Hyphal mediated transport processes of the allelochemical juglone in soil

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Foreword

This dissertation is a cumulative work of two manuscripts, either submitted or in preparation for submission.
Therefore, this thesis is based on following papers, which are referred by their Roman numerals.

I. **Michaela Achatz**, E. Kathryn Morris, Frank Müller, Monika Hilker, Matthias C. Rillig: Soil hypha mediated movement of allelochemicals: arbuscular mycorrhizae extend the bioactive zone of juglone (submitted to Functional Ecology)

II. **Michaela Achatz**, Matthias C. Rillig: Arbuscular mycorrhizal fungal hyphae enhance transport of the allelochemical juglone in natural soil (in preparation for submission to Soil Biology and Biochemistry)

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CHAPTER I

General Introduction

Allelopathy in its broader sense is defined as beneficial or detrimental effect by which a higher plant produces one or more natural products which influence other plants when released into the environment. In its literal sense, originating from the Greek-derived elements allelon ("of each other") and pathos ("to suffer") it means "mutual harm" or "suffering" (Rice, 1974). The term "allelopathy" passed through several subtleties of definition and was modified and specified over several decades. Hans Molisch (1937) originally characterized the term to relate to general biochemical interactions between all types of plants as well as microorganisms (Molisch, 1937); in the 70s the term "allelopathy" was proposed to be used for all chemical interactions among organisms. Rice (1984) felt that the term should include "any direct or indirect harmful effect by one plant (including microorganisms) on another trough the production of chemical compounds that escape into the environment". The International Allelopathy Society (IAS) defined 1996 allelopathy more generally as "any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influences the growth and development of agriculture and biological systems" (Reigosa et al., 2006). Nowadays scientists have come back to the original definition of allelopathy, meaning substances that are produced by one plant that inhibit another.

Allelopathy time and again became a target of criticism and an alternating subject of interest in plant ecology because its ecological relevance, especially the role in plant communities, was called into question. In the last years, biochemical mechanisms became more relevant in explaining ambiguous results in plant community structure and invasion biology (Callaway & Vivanco, 2007; Weir, 2007).

Recent studies emphasize the role of allelopathy in plant invasiveness, including trees like *Ailanthus* (Small et al., 2010) and *Eucalyptus* (Sasikumar et al., 2004) and the herbaceous *Centaurea stoebe* (Callaway & Ridenour, 2004). Both the inclusion of observational and experimental approaches and the rise of better analytical methods resulted in data of higher quality (Barto et al., 2011). Because of its complexity, allelopathy is seen as an important ecological phenomenon indeed, but its role in detailed ecological mechanisms is still discussed (Blair et al., 2005; Macias et al., 2007; Callaway et al., 2008).

Allelochemicals

Allelochemicals can be very different in their chemical composition, but they are mostly secondary metabolites, what means that they are ‘waste’ products of metabolic processes and not required for the allelopathic organism. There is a huge diversity in allelochemicals ranging from simple hydrocarbons to complex polycyclic compounds (alkaloids, terpenes) (Putnam, 1988). For many years, several allelopathic studies were carried out successfully under controlled laboratory conditions (e.g. Petri dishes or in vitro cultures, laboratory soil bioassays) by testing allelopathic effects on seed germination rates or seedling establishment (Bertin et al., 2003a; Wu et al., 2011). Absolutely clear demonstration of allelopathy in the field has always been difficult to document because it was on the one hand very hard to measure the nature of the plant-allelochemical-plant interaction and on the other hand to unambiguously distinguish between competition and allelochemical interference (Weidenhamer, 1996).

To overcome the problem to quantify allelochemical dynamics in the soil, the use of adsorbent materials (e.g. PDMS-based materials), originally applied to the analysis of organic pollutants, allowed the measurement of chemical flux rates in addition to static concentrations (Mohney et al., 2009; Weidenhamer et al. 2009). To demonstrate allelopathic effects conclusively, the chemical compound has to be released in the environment and stay there long enough to be available for uptake by the target plant. Additionally, the allelochemical must be detrimental to the target plant at typical concentrations and under realistic environmental conditions in order to play a significant ecological role (Choesin & Boerner, 1991). Allelochemicals are present in many types of plants, that are distributed over all plant families and are released into the environment by a variety of mechanisms, including degradation of plant residues, leaching from leaves and litter, volatilization and root exudation (Borner, 1960; Bertin et al., 2003a) (Fig. 1).

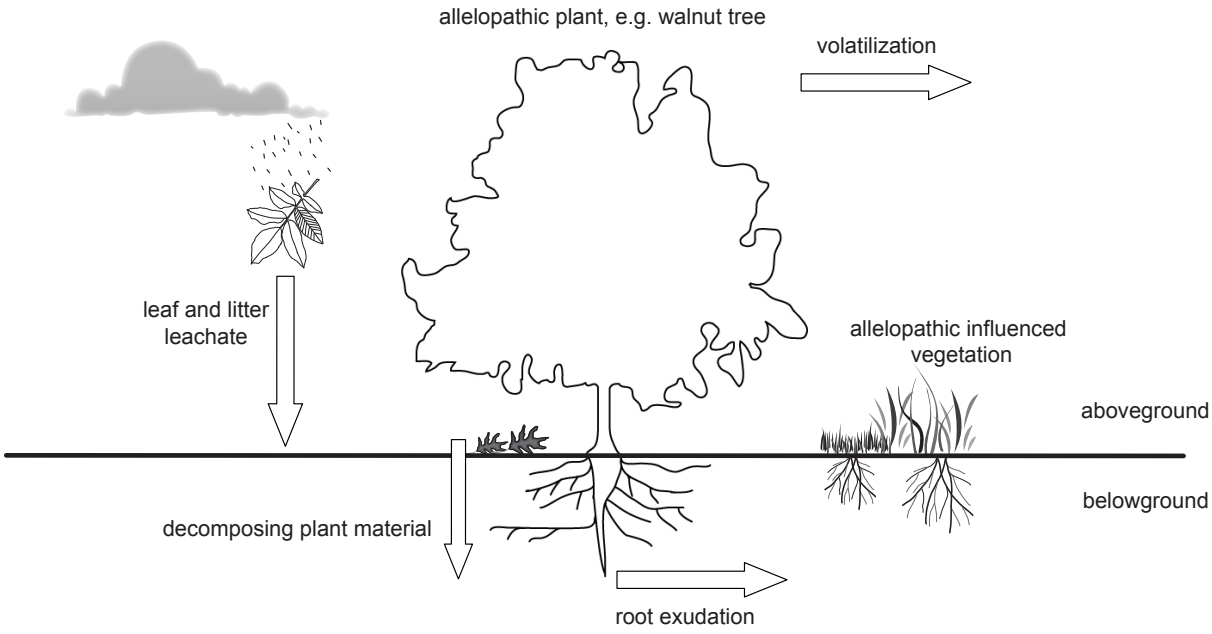

Fig.1. Sources of allelochemical release in the environment, like volatilization, leaf litter leachate, decomposition of plant material and rhizodeposition.

Juglone

One of the oldest examples of allelopathy and also one of the most thoroughly documented cases, involves the walnut, of which the American black walnut (*Juglans nigra* L.) is the most commonly studied representative (Willis, 2000). Since antiquity, the walnut tree is known as a source of substances detrimental to other organisms. Early writings of harmful effects relating to *Juglans regia* L. came from the Roman authors Varro and Pliny, who pointed to “natural antipathy” between oak, olive and pine trees and walnut (Willis, 2000). Documentations in American history state that rotting fruits were used to stun fish or that leaves acted as repellent for ants and flies. Altogether, knowledge about harmful effects of walnut concerning animals, woody, herbaceous and aquatic plant species has been recorded for over 2000 years (Rietveld, 1983; Hejl et al., 1993).

The chemical compound responsible for its noxious effects is juglone (5-hydroxy-1, 4 naphthoquinone) (Rice, 1974), and has also been isolated from many other plants in the walnut family (Juglandaceae), including *Juglans regia* (Daglish, 1950; Prataviera et al., 1983; Ponder & Tadros, 1985). This aromatic phytotoxic compound occurs naturally in all parts of the plant, but particularly in leaves and roots (Segura-Aguilar et al., 1992). They contain large amounts of nontoxic and colorless hydrojuglone, which

is transformed in the soil after cleavage of the glycosidic bond and oxidation of the aglycone to the toxic and orange juglone (Duroux et al., 1998; Bertin et al., 2003b) (Fig. 2).

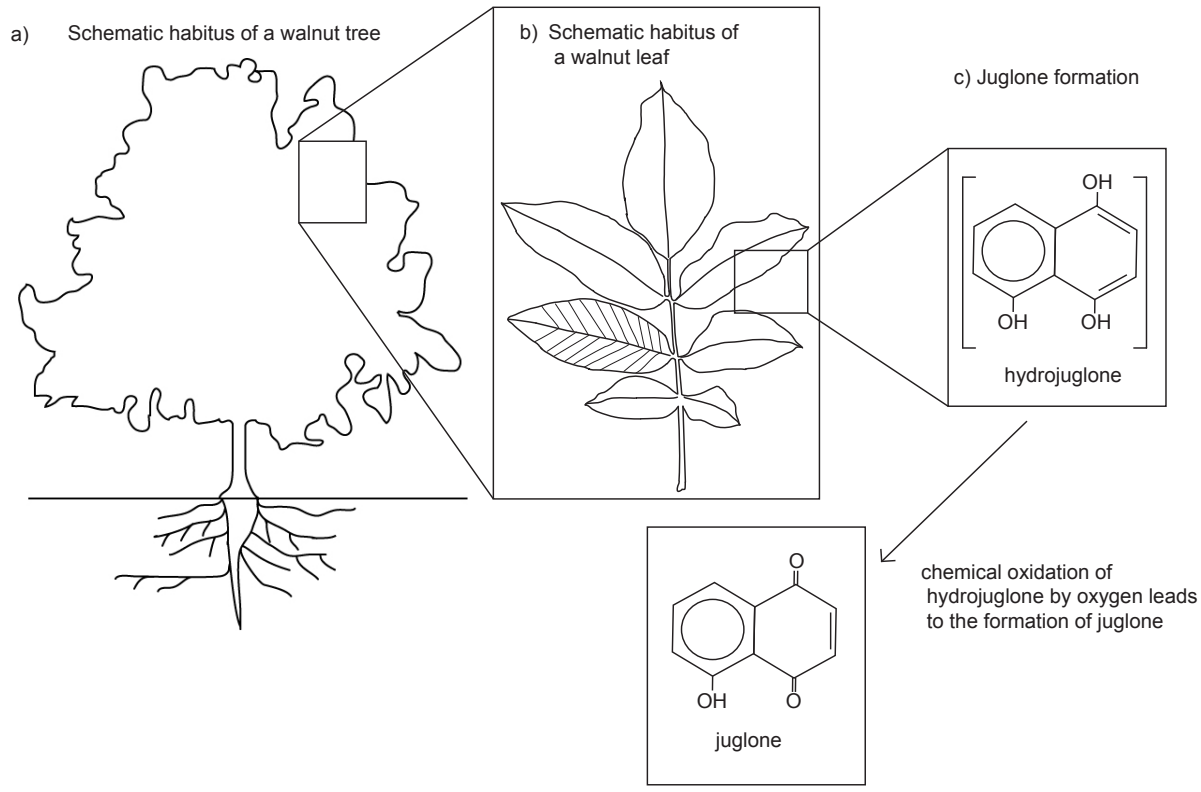

Fig. 2. Schematic habitus of a walnut tree (a) and a walnut leaf (b). c: Juglone formation by the chemical oxidation of hydrojuglone by oxygen to juglone.

The allelochemical is introduced into soil through several pathways like rhizodeposition and leaching out of decomposing leaves or other parts of the plant (hulls, fruits, bark), thereby affecting neighboring plants (Rietveld, 1983). The effects of juglone on plants are generally toxic, causing various growth restrictions and damages. Juglone inhibited *Lemna minor* L. growth, chlorophyll content, and net photosynthesis and influenced mitochondrial functions in soybean which contributed to plant growth reductions (Hejl et al., 1993). Hejl additionally showed that water uptake and acid release decreased for corn (*Zea mays* L.) and soybean (*Glycine max* Merr.) seedlings treated with juglone hence influencing the metabolism of root cells (Hejl & Koster, 2004). Jose and Gillespie (1998) also documented growth inhibition at micromolar concentrations (10^{-4}-10^{-6}M), inhibitory effects of leaf photosynthesis, transpiration, stomatal conductance and both leaf and root respiration on hydroponically grown corn and soybean (Jose & Gillespie, 1998). Both woody and herbaceous plant habits ranging from agricultural relevant

plants like tomato, potato, cucumber, apple, corn and soybean to ornamental species like rhododendron and azalea are especially sensitive to the harmful effects of juglone (Crist & Sherf, 1973, Rietveld, 1983).

Influence of soil conditions and microbes on plant-produced allelochemicals

The nature of the toxic compound by itself is a tremendous factor in allelochemical toxicity, but additionally, physical and chemical factors as well as biotic factors, like the presence of soil organisms play also an important role in its availability and effectiveness.

General soil conditions like soil moisture (Blair et al., 2006) and soil structure (Schmidt & Ley, 1999), pH, as well as the content and composition of organic matter (Inderjit, 2001) might influence the degree of impact an allelochemical can have on plant community dynamics. Soil moisture appears to play a significant role in whether or not for example catechin degrades rapidly or remains in the soil. Closely related to this are diffusion rates and the sorption of chemical compounds to mineral (e.g. clay particles) or organic matter what have been found to affect the phytoavailable concentration of allelochemicals (Dalton et al., 1989; Tharayil et al., 2006).

Another important factor which influences bioactivity of allelochemicals is the presence and the dynamics of microorganisms in soil (Blum, 2004; Cipollini et al., 2012). Microbes may both toxify and detoxify chemical compounds by metabolization after entering the soil (Inderjit, 2005). However, several studies showed in comparing sterile to natural soils, that allelopathic effects can be limited in presence of microbial communities (Rudgers & Orr, 2009; Zhu et al., 2011). Microbial modifications, as well as chemical breakdown processes influence accumulation of allelochemicals to phytotoxic levels (Kaur et al., 2009). Altogether, degradation processes lower the amount and diffusion rate of allelochemicals and therefore reduce the bioactive radius and its growth-retarding effects on target plants.

Mycorrhizal fungi and CMNs

Mycorrhizal fungi are an important functional group of soil microorganisms with strong ecological signficance. More than 80% of all vascular plants as associated with AMF and the formation of this unique obligate symbiotic relationship enables the exchange of nutrients, particularly phosphorus to the host plant in exchange for carbon to the fungus (Smith & Read, 1997). The colonization of plant roots by mycorrhizal fungi is not host specific and only obligate for the fungus, what can result in large fungal mycelia extending from one plant's roots to another to form common mycelial networks (CMNs)

and hence interconnect plants of different species (Selosse et al., 2006). Additionally, compatible hyphae can anastomose, by which branches of the same or different fungal individuals fuse to form mycelial networks (Giovannetti et al., 1999; Voets et al., 2006; Mikkelsenet al., 2008). These belowground hyphal connections could also play a very important role in allelopathic research, because taking arbuscular mycorrhizal fungi and their role in transport processes into consideration might elucidate conflicting results of previous allelopathic research.

Bioactive zones of allelopathic compounds could be enlarged in natural soils in the presence of common mycorrhizal networks (CMNs), described as the Network Enhanced Bioactive Zone model (NEBaZ), where CMNs act as “superhighways” connecting plants below ground (Barto et al., 2012). In general, CMNs can be formed by ecto-and endomycorrhizas, and play both important roles in several other transport processes, but hyphal mediated infochemical transport is only known for arbuscular mycorrhizal fungi until now.

