Technische Universität München

Lehrstuhl für Ökophysiologie der Pflanzen

Ectomycorrhizae and drought: effects of carbon allocation and nutrient relations on functional traits

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. J. Kollmann
Prüfer der Dissertation:	1. Priv.-Doz. Dr. K. Pritsch
	2. Prof. Dr. J. Durner

Die Dissertation wurde am 05.02.2018 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München am 08.05.2018 angenommen.

Es gibt nichts praktischeres als eine gute Theorie.

Kurt Lewin (1890 - 1947)

Abstract

In the present work, a form of symbiosis between fungi and plant roots, the so-called ectomycorrhiza, is investigated. In this system, water and nutrients are transferred from the fungus partner to the plant roots and in return, carbon in the form of sugars is transferred from the plant root to the fungal mycelium. Thus, the absorption capacity of the root system is increased and the carbon supply of the heterotrophic fungi is provided. Ectomycorrhizas are mainly found in association with woody plants in temperate and boreal climate zones.

The forests of these zones are threatened by climate change, especially by severe summer drought. Ectomycorrhizae can mobilize nutrients in the soil and access pore water, which would not be possible for tree roots without ectomycorrhizae. They are therefore of great importance for the ability of trees to survive drought and recover afterwards. This dissertation examines how ectomycorrhizae react to drought. It is not only important to characterize processes during a dry period, but also to observe the recovery phase after a dry period, because recovery is the decisive factor in determining whether an ecosystem can return to its original state after a disturbance. In this dissertation three important questions are examined on different experimental scales on the basis of two functional properties of ectomycorrhizae (nutrient mobilisation and soil exploration) which are expected to improve the understanding of the effects of summer drought on the ectomycorrhizal fungal community of different tree species.

1st What is the influence of summer drought on the ectomycorrhiza community?

It was found that the allocation of carbon from recent photosynthesis in ectomycorrhizae was greatly reduced. However, a carbon limitation of ectomycorrhizae was unlikely. The species community of fungi changed towards drought tolerant species. The relative proportion of mycorrhizal fungi, which are able to transport water over distances of several decimetres, increased. The potential for nutrient mobilisation remained remarkably stable and even increased slightly, although this qualitative stability could not compensate for quantitative losses of vital ectomycorrhiza due to drought. Nevertheless, the observed adaptation could be decisive in determining whether or not a forest ecosystem succumbs to drought.

2nd What is the influence of the respecitve tree species *Fagus sylvatica* (European beech) and *Picea abies* (Norway spruce) and their mixture on the ectomycorrhizal fungal community under repeated, severe summer drought?

The mixture itself changed the localisation of fine root production, with the fine roots of Norway spruce growing upwards and the fine roots of European beech shifting downwards. This effect was enhanced by drought. The decline in diversity of the ectomycorrhizal fungal community due to repeated summer drought was less pronounced in the mixed stand than in the respective pure stands. This increased the potential for recovery after drought. With regard to soil exploration and nutrient mobilisation, there were no clear effects between the two tree species and the mixture.

3rd What is the influence of nutrient ratios on the recovery phase after the end of drought?

One week after rewatering, a strongly increased carbon allocation from recent photosynthates was observed. This effect was reduced more by nitrogen fertilisation and less by phosphorus fertilisation. A comparable assimilation rate between recovering and control plants suggested a sink control of this carbon flux. As already during drought, increased potential for nutrient mobilisation and a relatively high proportion of mycorrhizal fungi capable of transporting water over long distances were found during the recovery phase.

The results of this work contribute to the mechanistic understanding of drought stress regulation in ectomycorrhizal fungal communities. There has been a shift in the ectomycorrhizal fungal community towards a higher proportion of long-distance exploration types. At the functional level, qualitative stability could not compensate for quantitative losses. The sink control of the allocation of carbon from recent photosynthates seems to be a general principle on different experimental scales.

Zusammenfassung

Ektomykorrhiza ist eine Form der Symbiose zwischen Pilzen und Pflanzenwurzeln. Hierbei werden Wasser und Nährstoffe vom Pilzpartner an die Pflanzenwurzeln abgegeben, und im Gegenzug wird Kohlenstoff in der Form von Zuckern von der Pflanzenwurzel an das Pilzmyzel abgegeben. Somit wird die Absorptionsleistung des Wurzelsystems erhöht und die Kohlenstoffversorgung der heterotrophen Pilze gewährleistet. Ektomykorrhizen sind hauptsächlich an holzigen Gewächsen in temperatem und borealem Klima zu finden.

Die Wälder dieser Zonen sind vom Klimawandel, besonders von starker Sommertrockenheit bedroht. Ektomykorrhizen können im Boden Nährstoffe mobilisieren und auf Porenwasser zugreifen, was für Baumwurzeln ohne Ektomykorrhizen nicht möglich wäre. Sie sind deshalb von großer Bedeutung für die Fähigkeit der Bäume, Trockenheit zu überleben und sich danach wieder zu erholen. Bei Untersuchungen wie Ektomykorrhizen auf Trockenheit reagieren ist es nicht nur wichtig Prozesse während einer Trockenperiode zu charakterisieren, sondern auch die Erholungsphase nach einer Trockenperiode zu beobachten. Denn die Erholung gibt letztendlich den Ausschlag, ob ein Ökosystem nach einer Störung in seinen ursprünglichen Zustand zurückkehren kann. In der vorliegenden Dissertation werden auf verschiedenen experimentellen Skalen anhand zweier funktioneller Eigenschaften von Ektomykorrhizen (Nährstoffmobilisation und Bodenexploration) drei wichtige Fragen betrachtet, welche das Verständnis der Effekte von Sommertrockenheit auf die Ektomykorrhiza-Gemeinschaft verschiedener Baumarten verbessern sollen.

1. Welchen Einfluss hat Sommertrockenheit auf die Ektomykorrhiza-Gemeinschaft?

Es zeigte sich, dass die Allokation von Kohlenstoff aus der aktuellen Photosynthese in Ektomykorrhizen stark verringert war. Eine Kohlenstofflimitierung der Ektomykorrhizen war jedoch unwahrscheinlich. Die Artgemeinschaft der Pilze veränderte sich in Richtung trockenheitstoleranter Arten. Dabei nahm der relative Anteil von Mykorrhizapilzen zu, die in der Lage sind, Wasser über Distanzen von mehreren Dezimetern zu transportieren. Das Potential zur Nährstoffmobilisation blieb bemerkenswert stabil und nahm sogar leicht zu, wobei diese qualitative Stabilität den quantitativen Verlust vitaler Ektomykorrhizen durch Trockenheit nicht kompensieren konnte. Trotzdem könnte die beobachtete Anpassung entscheidend dafür sein, ob ein Waldökosystem der Trockenheit erliegt oder nicht.

2. Welchen Einfluss haben die Baumarten *Fagus sylvatica* (Rotbuche) und *Picea abies* (Gemeine Fichte) und deren Mischung auf die Ektomykorrhiza-Gemeinschaft unter wiederholter, starker Sommertrockenheit?

Die Mischung an sich veränderte die Lokalisation der Feinwurzelproduktion, wobei die Feinwurzeln der Fichte sich nach oben und die der Buche sich nach unten verlagerten. Dieser Effekt wurde durch Trockenheit noch verstärkt. Der durch wiederholte Sommertrockenheit bedingte Rückgang der Diversität der Ektomykorrhiza-Gemeinschaft war im Mischbestand weniger ausgeprägt, als in den jeweiligen Reinbeständen. Dadurch wurde das Potential zur Erholung nach einer Trockenperiode erhöht. Im Gegensatz dazu zeigten sich hinsichtlich Bodenexploration und Nährstoffmobilisation zwischen den beiden Baumarten und der Mischung keine eindeutigen Effekte.

3. Welchen Einfluss haben Nährstoffverhältnisse auf die Erholungsphase nach Beendigung der Trockenheit?

Eine Woche nach Wiederbewässerung zeigte sich eine stark erhöhte Kohlenstoffallokation aus aktuellen Photosyntheseprodukten zu den überlebenden Ektomykorrhizen. Dieser Effekt wurde durch Stickstoffdüngung mehr und durch Phosphordüngung weniger stark reduziert. Eine vergleichbare Assimilationsleistung zwischen sich erholenden und Kontrollpflanzen legte eine Senkensteuerung dieses Kohlenstoffflusses nahe. Wie schon während der Trockenheit, zeigte sich auch während der Erholungsphase ein erhöhtes Potential zur Nährstoffmobilisierung und ein relativ hoher Anteil an Mykorrhizapilzen, die zu Langstreckentransport von Wasser fähig sind.

Die Ergebnisse dieser Arbeit leisten einen Beitrag zum mechanistischen Verständnis der Trockenstressregulation in Ektomykorrhiza-Gemeinschaften. Es fand eine Verschiebung der Ektomykorrhiza-Artgemeinschaft hin zu einem höheren Anteil an Langstrecken Explorationstypen statt. Auf funktioneller Ebene konnte die qualitative Stabilität nicht die quantitativen Verluste ausgleichen. Die Senkensteuerung der Allokation von Kohlenstoff aus aktuellen Photosyntheseprodukten scheint ein generelles Prinzip auf verschiedenen experimentellen Skalen zu sein.

List of original articles

Articles are listed chronologically. Please note the change of the surname due to marriage.

I Hagedorn F, Joseph J, Peter M, Luster J, Pritsch K, Geppert UT, Kerner R, Molinier V, Egli S, Schaub M, Liu J-F, Li M, Sever K, Weiler M, Siegwolf RTW, Gessler A & Arend M (2016): Recovery of trees from drought depends on belowground sink control. Nature plants, 2, 16111. doi:10.1038/nplants.2016.111.

II Goisser M, Geppert UT, Rötzer T, Paya A, Huber A, Kerner R, Bauerle T, Pretzsch H, Pritsch K, Häberle K-H, Matyssek R & Grams TEE (2016): Does belowground interaction with *Fagus sylvatica* increase drought susceptibility of photosynthesis and stem growth in *Picea abies*? Forest Ecology and Management, 375, 268-278. doi: 10.1016/j.foreco.2016.05.032.

III Nickel UT, Winkler JB, Mühlhans S, Buegger F, Munch JC & Pritsch K (2017): Nitrogen fertilisation reduces sink strength of poplar ectomycorrhizae during recovery after drought more than phosphorus fertilisation. Plant and Soil, 419(1), 405–422. doi:10.1007/s11104-017-3354-2.

IV Nickel UT*, Weikl F*, Kerner R, Schäfer C, Kallenbach C, Munch JC & Pritsch K (2017): Quantitative losses vs. qualitative stability of ectomycorrhizal community responses to 3 years of experimental summer drought in a beech-spruce forest. Global Change Biology. doi: 10.1111/gcb.13957

* These authors contributed equally to the work.

Author's contribution

I Uwe Nickel wrote those parts of introduction, material and methods, results and discussion that deal with ectomycorrhizae. He planned the experiments together with the co-authors and performed sampling and morphotyping of ectomycorrhizal roots. He carried out the lab work and statistical analyses and interpreted the results together with the co-authors.

