ in C_4 plants due to enzymatic discrimination (Ballentine et al. 1996, 1998).

Overall, the composition of fatty acids of Collembola in the present experiment is consistent with previous studies demonstrating Collembola to contain high amounts of palmitic (16:0), stearic (18:0) and oleic acid (18:1ω9c) (Haubert et al. 2004). Oleic acid has been proposed as marker for the incorporation of plant material in Collembola (Ruess et al. 2005, Chamberlain et al. 2005). Differences in $\delta^{13}C$ ratios of 18:1ω9c in Collembola and roots were particularly low in plant treatments without litter suggesting that Collembola indeed predominantly fed on roots. Unexpectedly, Collembola lacked linoleic acid (18:2 ω6,9) the marker fatty acid for fungi in the present study. Previous studies demonstrated linoleic acid to be among the most dominant fatty acids in Collembola (Haubert et al. 2004). In fact, the presence of litter increased the proportion of 18:2 ω6,9 in soils from 14% to 44% suggesting that fungal biomass strongly increased due to litter addition. The absence of 18:2 ω6,9 in Collembola further suggests the conclusion that in treatments with plants Collembola did not feed on fungi but rather on roots.

Overall, the results indicate that Collembola benefit to a similar extend from the availability of litter or plants. However, if both resources are offered Collembola preferentially feed on roots, thereby incorporating both nitrogen and carbon from plants. Collembola *(P. fimata*), being almost uniformly classified as decomposers, predominantly incorporate root resources if available, suggesting that they directly ingest fine roots and/or root hairs. This feeding activity on live plant biomass rather classifies the analyzed species as herbivorous. In contrast to root herbivores (such as nematodes, elaterid and curculionid larvae) feeding on plant roots by Collembola does not detrimentally affect plant performance, rather plant growth remained unaffected in the present experiment.

CHAPTER 5

DECOMPOSER ANIMALS (COLLEMBOLA) INDUCE THE EXPRESSION OF DEFENCE AND AUXIN GENES IN PLANTS

5.1 Abstract

The effects of decomposers on plant growth and performance are generally ascribed to enhanced nutrient availability to plants. However, decomposers influence plant performance by several indirect and direct pathways. Collembola, being among the most abundant soil decomposers, alter root morphology with the biomass and nutrient content of plants remaining unaffected. The genes involved in the reprogramming of plant development by Collembola are unknown. We linked for the first time plant phenotypic responses to decomposers with gene expression patterns using a custom-made DNA array focussing on genes related to plant secondary metabolism and defence.

Collembola (*Protaphorura fimata*) reduced the rosette diameter of *Arabidopsis thaliana* during early stages of rosette growth but this decrease was compensated later. The reduction of rosette growth by Collembola was accompanied by a strong induction of genes related to defence and plant hormone production with contrasting expression patterns in shoots and roots. In shoots Collembola elicited a strong response of plant defence genes. The enhanced investment in defence compounds likely was responsible for the transitory reduction in plant growth (rosette diameter). Compensation of the retarded plant growth later presumably was caused by the induction of plant growth hormones (auxin) by Collembola resulting in root proliferation and promotion of plant nutrient uptake. The results suggest that Collembola prime plants for later herbivore attack and concomitantly compensate for

the associated reduction in plant growth by stimulating root growth and nutrient exploitation.

5.2 Introduction

Decomposer animals alter plant growth by a multitude of different mechanisms. Their influence on plant growth is primarily ascribed to changes in nutrient availability to plants. Besides this predominant effect, decomposers influence plants by different indirect and direct mechanisms e.g. by affecting root pathogens or inoculating plant roots with mycorrhiza (Gormsen et al. 2004, Friberg et al. 2005). Decomposers, such as earthworms, further affect plant growth and development by hormone-like effects (Tomati et al. 1988). These findings indicate that decomposer activity may induce changes in plant metabolism that ultimately affect plant performance. In fact, recent studies demonstrated that decomposers influence plant secondary metabolism and induce the production of secondary compounds (Blouin et al. 2005, Wurst et al. 2006).

Collembola, being among the most abundant soil decomposers, are known to affect plant fitness parameters via indirect effects (Scheu et al. 1999). By affecting the activity and growth of microorganisms, Collembola change nutrient mineralisation and distribution (Tiunov and Scheu 2005, Chamberlain et al. 2006b) and thus affect plant nutrient uptake and tissue nutrient concentration (Cole et al. 2004a), which ultimately results in changes in plant growth (Bardgett and Chan 1999, Scheu et al. 1999, Lussenhop and BassiriRad 2005). Detailed analyses of the structure of the root system showed that plants respond to Collembola by increasing root elongation and branching even though total biomass and nutritional status may remain unaffected (Endlweber and Scheu 2006, 2007).

Effects of Collembola on plant fitness are likely caused by changes in plant metabolism. Therefore, information on the effect of Collembola on plant metabolism is essential for understanding Collembola - plant interactions. Linking phenotypic responses to changes in gene expression patterns might provide insight into the reaction of plant metabolism to Collembola presence.

Effects of soil organisms on gene expression profiles of plants have been rarely investigated. The majority of studies focus on the effect of plant pathogens or root feeding nematodes (Schenk et al. 2000, Jammes et al. 2005). Given the fact that decomposers affect secondary metabolism and thus are involved in plant defence (Wurst et al. 2004, 2006) studies on their effect on plant gene expression are needed to unravel the mechanisms responsible for these changes. The analysis of decomposer-induced reprogramming at the transcriptional level is necessary to disentangle the complex linkages between below- and aboveground processes (Scheu 2001).