The role of mycorrhizae in other transport functions has already been documented, for example the movement of water within or along hyphae during drought (Querejeta, et al., 2003; Egerton-Warburton et al., 2007) or surficial hyphal water movement along water potential gradients (Allen, 2007). The transfer of nutrients such as nitrogen, phosphorus and metals (He et al., 2003; Meding & Zasoski, 2008; Mikkelsen et al., 2008) is also enhanced by mycorrhizal transport processes, but not necessarily for carbon. Ecto-and ericoid mycorrhizal networks support the movement of carbon (Selosse & Roy, 2009), but clear evidence for the role of AMF in mycorrhizal carbon transfer is missing.

Some studies have documented the potential transport of infochemicals via CMNs, and thus the extension of bioactive zones of for example signal induced intraspecific plant communication in pathogen infected tomato plants (Song et al., 2010), or allelochemicals in soil (Barto et al., 2011). CMN hyphae appear to enhance transport rates of chemicals in the soil matrix probably by the moving along or in the hyphae instead of only diffusing through the bulk soil. Additionally, reduced transit times would result in shorter exposure to the soil environment and therefore potentially protect allelochemicals from degradation. As a consequence of higher flow rates allelochemicals can reach levels that induce growth inhibition sooner (Barto et al., 2012)

The aim of two studies (chapter II and III) comprising this doctoral thesis is to elucidate the role of hyphal mediated transport processes of juglone, a model allelopathic compound used in all studies. The combination of an observational study with high ecologi-

cal realism and several field and garden as well as manipulative greenhouse experiments should show the role of hyphal mediated processes and point to the importance of integrating mycorrhizal components of soil to further research on allelopathy.

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CHAPTER II

Soil hypha mediated movement of allelochemicals: arbuscular mycorrhizae extend the bioactive zone of juglone

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Summary

1. Allelopathy is a phenomenon where plants have deleterious effects on growth of surrounding plants through the production of chemical substances. Soil hyphae of arbuscular mycorrhizal fungi may enhance transport processes of allelochemicals by providing “highways” connecting plants belowground.

2. In three studies ranging from high ecological realism to experimental control we showed that the presence of mycorrhizal hyphae may strongly contribute to the transport of allelochemicals. We analyzed the accumulation of naturally released juglone in the field in intact or disrupted hyphal connections and determined its growth reducing effects on sensitive target plants in a bioassay. Second, we tested the effects of *Juglans regia* leaf litter addition in the presence or absence of the mycorrhizal fungus *Rhizophagus irregularis* on target plants and finally we added pure juglone to *Lycopersicon lycopersicum* plants in the presence or absence of *Rhizophagus*.

3.Throughout we found increased juglone transfer if mycorrhizal hyphae were present, resulting in reduced growth of target plants.

4. Our results point to mycorrhizal hyphae playing an important role in extending the bioactive zone of allelochemicals. We suggest that hyphal networks increase the effectiveness of allelochemicals in natural systems and play a crucial role in chemical interaction processes and hence influence community structure.

Keywords:

Allelopathy; arbuscular mycorrhiza; *Juglans*; mycorrhizal functioning; plant–soil interactions; rhizosphere; transport

Introduction

Allelopathy is widely understood as the biological phenomenon by which a plant produces one or more natural products that are released into the environment and that influence the germination, growth, development, reproduction and distribution of a number of plant species. These allelochemicals must accumulate and persist at phytotoxic levels and come in contact with the target plant to be ecologically relevant in the field (Choesin & Boerner, 1991).

Because of its complexity, the importance of allelopathy in plant-plant interactions is controversially discussed and its role in community structure processes is still unclear (Blair, Hanson, Brunk et al., 2005, Macias, Galindo & Galindo, 2007, Callaway, Cipollini, Barto et al., 2008). Allelochemicals are present in many types of plants and are released into the rhizosphere by a variety of mechanisms, including decomposition of residues, volatilization and root exudation (Bertin, Yang & Weston, 2003).

One of the oldest examples of allelopathy, and also one of the most thoroughly documented cases, involves the walnut, of which the American black walnut (*Juglans nigra* L.) is the most commonly studied representative (Willis, 2000). The chemical responsible for its allelopathy is juglone (5-hydroxy-1,4 naphthoquinone) (Rice, 1974), which has also been isolated from many other plants in the walnut family (Juglandaceae), including *Juglans regia* (Daglish, 1950, Prataviera, Kuniyuki & Ryugo, 1983, Ponder & Tadros, 1985). This aromatic phytotoxic compound occurs naturally in all parts of the plant, but particularly in leaves and roots (Segura-Aguilar, Hakman & Rydstrom, 1992). They contain large amounts of nontoxic and colorless hydrojuglone, which is transformed in the soil after cleavage of the glycosidic bond and oxidation of the aglycone to the toxic and orange juglone (Bertin et al., 2003).

The effects of juglone on plants are generally toxic (but see Whittaker & Feeny, 1971) causing growth inhibition at micromolar concentrations (Hejl, Einhellig & Rasmussen, 1993, Jose & Gillespie, 1998), disruption of photosynthesis and respiration (Jose et al., 1998) and impairment of water uptake (Hejl & Koster, 2004). Several garden plants including tomato, potato, pea, apple, cucumber, watermelon, bean, garden cress, corn and ornamental ericaceous species such as rhododendron and azalea are especially sensitive to the deleterious influence of juglone (Crist & Sherf, 1973).

Both rhizodeposition and leaching of juglone and its precursor out of decomposing leaves and other parts of the plant play an important role in introducing the allelochemical into the soil and thus affecting neighboring plants (Rietveld, 1983).

Recent studies emphasize the general role of allelopathy – beyond juglone – in plant invasiveness, including trees like *Ailanthus* (Small, White & Hargbol, 2010) and *Eucalyptus* (Sasikumar, Vijayalakshmi & Parthiban, 2004) and the herbaceous *Centaurea stoebe* (Callaway & Ridenour, 2004) as well as *Solidago canadensis* (Abhilasha 2008).

Additionally, physical and chemical factors as well as soil organisms play an important role in the magnitude of allelochemical toxicity, availability and effectiveness. Soil moisture (Blair, Nissen, Brunk et al., 2006), soil structure (Schmidt & Ley, 1999), the content and composition of organic matter (Schmidt et al., 1999), and the presence and dynamics of microbial communities (Blum, 2004, Cipollini, Rigsby & Barto, 2012) might play a role in the extent to which allelopathy contributes to plant community dynamics. The availability of allelochemicals in the soil might be influenced by diffusion rates, sorption of chemical compounds to soil particles (both mineral and organic matter), and chemical or microbial degradation (Rettenmaier, Kupas & Lingens, 1983, Schmidt, 1988, Schmidt et al., 1999, Kaur, Kaur, Kaur et al., 2009). The latter is probably especially important in allelopathic functions, because high rates of microbial degradation may additionally lower the amount and diffusion rate of chemical compounds in the soil matrix and therefore reduce the bioactive radius of action and its growth-retarding effect on target plants.

One of the ecologically most important groups of soil microbes are mycorrhizal fungi, which represent a unique functional group in the soil. Taking arbuscular mycorrhizal fungi into consideration might reconcile conflicting results of previous research.

Bioactive zones of allelopathic compounds could be extended in natural soils in the presence of common mycorrhizal networks (CMNs), described as the Network Enhanced Bioactive Zone model (NEBaZ), where CMNs act as “superhighways” connecting plants belowground (Barto, Weidenhamer, Cipollini et al., 2012). They can be formed by ecto-and endomycorrhizas and be established by either hyphal growth from plant to plant or via anastomoses by which different branches of the same or different individuals fuse to form a mycelial network (Giovannetti, Azzolini & Citernesi, 1999, Voets, de la Providencia & Declerck, 2006, Mikkelsen, Rosendahl & Jakobsen, 2008). These fungal mycelia can extend from one plant’s roots to another to form CMNs and therefore commonly interconnect plants of different species.

Several other transport functions of CMNs have been investigated, like the movement of water (Querejeta, Egerton-Warburton & Allen, 2003, Egerton-Warburton, Querejeta & Allen, 2007, Plamboeck, Dawson, Egerton-Warburton et al., 2007), the transfer of nutrients such as nitrogen, phosphorus and metals (He, Critchley & Bledsoe, 2003, Meding & Zasoski, 2008, Mikkelsen et al., 2008).

However, very few studies have examined the potential transport of infochemicals via mycorrhizal hyphae. Studies have addressed signal-induced intraspecific plant communication (Song, Zeng, Xu et al., 2010), underground signals warning neighboring plants of aphid attack (Babikova, Gilbert, Bruce et al., 2013) or allelochemicals in soil (Barto, Hilker, Mueller et al., 2011). CMN hyphae appear to make chemicals move more quickly probably by the moving along or in the hyphae instead of only diffusing through the bulk soil matrix. As a consequence of higher flow rates, allelochemicals reach levels that induce growth inhibition sooner.

The aim of our study was to combine ecological realism with more controlled approaches in a set of field studies and manipulative greenhouse experiments focused on the effects of a single allelochemical. The potential for allelopathic inhibition of plant growth has been demonstrated repeatedly in the laboratory and under natural conditions, but we believe this is the first study to demonstrate the role of hyphal mediated transfer enhancing allelopathic interactions in a natural system (*Juglans* and juglone) across such a range of scales and sensitivities.

We therefore first wanted to test whether or not mycorrhizal hyphae play a role in facilitating the transport of the naturally occurring allelochemical juglone under field conditions by creating and maintaining continuous and interrupted hyphal connection treatments for a whole growing season. Next, we determined how the growth inhibiting effect of the allelochemical was affected by the presence of mycorrhizal hyphae in a bioassay experiment by using the target plant tomato (known to be sensitive to juglone). Additionally, we used a manipulative approach by adding *Juglans regia* L. leaf litter outside a root exclusion compartment (REC) to mycorrhizal or non-mycorrhizal target plants, inoculated with *Rhizophagus irregularis* or sterile inoculum, in the greenhouse. By using leaf litter we mimicked a continuous amount of juglone entering the soil by leaching, and imitated one potential natural pathway of allelochemical release. Finally, we performed an experiment in which we added the pure substance juglone to tomato plants in the presence or absence of mycorrhiza to directly assess the effect of mycorrhiza on juglone action.

Thus, in this study we wished to test the potential functional role of fungal mycelia in

enhancing bioactive zones of juglone.

Materials and methods

Experiment 1. Hyphal mediated transport of naturally occurring juglone in the field

The study was carried out in a natural grassland field plot (Botanical Garden Berlin) containing *Juglans regia* trees, by using a modified in-growth core design (Johnson, Leake & Read, 2001) consisting of paired root exclusion compartments (REC) which were either rotated (interrupted mycelium connection) or static (Fig. 1). The RECs were prepared by cutting 8 windows in a laboratory plastic bottle (Volume 500 ml) and covering the sides with 30 µm mesh (Sefar Nitex 03-30/18, Sefar GmbH, Edling, Germany) to allow only hyphae to grow in (Fig 1, a). The RECs were filled with steamed (90°C, 4 hours), sieved (1 mm), sandy soil (sand = 74%, silt = 18%, clay = 8%; pH = 7.1; organic C = 1.87%, N = 0.12%) from an experimental field of the Institute of Biology of Freie Universität Berlin.

To take advantage of a pre-existing hyphae in the grassland field plot, RECs were placed in the soil, choosing an area with the highest expected juglone concentration, i.e. about 1m from the outside edge of the tree base (DeScisciolo, Leopold & Walton, 1990, von Kiparski, Lee & Gillespie, 2007). After placing the RECs in the soil, they were closed tightly with a plastic cap on the top to avoid contact with leaf litter (which could otherwise be an additional juglone source through leaching from the top). Diffusion of juglone from the bulk soil through the mesh can still occur in our rotated control REC; we were thus interested in differences of juglone content in the paired REC treatments.

We randomly picked five *Juglans regia* trees to serve as juglone donor trees and placed 2 RECs (one rotated, one static; see below) around each tree (Fig.1, b). *Juglans regia* is, as well as other members of the family Juglandaceae, ectomycorrhizal, but also colonized by arbuscular mycorrhizal fungi (Harley & Harley, 1987, DolcetSanjuan, Claveria, Camprubi et al., 1996, Wang & Qiu, 2006) likely forming hyphae in the natural grassland field plot. For our study it was not chiefly important where the hyphae originated, most likely they were mainly contributed by the meadow vegetation surrounding the tree, rather than the tree itself. To enable a connection to the existing mycorrhizal network, half of the compartments were kept static after placing in the soil

(depth: ca. 15 cm), the others were rotated three times per week by 1-2 mm to ensure severing of hyphae attempting to cross into the RECs. The movement of half of the pots was carried out very carefully to prevent major disturbance, formation of an air gap between soil and compartments, or damage to the mesh. After 12 months RECs were removed and the soil was immediately frozen or dried for further analyses.

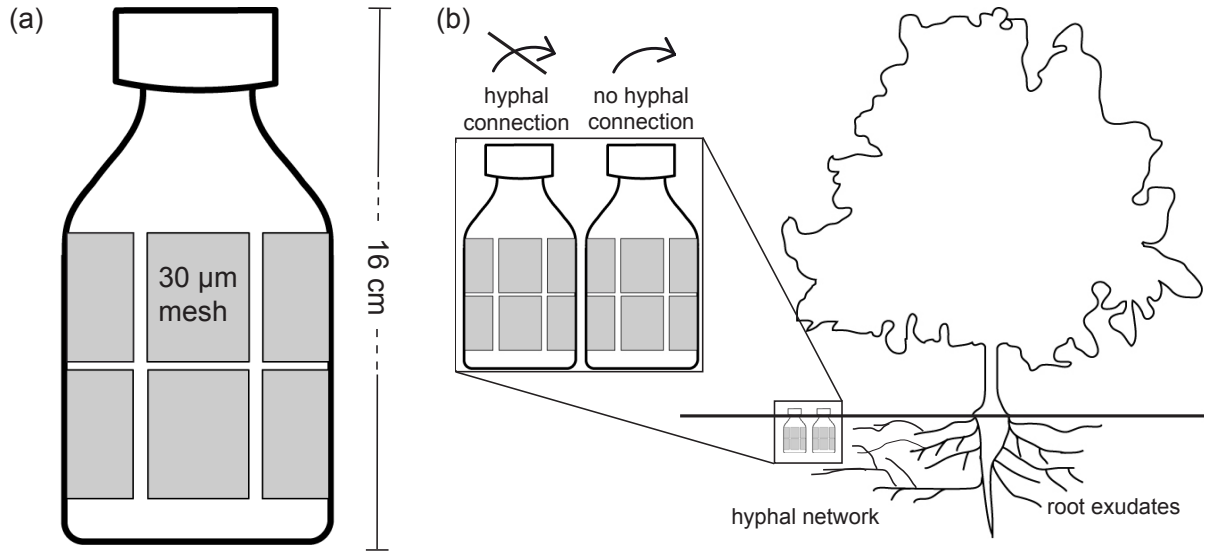

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Fig. 1. (a) Experimental unit of a modified in-growth core design. Root exclusion compartments (RECs) prepared with 8 windows, covered with 30µm mesh, to avoid root penetration.
(b) Experimental setup in the field. Placement of 2 RECs each under *Juglans regia* donor trees (n=5). Experimental pots were not moved after placing into the soil, controls were moved three times per week to cut off any hyphal connections across the mesh.

We determined pH in water (McKeague, 1978) and soil moisture gravimetrically (Hesse, 1971), as well as hyphal length in the soil (Jakobsen, Abbott & Robson, 1992) (Supporting Information Table S1).