II Uwe Nickel wrote those parts of introduction, material and methods, results and discussion that deal with ectomycorrhizae. He planned the experiments together with the co-authors and performed sampling and morphotyping of ectomycorrhizal roots. He carried out the lab work and statistical analyses and interpreted the results together with the co-authors.

III Uwe Nickel wrote the paper. He developed the idea and performed the experimental work and statistical analyses. He planned the experiment together with the co-authors and interpreted the results together with the co-authors.

IV Uwe Nickel wrote the paper draft. He and Fabian Weikl share first authorship. Uwe Nickel performed sampling and morphotyping in 2013, and 2014 together with René Kerner. Uwe Nickel performed sampling and sample processing in 2015 and 2016, and processed data from all these campaigns except for HTS-sequencing. Fabian Weikl performed high-throughput sequencing and data analysis thereof. Uwe Nickel was responsible for statistical analyses for which Prof. Matthies gave support. Uwe Nickel wrote the morphotyping part of the paper and assisted in writing the high-throughput part together with Fabian Weikl, as well as the enzyme activity part with Karin Pritsch. Uwe Nickel interpreted the results together with the co-authors.

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Abbreviations and acronyms

C	carbon
cm	centimetres
EA	exoenzyme activity
ECM	ectomycorrhizal
ITS	internal transcribed spacer
Ma	million years
mm	millimetres
N	nitrogen
P	phosphorus
SOM	soil organic matter

1 Introduction

Models of future climate scenarios predict warmer and drier summers and more variability in precipitation for the northern hemisphere (Meehl & Tebaldi 2004; Field 2012). These scenarios result in short, repeated, very dry periods. Thus, research on the effects of drought is an important issue. Trees and forests are ecologically and economically important by providing multiple ecosystem services, but are endangered by this climate change-type drought (Dale *et al.* 2001; Carnicer *et al.* 2011; Young *et al.* 2017). For example, they are important carbon sinks (Goodale *et al.* 2002; Pan *et al.* 2011), diverse ecosystems, sources for timber and drinking water (Núñez *et al.* 2006) and they serve recreational purposes (Weyland & Laterra 2014). Most temperate and boreal forest tree species are associated with ectomycorrhizal (ECM) fungi (Courty *et al.* 2010a). DNA sequences of more than 100 ECM fungal species can be found in a single forest stand (Jumpponen *et al.* 2010; Hui *et al.* 2011). ECM fungi ensheath fine roots of trees, forming ectomycorrhizae which are most efficient organs for water and nutrient uptake by soil exploration via emanating hyphae with ECM fungal species-specific properties. For example, they mobilise nitrogen (N) and phosphorus (P) by mineral weathering (Hoffland *et al.* 2004; van Schöll *et al.* 2008) and enzymatic processes (Pritsch & Garbaye 2011). Roots alone mostly are not able to perform these functions with the same efficiency. Therefore, ectomycorrhizae are integral parts of trees and forest ecosystems, adding numerous functions to root tips of individual trees. However, they are sensitive to changes in soil water content as experienced by drought (Swaty *et al.* 2004; Richard *et al.* 2011) and may thus play a decisive role whether trees succumb to climate change or not. Some ectomycorrhizae are also sensitive to anthropogenic soil nutrient additions such as atmospheric N-deposition or mineral fertilisation in short rotation forestry, reducing mycorrhization rate and ECM fungal diversity (Baum & Makeschin 2000; Lucas & Casper 2008). The available data strongly suggest that ectomycorrhizae play an important role in how forests survive climate change. Therefore, the present thesis investigates the influence of drought on functional traits of ectomycorrhizae and as a side aspect, how they are influenced by nutrient availability.

1.1 Role of mycorrhizae in ecosystems

Mycorrhizae are symbiotic associations between roots of a vascular plant and a fungus (Smith & Read 2007). The term derives from Greek μυκός *mykós*, "fungus", and ῥίζα *rhiza*, "root". So, they are literally fungus-roots and in fact, these are the organs by which plants acquire water and nutrients, forming the interface between plants and soil (Smith & Read 2007).

To develop exchange processes, both partners should have an excess of a good to trade it in for a limiting resource. The first land plants had a surplus of C, but not yet a functional root system to acquire nutrients which are less available in oxidative soils than in aquatic surroundings (Gryndler 1992). Fungi, on the other hand developed mechanisms to obtain these nutrients, as pointed out above. However, they depend on an external C source as they are not autotroph.

During evolution of mycorrhizal associations, several types of mycorrhizae have established. In recent ecosystems, the most important ones are arbuscular mycorrhizae, ectomycorrhizae, ericoid mycorrhizae and orchid mycorrhizae. About 80% of all land plants all over the globe are associated with arbuscular mycorrhizal fungi (Trappe 1987) belonging to the Glomeromycota (Schüβler *et al.* 2001), whereas ectomycorrhizae are dominant in temperate forest ecosystems (Tedersoo *et al.* 2014) occurring mainly on woody species in some families of gymnosperms, dicotyledons and one monocotyledon genus (Brundrett 2002) and are characterised by an enormous fungal diversity of > 6000 species. Orchid and ericoid mycorrhizae only occur in Orchidaceae and Ericaceae, respectively (Smith & Read 2007).

Mycorrhizae benefit plants in numerous ways. They increase plant nutrient supply by extending the accessible soil volume and by mobilising nutrients that would not be available to plants without mycorrhizae (Schweiger *et al.* 1995; Kahiluoto & Vestberg 1998). Further, they provide non-nutritional benefits to the extent that they can protect roots from parasitic fungi and nematodes (Little & Maun 1996; Cordier *et al.* 1998; Morin *et al.* 1999), induce changes in plant water relations, phytohormone levels, photosynthesis, etc. (Brundrett 1991). This can result in higher yields and/or reproductive success of mycorrhizal plants (Stanley *et al.* 1993). Additionally, C transfer between plants via networks of mycorrhizal mycelia has been observed (Simard *et al.* 1997) which may facilitate establishments of seedlings or growth of understorey vegetation shaded by dominant plants (Högberg *et al.* 1999) and thus stabilising

and diversifying ecosystems. At ecosystem level, mycorrhizae play an important role in nutrient cycling (Read & Perez-Moreno 2003), they are food sources for soil faunal organisms (Setälä 1995), and influence soil microbial populations in the mycorrhizosphere (Pion *et al.* 2013). Moreover, mycorrhizal soil hyphae are considered to contribute to soil structure (Griffiths *et al.* 1994; Rillig 2004) and play a major role in below-ground C sequestration (Clemmensen *et al.* 2013).

The present work focuses on ectomycorrhizal associations which are dominant on the tree species studied here. Phylogenetic research shows that ECM associations have developed at least eight times independently in different lineages of the Basidio- and Ascomycota (Hibbett & Matheny 2009; Tedersoo *et al.* 2012; Burke *et al.* 2014). Fossil records of ectomycorrhizae have been found from the Middle Eocene (ca. 50 Ma ago) (LePage *et al.* 1997), but molecular clock analyses indicate that the origin of ectomycorrhizae may be about 105 Ma ago (Hibbett & Matheny 2009). Literally, ectomycorrhiza means "external fungus-root", reflecting their morphology: Hyphae of ECM fungi form a hyphal mantle around young primary fine roots of their host plants, constituting a functional extension of the root system into the soil thus being influenced by the two "environments" plant and soil.

Trees have a wide variety of practical and commercial uses. Two common use practices are commercial timberland and agroforestry. In commercial timberland, traditionally monocultures were planted. However, it has been shown that mixed species stands often show over-yielding compared to monospecific stands (Morin et al. 2011; Pretzsch et al. 2013). Regarding climate change related drought periods in summer, Norway spruce (*Picea abies* (L.) Karst.) is considered to be challenged in monoculture (Neuner et al. 2015), because of its shallow root system and its low drought tolerance (Boden et al. 2014). Also, European beech (*Fagus sylvatica* L.) is a widely planted tree species threatened by climate change (Gessler *et al.* 2007). Both species form monospecific and mixed forest stands, with Norway spruce mostly exhibiting increased overall productivity when growing in mixture (Pretzsch et al. 2014). These positive mixed stand effects have been attributed to improved soil properties and increased overall biodiversity by European beech (Ammer et al. 2008). In addition, below-ground resource partitioning is likely to contribute to the positive effects of mixture as European beech shifts its fine roots from upper to lower soil depths when growing alongside Norway spruce because of competition (Bolte & Villanueva 2006; Goisser et al. 2016). Under severe summer drought conditions, Norway spruce can adapt by decreasing its fine-root

growth (Puhe 2003) while maintaining its standing fine-root biomass (Nikolova et al. 2010), whereas European beech exhibited slightly increased fine-root growth during the severe summer drought of 2003 (Nikolova et al. 2010). Thus, water limitation evokes different below-ground responses in these species (Schume et al. 2004), with Norway spruce decreasing water consumption and growth in the early stages of drought (Dobson et al. 1990; Maier-Maercker 1998) and European beech continuing to grow (Burkhardt & Pariyar 2016). The distinct physiological responses of these tree species to drought suggest that their ectomycorrhizae will be exposed to different conditions under the same drought scenario. In the present work this aspect is investigated in article IV.

Agroforestry is a well-established agricultural system in the tropics and subtropics. In temperate regions, it was a common practice until the industrial revolution, when timber production became the main way in which humans utilised forests (Nair 1993). However, in the second half of the 20th century, farmers again became aware of the benefits of mixing crops and trees, such as reducing the losses of soil, water and nutrients (Nair 1993). In addition to the agricultural benefits of agroforestry systems, they are important for sequestering C (Montagnini & Nair 2004). C sequestration is not only due to the production of above-ground biomass–wood in this case–but also due to the storage of assimilated C below-ground in roots, microorganisms of the rhizosphere including mycorrhizal fungi and finally SOM (Rillig *et al.* 2001; Treseder *et al.* 2007; Nehls *et al.* 2010). In Central European agroforestry systems, poplars are among the most commonly planted trees (Reisner *et al.* 2007). They can form both ECM and arbuscular mycorrhizal associations. For the nutrition of forest trees, the EAs of their ectomycorrhizae play a major role (see chapter 1.3.2). Trees in agroforestry systems, in contrast, receive additional nutrients through fertilisation of the adjacent field crops, which may either favour ECM fungi that are adapted to nutrient uptake from inorganic rather than organic sources, or alter nutrient acquisition strategies of ectomycorrhizae. In the present work this aspect is studied in article III.

1.2 The ectomycorrhizal fungal environment

1.2.1 The plant – trading carbon and nutrients

Associations of plant fine root tips with ECM fungi are generally considered to be mutualistic. Indeed, these associations evolved because both partners could increase their

fitness by provision of goods required by the respective other partner in nutrient poor conditions where nutrients are distributed patchily and often difficult to access because of surrounding organic material (Read & Perez-Moreno 2003; Lambers *et al.* 2009). In fact, however, it is a mutualism–parasitism continuum depending on the environmental factors such as the availability of nutrients or light that influence net costs on either side of the association (Johnson *et al.* 1997).

The plant-fungus interface of ectomycorrhizae is called Hartig net, consisting of hyphae that penetrate the apoplast of the root epidermis in angiosperms (Peterson *et al.* 2004) and additionally the root cortex in gymnosperms (Kottke & Oberwinkler 1986). In contrast to arbuscular mycorrhizal hyphae, ECM hyphae normally do not penetrate cells of their host plants. The exchange of substances between plant and fungus takes place through this interface.