Using a DNA microarray approach we investigated the effect of Collembola on plant gene expression profiles of *Arabidopsis thaliana* L. We employed a custom-made DNA array covering about 1000 gene-specific target sequences involved in plant stress response, signalling and the biosynthesis of secondary metabolites (Glombitza et al. 2004).

5.3 Materials and Methods

Two experiments were set up, the one investigating plant growth as affected by Collembola, the second investigating gene expression patterns. Both experiments were set up in parallel but the first was run until the plants produced seeds, i.e.

reached maturity, whereas the second was terminated after 6 days, i.e. targeting the short-term gene expression profiles as affected by Collembola.

5.3.1 Plant growth experiment

The experiment was set up in plastic pots (height 10 cm, width 6.5 cm, thickness 6.5 cm) filled with 170 g sand (grain size 2 mm). Each container received 0.3 g litter (shoots of *Lolium perenne* L.) which had been milled in a ball mill (Retsch, Haan, Germany) to allow homogeneous mixing with sand.

A. thaliana seeds were sterilised in 1% CaOCl and subsequently in 70% ethanol. After sterilisation the seeds were washed with sterile deionised water. Seeds were sown in Petri dishes filled with Gambourg media (0.5% plant agar, 0.05% glucose). After vernalisation at 4°C for 3 d the petri dishes were transferred to a growth chamber (day/night temperature 22/18°C; 10 h light, 120 µM $s^{-1}m^{-2}$). After 3 weeks seedlings were transferred to the experimental pots. Half of the experimental containers received 100 individuals of the Collembola species *Protaphorura fimata* Gisin. The treatments were replicated ten times. Every other day the pots were watered with about 2 ml deionised water keeping the water content at a constant level. For analysing plant growth the diameter of leaf pairs of *A. thaliana* seedlings were measured and summarised for each plant. Leaf pair diameters were measured every other day during the first two weeks. After 8 weeks plants were harvested, dried at 60°C for three days, the dry weight of each shoot was determined and the number of seeds per plant was counted.

5.3.2 Microarray experiment

The above experiment was replicated for microarray analysis. Control and Collembola treatments were replicated 72 times. Pots were watered and diameters of

rosette leaves were measured every other day as described above. Half of the plants were harvested after 72 and the other half after 144 h. Plant roots and shoots were separated, weighed and frozen in Eppendorf tubes in liquid nitrogen. Samples were stored at -80°C until analysis. The plant material of twelve plants was pooled to one sample for RNA extraction. Plant material was ground to powder in liquid nitrogen and placed in lysis buffer (LiCl precipitation; 100 mM Tris, 500 mM LiCL, 10 mM Na_2EDTA, 1% LiDS, 5 mM DTT, pH 8). Samples were centrifuged and the supernatant transferred to a new tube. Dynabeads Oligo$(dT)_{25}$ (Invitrogen, Dynal AS, Oslo, Norway) were added to the supernatant. The sample was incubated for 5 min at room temperature to allow binding of mRNA to Oligo $(dT)_{25}$ Dynabeads. The paramagnetical properties of the Dynabeads allow separation of the beads from the solution and transfer to a new solution by magnet. RNA bound to the beads was isolated and washed twice in buffer with lithium-dodecylsulfate (10 mM Tris, 150 mM LiCl, 1 mM Na_2EDTA, 0.1% LiDS, 0.05% Tween, pH 8). RNA was transferred to a new tube and washed twice in buffer without LiDS (10 mM Tris, 150 mM LiCl, 1 mM Na_2EDTA, 0.05% Tween, pH 8).

For cDNA synthesis beads were washed twice in 1x RT buffer and transferred to a new tube filled with 1x RT buffer. The 1x RT buffer was removed and RT-Mix added to the RNA (5x RT buffer, 100 mM DTT, 2 mM dNTPs, RNase Inhibitor, Diethylpyrocarbonat (DEPC)). SuperScript II (Ambion, Hamburg, Germany) was added and the samples incubated at 42°C for 1 h. Then, the RNA was washed twice in RT buffer (with Tween) and transferred to a new tube. TE buffer (10 mM Tris, 0.5 mM EDTA, pH 8.5) was added and the sample incubated at 95°C for 2 min. The supernatant containing the mRNA was transferred to a new tube. Elution was repeated once and mRNA samples were stored at -80°C until further analysis. The cDNA bound to the beads was washed twice in TE buffer.

Samples were washed twice with ddH_2O and denatured in ddH_2O and 10x Decamer solution (DECAprime II kit, Applied Biosystems, Foster City, USA) at 95°C for 2 min. Samples were kept on ice until second-strand synthesis was performed by addition of [α-^{33}P] dATP and Exonuclease free Klenow (Ambion, Huntington, UK). Second-strand synthesis was performed at 37°C for 2 h. After incubation 1x SSC was added and the supernatant removed. TE-buffer was added and the samples incubated at 95°C for 3 min for denaturing double-stranded cDNA. The supernatant containing [α-^{33}P]-labelled cDNA was transferred to a new tube and the process repeated once. The samples were filtered through an Anapore filter (Whatman, Maidstone, England).

5.3.3 DNA array hybridisation

Gene-specific PCR-amplified DNA fragments had been spotted onto Nylon Hybond N+ membranes (Amersham, Freiburg, Germany) in duplicate as described by Glombitza et al. (2004). Before hybridization to labelled cDNA each membrane was hybridized with a reference oligonucleotide targeting a common sequence derived from flanking vector sequences used for PCR amplification (Thimm et al. 2001, Glombitza et al. 2004).