In order to determine whether the experimental interruption of the hyphal connection affected juglone concentrations in the soil, we compared concentrations of samples with and without connection by extracting soil samples (100 g) with 100 ml chloroform (DeScisciolo et al., 1990) and shaking them for 1 hr in a rotation shaker (Heidolph). After centrifugation (5 min, 1000 rpm), the supernatant was vacuum filtered (Whatman No 1), the solvent evaporated, and the pellet was re-extracted in 500µl methanol/ dichloromethane (2:1, v:v) with the internal standard plumbagin (1ng/µl, Aldrich,

Steinheim, Germany). The re-extracted sample was analyzed by coupled gas chromatography – mass spectroscopy (GC-MS) (Fisons model 8060 GC coupled to an MD 800 quadrupole mass spectrometer; Thermo Finnigan, Engelsbach, Germany). A 1µl aliquot of a sample was injected at 240°C (injection port temperature) and separated on a DB-5 column (30 m x 0.32 mm i.d.; 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA). The oven temperature program was started at 130 °C for 4 min, and then heated to 280 °C temperature at a rate of 10 °C min^{-1} (solvent delay: 10 min). Helium was used as carrier gas with an inlet pressure of 10 kPa. Electron impact ionization (EI) was 70 eV. Juglone was identified by comparison with retention time and full mass spectrum of authentic juglone (Aldrich, Steinheim, Germany). In order to quantify juglone in soil samples, no full mass spectra were recorded, but the mass spectrometer was set to selective ion mode: m/z 174 for juglone and m/z 188 for plumbagin (the internal standard). Peak areas of these ions were determined. A calibration curve was recorded with juglone quantities ranging from 25 pg to 1 ng; plumbagin: 1 ng. Juglone concentrations in soil samples were determined by comparing the juglone/plumbagin peak area ratios obtained from soil sample analyses with those obtained from the calibration.

Bioassay with juglone-sensitive *Lycopersicon lycopersicum*

After harvesting the RECs, soil from both treatments was used for a bioassay (n=5) to test for the effect of juglone in the soil on a sensitive phytometer plant (*Lycopersicon lycopersicum*). The bioassay experiment was conducted in small “Cone-tainers” (Stuewe & Sons, Oregon, USA, volume 100ml) for three weeks in a growth chamber (18–20°C, 16 hr day length). Seeds of *L. lycopersicum* were pregerminated in glass beads and one seedling was transplanted into each pot and harvested four weeks later. Dry root and shoot biomass was determined, and roots were stained with India ink to measure colonization by AMF (Vierheilig, Coughlan, Wyss et al., 1998) (Supporting Information Table S1).

All data analyses were performed in R version 1.14. We used a linear mixed effect model (nlme package), where status of connection to the mycorrhizal network was a fixed effect while blocks (here walnut trees) were random factors. To incorporate different variances in the model, we used the function VarIdent (Zuur, 2009). To meet assumptions of normality, data were transformed as needed (see details of transformations in Supporting Information Table S1-S3).

Experiment 2. Transport of juglone via *Rhizophagus irregularis* hyphae after adding walnut leaf litter (greenhouse experiment)

The completely randomized experiment was conducted for three months (May-July) in a greenhouse of the Botanical Garden Berlin (temperature: 23-27°C). We filled 150 “Cone-tainers” (volume 500 ml) with twice-autoclaved (121°C, 20 min), sieved (1 mm), sandy soil (sand = 74%, silt = 18%, clay = 8%; pH = 7.1; organic C = 1.87%, N = 0.12%). We placed a mesh bag (pore size 30µm) in the center of every pot (Sefar Nitex 03-30/18, Sefar GmbH, Edling, Germany) to create a root exclusion compartment in which we placed the sensitive target plant *L. lycopersicum* (Fig. 2). Half of the mesh bags were filled with about 50 g twice-autoclaved soil mixed with mycorrhizal pellets (10 g), containing the arbuscular mycorrhizal fungus *R. irregularis* C. Walker & Schuessler (formerly *Glomus intraradices*) (Biomyc®, Germany) to provide a mycorrhizal treatment (m) or twice-autoclaved pellets for the non mycorrhizal control (nm) and microbial wash to all pots. After two weeks of target plant establishment, we added to half of the non-mycorrhizal and mycorrhizal pots 4.0 g of cut and sieved (2 mm) *Juglans regia* L. (Botanical Garden Berlin) leaf litter outside the root exclusion compartment (Fig. 2) or no leaf litter as controls. 100g of leaf litter contained 0.410 g (±0.03) juglone, as determined by HPLC analysis (see below).

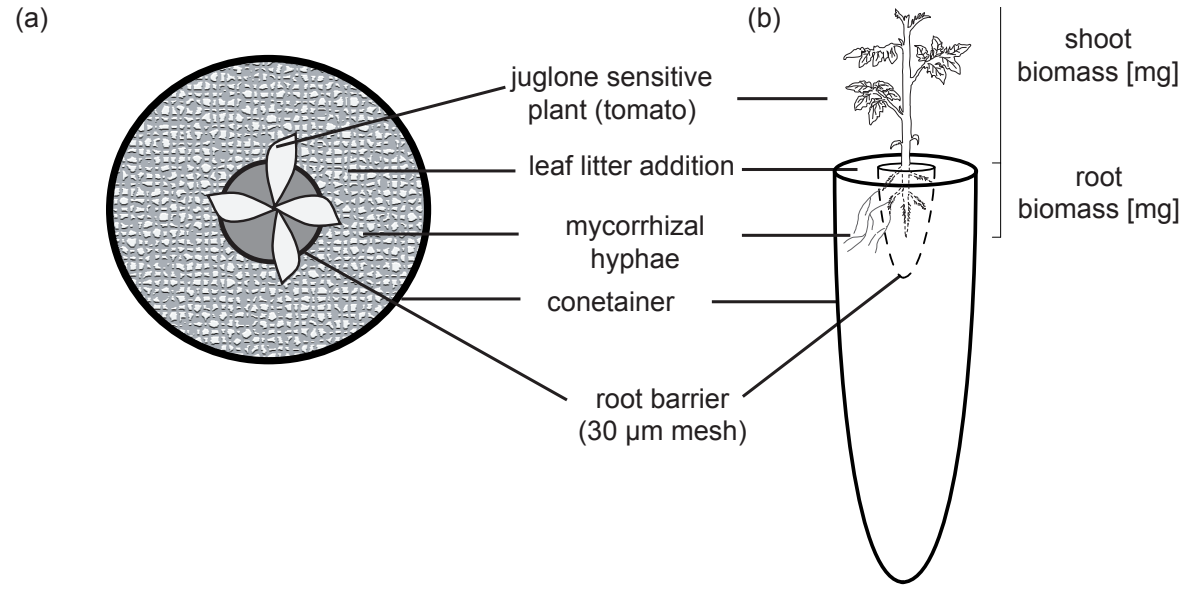

Fig. 2. (a) Top view of an experimental unit (experiment 2) showing root barriers, location of crushed *Juglans regia* leaf litter and mycorrhiza addition. Controls are without leaf litter, mycorrizal inoculation or both (n=8).
(b) Side view of an experimental unit. After harvesting we determined shoot and root biomass [mg], as well as mycorrhizal and nutritional parameters.

After 10 weeks the experiment was terminated and samples were taken. To test the effects of leaf litter addition (containing juglone), we determined dry shoot and root biomass of the target plants. Percent colonization (Vierheilig et al., 1998) was measured at 200x to verify presence of AMF hyphae in the mycorrhizal treatment and absence in the non mycorrhizal control (Supporting Information Table S2).

We measured the percentage of total C, N using a Euro EA analyzer (HEKAtech GmbH, Wegberg, Germany) to exclude that changes in C/N ratio, possibly caused by adding organic matter to soil and its breakdown through microbes, have affected our results. To take into account possible growth effects on target plants in the root exclusion compartments induced by nutrient levels affected by the treatments (mycorrhization and addition of leaf litter), we analyzed plant available soil P. This was done using the calcium-acetate-lactate method according to the German standard method DIN 3.4.1.30.2a (Deutsches Insitut für Normung, 2000) (Supporting Information Table S2).

To determine juglone content in leaf litter, we extracted 300 mg leaf litter (n=4) in 10 ml chloroform for 1 h in the dark (Girzu, Fraisse, Carnat et al., 1998). After centrifugation (5 min, 1000 rpm) the chloroform solutions were vacuum filtered (nylon membrane filter, pore size 0.2 µm, A. Hartenstein) and evaporated overnight. The dried extracts were dissolved in 2 ml methanol and used for HPLC analysis. Samples were measured with an Agilent 1100 HPLC-System (Agilent 1100 HPLC, Agilent Technologies, Santa Clara, CA, USA) and an UV diode array detector scanning the spectra of wavelength from 220 to 360 nm. Separations were carried out using an Agilent Eclipse XDB-C18 column (5µm, 4.6 x 150 mm), with a flow rate of 1 ml min^{-1}, and an injection volume of 20 µl. Solvent A was H_2O (millipore) and solvent B acetonitrile, which followed a gradient of 90% A to 45% A (10 min), 45% A to 0% (5 min), 0% A (5 min) and from 0% to 90% A (1 min). The total run time was 26 min including 5 min of equilibration treatment (90% A). Identification and quantification of juglone was achieved by first comparing the UV spectra with those obtained from standards. The spectra of samples were recorded from 280-360 nm, the detection wavelength was 254 nm with a retention time of 11.5 min for juglone. Quantification was achieved according to the concentrations of a corresponding external standard (0.00037-0.06 mg/ml) (5-hydroxy-1,4-naphthochinon, Sigma Aldrich Germany, CAS No. 481-39-0).

Experiment 3. Transport of juglone via *Rhizophagus irregularis* hyphae after adding the allelochemical directly (greenhouse experiment)

The experiment was conducted for 2 months in a greenhouse (temperature: 23-27°C). We filled 40 conetainers (volume 500g) with twice-autoclaved, sieved (1 mm), sandy soil (sand = 74%, silt = 18%, clay = 8%; pH = 7.1; organic C = 1.87%, N = 0.12%). We placed a mesh bag in every pot´s center (Sefar Nitex 03-30/18, Sefar GmbH, Edling, Germany) to create a root exclusion compartment in which we placed the sensitive target plant *Lycopersicon lycopersicum*. Half of the mesh bags were filled with about 50 g twice-autoclaved soil mixed with mycorrhizal pellets (10 g), containing *Rhizophagus irregularis* C. Walker & Schuessler to provide a mycorrhizal treatment (m) or twice autoclaved pellets (nm) for the non-mycorrhizal control as well as microbial wash to each pot. After two weeks of target plant establishment, we added 10 ml juglone (100 µM final concentration, 15% ethanol, 5-Hydroxy-1,4-naphthochinon, Sigma Aldrich Germany, CAS No. 481-39-0) (Hejl et al., 2004) or 10ml water as control on 10 consecutive days, to the edge of the pot outside the root exclusion compartment. The experiment was harvested 10 weeks later and root and shoot biomass of target plants was determined. Percent colonization was examined at 200x to quantify the presence of AMF hyphae in the mycorrhizal treatment and to confirm absence in the non-mycorrhizal control (Supporting Information Table S3). Additionally we measured soil pH as well as plant available soil P and plant tissue P to take into account differences in colonization rates caused by pH changes or possible growth effects on target plants induced by nutrient levels affected by the treatments.

Results

Experiment 1. Hyphal mediated transport of naturally occurring juglone in the field

In our first experiment we investigated the hyphal mediated transport of naturally occurring juglone released by *Juglans regia* donor trees under natural conditions in the field by using a modified rotated/static core array. We determined the amount of extractable soil juglone, which was on average 271% higher if the soil was connected (Fig. 3, $F_{1,10}$=15.1, p=0.02). No significant differences were found in soil properties (pH and moisture). We also found no significant differences in non-AMF soil hyphal length,

but there were differences in AMF hyphal length; however, the latter subsequently did not result in different colonization rates in bioassay plants (Supporting Information Table S1).

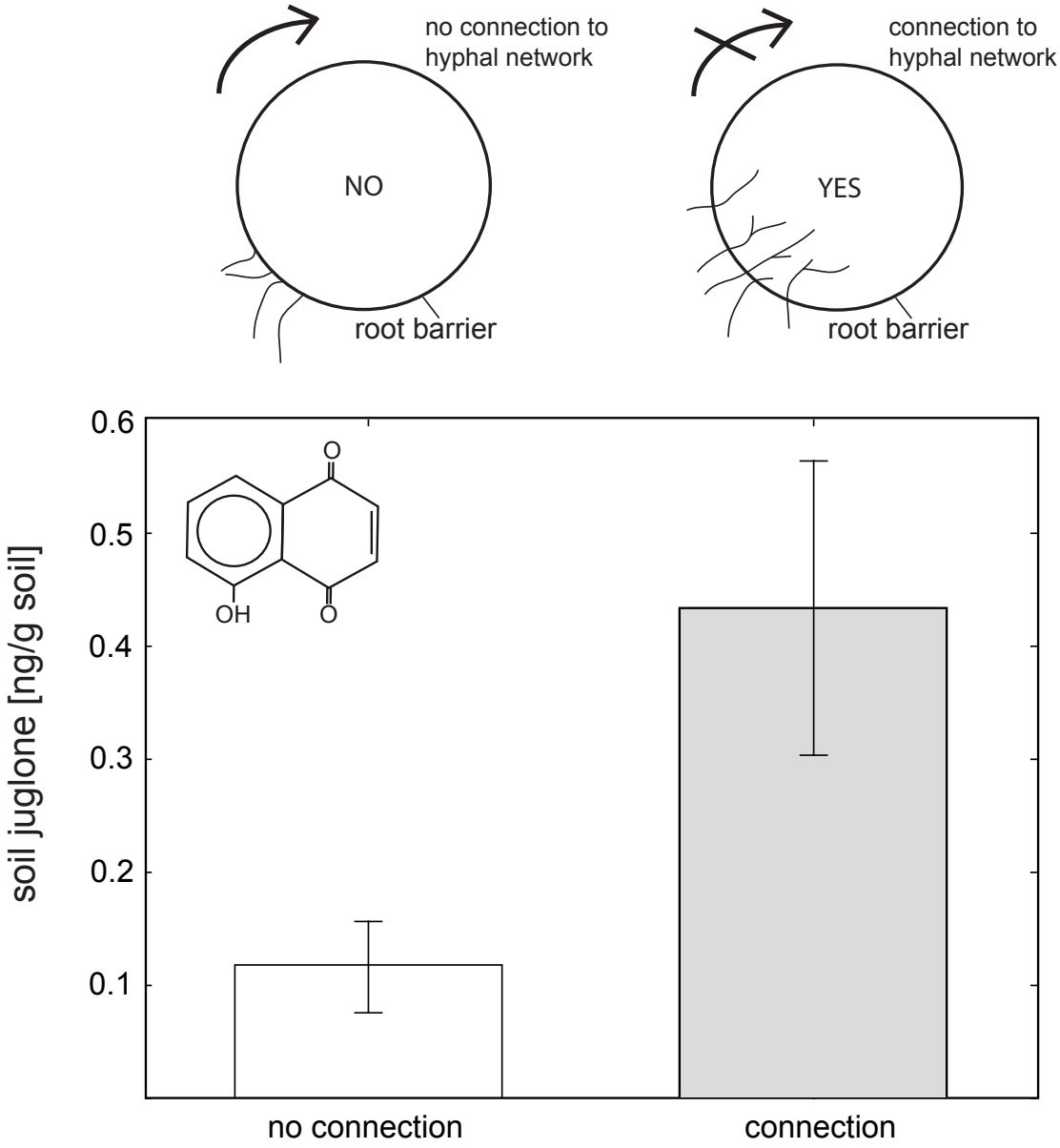

Fig. 3. Results from experiment 1 (means ± SE). White bar show extractable soil juglone [ng/g soil] of RECs with interrupted hyphal connection, gray bar show soil juglone of units with established hyphal connection (lme model, α=0.05, $F_{1,10}$=15.12; p=0.02).