C in the form of sucrose from the plant is exchanged for water and nutrients from the fungus. Therefore, sucrose and invertase are released into the apoplast by the plant, where invertase cleaves sucrose into fructose and glucose (both hexoses) (Nehls *et al.* 2001). Hexose transporters are located in both, the plant and the fungal cell walls. They carry fructose and glucose into fungal cells and also back into plant cells, which constitutes a control mechanism for C drain on the plant side by competition for hexoses together with the expression of invertase by the plant (Nehls *et al.* 2001). Thus, the plant controls C fluxes to the fungus. For arbuscular mycorrhizal fungi it has been shown that reciprocal rewards stabilise cooperation in symbiosis (Kiers *et al.* 2011) and that fungal nutrient allocation in common mycorrhizal networks is regulated by the carbon source strength of individual host plants (Fellbaum *et al.* 2014) leading to the concept of microbial market theory (Werner *et al.* 2014). In microbial market theory, cooperative behaviours of microbes with hosts and other microbes are described in economic terms which may be useful to generate predictions about microbial interactions (Werner *et al.* 2014). The first indications of a plant control of carbon drain derive from experiments with mineral fertiliser. After N fertilisation reduced extramatrical mycelium, less fruiting bodies and lower colonisation of roots tips was observed (Wallenda & Kottke 1998; Nilsson & Wallander 2003). Thus, N fertilisation seems to reduce plant dependency on ECM fungi which in turn may lead to reduced C allocation towards ectomycorrhizae. As pointed out above, mechanisms by the plant to prevent C drain include the control of sucrose and invertase export into the common apoplast and thereby sucrose

hydrolysis and competition for hexoses present in the Hartig net region of the apoplast. As exudation of sugars and other uncharged molecules occurs passively along concentration gradients, C efflux may be difficult to control for the plant (Jones & Darrah 1996). Acid invertase activity in *Betula* and *Populus* is increased upon ectomycorrhiza formation (Wright *et al.* 2000; Nehls 2008), indicating a candidate step for controlling C support to ECM fungi. However, in Norway spruce no such increase in invertase activity was detected in ECM plants (Schäffer *et al.* 1995). Hexose transporters are upregulated in poplar roots upon formation of ectomycorrhizae (Grunze *et al.* 2004; Nehls *et al.* 2007) indicating strong competition with fungal hexose transporters. However, post-translational regulation of poplar hexose transporters such as phosphorylation has been proposed (Grunze *et al.* 2004) which would allow fine tuning of the activation status of selected transporters in specific root cells. Indeed, it has been shown that transgenic poplar plants that overexpress an additional hexose importer gene engage less ECM symbioses which are eventually terminated abnormally (Nehls unpublished). In the present work this aspect is studied in articles I and III which determine ECM sink strength during drought (I) and in the phase of recovery (I & III) using $^{13}CO_2$ as tracer for assimilates in the plant. In article III, different N:P stoichiometries in the substrate were applied additionally.

The C necessary for exchange is produced by photosynthesis. Experiments showed that up to 30% of the net photosynthesis products (Söderström & Read 1987; Farrar & Jones 2000), or 47–59% of the total below-ground carbon allocation (McDowell *et al.* 2001) are needed for ECM symbiosis. For comparison, regular root exudation only constitutes about 3–5% of net photosynthesis (Pinton *et al.* 2001). To produce these high amounts of C, mycorrhizal plants can increase their photosynthetic capacity (Lamhamedi *et al.* 1994; Loewe *et al.* 2000). Once hexoses are taken up by Hartig net hyphae, they are used for metabolism such as ATP generation and amino acid synthesis, and for the formation of fungal carbohydrate storage compounds. Both of these mechanisms generate high C sink strength of the ECM fungus in symbiosis. The most important C storage compounds in ECM fungi are trehalose, sugar alcohols like mannitol, arabitol and erythritol and the long chain carbohydrate glycogen (Nehls 2008). Trehalose and sugar alcohols probably are used for long distance C transport to support the extramatrical mycelium, whereas glycogen serves as long term storage and, in Hartig net hyphae, temporal formation of glycogen granules may act as flux control mechanism (Nehls 2008). Experiments investigating ECM fungal sugar metabolism showed

high variability in the preference of metabolites and transporters (see Nehls 2008 for a review) even within Basidio- and Ascomycota, supporting the multiple and independent evolution of ECM symbiosis from different phylogenetic groups.

In exchange for C the plant receives water and nutrients from the fungus, especially N and P. The uptake of N by most ECM fungi occurs in the form of ammonium (NH_4^+) which is preferred, or in the form of nitrate (NO_3^-) which first has to be reduced to ammonium to be synthesised to glutamine together with glutamate (Javelle *et al.* 2003). Transport to the plant occurs either via glutamine or glutamate, depending on the available transporter proteins in the cell membranes of either partner of the symbiosis (Smith & Read 2007). The uptake of P occurs in the form of inorganic phosphate ions which are transformed to polyphosphate inside the fungal cells for transport through hyphae via a motile tubular vacuole system (Ashford & Allaway 2002). However, P in the form of polyphosphate is inaccessible for plants. So, it has to be transformed back into single phosphate ions to be taken up by the plant. This constitutes a control mechanism on the fungal side. For example, Perez-Moreno & Read (2000) showed in an experiment with *Betula pendula* and *Paxillus involutus* that only 34% of the P extracted from substrate material was transferred from the fungus to the plant.

Often underestimated is the importance of ectomycorrhizae in plant water relations. A prerequisite for functioning water transport is a soil-plant-air continuum. If more water is transpired than can be taken up by roots, the risk of cavitation in the xylem arises. As water in plants mainly moves through hydrostatic forces produced by transpirational water loss, xylem embolism eventually leads to xylem failure by loss of conductivity and drying of affected parts of the plant (Arango-Velez *et al.* 2011; Barigah *et al.* 2013). Therefore, transpiration has to be regulated by the plant via opening or closing of the stomata. However, closing stomata to reduce transpiration implies that CO_2 uptake for C fixation is also reduced. The outcome of this compromise is called water use efficiency, expressed as mmol CO_2 assimilated per mol H_2O transpired. If assimilation rate decreases due to stomatal closure, less recent photosynthates are available to support ECM symbiosis (Hagedorn *et al.* 2016). In this case, ectomycorrhizae have to be supported by mobilisation of C reserves like starch or by higher activity of C releasing enzymes of ECM fungi (chapter 1.3.2). If this is not possible, symbiosis may be impaired.

It has been shown that certain extramatrical mycelial structures (chapter 1.3.1) are able to transport water (Duddridge *et al.* 1980) and that the amount of water transported in this

way can be sufficient to make the difference between survival and death of tree seedlings (Boyd *et al.* 1986). However, the mycorrhizal pathway is not the major way to take up water, but may be particularly beneficial under drought stress conditions, similar to the mycorrhizal benefits concerning plant nutrition, by providing the minimal requirements for plant survival during drought (Read & Boyd 1986). One of the mechanisms involved may be enhanced aquaporin expression in ECM compared to nonmycorrhizal plants which is suggested to be particularly important during drought stress (Marjanović *et al.* 2005a, b). Aquaporins are membrane intrinsic proteins forming water channels which can open and close to regulate water flow through the membrane. Posttranslational regulation mechanisms are mainly phosphorylation and glycosylation. A well-known indirect effect on plant water relations is the enhanced nutrient status of the plant which can increase water use efficiency (Gessler *et al.* 2016).

1.2.2 The soil – nutrients and moisture

Soil is a complex and extremely diverse ecosystem in which biotic and abiotic factors interact with each other. Its properties vary with bedrock type, age, vegetation and climate which in turn influence biotic processes. Soils store water and nutrients to various extents depending on their history of origin (Weil *et al.* 2016). Although weathering of parent material includes physical and chemical processes, plants play a major role in soil formation (Taylor *et al.* 2009). They are sources of organic matter and provide C to soil microorganisms by organic matter and rhizodeposition (release of C compounds from vital plant roots into the surrounding soil) and have high impact on the weathering of minerals (Taylor *et al.* 2009). Microorganisms play a key role in the interactions of plants and soil by mediating fluxes between different inorganic and organic nutrient (particularly N and P) pools (Turner *et al.* 2013; Vincent *et al.* 2013). Especially in forest ecosystems, ectomycorrhizae are significant contributors to mineral weathering (Hoffland *et al.* 2004; van Schöll *et al.* 2008; Taylor *et al.* 2009). For example, carbonate rock weathers faster under ECM trees than under arbuscular mycorrhizal trees (Thorley *et al.* 2015). During soil aging, different proportions of the main nutrients N and P are available. In very young soils lots of mineral P is present in the form of apatite, whereas N contents are very low due to low biological activity. P contents decline consistently during soil development, whereas N contents peak in middle-aged soils (microbial N_2 fixation and atmospheric N deposition). Very old and highly weathered soils

(several millions of years old) contain only little amounts of P and N due to leaching and erosion (Lambers *et al.* 2008; Turner *et al.* 2013).

Any kind of mycorrhiza is particularly useful in soils where nutrients are largely immobile (i.e. low in soil solution) and not directly accessible for plant roots, but still available in minerals or organic macromolecules. Arbuscular mycorrhizal plant nutrition strategies (uptake of soluble inorganic N and P forms and few soluble organic N forms from soil solution) are partly replaced by ECM strategies in middle aged soils where N and P amounts are intermediate and largely bound in organic and/or mineral forms. Such soils are found in temperate and especially boreal regions of the northern hemisphere due to soil rejuvenation by glaciers of the last ice ages (132,000 to 12,000 years old). ECM fungi are able to access N and P bound in organic molecules. For example, in the Alaskan tundra 61–86% of N in plants is derived from ECM symbioses (Hobbie & Hobbie 2006) and ECM hyphae have been shown to preferentially colonise apatite, a mineral rich in P (Wallander & Thelin 2008; Smits *et al.* 2012).

Besides the effects that ectomycorrhizae have on soil formation, soil properties like nutrient content and soil moisture feed back on ECM fungal community structure. The impacts of these effects largely depend on the ECM fungal species composition. For example, the occurrence of *Cenococcum geophilum* and *Rhizopogon vinicolor* is positively correlated with available P content, whereas occurrence of other species is more strongly influenced by soil depth and thereby organic matter content (Twieg *et al.* 2009) or by inorganic N content (Lucas & Casper 2008).

A low soil water content also influences ECM fungal community composition towards species that are more drought tolerant (Shi *et al.* 2002; Swaty *et al.* 2004; Cavender-Bares *et al.* 2009; Richard *et al.* 2011; Nickel *et al.* 2017a). Drought tolerance differs among ECM fungal species (Lehto & Zwiazek 2011) and probably also intraspecifically (Lamhamedi *et al.* 1992). For example, *C. geophilum* is known to tolerate low water potentials (Kerner *et al.* 2012), whereas the thin cell walls of *Lactarius* spp. are prone to loose integrity under dry conditions (di Pietro *et al.* 2007). Not only ECM fungal community diversity is altered and reduced by drought, but also absolute abundance of ectomycorrhizae as more and more fine roots die due to water shortage (Nickel *et al.* 2017a, b). However, certain ectomycorrhizae are able to transport water over distances of several centimetres via their extramatrical mycelium (Duddridge *et al.* 1980; Cairney 1992), the organisation of which is described in detail in chapter 1.3.1.