Filters were prepared for hybridisation by addition of hybridisation buffer (20x SSC, 100x Denhardt, 10% SDS SSDNA, ddH_2O) at 65°C. Samples were added to hybridisation buffer and filters were hybridised at 65°C for 20 h. After hybridisation filters were washed twice in 2x SSC, 0.1% SDS and once in 0.2x SDS, 1% SDS. Filters were scanned employing a FLA-3000 image reader (Fuji, Düsseldorf, Germany) and analysed using ArrayVision 8.0 software (Imaging Research Inc., Haverhill, UK). The local background signal of the filters was subtracted from corresponding expression ratios. Expression ratios of controls were subtracted from

corresponding treatments. Expression ratios in the range between 0.6 and 1.8 were excluded from further analysis. Genes with ratios > 1.8 were assumed to be induced those with ratios < 0.6 to be downregulated by Collembola.

5.3.4 Statistical Analysis

Data on rosette diameter were analysed by a repeated measures ANOVA (SAS 9.1, Cary, Florida, USA). Data on induction ratios were analysed by t-tests performed in Excel (Microsoft Office). Results of the multiple t-tests are interpreted with care in context of regulatory pathways.

5.4 Results

5.4.1 Plant growth

Rosette diameter of *A. thaliana* was not significantly affected by Collembola during the first five days after transplantation to experimental pots. Starting with day six Collembola reduced rosette diameter significantly ($F_{5,90}$=80.84, p<0.0001). The growth rate in the control treatment increased strongly whereas rosette diameter stayed constant in the Collembola treatment during the first week after transplantation. During the following eight weeks plants compensated the reduced growth rate. At the end of the experiment biomass and the number of seeds per plant did not significantly differ between treatments with and without Collembola.

5.4.2 Gene expression

Changes in plant growth by Collembola were accompanied by changes in gene expression patterns. Expression patterns were little affected three days after transplantation, whereas they were altered strongly after six days. Changes in gene expression patterns by Collembola differed markedly between shoots and roots; induced or suppressed genes in shoots and roots hardly overlapped (Fig 1 a,b).

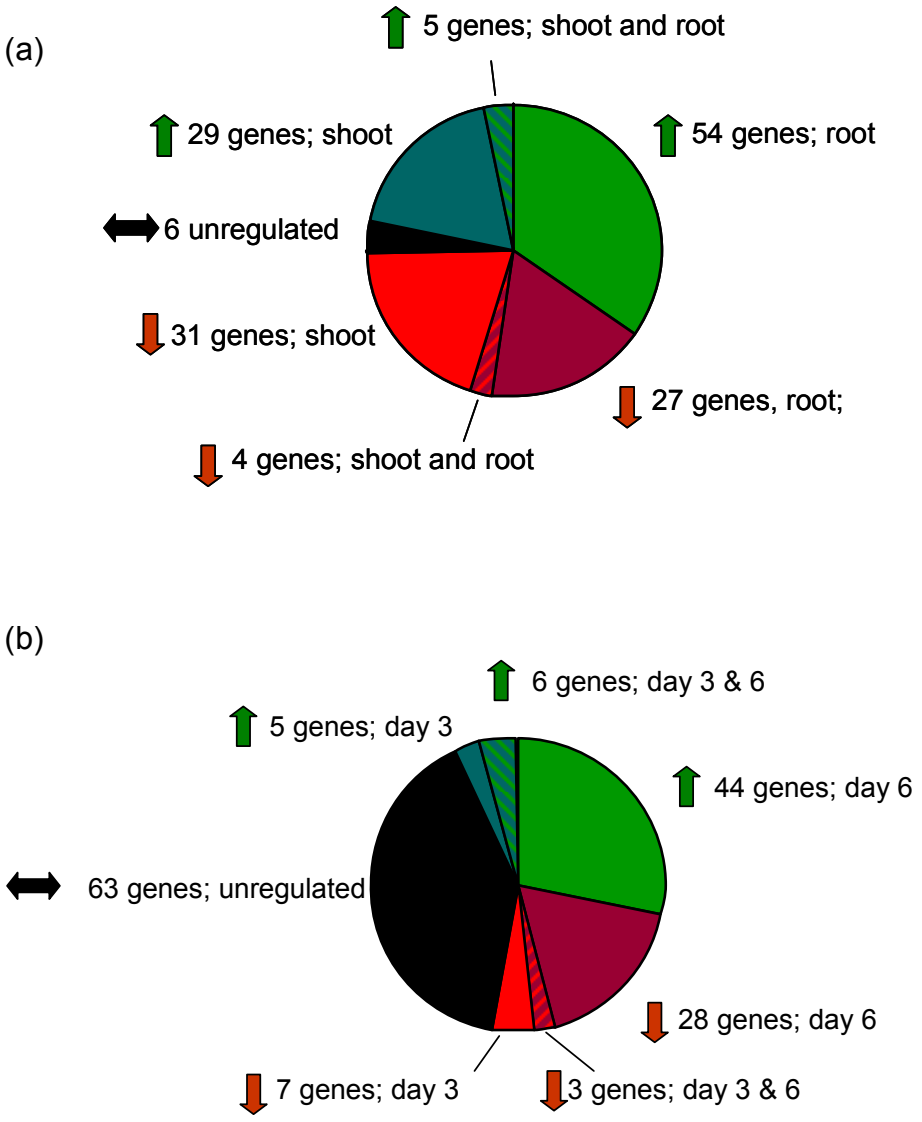


Figure 1. Comparison of genes regulated (see Methods) in presence of Collembola at day 6 in *A. thaliana* roots and shoots (a) and in *A. thaliana* roots at day 3 and 6 (b).

Genes in shoots:

Gene expression was not significantly affected by Collembola at day 3 after transplantation. In contrast, a total of 68 genes were regulated in presence of Collembola at day 6 with 34 genes being upregulated and 34 genes being downregulated (Fig. 2). The majority of the induced genes are involved in plant defence, including genes for plant defensin proteins PDF1.1, PDF1.2 and a hevin-like protein as well as beta-1,3-glucanase-like proteins.