We conducted the bioassay using the soil from inside the RECs to test the effect of juglone on a sensitive plant (*Lycopersicon lycopersicum*). Total biomass of bioassay plants was 31 % less compared to the corresponding control when they were grown in soil that was connected to a hyphae than when grown in a soil without a hyphal

connection. Root biomass was marginally significantly diminished by 36 % (Fig. 4, $F_{1,10}$=6.2, p=0.07), while shoot biomass (28% growth reduction) of bioassay plants was not affected (Fig 4, $F_{1,10}$=1.2, p=0.34) in these soils with higher juglone contents (Fig. 3).

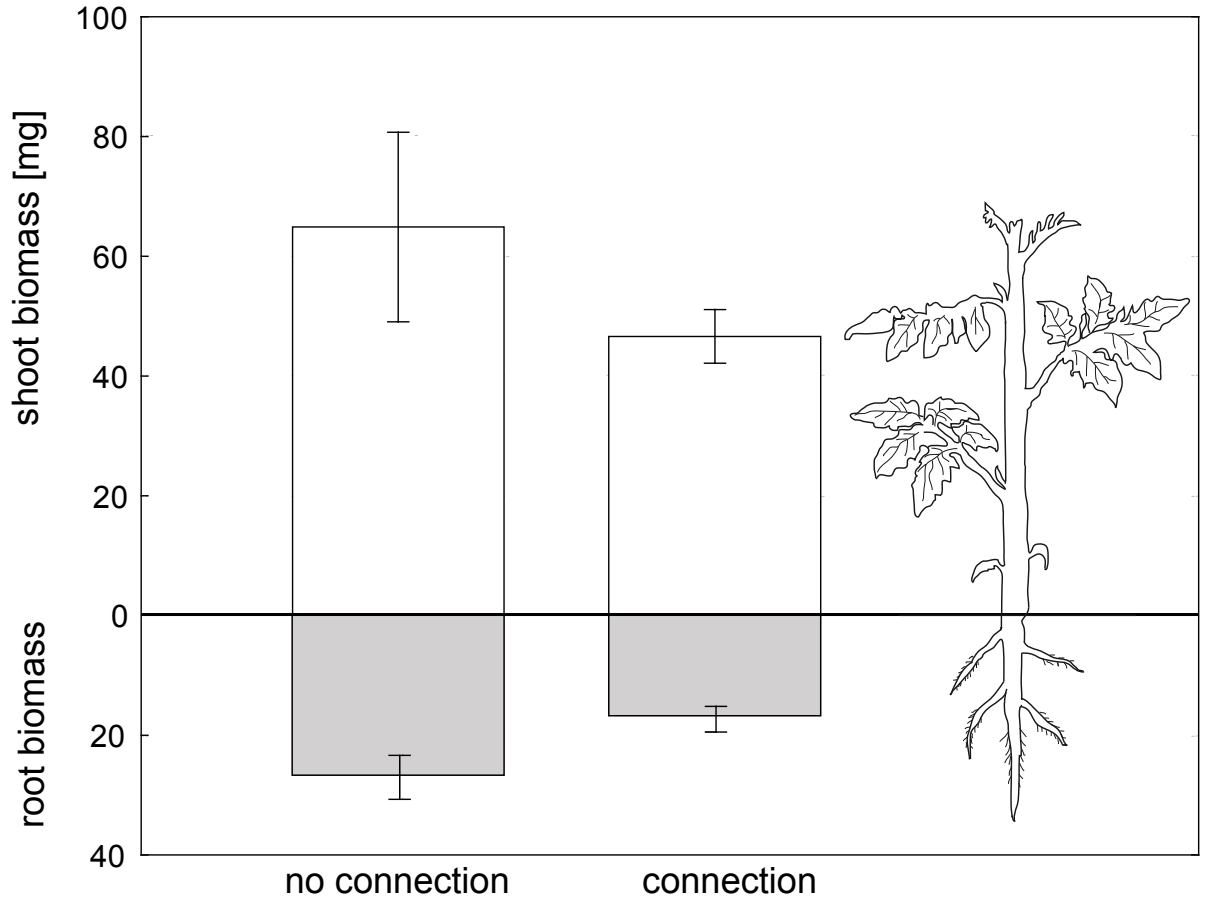

Fig. 4. Results from bioassay experiment (means ± SE). White bars show shoot biomass [mg], gray bars indicate root biomass [mg] of bioassay plants (tomato) (log transformation, lme model, α=0.05, shoot: $F_{1,10}$=1.2, p=0.34, root: $F_{1,10}$=6.2, p=0.07).

Experiment 2. Transport of juglone via *Rhizophagus irregularis* hyphae after adding walnut leaf litter (greenhouse experiment)

We conducted a greenhouse experiment to test the effects of allelochemical released from leaf litter in the presence of the mycorrhizal fungus *Rhizophagus*. For this we used *L. lycopersicum* (tomato) as a juglone sensitive target plant inoculated with *Rhizophagus* and the addition of *Juglans* leaf litter. Total biomass of tomato was reduced by 23% when litter was added in the presence of mycorrhizal hyphae compared to the corresponding control. Shoot biomass significantly decreased by 17% with leaf litter addition alone (Fig. 5, $F_{1,20}$=7.4, p=0.01), and root biomass by 35% (Fig. 5, $F_{1,20}$=38.1, p<0.01). The presence of mycorrhizal hyphae alone had no significant effects on

shoot and root biomass (Fig. 5, shoot: $F_{1,20}$=1.1, p=0.30, root: $F_{1,20}$=1.3, p=0.24). The interaction of mycorrhization and addition of leaf litter was highly significant for root biomass ($F_{1,20}$=8.4, p=0.01) but had no significant effect on shoot biomass (Fig. 5, $F_{1,20}$=1.1, p=0.32).

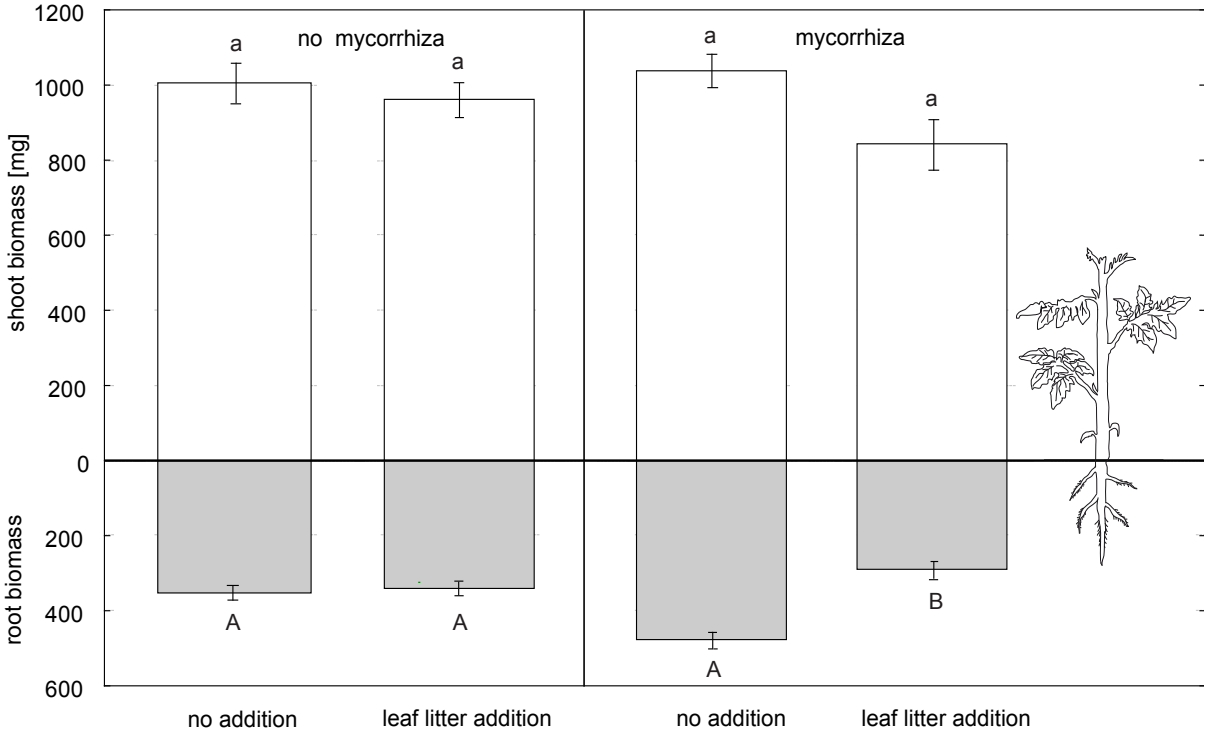

Fig. 5. Results from experiment 2 (means ± SE). Open bars show shoot biomass [mg], gray bars show root biomass [mg] (n=8). Bars with different letters indicate significant differences at α=0.05 using Tukey HSD test based on a two-factorial ANOVA with litter addition and mycorrhization as factors.

Nutrient contents of soil inside the root exclusion compartments were analyzed because the addition of litter outside the compartment could increase nutrient contents in soil. No significant differences were found in C or N, nor in plant available soil P or in AMF colonization rates (Supporting Information Table S2).

Experiment 3. Transport of juglone via *Rhizophagus irregularis* hyphae after adding the allelochemical directly (greenhouse experiment)

The strongest effects could be shown in a greenhouse experiment, where purified juglone was added outside the root exclusion compartments to mycorrhizal target plants tomato (or not as control). Total biomass was reduced by 65% in the presence of mycorrhiza relative to the corresponding control (Fig. 6, $F_{1,8}$=89.3, p<0.001) where shoot biomass was significantly reduced by 69.5% (Fig. 6, $F_{1,8}$= 118.9, p<0.001) and root biomass by 19% (Fig. 6, $F_{1,8}$=13.1, p<0.001). The presence of mycorrhiza alone had no significant effect on total biomass (Fig. 6, $F_{1,8}$=0.1, p=0.81). Accounting for shoot and root biomass separately, we found that shoot biomass was also not affected significantly by mycorrhization alone (Fig. 6, $F_{1,8}$=3.5, p<0.07) but root biomass was (Fig. 6, $F_{1,8}$= 5.6, p=0.02). The interaction between mycorrhization and addition of juglone was significant for shoot biomass (Fig. 6, $F_{1,8}$ =6.7, p=0.01) but not for root biomass (Fig. 6, $F_{1,8}$=0.3, p=0.59).

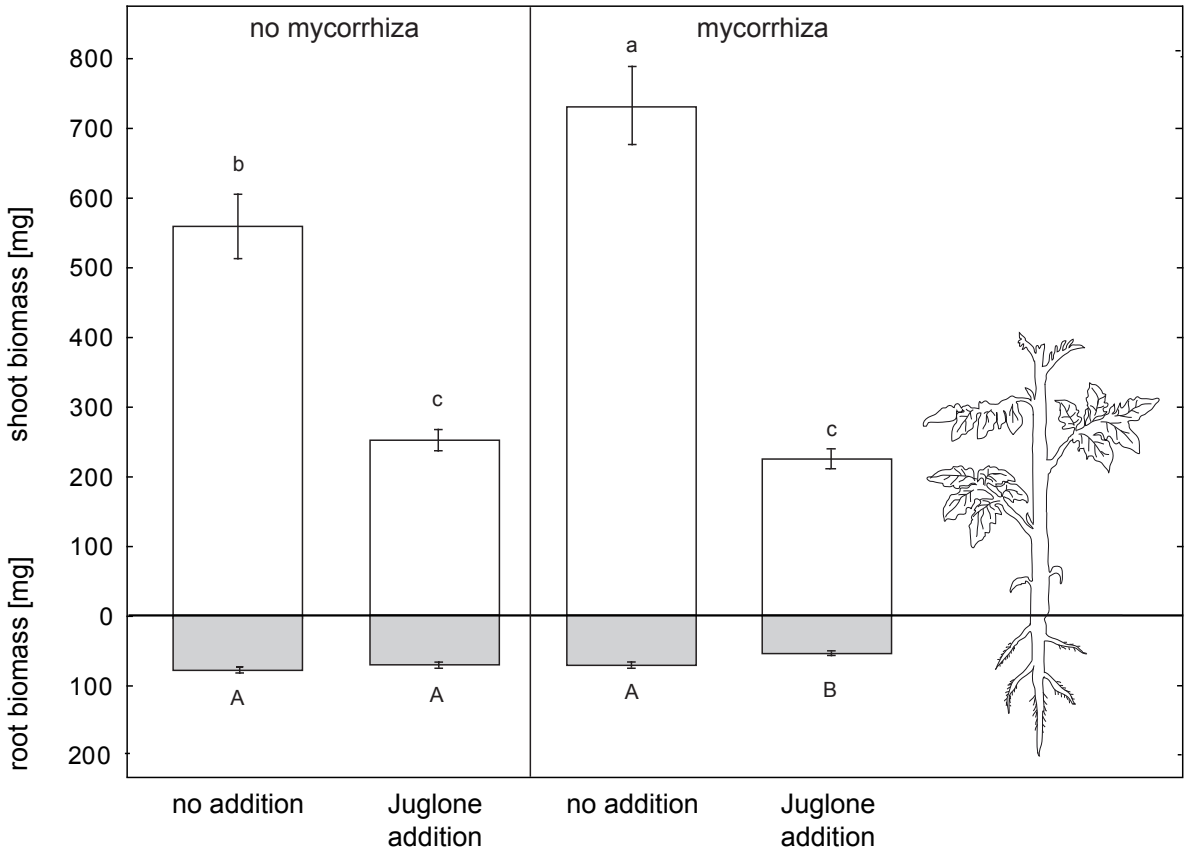

Fig. 6. Results from experiment 3 (means ± SE). Open bars show shoot biomass [mg], gray bars show root biomass [mg] (n=8). Bars with different letter indicate significant differences at α=0.05 using a two-factorial ANOVA with juglone addition and mycorrhization as factors.

Nevertheless, root length was significantly reduced if mycorrhiza and juglone were present ($F_{1,8}$= 17.6, p<0.01) but roots tended to be thicker (Supporting Information Table S3). Mycorrhizal colonization rates tended to be higher if juglone was added but were not affected significantly by the treatment (Supporting Information Table S3).

Plant available soil phosphorus and pH inside the root exclusion compartments were determined to test if juglone addition changed these parameters. There were no significant differences in soil pH (Supporting Information Table S3), but there was a significant effect on plant available P by juglone addition as a main effect (p<0.01), although not in the interaction between mycorrhization and juglone addition (p=0.92). Significant differences were also found in plant phosphorus, where we found the lowest phosphorus amounts in mycorrhizal plants (68% colonization with 233.04 mg 100 g^{-1} P) (p<0.01), and there was an interaction effect of mycorrhization and juglone addition on plant P (p<0.01).

Discussion

The role of allelopathy in plant-plant interactions has been challenged over the last decade, because it was argued that activity of the responsible compounds would be diminished under natural conditions (e.g. through sorption, chemical and microbial degradation) (Schmidt & Ley, 1999) to such an extent that its ecological significance was rendered questionable. However, the observation that allelopathic effects have often been difficult to show in a soil matrix may be partially explained by the fact that microbial (Inderjit, 2005, Kaur et al., 2009) and especially mycorrhizal influences have been omitted by using sterile soils in experimental approaches.

This realization has led to the development of the Network Enhanced Bioactive Zone model (NEBaZ) (Barto et al., 2012), which postulates a central role of soil mycorrhizal hyphae in mediating allelopathic effects. In a combination of complementary field work, plant bioassays and greenhouse studies we provide strong support for this conceptual model. Our field study enabled us to relate movement of naturally released juglone at realistic concentrations to the presence of hyphae most likely consisting of different fungal species. Two complementary greenhouse experiments with higher experimental control yielded results pointing in the same direction. Also, in order to not rely just on the REC design we also employed inoculation as an alternative method to establish mycorrhizal treatments.