Water movement through soil normally occurs through spaces between soil particles. When the topsoil dries between rain events the narrow spaces between these particles create a capillary force that can lift water from deeper soil layers to the topsoil. Besides, a local soil water deficit can be attenuated by hydraulic lift (Caldwell *et al.* 1998): Water moves passively through roots of deep rooting trees such as European beech from deep soil layers (higher water potential) to shallow soil layers (lower water potential), or laterally along the water potential gradient. This way of water transport is faster than water movement through soil and in coarse soils it is virtually the only way for water transport. Hydraulically lifted water can directly enhance survival and activity of ECM roots in dry soils (Querejeta *et al.* 2003). As ion movement and absorption processes in the root are strongly reduced by drought (Russell 1973; Dunham & Nye 1976), leakage of hydraulically lifted water and resorption thereof by fungal hyphae (Sun *et al.* 1999) is important for continued nutrient uptake by ectomycorrhizae.

There are numerous studies investigating the effects of drought on ecosystems (Breda *et al.* 2006; Penuelas *et al.* 2007; Sowerby *et al.* 2010; Jactel *et al.* 2012). However, drought recovery determines how fast and if at all ecosystems return to their pre-drought functional state. Still, the mechanisms controlling ecosystem resilience are uncertain (Reichstein *et al.* 2013), but the time between drought events may become shorter than the time needed for recovery (Schwalm *et al.* 2017). Most probably, ectomycorrhizae play a crucial role in tree recovery from drought. Hagedorn *et al.* (2016, article I) showed in a $^{13}CO_2$ tracer experiment that recent assimilates are preferentially allocated to vital ectomycorrhizae during recovery after drought. Further, N addition seems to reduce this effect (Nickel *et al.* 2017, article III).

1.3 Functional traits of ectomycorrhizae

Functional traits are considered to play a major role in community ecology because they are more informative than determining only species richness and abundance (McGill *et al.* 2006). Literature distinguishes between response and effect traits. Response traits influence the response of an organism to its environment (e.g. the ability to survive dry periods), whereas effect traits influence ecosystem function (e.g. the rate at which ECM fungi decompose litter) (Lavorel & Garnier 2002). Environmental change can have direct or indirect effects on ecosystem functioning. If we consider drying as environmental change and nutrient cycling as ecosystem function, the direct effect would be a slowdown of nutrient cycling due to soil water deficit (Moore 1986). However, drought would change ECM fungal community

composition (Swaty *et al.* 2004) towards species that are drought tolerant, which in turn influences decomposition rates. If those ECM fungal species that will persist in this scenario produce litter that is more recalcitrant, this would further slow down nutrient cycling. If drought would establish fast with no time for community adaptation, direct effects would predominate. If drought and change in community composition occur simultaneously, it would be hard to disentangle direct and indirect effects of environmental change on ecosystem functioning. However, there are certain traits serving as effect and response traits at the same time. Such traits have big effects on ecosystem functioning, because they are at the same time filtered by the environment and in turn influence their environment. They further appear in high frequencies due to ecological filtering. In the present work two types of traits that are considered dual response–effect traits according to Koide *et al.* (2014) are analysed: soil exploration and exoenzymatic activities (EAs).

1.3.1 Soil exploration

ECM fungi form extensions of plant root systems, being mediators between plant and soil. Due to the low diameter of hyphae (< 10 μm) (Agerer 1997), fungi are able to explore even small soil pores that contain soil solution but are not accessible for roots or even root hairs. The totality of hyphae that emanate from the hyphal mantle around the fine root tip is called extramatrical mycelium. The extent to which ECM fungi can explore the volume around the root tip they are associated with is an ecologically relevant feature and was categorised by Agerer (2001).

In the present work, four of these five exploration strategies (Agerer 2001) play a role. First, there is the contact exploration type, possessing only few extramatrical hyphae that reach less than 0.5 mm into the surrounding soil. Second, the short-distance exploration type with abundant emanating hyphae reaching up to 10 mm into the soil matrix. Then there exists the medium distance type with three subtypes, each exploring the soil up to several centimetres from the root tip with so called rhizomorphs. Rhizomorphs are bundles of hyphae with different levels of differentiation (Agerer 2006), able to transport water with similar velocity (27 cm h^{-1}) as measured for transport in xylem vessels (Duddridge *et al.* 1980; Brownlee *et al.* 1983). Emanating hyphae of the medium-distance fringe subtype form fans and highly branched rhizomorphs that show frequent anastomosis. Medium-distance mat subtype ectomycorrhizae form mats of undifferentiated rhizomorphs, as the name suggests.

Rhizomorphs of the medium-distance smooth subtype appear rather smooth with only few emanating hyphae. Finally, the long-distance exploration type ectomycorrhizae form few but highly differentiated rhizomorphs that reach up to several decimetres into the soil. Thus, they are able to bridge areas in soil devoid of nutrients and/or water. Rhizomorphs can contain large tube-like hyphae with diameters up to 20 μm often lacking cytoplasmic contents and cross walls, which are surrounded by thinner, densely cytoplasmic hyphae with thick cell walls (Duddridge *et al.* 1980; Brownlee *et al.* 1983). However, it is not entirely clear if these tube-like hyphae are important for water movement, because there are only few hints for a mechanism for solute accumulation which is necessary to create a hydrostatic pressure that is high enough for considerable translocation (Cairney 2005).

Hydrophilic and hydrophobic cell walls of ECM fungi probably make a difference for water transport in extramatrical mycelium (Unestam 1991; Unestam & Sun 1995). Hydrophilic species like many *Laccaria*, *Lactarius*, *Russula* and *Hebeloma* species are thought to transport water in the apoplast, whereas hydrophobic fungi such as *Paxillus involutus* and *Suillus* species form rhizomorphs where water may be transported in the symplast (Unestam & Sun 1995). However, hyphal tips of hydrophobic species also show hydrophilic properties to be able for water uptake (Unestam & Sun 1995). Generally, most contact type fungi are hydrophilic whilst most long-distance type fungi have hydrophobic hyphae (Agerer 2001).

It is not surprising that biomass and therefore maintenance costs vary highly between the different exploration types. Long-distance types have been found to have up to ten times higher biomass than short- and medium-distance types, respectively (Weigt *et al.* 2012). Thus, the balance of carbon cost and benefit provided by ectomycorrhizae may influence ECM fungal community composition and functions.

1.3.2 Exoenzymatic activities

Nutrient availability is mainly determined by soil chemical properties. One exception is N, which can be fixed biologically by prokaryotes. There are several mechanisms for ECM fungi to mineralise these nutrients. Nutrient mobilisation from minerals can be achieved by organic acids, as well as siderophores (Hoffland *et al.* 2004). However, the present thesis focuses on nutrient mobilisation from organic substances via excreted enzymes being important catalysers in forest nutrient cycling (articles III & IV).

Due to their origin within saprotrophic groups of white and brown rot fungi (Hibbett & Matheny 2009; Tedersoo *et al.* 2012; Burke *et al.* 2014), many ECM fungi possess the ability to secrete exoenzymes enabling them to mobilise nutrients. For example, experimentally reduced photosynthesis via defoliation revealed an increase in activity of enzymes typical for wood degrading fungi in ECM tips of *Suillus granulatus* (Cullings *et al.* 2008). Among those enzymes are cellulases, phosphatases, chitinases, proteases and laccases (Pritsch *et al.* 2004, 2011; Pritsch & Garbaye 2011). As pointed out above, patchily distributed nutrients are often protected by surrounding organic material which can be broken down by these enzymes.

The litter layer in forests mainly consists of dead plant material, but also of remnants of prokaryotic, fungal and animal origin. Although some ECM fungi are apparently able to mobilise C from SOM, they mainly receive C from photosynthesis of their host plant (chapter 1.2.1) (Treseder *et al.* 2006) or, to a lesser extent, of another plant in the common mycorrhizal hyphal network (Simard *et al.* 1997). While dwelling side by side, ECM fungi preferentially take up N from soil, whereas saprotrophic fungi also use soil C (Hobbie & Horton 2007). However, stable isotope measurements (^{13}C) of ECM fungal fruit bodies yielded values intermediate between what would be expected from pure saprotrophs (SOM as only C source) or photosynthesis as single C source (Hobbie *et al.* 2001; Taylor *et al.* 2003). Thus, there are apparently differences in the saprotrophic potential of different ECM fungi, as also evidenced by comparing the endowment with CAZymes (carbohydrate-active enzymes) of different ECM fungi (Martin *et al.* 2016). Although the saprotrophic capacity of ECM fungi has been questioned due to clear differences in enzymatic potentials of ECM and saprotrophic fungi (Baldrian 2009), it has been assumed that ectomycorrhizae form a saprotrophy-biotrophy continuum (Koide *et al.* 2008; Cullings & Courty 2009). However, recent studies suggest the term "saprotrophs" to be avoided in favour of "decomposers" because ECM fungi can degrade organic materials but without using the C contained therein (Lindahl & Tunlid 2015).

In natural forest soils, the main nutrient elements N and P are sequestered in organic macromolecules which have to be broken down in order to mineralise N and P and therefore make these nutrients accessible for plants and fungi. Sources of organic P accounting for 30–65% of total P in soils (Vincent *et al.* 2013) are for example nucleic acids, phospholipids and phytate. Phosphomonoesterases that are common among ECM fungi (Courty *et al.* 2006; Hrynkiewicz *et al.* 2009; Burke *et al.* 2014) are able to cleave phosphate groups from organic phosphates. Phytate is a rather recalcitrant source of P (Lim *et al.* 2007), but there are also ECM

fungi with phytase activity (Antibus *et al.* 1992). Sources of organic N in soils are proteins and especially in forest soils chitin. In soils, proteins often form protein-phenol complexes being poorly degradable. Phenoloxidases are able to cleave protein-phenol complexes and have been demonstrated in several ECM fungi (Wu *et al.* 2003; Joanisse *et al.* 2009). Fungal cell walls and exoskeletons of arthropods account for most of the chitin in forest soils. Chitinase activity is widespread in ECM fungi (Leake & Read 1990; Lindahl & Taylor 2004), but also in plants where they serve among others defence against fungal pathogens and engagement of symbiotic interactions with N-fixing bacteria or mycorrhizal fungi (Kasprzewska 2003). Additionally, cellulases, hemicellulases, pectinases and possibly laccases can be used to degrade cell walls of plants. This enables to access nutrients that are trapped inside dead tissues or bound to or embedded in cell walls (Perez-Moreno & Read 2000; Leake *et al.* 2002).