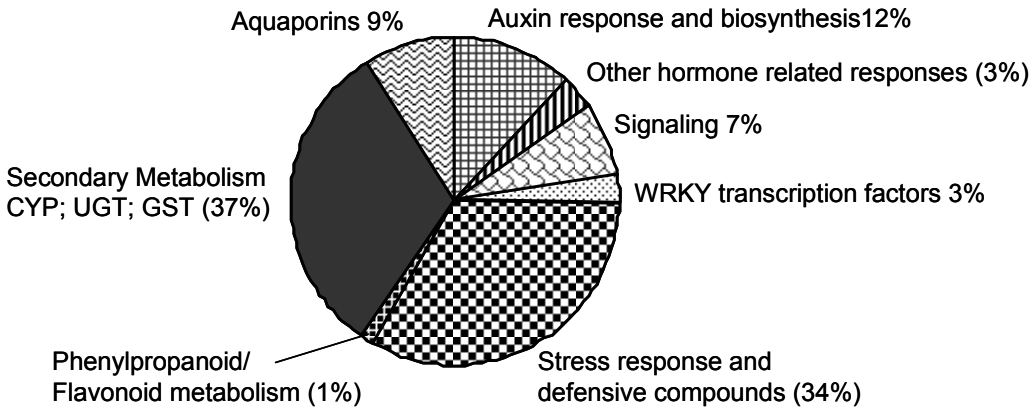


Figure 2. Genes regulated (see Methods) in presence of Collembola in shoots of *A. thaliana* at day 6. Genes are combined to functional categories. Total number of genes regulated 156 out of 1054. The fraction of regulated genes per functional group is given as percentages.

Other genes induced by Collembola comprise glutathione-S-transferases and genes encoding for enzymes related to signalling or hormone response, e.g. cytochrome P450 monoxygenases (CYP) CYP79B2, CYP79B3, and the tryptophane synthase subunits A and B (TSA1, TSB1). Furthermore, genes encoding UGT73C5, AHP3 and the pathogen and wounding induced transcription factor WRKY60 were induced in presence of Collembola. In contrast, Collembola suppressed the expression of several genes involved in signalling, including CDPK5, MPK15 and KRP1, and those encoding for other (CYP) members, glycosyltransferases and glutathion-S-

transferases. Among those CYP83A1 is involved in auxin homeostasis and glucosinolate biosynthesis. Also, the expression of genes encoding aquaporins was suppressed as well as a series of stress responsive and defensive compounds, including CAT2 and two lipid transfer proteins. Furthermore, IMS1 and IMS2 involved in aliphatic glucosinolate production and leucine biosynthesis were suppressed.

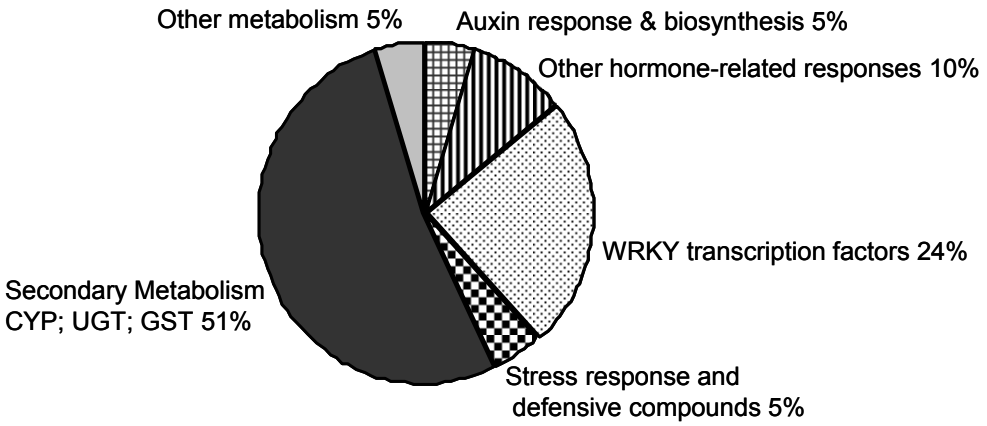
Genes in roots:

Collembola had only minor influence on gene expression patterns in roots at day three after transplantation; 11 genes were upregulated whereas ten genes were downregulated (Fig. 3a). The upregulated genes comprised several WRKY transcription factors, cytochrome P450 monoxygenase and five glycosyltransferases including UGT73C5. The downregulated genes comprised glutathione-S-transferases, glycosyltransferases and cytochrome P450 monoxygenase including CYP79B2; furthermore, AHP6, WRKY1 and a beta-1,3-glucanase-like protein.

Changes in gene expressions in presence of Collembola were much more pronounced at day 6; 60 genes were upregulated and 31 genes were downregulated (Fig. 3b). The induction was highest in auxin induced genes including GCN5-related N-acetyltransferase and ACS4, a key regulatory enzyme in the biosynthesis of ethylene. Several of the induced genes are involved in signal transduction, e.g. ABF1 and UGT73C5, or are related to pathogen defence, e.g. NPR1, PDF1.2 and a beta-1,3-glucanase. Also, the presence of Collembola affected the expression of a series of enzymes including glycosyltransferases (UGT) that are involved in secondary metabolism or activated by pathogen attack or wounding. Further, genes related to the phenylpropanoid and flavonoid metabolism were induced by Collembola as well as a series of WRKY transcription factors. Furthermore, glutathione transferases and cytochrome P450 monoxygenases were induced. Genes suppressed by Collembola at day 6 encode for several cytochrome P450 monoxygenases, glutathione-

transferases and glycosyltransferases. However, also a number of genes related to stress response as the putative lipid transfer protein GSH peroxidase were down-regulated in roots.