Hyphal mediated transport

Transport via mycorrhizal networks is expected to be much quicker than bulk soil flow, because the length of the flow path is shorter (Allen, 1996). Infochemicals that mediate information exchange among organisms may be transported in the gas phase by wind or diffusion and in the aqueous phase via water movements or diffusion. For plant – plant communication, a fungal hyphal network provides a further path for infochemical transport. Several pathways for infochemical flow via hyphae are possible, e.g. surficial, apoplastic or cytoplasmic paths as well as hyphal cord interior transport or optimized hyphosphere diffusion (Barto et al., 2012). The movement of juglone through a hyphal mycelium could occur most likely via water flowing along hyphae (Querejeta et al., 2003) because juglone is slightly hydrophilic (solubility in water at 25°C c log P= 1709 (http://www.chemexper.com)) and could therefore dissolve in a liquid layer surrounding hyphae in the soil matrix. Juglone might also partly be transported cytoplasmically by entering the hyphae actively or by diffusion. Additionally, altered hyphosphere conditions such as increased soil aggregation, conductivity or microbial community modifications could also play a role in accelerating allelochemical movement.

Recently Duhamel, Pel, Ooms et al. (2013) provided evidence that transport via hyphae could also be advantageous to the hyphae themselves by potentially protecting them from fungivores. Our experiments were not designed to differentiate between the transport mechanisms, and a goal of future work is to test if the transport was active or merely passive; however, the functional significance of this hypha-mediated movement is becoming increasingly clear through our set of studies.

Other possible influencing factors

Independently of its allelochemical transport functions the presence or absence of fungal hyphae itself can potentially affect plant growth. The absence of hyphae might influence target plant growth negatively through reducing the efficiency of nutrient foraging by AMF or increased costs to the host plant in maintenance of its AMF symbiont. This seemed to play no role in experiment 1 because even when the mycorrhizal connection was interrupted repeatedly, there were no significant differences in AMF colonization rates of bioassay plants (Supporting Information Table S1). This suggests that differences in bioassay plant growth were likely not influenced by a reduced inoculum potential caused by interrupted hyphal connections; however, in this field study this possibility cannot be completely excluded. Therefore, we conducted two further experiments in the greenhouse. In experiment 2 and 3 we used autoclaved soil

with a microbial wash as control and inoculated the other half of the pots with a single mycorrhizal fungus to generate hyphae. In both experimental approaches colonization rates were not significantly different within the mycorrhizal treatment (adding leaf litter in experiment 2 and juglone solution in experiment 3) which suggests that differences in plant biomass were likely not caused by variable inoculum potentials.

Connectivity to a hyphal network generally can have variable effects on target plants due to nutrient availability, especially influencing P uptake (Smith & Read, 1997) and carbon fluxes between plants and mycorrhizal fungi. Connection to hyphae could lead to reduced seedling growth caused by increased carbon flow from the seedling to the fungal mycelium (Nakano-Hylander & Olsson, 2007). Increasing plant available soil P levels could point to effects of AMF on nutrient levels. Considering carbon fluxes we see that all bioassay plants were exposed to AM hyphae and had similar colonization rates (Supporting Information Table S1) and therefore similar potential carbon costs of supporting the fungal mycelium. Differences in plant biomass caused by carbon investment from seedlings into fungal mycelium hence likely played no role in experiment 1. In experiment 2 we measured C and N contents as well as plant available P, because by using leaf litter as a treatment, a possible additional input of nutrients to the soil could result in plant growth differences (Paul & Clark, 1989). We found neither significant differences in C and N contents nor in plant available soil P. Differences in plant growth caused by carbon fluxes from seedlings into the fungal mycelium seem to play no role, because there were no biomass reductions in control plants with connections to soil mycorrhizal hyphae (Fig. 5).

We used, as mentioned before, autoclaved soil in this experimental approach, which in consequence resulted in relatively high phosphorus content (Endlweber & Scheu, 2006). In nutrient poor soils mycorrhizal plants are more likely to be dependent on nutrient supply by their symbionts (Smith & Read, 1997). However, at adequate soil phosphorous levels, the importance of fungal mycelia in nutrient availability decreases and therefore we suggest that in experiment 2 effects of AMF on nutrient levels would have been small and unlikely to cause growth reductions in tomato plants.

In experiment 3 we found no significant differences in plant available soil P considering only mycorrhization or the interaction of mycorrhization and juglone addition, but juglone addition alone resulted in significant differences in soil P levels ($p<0.01$). The addition of juglone solution could influence the mobilization and availability of P in soil by influencing the microbial community (Sun, Yang, Wang et al., 2013). We also analyzed plant phosphorus contents and found differences concerning mycorrhization ($p<0.01$) and the interaction of mycorrhization and juglone addition ($p<0.01$). Here,

carbon and phosphorus fluxes between plant and fungal mycelium may have played a role (Hammer, Pallon, Wallander et al., 2011) because if the photosynthetic activity of the plant was reduced due to juglone addition (Hejl et al., 1993), less carbon would be available to support the fungal mycelium and therefore less phosphorus might be available for the plant. Higher colonization rates (68%) as well as higher number of vesicles (data not shown) might point to increased phosphorus storage in the fungal mycelium. Carbon drain from seedlings to a fungal mycelium seems, as in experiment 2, unimportant because there is no decrease in plant growth when comparing non-mycorrhizal to mycorrhizal control plants.

Conclusions

We have shown here that there is strong influence of fungal mycelia in facilitating transport processes and therefore extending the bioactive zone of allelochemicals in the soil, described as the Network Enhanced Bioactive Zone model (NEBaZ) (Barto et al., 2012).

In line with those findings we have shown that juglone can accumulate and persist in soil at high levels, and its accumulation results in more effective target plant growth reductions when fungal mycelia are present. We have shown this in complementary experimental approaches and a field study, supporting the notion that mycorrhizal transport processes are important in plant communication processes in natural settings.

Our results suggest that hyphal mediated transport processes can affect allelopathic effects, with potentially profound consequences for plant community ecology. Further research on allelopathy should thus explicitly consider the mycorrhizal component of soils.

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Supporting Information

Table S1. Differences in soil and fungal properties between experimental units with established or interrupted hyphal (control) connection. Values are means and standard errors (n = 5). Significant p values (< 0.05) are bolded.

Response variable		no connection to CMN	connection to CMN	transformation	F-value	p-value
Soil properties	pH	7.26 (0.04)	7.26 (0.02)	-	0.27	0.56
	soil moisture [%]	24.78 (1.40)	24.22 (1.00)	-	0.04	0.72
Fungal properties	AMF hyphal length [m g^{-1} soil]	1.51 (0.26)	2.55 (0.37)	-	5.24	**0.03**
	non AMF hyphal length [m g^{-1} soil]	8.54 (1.11)	8.99 (0.76)	-	0.10	0.72
	AMF colonization [%]	12.09 (3.23)	18.10 (4.52)	-	1.55	0.11

Table S2. Differences in soil and fungal properties between experimental units with or without (= control) mycorrhiza and leaf litter addition. Values are means and standard errors (n = 6). Significant p values (< 0.05) are bolded. Soil properties were analyzed using a factorial ANOVA with mycorrhiza and litter addition as factors. AMF colonization [%] data were analyzed using a Wilcoxon rank sum test.

Response variable		no mycorrhiza		mycorrhiza		transfor-mations	F-value			p-value		
Litter addition		0g	4g	0g	4g		CMN	litter addition	interaction	CMN	litter addition	interaction
Soil properties	C [%]	2.55 (0.33)	2.97 (0.23)	3.75 (0.54)	3.21 (0.40)	-	3.32	0.03	1.47	0.08	0.87	0.23
	N [%]	0.19 (0.03)	0.18 (0.02)	0.26 (0.04)	0.21 (0.03)	-	2.47	0.77	0.16	0.13	0.39	0.69
	plant available P [mg g $soil^{-1}$]	3.86 (1.55)	7.22 (1.03)	7.16 (0.94)	3.83 (1.24)	-	3.37	3.56	0.84	0.08	0.07	0.37
Response variable		no CMN (control)		CMN			W-value			p-value		
Litter addition		0g	4g	0g	4g							
Fungal properties	AMF colonization [%]	0	0	49.52 (4.64)	39.11 (6.48)	-	-	26.5		-	0.20	

Table S3. Differences in soil and fungal properties between experimental units with or without (= control) mycorrhiza and juglone addition. Values are means and standard errors (n = 8). Significant p values (< 0.05) are bolded. Soil and plant properties were analyzed using a factorial ANOVA with mycorrhiza and juglone addition as factors. AMF colonization [%] data were analyzed using a Wilcoxon rank sum test.

Response variable		no mycorrhiza (control)		mycorrhiza		transformations	F-value			p-value		
Juglone addition		no	yes	no	yes		mycorrhiza	juglone	interaction	mycorrhiza	juglone	interaction
Soil and plant properties	soil pH	**6.94 (0.05)**	**6.88 (0.03)**	**6.98 (0.03)**	**6.95 (0.03)**	-	**1.45**	**0.89**	**0.11**	**0.24**	**0.33**	**0.74**
	plant available P [mg g soil $^{-1}$]	12.77 (0.69)	10.36 (1.82)	14.18 (1.11)	13.51 (0.01)	-	1.09	7.97	0.01	0.31	<0.01	0.92
	plant P [mg g $^{-1}$]	**31.97 (2.30)**	**34.88 (1.78)**	**29.98 (1.67)**	**23.30 (2.25)**	-	**19.47**	**3.04**	**11.07**	**<0.01**	**0.09**	**<0.01**
	root length [cm]	153.94 (15.75)	259.31 (17.07)	219.20 (28.22)	146.62 (21.48)	-	1.25	0.60	17.63	0.27	0.45	<0.01
	root diameter [cm]	**0.45 (0.05)**	**0.58 (0.01)**	**0.58 (0.01)**	**0.67 (0.01)**	**log**	**15.88**	**25.55**	**1.25**	**<0.01**	**<0.01**	**0.27**
Response variable		no mycorrhiza (control)		mycorrhiza			W-value/F-value			p-value		
Juglone addition		no	yes	no	yes							
Fungal properties	**AMF colonization [%]**	**0**	**0**	**25.13 (6.10)**	**68.22 (14.13)**	-		**19**			**0.06**	
	AMF hyphal length [m g soil^{-1}]	0.43 (0.09)	0.61 (0.08)	0.52 (0.06)	0.63 (0.03)	-	4.34	0.64	0.28	0.06	0.44	0.61
	non AMF hyphal length [m g soil^{-1}]	**0.83 (0.04)**	**0.94 (0.11)**	**0.75 (0.10)**	**0.77 (0.08)**	-	**0.06**	**0.42**	**0.02**	**0.81**	**0.52**	**0.88**

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CHAPTER III

Arbuscular mycorrhizal fungal hyphae enhance transport of the allelochemical juglone in natural soil

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Summary

1. Allelopathy is a biological phenomenon where plants have harmful effects on growth of surrounding plants through the production of chemical substances. Here we focus on allelochemical processes which operate belowground, can influence plant interactions and therefore potentially affect plant community structure.

2. Soil hyphae of arbuscular mycorrhizal fungi (AMF) may enhance transport processes in the soil matrix by providing direct connections between plants facilitating infochemical exchange.

3. In a two-component field study we showed that soil hyphae play a crucial role in movement of allelochemicals in natural soils and greatly expand bioactive zones by providing effective transport pathways for chemical compounds. First, we tested the effects of *Juglans regia* leaf litter extract addition in intact or disrupted hyphal networks and simultaneously determined juglones growth reducing effects on sensitive *Lycopersicon lycopersicum* plants. Second, we analyzed the effect of allelochemical juglone on tomato by adding leaf litter. In both approaches we found an increase of juglone transport if a hyphal network was present, resulting in reduced growth of target plants.

4. Our results point to hyphae of soil fungi playing an important role in the transfer of allelochemicals and effectively acting as transport highways in the field. We suggest that hyphal networks, mostly formed by AMF, increase the effectiveness of allelochemicals in natural systems and play a crucial role in chemical interaction processes in the soil.

Keywords:

Allelopathy; arbuscular mycorrhiza; CMNs; *Juglans*; mycorrhizal functioning; plant–soil interactions; rhizosphere; transport

Introduction

Allelopathy is a controversially discussed topic of great interest to plant and soil ecologists. The biological phenomenon in its broader sense is understood as the direct or indirect harmful effect of one plant on another through the production of chemical compounds that are released into the environment (Rice, 1974). These allelochemicals must accumulate and prevail at phytotoxic levels and reach the target plant to be ecologically relevant in the field (Choesin and Boerner, 1991).

The allelopathic influence ranges from affecting germination, growth, development, reproduction and distribution of a number of plant species to plant-plant interactions and eventually might play an important role in community structure (Bais et al., 2003; Blair et al., 2005; Inderjit et al., 2005). Allelochemical compounds are found in many types of plants releasing them by a variety of mechanisms into the rhizosphere, including decomposition of residues, volatilization and root exudation (Bertin et al., 2003).

Growth inhibition caused by the presence of walnut trees in the landscape is known since the Ancient World and is therefore one of the oldest classical examples of allelopathy. American black walnut (*Juglans nigra* L.) is the most commonly studied species (Willis, 2000), but harmful influences on the environment were also observed in other members of the familiy Juglandaceae including *Juglans regia* (Daglish, 1950; Prataviera et al., 1983; Ponder and Tadros, 1985), the plant used in our study. Walnut toxicity is related to the aromatic phytotoxic compound juglone (5-hydroxy-1, 4 naphthoquinone) (Rice, 1974), and can be found in all parts of the plant, but particularly in leaves and roots (Segura-Aguilar et al., 1992). In living tissues juglone is mostly found in a reduced, nontoxic and colorless form, the so-called hydrojuglone, which is transformed in the soil after cleavage of the glycosidic bond and oxidation of the aglycone to the toxic and orange juglone (Bertin et al., 2003).

The effects of juglone on woody and herbaceous plants are generally harmful (but see (Whittaker and Feeny, 1971) responding with stunting, wilting and necrosis caused by growth inhibition at micromolar concentrations (Hejl et al., 1993; Jose and Gillespie, 1998), disruption of photosynthesis and respiration (Jose and Gillespie, 1998) and interference with water uptake (Hejl and Koster, 2004). Several ornamental and agricultural plants including tomato, potato, pea, apple, cucumber, watermelon, bean, garden cress, corn and ericaceous species such as rhododendron and azalea are especially sensitive to the deleterious influence of juglone (Crist and Sherf, 1973).

Both rhizodeposition and leaching of juglone and its precursor out of decomposing leaves and other parts of the plant are important mechanisms by which the allelochem-

ical arrives in the soil and can hence influencing neighboring plants (Rietveld, 1983).

Factors which shape soil complexity like soil composition and structure (Schmidt and Ley, 1999; Inderjit, 2001), soil moisture (Blair et al., 2006) and the content and composition of organic matter (Schmidt and Ley, 1999), as well as the presence and dynamics of microbial communities (Blum, 2004; Cipollini et al., 2012) can influence the extent to which allelopathy contributes to plant community dynamics. Furthermore diffusion rates, sorption of chemical compounds to mineral and organic matter and chemical as well as microbial degradation can affect the functioning of allelochemicals in the soil (Rettenmaier et al., 1983; Schmidt and Ley, 1999; Kaur et al., 2009).

Microbial decomposition is probably particularly important in allelopathic effectiveness because high rates of degradation in the rhizosphere and beyond decreases the amount of allelochemicals in the soil and thus shrinks the bioactive zone of the respective compound.

Most studies of allelopathy have not taken into account the existence of soil hyphal networks, mostly formed by arbuscular mycorrhizal fungi (AMF) (Barto et al., 2012). These fungi colonize the roots of the vast majority of plant species, but also form a mycelial network in the soil, potentially linking root systems of multiple plant species in a community (Giovannetti et al., 2001; Voets et al., 2006; Mikkelsen et al., 2008). These common mycelial networks (CMNs) may act as “superhighways” connecting different plants belowground (Barto et al., 2012) and could enlarge bioactive zones of allelopathic compounds in natural soils, described in the Network Enhanced Bioactive Zone model (NEBaZ) (Barto et al., 2012).