Proteins forming enzymes are synthesised on ribosomes into the rough ER where they are glycosylated. Then they are modified if necessary during transport in Golgi vesicles and finally transported to the plasma membrane (Peberdy 1994). To be effective outside the cell, exoenzymes have to pass the cell wall after synthesis. As the molecular threshold of fungal cell walls is lower than molecular weights of many secreted enzymes, it is suggested that secretion of most enzymes takes place at hyphal tips where also the cell wall is formed (Peberdy 1994). Enzymes brought by vesicles are placed on the outer surface of the cell membrane and become included in the cell wall polymers that are increasingly crosslinked as hyphal growth proceeds. If they are released into the surrounding, they can break down their substrates. However, this is only observed in nutrient-rich environments, but this strategy would be a waste of resources when other soil microorganisms compete for the same nutrient sources. Further, freely released enzymes are prone to be degraded by other enzymes meaning costly resources are lost, or their products are taken up by other organisms. Therefore, several mechanisms evolved to keep excreted enzymes close to the producing cell: they can be attached to the cell wall with a glycosylphosphatidyl inositol (also called GPI) anchor, or by transglycosylation (Latgé 2007). Other mechanisms include weak ionic forces, disulphide bridges and the embedment in extracellular polysaccharide matrices (Pitarch *et al.* 2002; Rast *et al.* 2003; Pritsch & Garbaye 2011). In intact mycorrhizae, predominantly tight bonds of enzymes were found as revealed by repeated rinsing with buffer (Pritsch *et al.* 2004) or by using sequential extraction of extracellular bound fungal enzymes (Pérez-de-Mora *et al.* 2013).

However, not only ECM fungi may be the source of excreted enzymes in ectomycorrhizae. Prokaryotes are omnipresent in soils and also occur on the surface of hyphae (Mogge *et al.* 2000). They are also able to secrete a wide spectrum of enzymes such as cellobiohydrolase, glucosidase, xylosidase, glucuronidase N-acetylglucosaminidase, leucine aminopeptidase, phosphatase and even laccase (Uroz *et al.* 2013). Thus, measuring the EAs of excised ECM root tips means measuring the functional unit ectomycorrhiza consisting of plant root, fungal hyphae and associated microorganisms. Ectomycorrhizae of *Quercus petraea* with *Xerocomus pruinatus* and with *Scleroderma citrinum* share similar bacterial communities (Uroz *et al.* 2012). For the ECM fungus *Morchella crassipes* it has been shown that the soil bacterium *Pseudomonas putida* is dispersed, fed with fungal exudates and finally bacterial C is harvested (Pion *et al.* 2013). Further, numerous so-called mycorrhiza helper bacteria have been described that assist mycorrhiza formation and interact positively with ectomycorrhizae (Frey-Klett *et al.* 2007).

Even though certain ectomycorrhizae share similar prokaryotic communities (Nguyen & Bruns 2015) which excrete enzymes on their own, this does not mean that these ectomycorrhizae also have similar EA profiles. Most EAs differ with ECM fungal species and are further influenced by environmental factors (Pritsch & Garbaye 2011). However, essential functions like phosphatase activity appear to be redundant while specific abilities like phenol oxidase activity seem to be restricted to few species (Buée *et al.* 2007; Rineau & Garbaye 2009). As ECM morphology seems to be mostly phylogenetically conserved (Agerer 2006), exploration types were recently considered a useful proxy to predict EAs (Tedersoo *et al.* 2012; Hupperts *et al.* 2017). But in the end, genetic composition originates from the respective phylogenetic background (Tedersoo *et al.* 2012; Tedersoo & Smith 2013). Thus, correlation of EAs with exploration types may be an artefact and should be considered with care.

At single species level the EA profile can be altered by environmental factors (Courty *et al.* 2005; Nickel *et al.* 2017b). However, taking into account the vast number of ECM fungal species in a temperate forest (Jumpponen *et al.* 2010; Hui *et al.* 2011) it is important to look at the EA profile of the whole community of ectomycorrhizae (Courty *et al.* 2005; Buée *et al.* 2007). In the present work, this aspect is studied in article IV.

Eventually, the ecologically relevant processes are the interplay of ECM fungal community composition, host tree performance and the environment. For example there are seasonal changes in ECM fungal community composition as well as in the EA profile due to

changes in soil moisture, nutrient availability und C supply by the host tree (Courty *et al.* 2008, 2010b; Kaiser *et al.* 2010). However, functional complementarity within ECM fungal communities has been shown such as seasonally complementary patterns of potential metabolic activity in five types of ectomycorrhizae during the course of a year (Buée *et al.* 2005), and seasonally complementary activities of laccase and phosphatase between three types of ectomycorrhizae (Courty *et al.* 2006). Even a single ECM type can balance its EA according to its abundance. For example, a negative correlation between leucine aminopeptidase activity and the abundance of *C. geophilum* ectomycorrhizae has been found (Herzog *et al.* 2013). There is also evidence that EAs are stimulated by the increasing amount of nutrients contained in recent plant litter in autumn (Mosca *et al.* 2007). This suggests EAs to be meaningful parameters when studying ECM community functions in ecosystems.

1.4 Aims of the thesis

The main research questions of this thesis are:

1. How do ECM fungal communities react under drought (articles I & IV)?
2. How does tree species mixture influence ECM fungal communities (article II) under drought (article IV)?
3. How does recovery after drought change sink strength and the capacity to mobilise nutrients of ectomycorrhizae (article I) and how is this influenced by different N:P stoichiometries in the substrate (article III)?

The present thesis aims to link the environmental factors drought and nutrient availability together with the plant-fungus interplay to two functional traits of ectomycorrhizae that are considered dual effect–response traits (Koide *et al.* 2014): exoenzymatic activities and soil exploration. This approach shall help to better deduce effects of environmental change (drought) on ecosystem functions (nutrient cycling in soils and water relations of host plants).

There are several studies about plants and ectomycorrhizae facing drought (Shi *et al.* 2002; di Pietro *et al.* 2007; Kilpeläinen *et al.* 2017). However, recovery from drought is decisive for ecosystem functioning. In articles I and III the phase of recovery after drought is investigated. Article I focuses on the source–sink C allocation during restoration of photosynthesis after drought. Article III adds the aspects of differential N:P stoichiometries in the substrate and ECM enzymatic potential to C allocation during recovery after drought in a

controlled greenhouse experiment. Thus, these two articles aim to contribute to the understanding of the plant fungus interplay during recovery after drought and how this affects soil nutrient cycling. Articles II and IV transfer the drought topic to a near natural ecosystem: the experimental site Kranzberger Forst where European beech and Norway spruce grow in mixed stands. As European beech and Norway spruce show different modes of rooting, article II investigates the effect of below-ground interaction of European beech and Norway spruce on drought susceptibility of Norway spruce. Article IV reports results from a three-year rain exclusion experiment simulating repeated severe summer droughts with focus on ECM fungal community composition and ECM potential EAs that influence nutrient cycling in the system studied.

Taken together, the present thesis presents studies of trees under drought conducted at different levels of experimental control. It reports how plants and their ECM fungal symbionts react to the environmental change of water shortage and how these reactions feed back on the environment, i.e. how they influence the ecosystem function of nutrient cycling.

2 Material and methods

Throughout the present work, values are given as mean ± 1 standard error (SE) if not indicated differently.

2.1 Overview of the methods and analyses in the articles

Detailed descriptions of experimental procedures and statistical analyses are given in the respective articles (I, II, III, IV). Here, a short summary of the work done by the author is given.

The experiments described in this thesis used different approaches regarding soil or substrate for ECM plants. The Kranzberg site (articles II & IV) is a mixed forest growing on a luvisol developed from loess over Tertiary sediments (eutric cambisols, FAO classification). This site is considered to have nearly natural soil conditions, although experimental plots were trenched and roofs were installed over half of the plots. A more controlled experiment was performed in the model ecosystems at WSL in Birmensdorf, Switzerland (article I). They consisted of respectively two lysimeters (3 m² plantable area each) filled with forest soil in open top chambers (3.5 m high) containing European beech trees that were up to 2.5 m tall. The most controlled experiment was performed in the greenhouse (article III). Here, poplar cuttings were planted into pots containing a substrate mixed from farmland soil adjacent to agroforestry plots and vermiculite. Thus, different levels of control and complexity of conditions can be compared.

The great advantage of controlled laboratory or greenhouse experiments is that most variables can be controlled. However, their major drawback is that they are artificial systems. Thus, they are very useful for detailed questions or extension of existing work, such as introducing a further variable. But it is hard to transfer findings of those experiments on ecological interrelationships to field conditions. This is why knowledge gained in the laboratory should be confirmed in the field. In the present work, the finding of preferential carbon allocation to ectomycorrhizae during recovery after drought from a medium controlled experiment (article I, open top chambers without illumination, heating and air humidity control) were amended by nutrient addition in a highly controlled pot experiment conducted in a greenhouse where environmental conditions were more controlled (article III, illumination, heating and air humidity control). The ultimate goal of the KROOF experiment

is to transfer findings of controlled experiments on drought recovery to ecosystem scale. The experiment was established in in 2011 with plot trenching; the drought treatment started in 2014 with throughfall exclusion and is intended to last for several years with the aim to push adult trees to their hydrostatical limits without killing them. Thus, the results presented here describe the status quo of the ECM fungal community before onset of drought (article II with focus on European beech-Norway spruce interaction, article IV with focus on soil exploration and EAs of ectomycorrhizae) and during three successive years of drought (article IV).

Morphotyping describes the visual (dissecting microscope) classification of ectomycorrhizae and was used in all articles. It is a method for preliminary morphological classification, as most ECM fungal species cannot be assigned an unequivocal species by looking at them, even with a dissecting microscope. Thus, species identification via Sanger sequencing of the ribosomal internal transcribed spacer (ITS) DNA region is necessary. In contrast to next generation sequencing (NGS) techniques that overestimate species richness by including the DNA of non-vital ECM fungi, single hyphae and resting stages (Medinger *et al.* 2010), morphotyping is prone to underestimate species richness even when including ITS information as it cannot distinguish between visually similar ectomycorrhizae (Erland *et al.* 1999). However, it is the best method to distinguish between dead and vital ectomycorrhizae and to select vital and intact ECM root tips for EA assays and stable isotope analysis.

Measuring EAs takes place *ex situ* in laboratory conditions and thus reveals rather potential activities than actual conversion rates of substrates *in situ*. However, it is a good measure of ECM C, N and P demands and it shows well functional adaptations of ectomycorrhizae in ecosystems. The method provides insights in the influence of disturbances on spatial and temporal dynamics of EAs involved in nutrient cycling (Buée *et al.* 2007; Mosca *et al.* 2007; Courty *et al.* 2011).

Stable isotopes can be used either as tracers to follow translocation or transformation of substances of interest, or natural abundances of stable isotopes are measured to infer metabolic or transport processes via knowledge about discrimination against certain isotopes in processes of interest. A great advantage of stable isotopes is that there is no radioactive decay which makes them secure and detectable for ever.