(a)



(b)

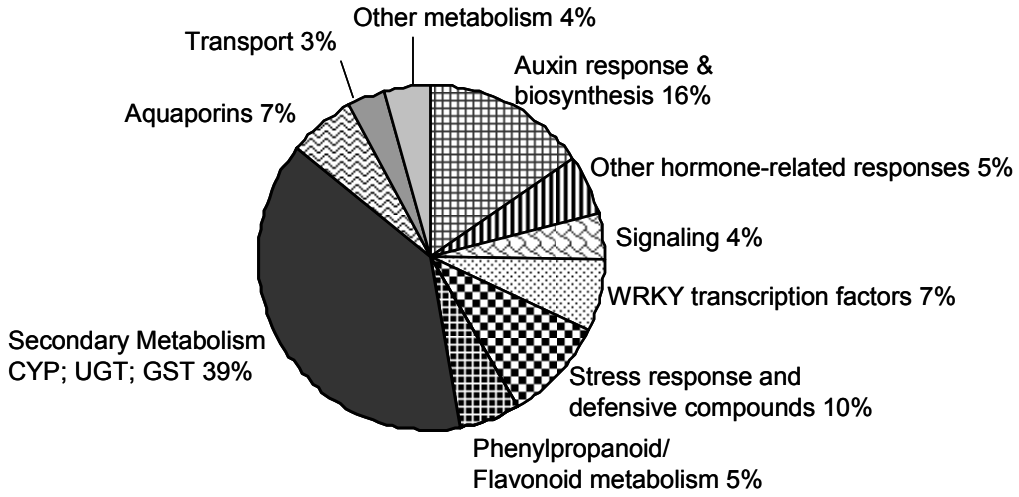


Figure 3. Genes regulated (see Methods) in presence of Collembola in *A. thaliana* roots at day 3 (a) and day 6 (b). Genes are combined to functional categories. Total number of genes regulated 156 out of 1054. The fraction of regulated genes per functional group is given as percentages.

5.5 Discussion

Collembola affect plant growth and performance by different mechanisms (Harris and Boerner 1990, Chamberlain et al. 2006b). A number of studies demonstrated that Collembola enhance the mineralization of nutrients in soil, plant nutrient content and plant biomass (Bardgett and Chan 1999). However, plants may also respond to Collembola by changes in root structure with the total biomass remaining unaffected (Endlweber and Scheu 2006, 2007). The modification of plant performance by Collembola commonly is ascribed to indirect effects, primarily the increased nutrient mineralization and subsequent increase in plant nutrient uptake (Cole et al. 2004a). The decrease in rosette growth of *A. thaliana* during the early stages of development in our experiment contrasts previous findings demonstrating an increase in shoot growth in presence of Collembola (Theenhaus et al. 1999, Kreuzer et al. 2004, Partsch et al. 2006). Gene expression patterns proved that Collembola elicited a strong and fast response of plant metabolism which presumably resulted in the observed deceleration of rosette growth. The rapid response of the plants and the detrimental effects contradict the assumption that effects of Collembola on plant growth are solely mediated via nutrients. In fact, analysis of gene expression patterns suggest that Collembola strongly affected plant secondary metabolism, in particular the expression of genes related to defence and stress. Thus, the investment of *A. thaliana* into rosette development likely was reduced by the allocation of metabolites to secondary metabolism (Heil and Baldwin 2002).

The strongest inductions were detected for genes encoding plant defensins PDF1;1 and PDF1;2, hevein-like protein, a putative lipid transfer protein, an endochitinase, a thionin, a thaumatin-like protein, several pathogenesis-related proteins as well as several ß-1,3-glucanase isoforms in shoots after six days. Surprisingly, there was no or only a marginal induction of these genes in roots. Two other lipid transfer protein

genes repressed in shoots, At2g15050 and At2g38540 are known to be downregulated by diverse biotic interactions in the Genevestigator database (Zimmermann et al. 2004). Obviously, Collembola elicit a strong defence response and thus provoke a major systemic response in plant shoots. Notably, the induction of defence-related genes in shoots occurred although Collembola predominantly altered gene expression patterns in plant roots. It is known that plant defence responses are not restricted to the attacked organ; rather, plants increase the production of defence related proteins throughout the whole plant (Stout et al. 1996). In fact, the strong upregulation of defence genes in shoots of *A. thaliana* suggests that decomposers in the rhizosphere, such as Collembola, elicit strong defence responses aboveground, i.e. provoke a systemic induced defence in unaffected plant organs.

Of the defence genes induced by Collembola particularly those related to fungal and pathogen defence were highly expressed in plant shoots, e.g. plant defensin proteins PDF 1.1 and PDF 1.2. Both genes are responsive to jasmonic acid and induce enhanced resistance to fungal pathogens (Larsen and Cancel 2004). Infection with fungal pathogens might be induced by Collembola functioning as a vector for plant pathogens by carrying microorganisms on their body surface or in their gut (Thimm et al. 1998, Dromph 2003). However, the induced transcription levels appear to be too high and too quick to be caused exclusively by pathogens transferred by Collembola, considering the short exposure of the plants to Collembola and the fact that Collembola carry predominantly propagules of saprophytic rather than pathogenic fungi (Visser 1987). Rather, the strong induction of a number of wound-inducible genes by Collembola suggests that Collembola themselves fed on roots, presumably fine roots and root hairs. However, induction of wound-inducible genes may also result from feeding of Collembola on hyphae and rhizosphere soil organic matter

thereby accidentally damaging fine roots and root hairs. Results of recent experiments suggest that Collembola in the rhizosphere of plants indeed acquire virtually all of their carbon from plant roots suggesting that they directly feed on roots (K. Endlweber, L. Ruess and S. Scheu, unpubl. data).