Several transport functions of CMNs have been studied like the movement of water (Querejeta et al., 2003; Egerton-Warburton et al., 2007) and nutrients (He et al., 2003; Mikkelsen et al., 2008; Walder et al., 2012) as well as metals (Meding and Zasoski, 2008), but very few studies have examined the potential transport of infochemicals via CMNs.

Studies have addressed signal-induced intraspecific plant communication (Song et al., 2010), underground signals warning neighboring plants of aphid attack (Babikova et al., 2013) or allelochemicals in soil (Barto et al., 2011). Recently it was shown that transport via hyphae could also be beneficial to the hyphae themselves by potentially protecting them from fungivores (Duhamel et al., 2013).

CMNs or parts of a CMN could mediate an accelerated movement of the allelochemical out of the rhizosphere of the producing plant, the place with highest microbial activity, thus reducing vulnerability to microbial degradation. While it is not clear if substances move within or along the hyphae of such networks, which is beyond the scope of our

study to disentangle, the presence of hyphae appears to accelerate the movement of allelochemicals compared to diffusing through the soil matrix. As a consequence of these higher flow rates, allelochemicals may reach levels that induce growth inhibition in the target plant.

The aim of our study was to experimentally test for AMF-mycelium mediated allelopathic effects under field conditions. The potential for allelopathic inhibition of plant growth has been demonstrated repeatedly under laboratory conditions or in greenhouse experiments, but rarely under field conditions, with in situ microbial communities of AMF and saprobes, and natural temperature and precipitation conditions. Our approach was to create continuous and interrupted hyphal networks using root exclusion compartments (RECs), combined fully factorially with addition of juglone. Our experiment had two parts, one with addition of *Juglans regia* L. leaf litter outside RECs, one with addition of an aqueous extract of walnut leaves outside the RECs.

Materials and Methods

General study design

The study was carried out in a semi-natural grassland field plot (Institute of Biology of Freie Universität Berlin, 10m x 1.8m), mainly consisting of *Lolium perenne* and *Poa annua* (colonization rates of 5 random root samples: 42.2±4%), to take advantage of a pre-existing hyphal network of AMF and the resident microbial community.

We used a modified in-growth core design (Johnson et al., 2001) consisting of root exclusion compartments (RECs) which were either static, to permit a connection to the surrounding mycelial network or rotated, to interrupt the mycelium connection (Fig. 1). Then RECs were prepared by covering the sides and the bottom of small plastic baskets (Poeppelmann Teku® 5 x 5 cm, G, 0.07 l, Poeppelmann, Germany) with 30 µm mesh (Sefar Nitex 03-30/18, Sefar GmbH, Edling, Germany) to exclude roots but permit hyphae to grow in (Fig. 1a). The RECs were filled with well-mixed, sieved (1 mm), sandy soil (sand = 74%, silt = 18%, clay = 8%; pH = 7.1; organic C = 1.87%, N = 0.12%) from an experimental field of the Institute of Biology of Freie Universität Berlin. To avoid differences in AMF inoculum potential or other soil properties between treatments (which may have been present in the field plot) all RECs contained the same soil.

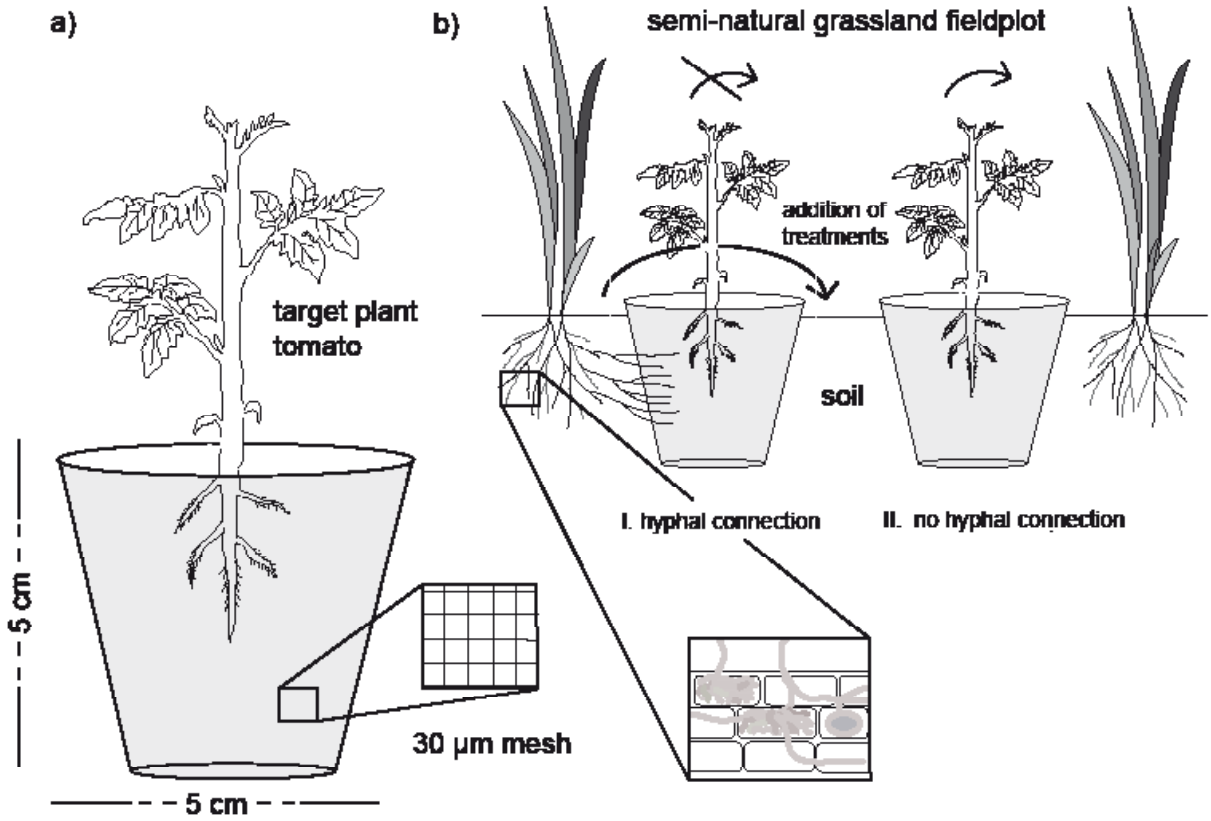

Fig. 1. a) Experimental unit of a modified in-growth core design with the target plant tomato in root exclusion compartments (RECs). Root exclusion compartments (RECs) were covered with a 30 µm mesh to avoid root penetration but allow connection to mycorrhizal hyphae in the surrounding soil.

b) Experimental setup in the field. Placement of RECs with tomato receiver plant in the soil in a block design. Arrows indicate that pots were rotated to interrupt the hyphal connection (= control) or left in place to permit a hyphal connection.

Tomato seeds were pregerminated in glass beads for 1 week before planting them into the field. Tomato seedlings of the same age were then planted into the RECs (n=8) just before inserting them into the field plot. We chose tomato because it is known to be sensitive to juglone (Crist and Sherf, 1973).

After placing the RECs in the soil, the whole experimental area was covered with a mesh (mesh size: 5mm) on wooden sticks (ca. 50 cm above soil) to prevent disturbance through nutrient input by birds or small mammals or feeding on tomato plants by snails. The experimental plot area was divided in two halves, one for each experiment. Each experiment had 4 subplots (1.2m x 1.8m), representing the control plot (no connection and connection to hyphal network) and the treatment plot (no connection and connection to hyphal network in combination with juglone addition or not).

For further statistical analysis the location of RECs were treated as blocks. Initially we started with a high number of replicates (n=20) because we expected a potential high seedling loss through external conditions in the semi-natural field plot. Despite of using a protection mesh, several seedlings had to be excluded for statistical analysis because they were damaged or eaten and we thus ended up with n=8 in experiment 1 and n=6 in xperiment 2.

To enable a connection to the existing mycorrhizal network in the field plot, half of the compartments were kept static after placing in the soil (depth: ca. 5 cm), the others were rotated three times per week by 1-2 mm to ensure severing of hyphae attempting to cross into the RECs. The movement of half of the pots was carried out very carefully to prevent major disturbance, formation of an air gap between soil and compartments, or damage to the mesh. Diffusion of juglone from the bulk soil through the mesh could still occur in our rotated control REC; we were thus interested in differences of juglone content being reflected in plant growth differences in the REC treatments. Juglone contents in soil were below detection limits with our methods.

Movement of juglone via hyphal networks in the field: leaf litter leachate

For experiment part 1 we used as juglone addition a litter leachate of fresh *Juglans regia* L. (Botanical Garden Berlin) leaves. 1 kg fresh leaves were soaked in 20 l dH_2O for 3 days, we filtered (53 µm sieve) the leachate afterwards to remove litter particles and stored it at 4°C in darkness (Piotrowski et al., 2008). We added 30 ml of the litter extract or dH_2O water as control 0.5 cm around the mycorrhizal and non mycorrhizal root exclusion compartments five times weekly. We chose an aqueous leachate because we assumed that the allelopathically effective compound will enter the soil matrix quickly and could therefore have an immediate and intense effect on bioassay tomato plants.

Movement of juglone via hyphal networks in the field: leaf litter

In experiment part 2 we used fresh leaves of *Juglans regia* L. (Botanical Garden Berlin), dried them for 1 week at 40°C, crushed and sieved (5mm) to obtain a homogenous leaf litter particle size. We added 8 g leaf litter per pot 0.5 cm around the mycorrhizal and non mycorrhizal root exclusion compartments and no leaf litter around the control compartments. We chose the addition of leaf litter to achieve a slow release of allelopathic compounds by gradual leaching into the soil. 100g of leaf litter contained 0.410 g (±0.03) juglone, as determined by HPLC analysis (for detailed analysis see Supplementary material).

Post-harvest measurements and statistical analysis

After 6 weeks of growth for both experiments, shoots and roots of tomato plants in RECs were removed carefully, dried for 1 week at 40°C and dry weight was measured. After this, a random subsample of roots was stained to measure AMF root colonization using the modified method of Vierheilig (1998) by maceration of the roots with KOH (10% for 10 min at 90°C) with subsequent acifidation and staining (10% ink in acetid acid 10% for 15 min at 90°C). Intersections were counted at 200 magnification for presence of AMF to determine percent root colonization (McGonigle et al., 1990).

We also determined pH of soil in water (McKeague, 1978) as well as hyphal length in the soil using an aqueous extraction and quantification method (Jakobsen et al., 1992).

Plant biomass data, root colonization data and soil AMF hyphal length were analyzed using linear mixed effect models (nlme package) in R 2.13.0 (R Core Team, 2012). Status of connection to the mycorrhizal network and addition of leaf litter/ litter extract were fixed effects in the fully factorial analysis while blocks were random effects. All residuals were tested for normality and homogeneity of variances (Shapiro test and Bartlett test, respectively). Differences were considered significant at $p<0.05$. In experiment 1 and 2 we used log transformations for AMF colonization data.

Results

To investigate the potentially enhanced transfer of juglone from the surrounding soil, where juglone-containing litter or extract were applied, into the root exclusion compartment by hyphal mediated movement, we measured effects of juglone on tomato target plants in the presence or absence of a hyphal connection. In both experiments we observed a significant growth reducing effect when the target plant was connected to a hyphal network and juglone was added. It is important to note that all our plants in this experiment were mycorrhizal and that there were no differences in mycorrhizal root colonization among treatments (Table 1, 2).

Leaf litter leachate effects

In experiment 1 the addition of leaf litter extract led to a 56% decrease in total biomass in the presence of a hyphal connection compared to the corresponding control. Both mycorrhizal network connection ($F_{1,32}$=4.9, p<0.01) and the factor leaf extract addition ($F_{1,32}$=5.8, p<0.01) were significant, and there was a highly significant interaction term ($F_{1,32}$=19.7, p<0.001).

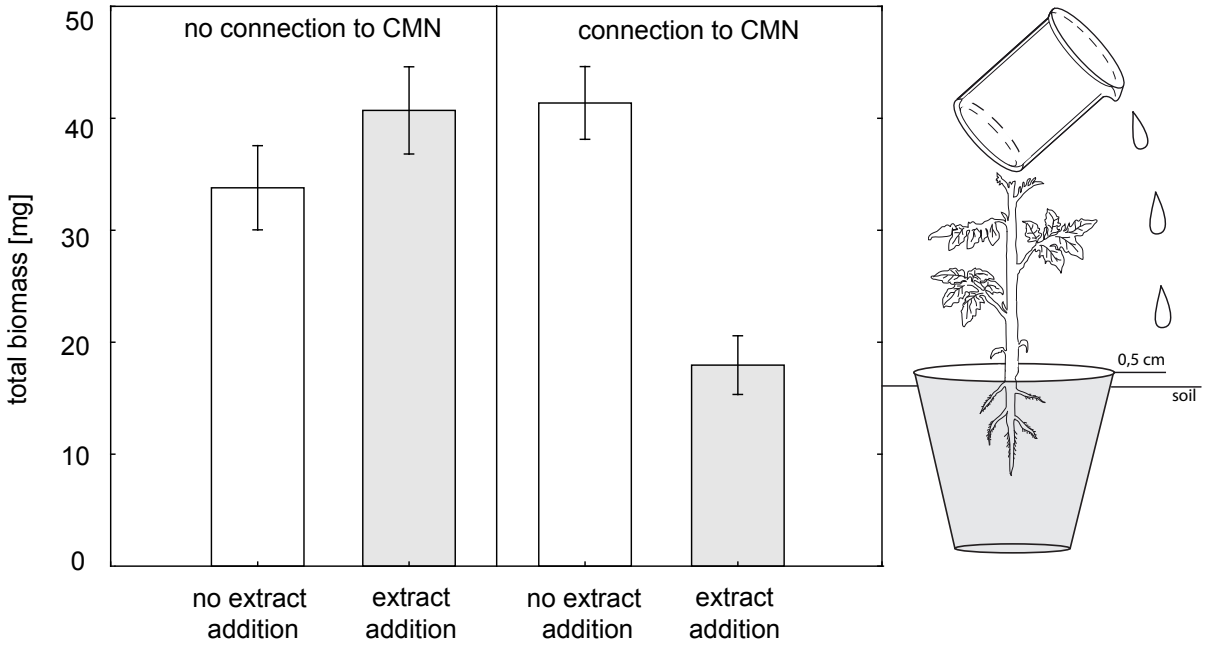

Fig. 2. Results from experiment 1 (means ± SE; n = 8). White bars show total biomass [mg] of tomato plants without extract addition, gray bars indicate total biomass [mg] of tomato plants with extract addition.

Soil pH was not significantly affected by treatments nor was there a significant interaction; the same was the case for root colonization rates of AMF (Table 1). There was a significant main effect of network connection on AMF soil hyphal length, with higher values in the treatment combinations with a connection. There was a significant interaction term, such that without network connection hyphal length decreased with extract addition, but with the network connection we observed the opposite pattern.

Table 1. Differences in soil and fungal properties between experimental units connected or not (= control) to a hyphal network with addition of juglone leaf litter extract (or not as control). Values are means and standard errors (n = 8). Significant p values (<0.05) are bolded.