3 Results

3.1 Overview of the results presented in the articles

3.1.1 Carbon allocation in European beech during recovery from drought (article I)

In the open-top chamber experiment with ca. 2.0–2.5 m tall European beech trees, net photosynthesis and soil respiration decreased during drought by 44% and 28%, respectively. The uptake of $^{13}CO_2$ decreased by 81% and assimilate translocation to ectomycorrhizae was strongly reduced. Soil efflux of $^{13}CO_2$ showed a stronger reduction (83%) than soil respiration (≈50%), indicating that other carbon sources, either related to heterotrophic soil respiration or tree internal carbon storages contributed to soil respiration but were less sensitive to drought. Under drought, the ^{13}C peak from the first pulse labelling in soil CO_2 was delayed by one day and mean residence times of assimilated ^{13}C in the plant–soil system increased (drought 76 h, control 30 h). The recovery of plant and ecosystem carbon fluxes after prolonged drought was examined by rewatering the plants. Soil respiration responded rapidly, reaching control values within the first 3 days and exceeding thereafter values in controls until the end of the growing season. This stimulation of soil respiration nearly compensated for the previous drought reduction, with the flux integrated over the entire growing season amounting to 98% of that in controls. Net photosynthesis responded similarly but the recovery was delayed by approximately one week because of metabolic limitation, as shown by impaired photosystem II chemistry. Further, the stimulation of net photosynthesis occurred later and compensated for only 82% of the previous drought reduction. A second $^{13}CO_2$ pulse label was applied to the tree canopies when soil respiration exceeded the values in controls but net photosynthesis was still slightly below that of controls. The previous drought exposure increased the translocation of recent assimilates to below-ground sinks compared to controls, as shown by higher ^{13}C signals in mycorrhizal roots and soil microbial biomass and by an enhanced $^{13}CO_2$ soil efflux. The latter signal was enhanced and represents exclusively autotrophic respiration, showing that the plant-driven carbon flux was primarily responsible for the observed stimulation of soil respiration. The greater allocation and use of recent assimilates in below-ground sinks after rewatering shows the high priority trees give to investing into their roots for recovery from drought.

3.1.2 Below-ground interaction of European beech with Norway spruce (article II)

When grown intraspecifically at the forest stand Kranzberger Forst under ambient conditions, European beech produced a greater proportion of roots within deeper soils (54% were deeper than 30 cm) compared to intraspecific Norway spruce root production, which concentrated its roots closer to the soil surface (34% ≤ 6–10 cm). Growing root tips of both species in intraspecific zones were found deeper than at the respective interspecific zones. Thus, both species reacted similarly to growing in mixture by decreasing their average rooting depth. Irrespective of depth, fine root production of Norway spruce was strongly reduced in mixture, whereas European beech produced equal amounts of fine roots in mixture and when neighbouring other European beech trees. When analysed in 10 cm depth increments, significant inter- vs. intraspecific differences in fine root production were observed within 0–10 and 11–20 cm depth increments only (Tukey's HSD test, $\alpha = 0.05$). In both European beech and Norway spruce, a higher abundance of intraspecifically growing root tips were found within 0–10 cm depths when compared to interspecifically growing root tips. This trend was also observed within 11–20 cm depths in Norway spruce, but the opposite was found in European beech pointing to interspecific complementarity in the vertical distribution of fine roots of both tree species.

Irrespective of tree species and mixture situation, fine root surface area ($m^2 \times m^{-3}$ soil) was higher in the upper soil (> 8.6 cm) than in the lower soil (8.6–25 cm). In the upper soil, fine root surface area was higher in European beech alone than in Norway spruce alone and intermediate in mixture zones. In the lower soil in mixture zones, fine root surface of European beech was higher than of Norway spruce, which was not the case in the upper soil, also indicating vertical stratification between European beech and Norway spruce in response to below-ground interaction.

Regarding ectomycorrhizae, ca. five times more ECM root tips were found without rhizomorphs compared to ECM root tips with rhizomorphs. This ratio was not significantly influenced by tree species and soil depth. For both tree species, relative abundances of ECM groups (with or without rhizomorphs) were not significantly different between upper and lower soil. Ectomycorrhizae without rhizomorphs were relatively more abundant in intraspecific Norway spruce in the upper soil compared to the respective interspecific situation, while ECM root tips with distinct rhizomorphs were relatively less abundant. There

were no significant differences among ECM groups of European beech in the upper, and in European beech and Norway spruce in the lower soil.

3.1.3 Fertilisation reduces C allocation to ectomycorrhizae after drought (article III)

Fertilisation with N and P enhanced plant growth in irrigated treatments of the greenhouse experiment with poplar under drought and different fertilisation regimes. Provision of N in drought-treated plants was significantly higher ($p < 0.01$) until the end of the experiment while irrigated plants had taken up the fertilised N in the first three quarters of the experiment. Soil moisture, stomatal conductance and water use efficiency were not significantly altered by fertilisation regime, indicating that bigger plants did not experience differential drought stress.

During severe drought with 25% irrigation compared to the control, net assimilation and hence source activity was significantly reduced by the irrigation regime ($p < 0.05$), while water use efficiency was significantly increased ($p < 0.001$). Drought reduced the relative number of fine-roots as below-ground sinks. At harvest, double the amount of non-vital ECM tips were found in the drought treatments compared to the irrigated treatments.

During recovery (100% irrigation of the control), water use efficiency of recovering plants was still significantly higher than that of control plants ($p < 0.05$), but there was no significant difference in net assimilation anymore ($p = 0.350$). After one week of recovery, below-ground sinks (ECM tips) of plants recovering from drought showed higher ^{13}C abundances (C allocation) than ECM tips from irrigated treatments ($p < 0.005$). This effect was highest in treatments without N fertilisation ($p < 0.005$). ECM tips of recovering plants that were fertilised with nitrogen did not show significant differences in ^{13}C abundances compared to ECM tips of irrigated plants. The non-fertilised treatment showed the greatest difference in ^{13}C abundance between ECM tips of irrigated and recovering plants.

In ECM tips of plants recovering from drought, activities of xylosidase, glucuronidase and cellobiohydrolase did not show significant differences among treatments. Laccase activity was not detectable. Yet, activities of chitinase, β-1,4-glucosidase (both degrading cell walls) and phosphatase (P mobilising) were significantly higher in ECM tips of plants recovering from drought compared with continuously irrigated treatments. The effect of drought on the activity of the N mobilising enzyme leucine aminopeptidase was significantly different depending on nitrogen fertilisation. In N-fertilised treatments, ECM tips of recovering plants

showed significantly higher activity than tips from irrigated treatments, while in treatments without N addition, the activity of recovering ECM tips was decreased compared with that in continuously irrigated treatments. Thus, ectomycorrhizae of recovering plants had a higher potential to release nutrients from soil organic matter, thereby influencing nutrient cycling.

3.1.4 Ectomycorrhizal responses to extended drought (article IV)

Based on morphotypes, drought was a strong predictor for the abundance of the contact, short- and medium-distance exploration type groups in the throughfall exclusion experiment at Kranzberger Forst. Repeated summer droughts led to a progressive decline in contact types relative to control ($p < 0.05$) and a strong decline relative to control in short-distance and medium-distance types ($p < 0.05$) causing a strong increase in relative abundance of long-distance types relative to the other types. By contrast, long-distance types were not affected in the first 2 years of drought, significantly decreasing in abundance relative to control only after 3 years ($p < 0.01$). Soil depth was also a major predictor for the abundance of all three exploration type groups, with ca. 90% vital tips occurring in the topsoil. By contrast, based on phylotype data, drought was a weak predictor for the abundance of exploration type groups, with only the abundance of short-distance and medium-distance types being decreased significantly in the final year ($p < 0.01$).

The effect of throughfall exclusion on ECM fungal diversity indices differed between European beech and Norway spruce, irrespective of the competitive situation. In the throughfall exclusion plots, the ECM fungal diversity indices remained unchanged in the first 2 years of drought for European beech but declined from the second year of drought onwards for Norway spruce. After three years of throughfall exclusion, there was a significant difference in the morphotype diversity indices between European beech and Norway spruce with a much stronger decline being observed in Norway spruce. Phylotype diversity indices responded less to drought, but also exhibited a pronounced decline in samples from the Norway spruce zone after the third drought period. ECM fungal diversity declined less upon drought when European beech and Norway spruce grew in mixture.

Overall, the most pronounced effect of throughfall exclusion on EAs and differences between the qualitative measure $EA_{per\ tip}$ and the quantitative measure $EA_{per\ vol}$ was observed in the topsoil. $EA_{per\ tip}$ was remarkably stable in Norway spruce and European beech

ectomycorrhizae while $EA_{per\ vol}$ decreased over repeated drought years, which was mainly caused by a decline of vital ectomycorrhizae on throughfall exclusion plots.

The EAs of some ECM fungi became dominant under throughfall exclusion, mainly because other morphotypes disappeared. This led to an increase of the cellulolytic potential of the ECM fungal community in throughfall exclusion plots. Laccase activity disappeared in Norway spruce ectomycorrhizae from the topsoil of the Norway spruce zones after three years of drought along with laccase-positive morphotypes.

3.2 Additional results not presented in the articles

3.2.1 ^{13}C signature and abundance of ectomycorrhizae during drought (data not shown in article I & IV)

Labelling with $^{13}CO_2$ in the open top chamber experiment reported in article I showed that during drought net assimilation and hence C allocation to ECM root tips was strongly reduced compared to the continuously irrigated control treatment (Fig. 1). Exploration types were differentially enriched with ^{13}C, but only two days after labelling and before rewatering. After these two days and after rewatering the measuring error enormously increased. In ectomycorrhizae of continuously irrigated trees, the amount of ^{13}C was positively correlated with the amount of extramatrical mycelium and hence with mycelial biomass (contact: 26.84 ± 14.92‰; short-distance: 77.64 ± 24.46‰; long-distance: 273.62 ± 85.02‰ V-PDB; Fig. 1). Among ectomycorrhizae of drought treated trees short distance types were most enriched (-13.76 ± 4.40‰ vs. contact: -28.45 ± 0.51‰ and long-distance: -23.64 ± 2.92‰ V-PDB; Fig. 1). There were no significant differences before rewatering, 6 days after labelling and at any time point after rewatering. The European beech trees in this experiment originated from sites with different natural precipitation regimes and were considered as different ecotypes. However, based on ^{13}C label no differences between ecotypes were detected.

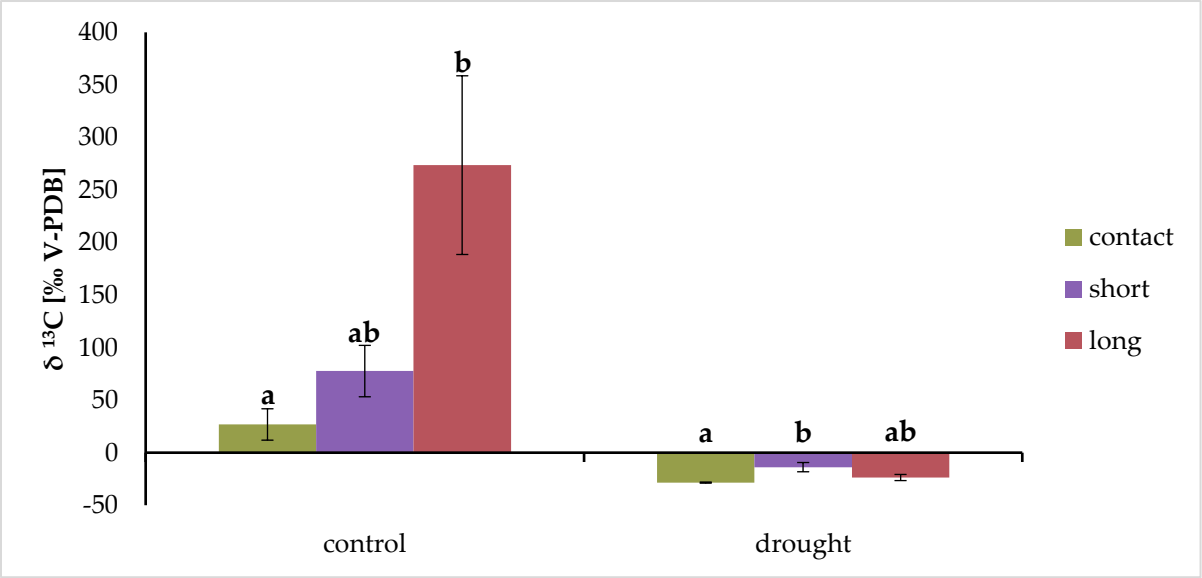


Figure 1: δ ^{13}C values of ectomycorrhizae of European beech trees from the experiment reported in article I. The values reported here show the situation two days after labelling in the drought phase of the experiment before rewatering. Different letters indicate significant differences between contact, short- and long-distance exploration types (α = 0.05; Tukey-HSD test following one-way ANOVA). Error bars indicate ± 1 SE.