The characteristics of induced defence vary depending on the site of attack. It is known that foliar defence compounds can be affected by root pathogens and mycorrhizal fungi (Blilou et al. 2000, Pieterse 2002). Bezemer et al. (2004) demonstrated that the defence response induced by foliar herbivores was limited to the youngest leaves, whereas defence proteins increased in all plant leaves following root herbivory. Despite the reduced growth during early development, *A. thaliana* might ultimately benefit from the elevated content of defence compounds. The induction of plant defence above ground might protect plants particularly during the vulnerable early stages of development. In fact, the induction of plant defence compounds by soil organisms affects foliar herbivore performance (Bezemer et al. 2003, van Dam et al. 2004, Wurst et al. 2006). Therefore, plants may ultimately benefit from an increase in secondary metabolism. Growth inhibition caused by enhanced production of secondary metabolites is likely to be compensated by increased phytohormone concentrations. Indeed, plants grown in presence of Collembola compensated the decelerated growth later; at harvest plant biomass and number of seeds did not differ between control and Collembola treatments. Therefore, the temporary inhibition of plant growth by Collembola presumably was negated by increased production of phytohormons and an associated Collembola-mediated increase in nutrient availability to plants.

In addition to defence genes the upregulation of wound-inducible glucosinolates by Collembola likely also caused fitness costs and contributed to the reduction of rosette growth (Siemens et al. 2002). Notably, glycosyltranferase *UGT73C5* was among the

few genes induced in both shoots and roots. Overexpression of *UGT73C5*, which is involved in brassinosteroid (BR) homeostasis, results in a typical brassinosteroid deficient phenotype with reduced leave size and decreased shoot growth (Gaspar et al. 1996, Poppenberger et al. 2005).

Generally, gene expression patterns induced by Collembola strongly differed between shoots and roots with the response in roots being more pronounced than that in shoots. In contrast to shoots, Collembola most strongly elicited genes encoding auxin induced IAA proteins in plant roots. The short lived AUX/IAA proteins are responsible for rapid responses to auxin (Abel et al. 1994). Functioning as activators or repressors of genes that mediate auxin responses of the plant, AUX/IAA proteins regulate auxin induced responses, e.g. cell growth and elongation (Park et al. 2002). Therefore, the expression of AUX/IAA proteins indicates enhanced auxin levels in plant roots (Zhao et al. 2001). Elevated auxin levels probably cause root proliferation. Therefore, the production of longer and thinner roots in presence of Collembola, as observed in previous experiments (Endlweber and Scheu 2006, 2007), presumably is due to Collembola-induced enhancement of auxin levels.

Changes in the expression of genes encoding auxin induced IAA proteins in roots presumably were triggered by Collembola-mediated changes in the expression of genes in shoots. The induction of cytochrome P450, *CYP79B2* and *B3* in shoots by Collembola suggests an increased production of IAA in stems and leaves of *A. thaliana*. Both enzymes catalyse the conversion of tryptophan (Trp) to indole-3-acetaldoxime (IAOx) (Pollmann et al. 2006). However, the wound-inducible cytochrome P450 *CYP79B2* may also catalyse the production of glucosinolates from IAOx and thus contribute to plant defence (Mikkelsen et al. 2000). Zhao et al. (2001) demonstrated that overexpression of *CYP79B2* induces the expression of IAA regulated genes, such as AUX/IAA. Therefore, the increased expression of AUX/IAA

proteins in presence of Collembola likely in fact was triggered by an increase in auxin production in *A. thaliana* shoots and subsequent transport to the roots. Increased auxin levels in presence of Collembola are also indicated by the expression of Nitrilase 1 catalysing a final step in IAA biosynthesis (Pollmann et al. 2006). However, the reduced rosette diameter of *A. thaliana* in Collembola treatments contradicts the increased production of auxin but, as outlined above, the effect of auxin likely was superposed by the production of secondary metabolites compromising plant growth.

5.6 Conclusions

Using a genomics approach this study for the first time showed that Collembola-mediated changes in plant performance likely are caused by changing the expression of genes reprogramming plant growth but also those inducing plant defence. The reprogramming of plant growth predominantly occurs in roots resulting in altered morphology of the root system and therefore in root foraging. These changes belowground are accompanied by the induction of plant defence genes in shoots thereby priming plants against herbivore and pathogen attack above the ground. Increased investment in plant defence initially was associated with reduced plant performance; however, this was compensated later most likely due to Collembola-mediated increase in plant hormone concentrations and nutrient availability. Decomposer invertebrates, such as Collembola, therefore not only affect plant growth by changing the amounts of nutrients available to plants but also by affecting in a complex way plant morphology, growth and defence against herbivores and pathogens. The results suggest that if we are to understand antagonistic and mutualistic interactions of plants with other organisms decomposers need much closer attention.

GENERAL DISCUSSION

Terrestrial ecosystems are characterised by interactions within and between the above- and belowground subsystems. Plants link both subsystems via their shoots and roots and mediate changes between the systems. Plant growth and performance depends on nutrients mineralised by soil decomposers; changes in nutrient cycling likely affect plant growth and performance above- and belowground (Setälä et al. 1998).