Response variable		no connection to CMN		connection to CMN		F-value (p-value)		
		no addition	extract addition	no addition	extract addition	CMN	extract addition	interaction
pH		6.73 ± 0.01	6.73 ± 0.02	6.76 ± 0.01	6.71 ± 0.02	0.18 (0.30)	4.45 (0.83)	3.35 (0.06)
AMF properties	hyphal length [m g^{-1} soil]	2.51 ± 0.34	1.41 ± 0.19	2.84 ± 0.32	3.46 ± 0.54	**10.21 (0.01)**	0.37 (0.05)	**5.23 (0.03)**
	colonization rate [%]	36.5 ± 7.8	35.3 ± 8.1	32.1 ± 7.8	45.4 ± 8.6	0.14 (0.36)	0.43 (0.84)	0.89 (0.36)

Leaf litter effects

In experiment 2 the addition of leaf litter led to a 36% decrease in total biomass (Fig. 3, $F_{1,24}$=6.01, p=0.03) in the presence of a hyphal connection. Both mycorrhizal network connection ($F_{1,24}$=5.88, p<0.01) and leaf litter addition ($F_{1,24}$=4.92, p<0.01) were significant effects for total biomass, and there was a significant interaction term ($F_{1,24}$=6.01, p=0.03).

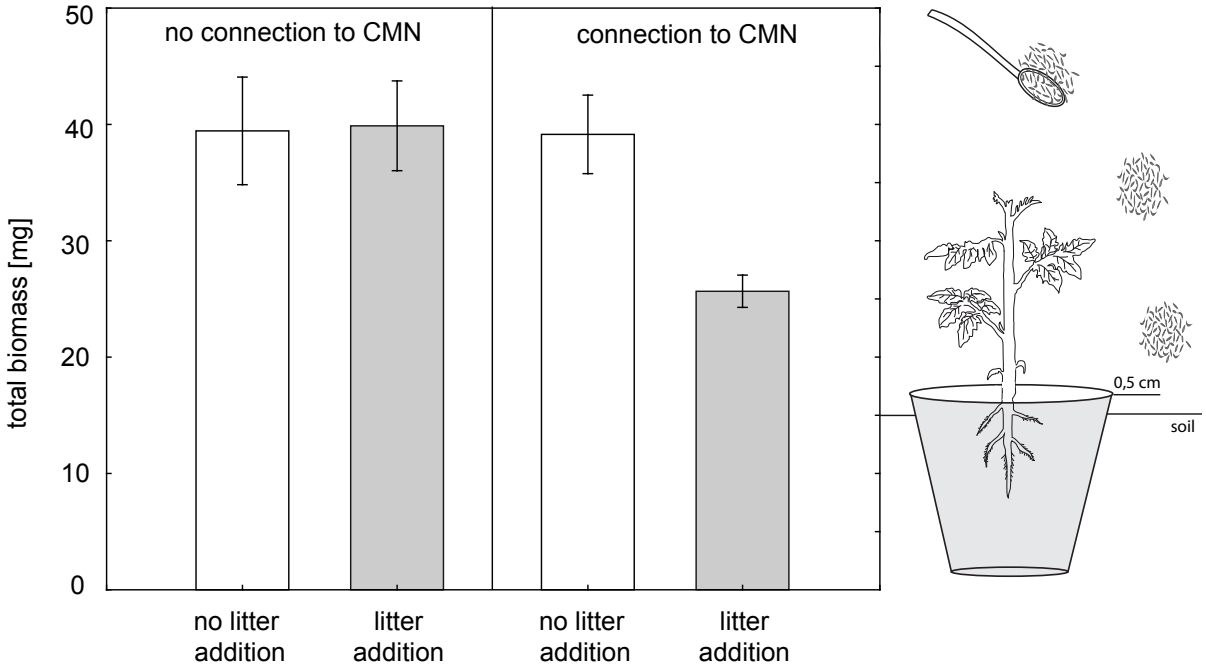

Fig. 3. Results from experiment 2 (means ± SE; n = 6). White bars show total biomass [mg] of plants without litter addition, gray bars indicate total biomass [mg] of plants with litter addition.

Soil pH was not significantly affected by treatments, neither were colonization rates of AMF (Tab. 2). We found significant differences in AMF fungal hyphal lengths in RECs, with a significant effect of network connection on AMF soil hyphal length, with higher values in the treatment combinations with a connection. There was a significant interaction term, such that without network connection hyphal length decreased with leaf litter addition as well as with network connection.

Table 2. Differences in soil and fungal properties between experimental units connected or not (= control) connected to a common mycorrhizal network with adding of juglone leaf litter (or not as control). Values are means and standard errors (n = 6). Significant p values (<0.05) are bolded.

Response variable		no connection to CMN		connection to CMN		F-value (p-value)		
		no addition	litter addition	no addition	litter addition	CMN	litter addition	interaction
pH		6.73 ± 0.02	6.75 ± 0.02	6.77 ± 0.02	6.77 ± 0.02	1.86 (0.45)	0.36 (0.87)	0.15 (0.67)
AMF properties	hyphal length [m g^{-1} soil]	2.58 ± 0.08	1.55 ± 0.06	2.78 ± 0.11	3.44 ± 0.17	**118.35 (<0.01)**	**14.14 (<0.01)**	**81.13 (<0.01)**
	colonization rate [%]	55.0 ± 0.11	44.8 ± 3.8	31.0 ± 3.5	35.2 ± 3.2	7.68 (0.20)	0.01 (0.48)	0.88 (0.35)

Discussion

The role of allelopathy in plant-plant interactions has been the subject of ongoing discussions, because it was called into question if the compounds responsible would be available in sufficient amounts to be ecologically effective under natural soil conditions in the face of sorption to soil surfaces and chemical and microbial degradation (Schmidt and Ley, 1999; Blum, 2004).

Results from our semi-natural experimental set-up are consistent with the hypothesis that juglone derived from either leaf litter or leachate was more effective with continuous network connectivity, as indicated by significantly reduced biomass of target plants (Fig. 2 & 3).

The demonstration of allelopathic effects has often been quite difficult but might be explained by the fact that in many experiments microbial and particularly mycorrhizal influences have been excluded a priori by using sterile soil in experimental set-ups (Inderjit, 2001, 2005; Kaur et al., 2009). Based on this, Barto et al. (2012) introduced the Network Enhanced Bioactive Zone model (NEBaZ) which emphasizes an important role of mycorrhizal hyphal networks in enhancing allelopathic effects. Evidence from

our semi-natural field experiments corroborates this model and emphasizes the importance of including mycorrhizal components in allelopathic studies.

The role of mycorrhizae – alternative explanations

Its potential transport functions aside, the presence or absence of mycorrhizae itself can also potentially affect plant growth. Mycorrhizal fungi in general play an important role in enhanced nutrient uptake, especially in facilitating plant available soil P uptake (Smith and Read, 1997) and in return carbon flows from plants to mycorrhizal fungi. The presence of a hyphal network connection might increase target plant growth by enhancing the possibility of nutrient foraging by AMF or by decreasing carbon costs to the host plant for maintenance of its AMF symbionts.

This might have played a role in experiment 1 because when the mycorrhizal connection was continuous, soil hyphal lengths were significantly higher ($p=0.01$) inside RECs translating also to higher plant biomasses (Fig. 2). However, considering the effects of connectivity to a CMN in combination with addition of leaf litter extract together (Fig. 2), we find significantly reduced plant growth in just those tomato plants receiving both treatments. In experiment 2 we also see that connectivity to a mycorrhizal network did result in increased hyphal length (Tab. 2) but did not significantly influence plant biomass (Fig. 3). This suggests that the potential beneficial role of mycorrhization did not play a role in the second experiment. Regarding here connectivity and litter addition together, we see, similar to experiment 1, an increase of hyphal length with highest values in RECs connected to a CMN and receiving leaf litter (Tab. 2) but we also find a significant decrease in total plant biomass.

These findings show that even if target plants benefitted through their network connection from a mycorrhiza-supported nutritional supply, the detrimental effect of juglone was predominant and ended up in significantly reduced target plant biomass (56 % in experiment 1 and 36 % in experiment 2).

Connection to a CMN could lead to reduced tomato seedling growth caused by increased carbon flow from the seedling to the fungal mycelium (Nakano-Hylander and Olsson, 2007). Considering carbon fluxes in experiment 1 and 2 we see that all bioassay plants were exposed to AM hyphae and had similar colonization rates (Tab. 1 & 2) and therefore likely similar potential carbon costs of supporting the fungal mycelium, even we did not measure this. Differences in plant biomass caused by carbon investment from seedlings into fungal mycelium hence likely played no role in both experiments.

The disruption of network connectivity might also lead to a reduced mycorhizal inocu-

lum potential, which could potentially be a factor of plant growth limitation in our experiments.

Our experimental units were filled with natural soil, most likely containing fungal propagules to ensure a mycorrhizal colonization of all tomato plants, independently of connection to an external mycorrhizal network. We found similar colonization rates throughout both experiments, suggesting that the inoculum potential remained stable and did not change among treatments.

The presence of fungal hyphae themselves can also indirectly influence the soil environment by changing soil structure (Rillig and Mummey, 2006) as well as by modifying hydraulic conductivity (Auge et al., 2001). Both of these indirect influences of hyphae would have happened in all RECs in a similar way, because all experimental units were equally colonized, pointing to the importance of a connection to a hyphal network and not merely the presence of hyphae.

All in all, while in a field study other explanatory factors can never be fully ruled out, available evidence suggests that we did mostly observe the effects of hyphal mediated transport of allelochemicals. This is particularly true considering that found similar effects when directly adding juglone as a purified substance, and when studying the effects of juglone accumulating in soil (Achatz, unpublished).

Hyphal mediated transport

We provided evidence that continuous mycorrhizal fungal connections could support transport of juglone, a moderately hydrophilic allelopathic compound, using two different modes of application to experimental units. We feel our study adds two important components to the discussion of the NEBaZ model.

First, the hyphal mediated transport took place under field conditions, by using a pre-existing, established mycorrhizal fungal network situation, presumably consisting of different mycorrhizal fungal species and the full complement of soil biota. This adds a strong element of ecological realism compared to many in vitro or greenhouse studies with selected players.

Secondly, the application of juglone was based on fresh walnut leaves, added in a fashion that would lead to “fast release” by using a fresh leaf litter leachate in experiment part 1, and slow release in experiment 2, as would be expected to occur in a natural setting. Under these conditions, both experiments resulted in reduced target plant growth in the presence of an intact hyphal connection.

Mycorrhizal enhanced transport processes are supposed to be faster than diffusion through the soil matrix, because the length of the flow path is shorter (Allen, 1996). Additionally, the movement through soil via hyphal networks may protect infochemi-

cals from decomposition, sorption, and complex formation by limiting their exposure to the soil environment (Barto et al., 2012). Several pathways for infochemical flow are possible, e.g. surficial, apoplastic or cytoplasmic paths as well as hyphal cord interior transport. For organic substances like juglone, even if it is moderately water soluble, water flow along hyphae is a plausible option for enhanced transport since water flow along hyphae has been shown (Querejeta et al., 2003).

Additional factors like modified hyphosphere conditions concerning soil aggregation, conductivity or changes in the microbial community could also influence movement of allelochemicals. Recently it was shown that transport via hyphae could also be beneficial to the hyphae themselves by potentially protecting them from fungivores (Duhamel et al., 2013).

Conclusions

Our field data suggest that connectivity to an established mycorrhizal fungal network may play a critical role in mediating movement and therefore enlarging the bioactive zone of allelochemicals in the soil, described as the Network Enhanced Bioactive Zone model (NEBaZ) (Barto et al., 2012). While we could not monitor movement of juglone itself, we observed significant target plant biomass reductions that were very likely attributable to juglone effects. Further research on allelopathy should thus explicitly consider the mycorrhizal component of soils.

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Supplementary material

HPLC analysis

To determine juglone content in leaf litter, we extracted 300 mg leaf litter (n=4) in 10 ml chloroform for 1 h in the dark (Girzu et al., 1998). After centrifugation (5 min, 1000 rpm) the chloroform solutions were vacuum filtered (nylon membrane filter, pore size 0.2 µm, A. Hartenstein) and evaporated overnight. The dried extracts were dissolved in 2 ml methanol and used for HPLC analysis. Samples were measured with an Agilent 1100 HPLC-System (Agilent 1100 HPLC, Agilent Technologies, Santa Clara, CA, USA) and an UV diode array detector scanning the spectra of wavelength from 220 to 360 nm. Separations were carried out using an Agilent Eclipse XDB-C18 column (5µm, 4.6 x 150 mm), with a flow rate of 1 ml min^{-1}, and an injection volume of 20 µl. Solvent A was H_2O (millipore) and solvent B acetonitrile, which followed a gradient of 90% A to 45% A (10 min), 45% A to 0% (5 min), 0% A (5 min) and from 0% to 90% A (1 min). The total run time was 26 min including 5 min of equilibration treatment (90% A). Identification and quantification of juglone was achieved by first comparing the UV spectra with those obtained from standards. The spectra of samples were recorded from 280-360 nm, the detection wavelength was 254 nm with a retention time of 11.5 min for juglone. Quantification was achieved according to the concentrations of a corresponding external standard (0.00037-0.06 mg/ml) (5-hydroxy-1,4-naphthochinon, Sigma Aldrich Germany, CAS No. 481-39-0).

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CHAPTER V

General Discussion

The results in this dissertation point to the direction that either the presence of mycorrhizal hyphae or the connection to an existing mycorrhizal network in soil enhance the transport of juglone and therefore enlarge the bioactive zone.
It was important to try to answer this question by performing multiple experimental approaches, to maximize the validity of our results. Like in many fields of plant ecological research, different experimental designs reaching from high experimental control to high ecological realism were essential to determine the importance of our findings.

I started with a realistic approach by using naturally released juglone soil concentrations (manuscript 1, experiment 1) , moving to more controlled conditions in two greenhouse studies, either using juglone leaf litter (manuscript 1, experiment 2) or the pure chemical compound (manuscript 1, experiment 3) in the presence or absence of a single mycorrhizal fungus. To support my findings in manuscript 1, I went back to more realistic conditions and additionally conducted a semi-natural field experiment (manuscript 2). I used a field plot with an existing fungal network, containing presumably different fungal species and added *Juglans*-leaf litter leachate (manuscript 2, experiment 1) or leaf litter (manuscript 2, experiment 2) to the root exclusion compartments.
Using combinations of experimental designs minimized the chance that results and their conclusions are based on artifacts caused by the experimental approach. That is why it is in general important for future allelopathic research to not only trust in one experimental approach, because nearly every experimental design has inevitable disadvantages.
The advantage of placing RECs in a naturally occurring hyphal network (manuscript 1 and 2), enables us to use all fungi generating a hyphal network and include the whole microbial community in soil, as well as constant moisture conditions; but the rotation of RECs might lead to air gaps or soil compression that could influence allelochemical flow. By using autoclaved soil, and inoculating the experimental units with a mycorrhizal fungus (manuscript 1, experiment 2 & 3) we increased the experimental control but results could be influenced by factors generated by the presence of mycorrhizal fungi, like plant growth effects caused by nutrient differences. To overcome this pattern, it is important to avoid nutrient limitations and additionally measure soil and target plant

nutrient levels. To monitor any nutrient influences, the use of additional treatments with activated carbon to remove allelochemicals could be helpful.