Results from natural ^{13}C signatures confirm the reduced flux of recent assimilates. In drought treatments of the open top chamber experiment communicated in article I, ectomycorrhizal δ ^{13}C values are higher (-27.85 ± 0.28‰ V-PDB) than in control treatments (-29.52 ± 0.23‰V-PDB; $p < 0.001$). Stable isotope measurements from the throughfall exclusion experiment Kranzberger Forst communicated in article IV showed similar results. After the third period of throughfall exclusion, ectomycorrhizae of drought treated European beech trees had a higher abundance of ^{13}C (-26.98 ± 0.21‰ V-PDB) than ectomycorrhizae of European beech trees that did not experience drought (-28.26 ± 0.19‰ V-PDB). In contrast, throughfall exclusion did not significantly alter ^{13}C abundance of Norway spruce ectomycorrhizae (control: -26.92 ± 0.21, throughfall exclusion: -26.84 ± 0.50‰ V-PDB; treatment × beech vs. spruce interaction: $p < 0.01$).

Regarding exploration type abundances in the open top chamber experiment reported in article I, drought increased relative abundance of long-distance types significantly (24 ± 4 vs. 48 ± 9%; $p > 0.05$). Relative abundance of contact types was also increased in drought treatments, yet not significantly (24 ± 4 vs. 40 ± 11%; $p = 0.173$), whereas relative abundance of short distance types was not markedly changed by drought (control: 30 ± 2%; drought: 29 ± 2%).

3.2.2 Effects of tree species mixture on ectomycorrhizae of European beech and Norway spruce under drought (data not shown in article IV)

In the first year of the throughfall exclusion experiment Kranzberger Forst before onset of drought, the abundance of ectomycorrhizae of all exploration types was lower in mixture compared to pure European beech and Norway spruce zones, respectively ($p < 0.05$). After one year of drought this was only true for short-distance types ($p < 0.01$), however, without an effect of throughfall exclusion. The other exploration types did not show significant differences between mixture and pure European beech and Norway spruce zones, respectively. After two years of drought, again ectomycorrhizae of all exploration types were significantly more abundant in pure European beech and Norway spruce zones, respectively, compared to mixture zones ($p < 0.05$) Even after three years of drought, the effect of tree species mixture did not depend on the throughfall exclusion treatment and only contact type ectomycorrhizae were less abundant in mixture compared to mono zones ($p < 0.05$). Soil depth also did not influence the effect of tree species mixture significantly.

Regarding qualitative EAs the effect of mixture depended on the throughfall exclusion treatment. While levels of cellobiohydrolase and glucosidase did not differ markedly between mixture and pure European beech and Norway spruce zones, respectively (see article IV) in control plots (cel_{Mix}: 4.63 ± 0.57, cel_{Mono}: 4.19 ± 0.54; gls_{Mix}: 14.72 ± 1.32, gls_{Mono}: 14.61 ± 1.26 pmol mm^{-2} min^{-1}), activities of both enzymes were increased in monospecific zones compared to the mixture zone after three years of experimental drought (cel_{Mix}: 6.01 ± 0.80, cel_{Mono}: 8.55 ± 1.16; gls_{Mix}: 19.61 ± 1.49, gls_{Mono}: 26.99 ± 2.74 pmol mm^{-2} min^{-1}; $p < 0.05$). This effect was more pronounced in the organic soil layers ($p < 0.05$). Quantitative EAs did not exhibit any mixture effects.

3.2.3 Effects of drought on enzyme activity profiles of ECM fungal species in a long-term experiment (data not shown in article IV)

During evaluation of data from the throughfall exclusion experiment Kranzberger Forst an interactive platform was established in the R computing environment (R-Core-Team 2016). This platform displays a spearman's rank correlation matrix containing qualitative EAs and ECM fungal species abundance. The user can specify year of experiment, treatment, soil depth and tree species. This enables inspection of interrelationships between qualitative EAs and ECM fungal species abundance as well as within these sets on the sample type scale. However,

clear trends were only detectable after aggregating data at ecosystem level. Yet, data analysis at this fine scale showed how transient and variable the examined system reacted to throughfall exclusion. Additionally, it revealed that there was great variability between the years within control plots. For example, in 2016 in the topsoil of control plots abundance of *X. pruinatus* (Xer_pru in Fig. 2 & 3) associated with fine roots of European beech was positively correlated with qualitative EAs of all (eight) enzymes measured (Fig. 2), whereas in the same type of sample in 2015 this positive correlation was found for qualitative EAs of only three enzymes measured (Fig. 3). Additionally, correlations among EAs originating from the same sample type of control plots as described in the first example was never negative in 2016 (Fig. 2), but in the year before there were also negative correlations (Fig. 3). Less variable between the years were correlations among ECM fungal species (Fig. 2 & 3) as certain species share similar environmental requirements. This was also reflected by data from throughfall exclusion plots (Fig. 4 & 5).

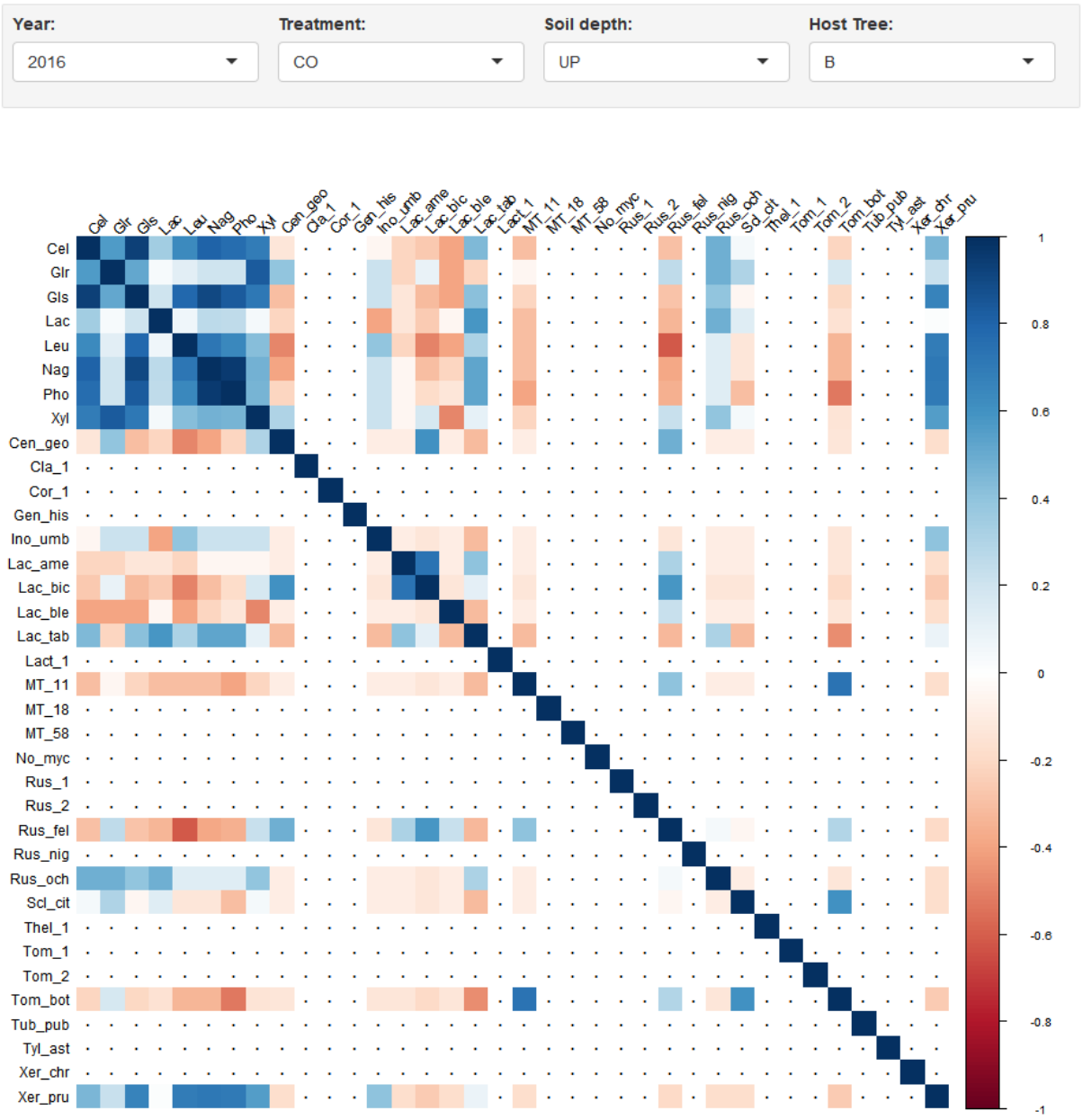


Figure 2: Spearman's rank correlation matrix of qualitative EAs and ECM fungal species abundances. The choice in the upper panel computed results for 2016 (3 years of throughfall exclusion) in the topsoil of control plots for ectomycorrhizae associated with European beech. Positive correlations are indicated by blue colours and negative correlations are indicated by red colours. The darker the colour the stronger the correlation. Dots appear where number of observations was not sufficient to calculate a correlation.