Collembola, being among the most abundant decomposers in soils, beneficially affect plant shoot and root growth (Harris and Boerner 1990, Cole et al. 2004a). Their influence is commonly ascribed to enhanced nutrient availability and subsequently increased nutrient uptake (Teuben 1991, Bardgett and Chan 1999, Scheu et al. 1999). Plant nutrient uptake is determined by the root system and thus plant species and functional group identity (Fransen et al. 1999, Schenk et al. 1999). Changes in root performance may affect plant nutrient acquisition and ultimately influence plant growth and competition between plants. The competitive relationship of *Cirsium arvense* and *Epilobium adnatum* was studied in a greenhouse experiment (Chapter 2). Although plant biomass and tissue nutrient content remained unaffected, Collembola influenced root performance of *E. adnatum* and *C. arvense*. Both plant species produced longer, thinner roots and a higher number of root tips, with the effect being most pronounced in *E. adnatum*. The limited effect of Collembola presence on shoot and leaf growth contradicts previous findings demonstrating a promotion of plant growth and nutritional status by Collembola (Scheu et al. 1999, Kreuzer 2004). Changes in plant biomass may need a longer experimental period allowing consolidation of nutritional effects caused by Collembola. Furthermore, the density of Collembola was comparatively low in the present study, as compared to

the study of e.g. Partsch et al. (2005), and Collembola density is an important factor determining the strength of their impact on plant growth (Klironomos and Ursic 1998). Root morphology may be affected by the inoculation with arbuscular mycorrhizal fungi (Gamalero et al. 2004). Plants inoculated with AM fungi invest less in the expansion of their root system since the fungus facilitates plant nutrient uptake in particular that of phosphorus (Bonkowski et al. 2001). Consequently, colonisation of plant roots with mycorrhizal fungi varies with plant nutrient content and plant species (Graham and Eissenstat 1994). By grazing on mycorrhizal fungi and by mobilizing nutrients Collembola affect the mycorrhiza-plant symbiosis and thereby plant growth (Bakonyi et al. 2002). Further, Collembola mediated changes in nutrient supply are likely to affect plant competitiveness. Investigating Collembola effects on mycorrhizal inoculation and plant competitiveness in a greenhouse experiment it was shown that Collembola indeed alter plant competition by reducing the competitive superiority of *Lolium perenne* over *Trifolium repens* (Chapter 3). This is consistent with previous findings demonstrating an increase in the competitive strength of legumes against grasses in presence of Collembola (Kreuzer et al. 2004, Partsch et al. 2006). However, in contrast to our initial hypothesis changes in the competitive relationship were not due to reduced mycorrhizal infection of plant roots in presence of Collembola. Rather, infection of roots of *L. perenne* by mycorrhiza exceeded that of *T. repens* in presence of Collembola. Collembola only reduced the infection of clover roots with mycorrhiza, although Collembola were less abundant in treatments with *T. repens* than in those with *L. perenne*. The effect of Collembola on mycorrhizal infection has generally been assumed to increase with Collembola density (Bakonyi 2002). Collembola presumably benefited from the higher root biomass in the ryegrass treatments providing more root exudates. The allocation of root exudates enhances microbial biomass; in particular it increases the biomass of saprophytic

fungi (Griffiths et al. 1999). Saprophytic fungi likely function as an additional food source for Collembola, as Collembola preferentially feed on saprophytic rather than mycorrhizal fungi (Klironomos et al. 1999).

Similar to the results of the first experiment, Collembola induced the production of longer and thinner roots in both plant species particularly in *L. perenne.* Root structure and morphology are determined by the accessibility and distribution of nutrients in soil (Rengel and Marschner 2005). Collembola probably affected root proliferation by changes in nutrient availability and distribution e.g. by the creation of nutrient rich patches and subsequent root proliferation towards those nutrient rich areas (Robinson et al. 1999). The increase in root tip numbers indicates the production of more roots (e.g. root branching) and the development of an expansive root system, being a prerequisite for effective root foraging. Therefore, Collembola may facilitate resource exploitation by changing root performance; an effect that is likely to increase with time and ultimately may affect plant nutrition and competitiveness. However, these conclusions are not supported by an increase in plant tissue nutrient concentration in the two experiments presented in Chapters 2 and 3.

The differential response of *E. adnatum* and *C. arvense* in the first experiment and of *L. perenne* and *T. repens* in the second suggests that the effect of Collembola on root morphology depends on plant species and functional group identity (differential response of grasses and legumes). This is consistent with previous studies demonstrating that plant responses to Collembola vary with plant species (Scheu et al. 1999, Kreuzer et al. 2004, Partsch et al. 2006). This variance of Collembola effects in different plant species likely affects the competitive relationship among plant species and ultimately alter plant community composition.

The results indicate that Collembola affect plant performance by several pathways. The changes in root morphology point to indirect effects via nutrient mobilisation and probably more direct interactions, e.g. ingestion or damaging of plant roots while feeding. Therefore, Collembola feeding habits and the types of food sources were investigated further by stable isotope analysis and compound specific ^{13}C analysis of fatty acids (Chapter 4). Collembola were cultured in a system consisting of maize (C_4-plant) growing in soil into which ^{15}N labelled C_3-litter was mixed. Collembola benefited from the availability of litter and plants with their body tissue carbon and nitrogen concentration as well as their total biomass being enhanced. Dietary mixing is assumed to be beneficial to consumers and mixing of low and high quality recourses may increase consumer fitness (Toft 1995, Scheu and Simmerling 2004). As indicated by their $\delta^{15}N$ and $\delta^{13}C$ ratios Collembola predominantly incorporated plant-born nutrients and fed very little on litter. This contradicted the initial hypothesis that Collembola obtain carbon and nitrogen from different resources, i.e. carbon from roots and nitrogen from litter, rather it suggests that they exclusively digested root derived carbon resources, i.e. either root tissue or mycorrhizal hyphae. Presumably, they benefited from the high nutrient turnover and increased accessibility of nutrients in close vicinity of plant roots. In fact, the almost identical $\delta^{15}N$ ratios of plants and Collembola suggest that they directly fed on plant roots. Therefore, they may have benefited from increased root growth and root branching in the vicinity of nutrient patches.