We could demonstrate both in field approaches and in greenhouse experiments the role of hyphal transport of juglone, but specific knowledge about the detailed physiological processes of allelochemical transport is still lacking. Different potential pathways of transport were proposed (Barto et al., 2012), but precise evidence if transport for example occurs inside (apoplastic or cytoplamatic) or along the outside of hyphae (surficial or hyphal cord flow), remains unclear and likely depends on the chemical structure (e.g. size, polarity) of the allelopathic compound.
In further research it would probably be possible to investigate detailed physiological processes by using sterile, mycorrhizal in vitro cultures (Rillig et al., 2010) and track the transport pathway of an allelochemical compound and even quantify the amount by using stable isotope labeling. Stable isotope labeling is currently used in quantification of metabolite concentrations and fluxes in root systems (Engelsberger et al., 2006) and could be used in a modified way in tracking mycorrhizal transport pathways of allelochemicals.
To generalize the role of mycorrhizal networks in soil in allelopathic transport processes, it is necessary to investigate more allellochemical compounds. The importance of mycorrhizal hyphae in transport processes of α-terthienyl, a Thiophene released by *Tagetes tenuifolia* was analysed in an experimental approach (Barto et al., 2011), but numerous other allelopathic plants, especially agriculturally important species, like rice, wheat or sunflower could serve as model plants. Especially sorghum (*Sorghum bicolor* L. Moench) with its allelopathic compound sorgoleone could be used as future allelopatic study plant, because its phytotoxic compound could easily be detected non destructively with modern analytical methods like polydimethylsiloxan (PDMS) tubings and even allelopathic dynamics in soil could be investigated (Weidenhamer, 2005; Weidenhamer et al., 2009). Additional modern analytical tools for detection and quantification of low infochemical concentrations, like the use of microdialysis techniques, should be improved (Sulyok et al., 2005), because PDMS tubings can only be used for non-polar allelochemicals.
Considering the role in allelochemical transport processes and the importance of mycorrhizal networks in natural soils, another important topic of future research should be the detection of the role of single mycorrhizal fungal species in comparison to fungal diverse communities. A higher fungal diversity could lead on the one hand to increased fungal biomass and hyphal length, which could enhance transport traits. On the other hand, a higher fungal diversity could lead to the formation of several independent hy-

phal networks and therefore lower network connectivity. To clarify this issue, one could conduct greenhouse and field experiments by using single fungal species and variable combinations of them to simulate different diversity levels. Knowledge about the importance of fungal diversity in soil on plant communities and its diversity and productivity (van der Heijden et al., 1998) leads to the idea that allelopathic processes could also be influenced by mycorrhizal fungal diversity.

In prospective research on allelopathic compounds and mycorrhizal networks and its general influence in natural surroundings, not only soil microbiota should be included, but also the role of macrobiota has to be examined. Soil organisms, which influence the disturbance of hyphal networks (Johnson et al., 2005) or plants, which are connected by hyphal networks, should be included in future studies. Both organisms feeding on hyphae like collembolans (Jonas et al., 2007) or mites (Hishi and Takeda, 2008) as well as animals, which influence soil structure (e.g. earthworms) or plants directly, should be included in future research, to obtain a broad and realistic view of the role of mycorrhizal hyphae in transport processes of allelopathic compound under natural conditions.

We have shown that hyphal networks can increase effectivity of allelochemicals in natural systems and hence play an essential role in chemical interaction processes which can impact the structure of plant communities. Belowground interactions in natural plant communities refer to intra- and interspecific plant interactions, and include both allelopathic mediated transfer processes via hyphae but also interactions concerning hormones and signal molecules passing the soil.

Mycorrhizal networks might as well extend the bioactive zones for those molecules and thus influence intra- and interplant communication processes in natural surroundings more than previously thought by acting beneficially or detrimentally. In recent studies common mycorrhizal networks acted as “defense networks” by being involved in disease resistant and pathogen protection processes in plants (Song et al., 2010) as well as plant-mediated protection of the mycorrhizal hyphal network (Duhamel, 2013) and played an important role in mediating multitrophic interactions in an agricultural ecosystem (Babikova et al., 2013). It is very likely that hyphal mediated transport processes of allelopathic compounds might contribute to the allelopathic influence on plant invasions (Inderjit et al., 2008) and in general play a role in impacting on natural selection, community integration, community coevolution by regulating the abundance and distribution of natural plant populations (Bais et al., 2004).

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CHAPTER VI

Summary

Allelopathy is in general a biological phenomenon by which plants release one or more naturally occurring chemical compounds into the environment and therefore influence neighboring plants negatively (Rice, 1974). Since the whole subject area of allelopathy is quite complex and multifactorial, the relevance of allelopathic processes in ecology, especially their role in plant-plant interactions, has been controversially discussed and frequently called into question (Macias et al., 2007; Callaway et al., 2008).
This complexity issues arise on the one hand from the fact that allelochemicals have to be present in the soil in adequate amounts to be effective (Choesin and Boerner, 1991). On the other hand, many other factors potentially influence the effectiveness of an allelopathic compound, like physical and chemical properties, as well as the influence of soil organisms (Inderjit, 2001, 2005).
One of the most important functional groups in soil are mycorrhizal fungi, which influence ecologically relevant processes in diverse ways (Allen, 1996) and actually seem to impact allochemical transport processes significantly with their hyphal network present in the soil. Mycorrhizal fungal hyphae play an important role in several other transport processes, like water (Egerton-Warburton et al., 2007) or nutrient transfer (He et al., 2003; Mikkelsen et al., 2008), as well as in underground signal induced plant communication (Song et al., 2010) and mycorrhizal based warning of herbivory (Babikova et al., 2013). Thus, the so-called NEBaZ model was developed, which suggests that bioactive zones of allelochemicals are extended by the presence of a mycorrhizal fungal network (Barto et al., 2011).

In this present dissertation the role of arbuscular mycorrhizal fungi in transport processes of the allelochemical juglone was investigated by using a variety of different experimental approaches.
Juglone has been known for several hundred years to have growth retarding effects on the surrounding vegetation; the chemical compound occurs in high amounts in the Black walnut (*Juglans nigra* L.) but also in other members of the Juglandaceae. Because of its toxicity, juglone was frequently the subject of allelopathic research (Crist and Sherf, 1973; Jose and Gillespie, 1998; Li et al., 2010).
To be able to arrive at a generally accepted and potential ecologically relevant statement concerning the role of mycorrhizal networks in transport processes using the

example of juglone, I tried to cover as broad spectrum of experimental approaches as possible. My studies reached from controlled greenhouse to field experiments lasting several weeks using variable experimental designs (RECs or mycorrhizal inoculation) with several ways of adding the allelochemical juglone (direct application of the pure chemical, leaf litter extract or leaf litter addition). Additionally, I conducted a field study for several months using the naturally occurring juglone concentration in soil for further analyses and a bioassay. The variation in the experiments should elucidate and support the general idea, that mycorrhizal networks play an important role in transport processes of juglone and emphasize the necessity to consider mycorrhizal fungal networks in further allelopathy research.

The most important findings were:

- The growth retarding effect of juglone on phytometer plant biomass (*Lycopersicon lycopersicum*) was shown in all experiments in presence of mycorrhizal hyphae (manuscript I and II).
- The amount of naturally released juglone by *Juglans regia* was significantly higher ($p=0.02$) in those RECs connected to the surrounding hyphal network. These higher juglone amounts negatively affected root biomass of plants in a subsequent bioassay experiment (experiment 1, manuscript I).
- In addition, the growth inhibiting effect of juglone was shown clearly in the presence of *Rhizophagus irregularis*, because both the addition of juglone by using leaf litter (experiment 2, manuscript I) and especially by adding the chemical compound juglone directly (experiment 3, manuscript II) resulted in significant growth retarding effects of root biomass ($p=0.01$, experiment 2, manuscript I) or shoot biomass ($p=0.01$, experiment 3, manuscript I), if mycorrhizal fungal hyphae were present.
- Additionally, two more field experiments (manuscript II) confirm the notion that mycorrhizal fungal hyphae relevantly enhance the transport of juglone in soil and therefore lead to reduced growth of sensitive phytometer plants. Both the addition of a *Juglans regia* leaf litter extract (experiment 1, manuscript II) and the application of leaf litter (experiment 2, manuscript II) resulted in significantly reduced total biomass ($p<0.001$, experiment 1, manuscript II; $p=0.03$, experiment 2, manuscript II) in the presence of an intact hyphal connection to the surrounding mycorrhizal network.

All results together show that the presence of mycorrhizal hyphae or the connection to an existing mycorrhizal network in soil influence the transport of juglone and therefore

enlarge its bioactive zone. Hyphal networks can increase effectivity of allelochemicals in natural systems and hence play an essential role in chemical interaction processes, which then again impact the structure of plant communities.

Zusammenfassung

Allelopathie wird im Allgemeinen als ein biologisches Phänomen gesehen, bei dem Pflanzen eine oder mehrere, in ihr natürlich vorkommende chemische Verbindungen in die Umwelt abgeben, und dabei andere in der Umgebung vorkommende Pflanzen negativ beeinflussen (Rice, 1974). Der ganze Themenbereich der Allelopathie ist hochkomplex und vielschichtig, weshalb die Bedeutung allelopathischer Prozesse in der Ökologie, insbesondere ihre Rolle in Pflanzeninteraktionen, immer wieder kontrovers diskutiert und in Frage gestellt wird (Macias et al., 2007; Callaway et al., 2008).
Die Komplexität kommt zum Einen dadurch zustande, dass allelopathische Verbindungen in ausreichenden Mengen im Boden vorhanden sein müssen, um wirksam zu sein (Choesin and Boerner, 1991), und zum Anderen, weil zahlreiche weitere Faktoren, unter Anderem physikalische und chemische Eigenschaften, sowie der Einfluss von Bodenorganismen, die Effektivität einer allelopathischen Substanz maßgeblich beeinflussen können (Inderjit, 2001, 2005).
Eine der wichtigsten funktionellen Organismengruppen im Boden, die arbuskulären Mykorrhizapilze beeinflussen auf vielerlei Weise ökologisch bedeutsame Prozesse (Allen, 1996) und scheinen mit ihrem Hyphennetzwerk im Boden auch eine wichtige Rolle in Transportprozessen allelopathischer Substanzen zu spielen. Dadurch, dass Mykorrhizanetzwerke bei vielen anderen Transportprozessen, wie zum Beispiel beim Wasser- (Egerton-Warburton et al., 2007) und Nährstofftransport (He et al., 2003; Mikkelsen et al., 2008), sowie bei der unterirdischen, signalinduzierten Pflanzenkommunikation (Song et al., 2010) oder mykorrhizabasierten Warnung vor Herbivorie (Babikova et al., 2013) eine maßgebliche Rolle spielen, entwickelte sich das NEBaZ-Modell, das besagt, dass die bioaktive Zone allelopathischer Stoffe in Anwesenheit von Mykorrhizanetzwerken ausgeweitet werden kann (Barto et al., 2011).

In der vorliegenden Doktorarbeit wurde die Rolle von arbuskulären Mykorrhizapilzen beim Transport der allelopathischen Substanz Juglon anhand unterschiedlicher Versuchsansätze untersucht. Juglon ist ein seit Jahrhunderten bekannter, allelopathisch hemmender Stoff, der in besonders großer Menge in der schwarzen Walnuss (*Juglans nigra*), aber auch anderen Vertretern der Familie der Juglandaceae vorkommt und aufgrund seiner lange bekannten Toxizität schon oft Gegenstand in der allelopathischen Forschung war (Crist and Sherf, 1973; Jose and Gillespie, 1998; Li et al., 2010).
Um eine möglichst allgemeingültige und potentiell ökologisch relevante Aussage über die Rolle von Mykorrhizanetzwerken bei Transportprozessen am Beispiel von Juglon

treffen zu können, habe ich im Rahmen meiner hier vorliegenden Arbeit versucht, ein möglichst breites Spektrum an Versuchsansätzen abzudecken. Meine Untersuchungen reichten von mehrwöchigen Gewächshaus- und Freilandexperimenten mit variierenden Designs (Verwendung von RECs oder Inokulation mit einer Pilzart) unter möglichst kontrollierten Bedingungen, bei denen die allelopathische Substanz Juglon auf verschiedene Art und Weise zugegeben wurde (direkte Zugabe der Chemikalie, Laubextrakt- oder Laubzugabe) bis zu einer mehrmonatigen Freilandstudie, bei der das Juglon schon auf natürliche Weise im Boden vorhanden war. Die Variation in den Experimenten sollte die allgemeine Idee, dass arbuskuläre Mykorrhizapilze eine wichtige, unterstützende Rolle beim Transport der Allelochemikalie Juglon spielen, stützen und die Notwendigkeit der Beachtung von Mykorrhizanetzwerken in der Allelopathieforschung bestätigen.

Die wichtigsten Ergebnisse der Arbeit sind:

- Die wachstumshemmende Wirkung von Juglon auf die Biomasse der Phytometerpflanzen (*Lycopersicon lycopersicum*) wurde in allen Versuchen (Manuskript I und II) in Anwesenheit von Mykorrhizapilzen deutlich.
- Das unter Walnussbäumen (*Juglans regia*) in realistischen Mengen, auf natürlichem Wege freigesetzte Juglon war in den Versuchseinheiten signifikant mehr vorhanden ($p=0.02$), wenn diese mit einem Hyphennetzwerk im Boden verbunden waren. Diese größere Menge Juglon wirkte in einem darauffolgenden Bioassayexperiment hemmend auf das Wurzelwachstum der Versuchspflanzen (Experiment 1, Manuskript I).
- Noch deutlicher wurden die wachstumshemmenden Effekte von Juglon in Anwesenheit von *Rhizophagus irregularis*. Sowohl bei der Zugabe von Juglon in Form von *Juglans regia*-Laub (Experiment 2, Manuskript I) als auch besonders bei der direkten Applikation der allelopathischen Substanz selbst (Experiment 3, Manuskript I) war in Anwesenheit eines pilzlichen Hyphengeflechts eine signifikante Wachstumsreduktion in der Wurzelbiomasse ($p=0.01$, Experiment 2, Manuskript I) oder der Sproßbiomasse ($p=0.01$, Experiment 3, Manuskript I) zu verzeichnen.
- Es konnte zusätzlich in zwei weiteren Experimenten (Manuskript II), diesmal unter Freilandbedingungen, bestätigt werden, dass Mykorrhizahyphen maßgeblich den Transport von Juglon im Boden beeinflussen und zu reduziertem Wachstum bei sensitiven Phytometerpflanzen führen. Sowohl die Zugabe eines Extraktes, der aus *Juglans regia*-Laub hergestellt wurde (Experiment 1, Manuskript II) also auch die Zugabe von Walnusslaub (Experiment II, Manuskript II) resultierte in signifikant

verminderter Biomasse ($p<0.001$, Experiment 1, Manuskript II, $p=0.03$, Experiment 2, Manuskript II), wenn eine Verbindung zum umliegenden Hyphennetzwerk im Boden gewährleistet war.

Alle Ergebnisse zusammengenommen zeigen, dass das Vorhandensein des Hyphennetzes eines Mykorrhizapilzes oder die Verbindung zu einem bereits existierenden Mykorrhizanetzwerkes im Boden Einfluss auf den Transport der allelopathischen Substanz Juglon nehmen und somit die bioaktive Zone allelopathischer Substanzen erweitern können. Hyphennetzwerke können daher die Effektivität von Allelochemikalien in natürlichen Systemen verstärken und spielen somit eine wichtige Rolle in chemischen Interaktionsprozessen, die sich wiederum auf die Struktur der Pflanzengesellschaft auswirken können.

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Contribution to the publications

I. **Michaela Achatz**, E. Kathryn Morris, Frank Müller, Monika Hilker, Matthias C. Rillig: Soil hypha mediated movement of allelochemicals: arbuscular mycorrhizae extend the bioactive zone of juglone (submitted to Functional Ecology)

Own contributions: I conceptualized the experimental designs (together with Kathryn Morris and Matthias Rillig), conducted the field and greenhouse experiments, performed laboratory work including HPLC analysis, soil parameter analysis, as well as plant and fungal measurements. I statistically analysed the data, carried out the graphical realization and interpretation of results and I wrote the manuscript.

II. **Michaela Achatz**, Matthias C. Rillig: Arbuscular mycorrhizal fungal hyphae enhance transport of the allelochemical juglone in natural soil (in preparation for submission)

Own contributions: I conceptualized the experimental designs, conducted the field experiments, performed laboratory work including HPLC analysis, analysis of soil parameters as well as plant and fungal measurements. I statistically analysed the data, carried out the graphical realization and interpretation of results and I wrote the manuscript.

Curriculum vitae

For reasons of data protection, the curriculum vitae is not included in the online version