The results of the experiments presented in Chapters 2, 3 and 4 indicate that Collembola are particularly active in the rhizosphere of plants. While feeding in close vicinity of roots Collembola may not be able to differentiate exactly between root associated soil organic matter, fungal hyphae and plant roots. Therefore, they likely directly ingest or at least damage plant roots, particularly fine roots and root hairs

(Chapter 4). Damaging roots probably induces changes in plant metabolism that subsequently result in modifications of root performance. This hypothesis was tested in a fourth study investigating Collembola induced changes in gene expression profiles of *Arabidopsis thaliana* by a DNA-microarray (Chapter 5). Gene expression patterns were correlated with changes in rosette growth of *A. thaliana*.

Gene expression patterns reflected the induction of genes related to plant defence and signalling by Collembola. Collembola mainly affected gene expression in plant roots but elicited the highest response of defence related genes in plant shoots. Plant defence is not restricted to the damaged organ but likely causes production of plant secondary metabolites and defence molecules throughout the whole plant (Stout 1996). Collembola apparently elicited a strong defence response in plant roots and thus induced a systemic response in plant shoots. The characteristics of systemic induced response may vary depending on the area of damage. Root herbivory particularly induces the production of defence molecules throughout the whole plant, whereas the response to shoot or leave herbivory is often particularly high in young plant shoots (Bezemer 2004). Several upregulated genes are mainly responsive to infection with pathogenic fungi. Collembola may have indirectly affected plant defence by transferring plant pathogens that infected plant roots and subsequently induced plant defence (Pieterse 2002). However, the abrupt and strong induction of plant defence contradicts a defence response elicited by root pathogens. More likely Collembola injured plant roots by feeding; this conclusion is supported by the high expression of wound-inducible genes in plants.

The defence response of the plants induced by Collembola was associated by a decelerated rosette growth of *A. thaliana.* Presumably, plants allocated more metabolites to secondary metabolism and subsequently reduced the investment in rosette growth (Heil and Baldwin 2002). However, a reduction in rosette growth in

young plants as induced by plant defence response can be compensated during further development (Heil and Baldwin 2002). In fact, *A. thaliana* compensated the growth reduction; at harvest (after 56 days) plant biomass and seed number did not differ between treatments with and without Collembola. This compensation of plant growth probably was facilitated by an increased production of growth promoting hormones in presence of Collembola, in particular auxin in *A. thaliana* shoots, as suggested by the microarray analyses.

The conclusions are supported by compound specific analysis of fatty acids indicating that Collembola preferentially incorporated fatty acids originating from plant roots. The depletion in $\delta^{13}C$ of Collembola fatty acids suggested that fatty acids were not directly incorporated from diet (Ruess et al. 2005). Rather Collembola synthesised fatty acids de novo causing depletion in $\delta^{13}C$ due to the enzymatic discrimination of the heavier isotope in lipid biosynthesis (DeNiro and Epstein 1977). Collembola contained high amounts of oleic acid (18:1 ω9c), being proposed as a marker fatty acid for incorporation of plant material (Ruess et al. 2005, Chamberlain 2005). The low difference in $\delta^{13}C$ signatures in 18:1 ω9c in Collembola and plant roots indicates that Collembola preferentially incorporated plant roots. Furthermore Collembola lacked linoleic acid (18:2 ω6,9), being a marker fatty acid for fungi (Hauber et al. 2004). This further supports the conclusion that Collembola fed rather on plant material than on fungi.

Overall, the results of Chapter 4 and 5 indicate that Collembola are closely associated with plant roots. Stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) and induced defence in *A. thaliana* suggest that Collembola directly feed on plant roots (Chapter 4 and 5). They may ingest roots hairs or injure roots while feeding on organic matter or microorganisms in the rhizosphere, rather than exclusively feeding on plant roots. The results demonstrate that the influence of Collembola on plant growth and

performance are not solely due to changes in nutrient cycling and availability. In fact, Collembola influence plant metabolism by direct interactions with plant roots. This contradicts the widely accepted categorisation of Collembola as fungivorous decomposers and the assumption that the influence of Collembola on plant performance is restricted to indirect effects (Filser 2002). Furthermore the findings challenge the hypothesis that decomposers are unlikely to affect plant secondary metabolism (van Dam et al. 2003). Collembola induce the production of secondary plant compounds with the effect being generally beneficial to plants (Wurst et al. 2005; Chapter 5). In contrast to feeding by root herbivores, the influence of decomposers, such as Collembola, is not detrimental to plants. Rather, plants benefit from the induction of plant defence that increases the protection of plants against herbivores. This interaction may be particularly important in the vulnerable early stages of plant development. Furthermore, the enhanced production of hormones that promote plant growth (e.g. auxin) induced by Collembola facilitates compensational growth (Chapter 5). The growth rate is further promoted by the production of an expansive roots system and an enhanced nutrient availability in presence of Collembola (Chapter 2 and 3).

The results of this study stress the importance of decomposer-mediated changes in plant performance. The various interactions between decomposer animals, such as Collembola, and plants as demonstrated by this study add to the complexity of interactions between the below- and aboveground system of terrestrial ecosystems.

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation ohne fremde Hilfe angefertigt und mich keiner anderen als die von mir angegebenen Schriften und Hilfsmittel bedient habe.

Darmstadt, 29.05.2007 Unterschrift