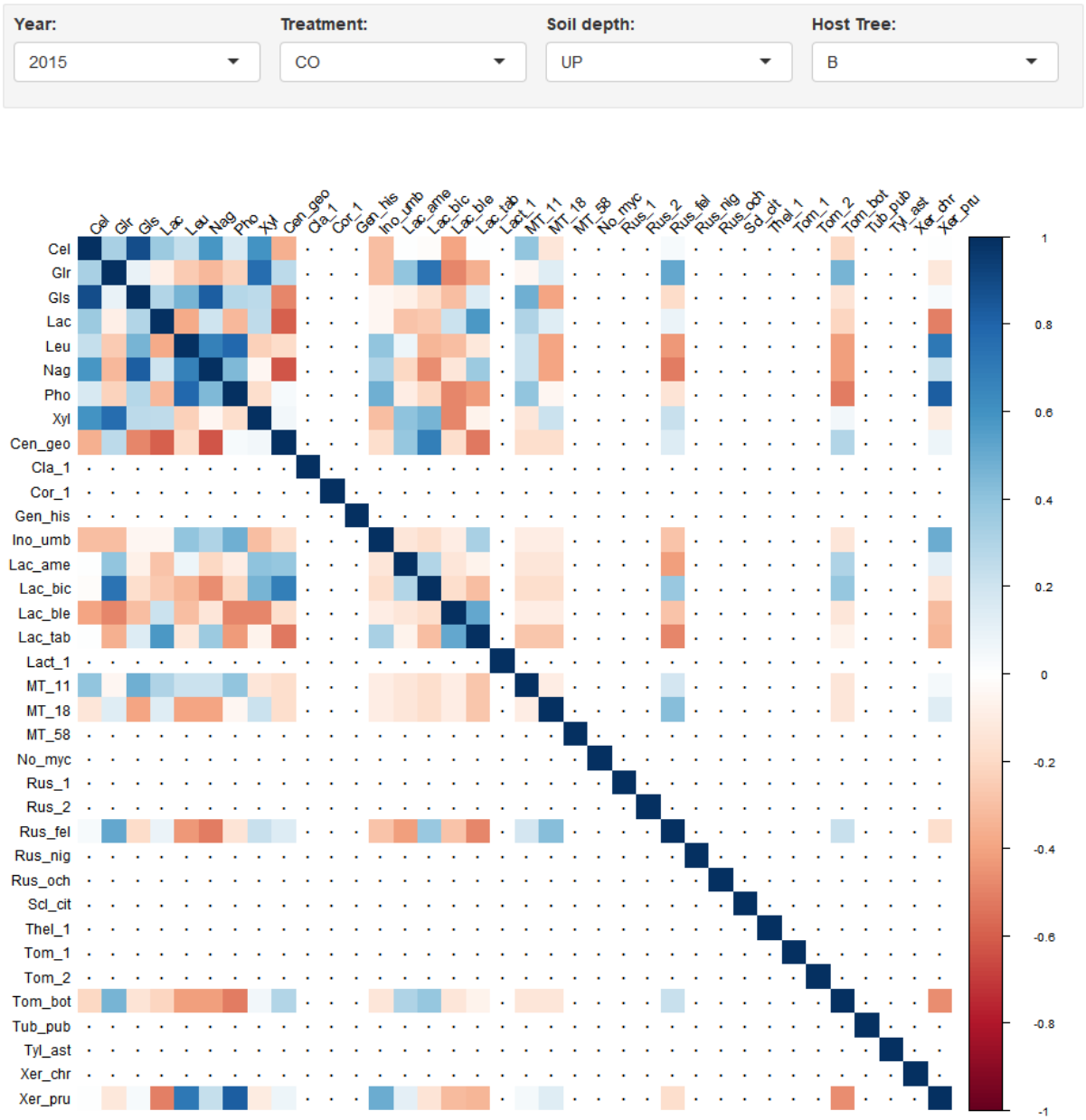
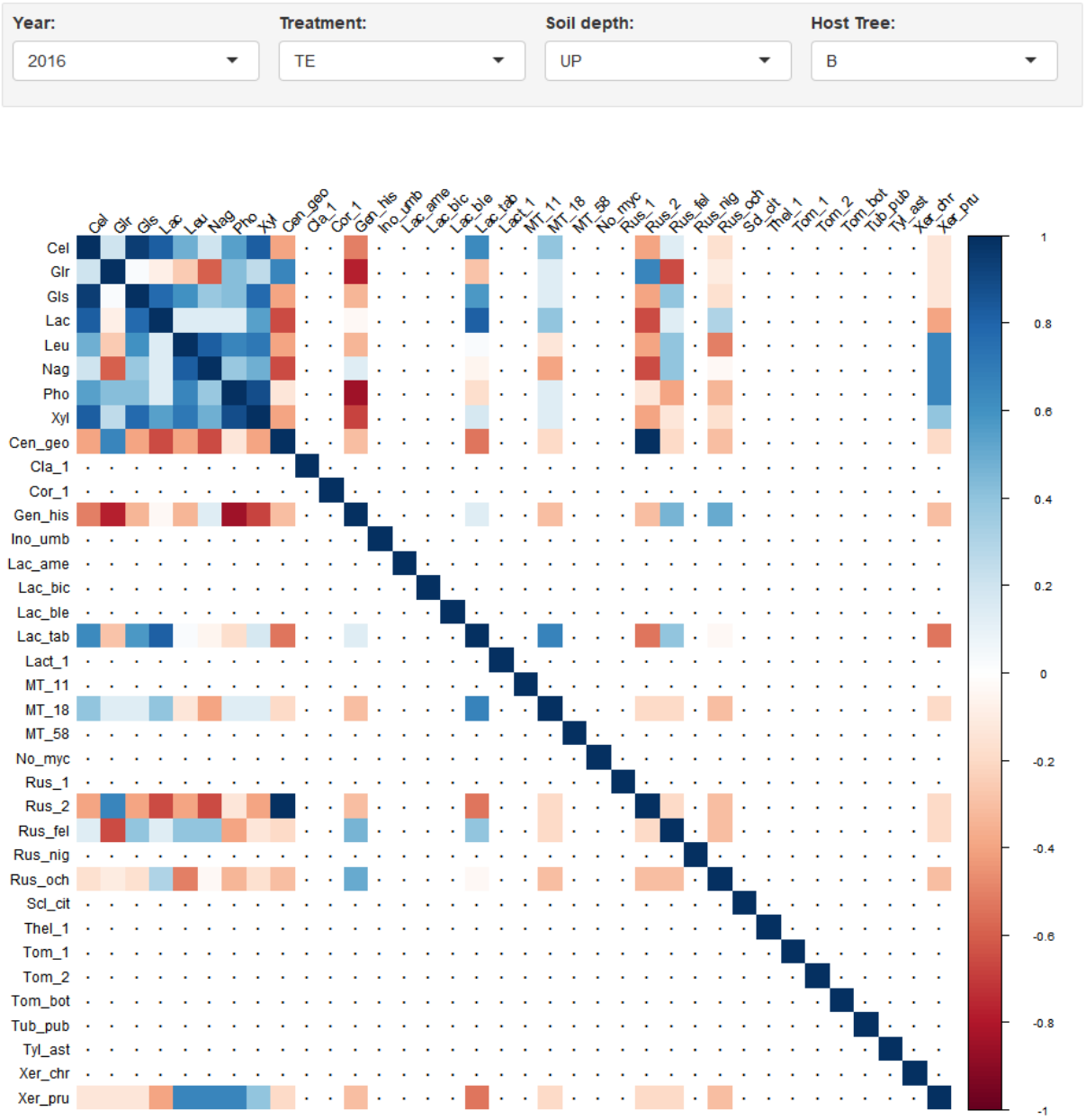


Figure 3: Spearman's rank correlation matrix of qualitative EAs and ECM fungal species abundances. The choice in the upper panel computed results for 2015 (2 years of throughfall exclusion) in the topsoil of control plots for ectomycorrhizae associated with European beech. Positive correlations are indicated by blue colours and negative correlations are indicated by red colours. The darker the colour the stronger the correlation. Dots appear where number of observations was not sufficient to calculate a correlation.



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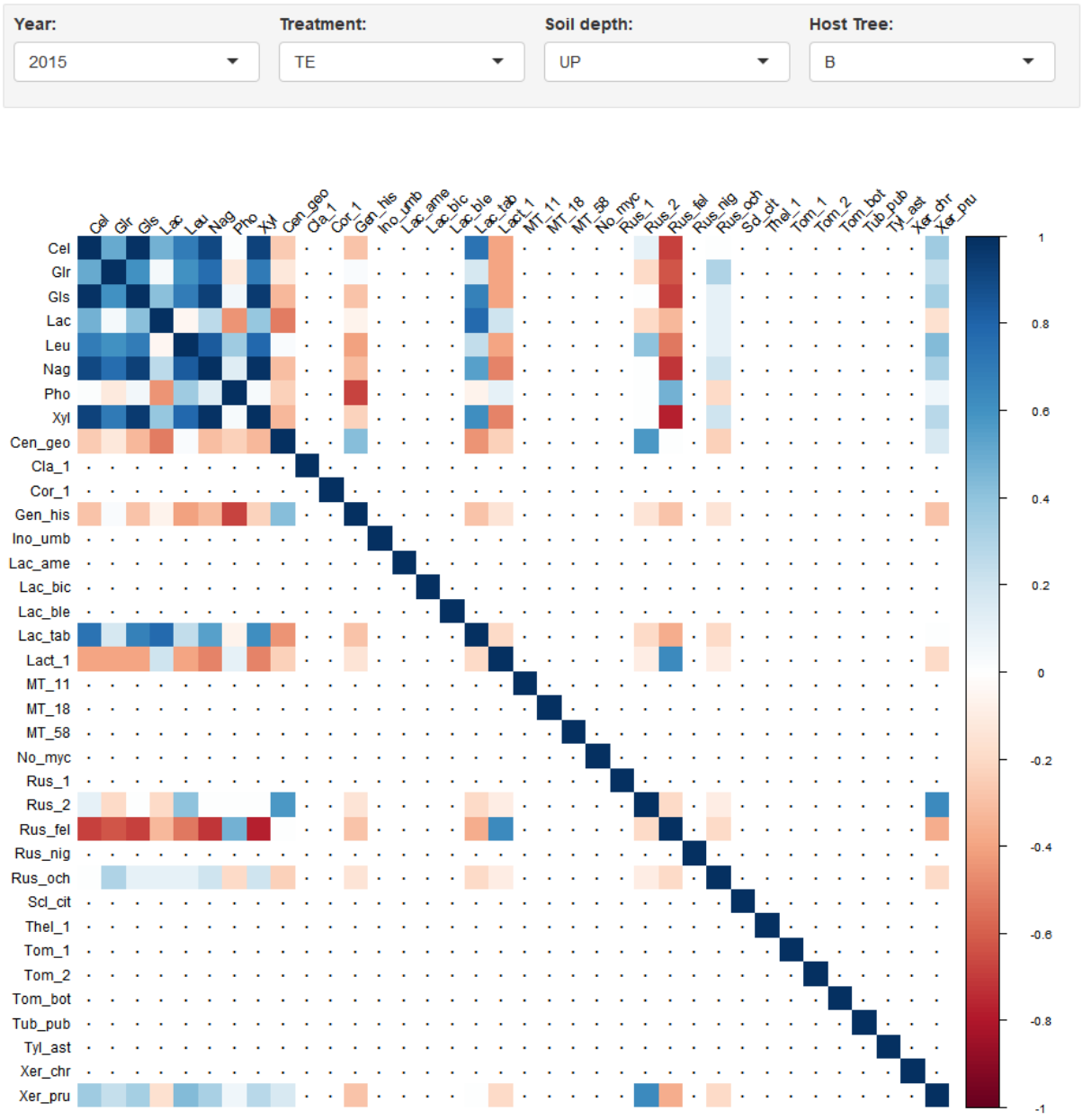

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4 Discussion

4.1 How do ECM fungal communities react under drought?

The results communicated in article IV clearly showed that during drought ECM fungal species composition shifted towards drought tolerant species. This effect was caused by the differential ability of ECM fungi to cope with low soil water potentials. For example, cell walls of *Lactarius* spp. are relatively thin and thereby prone to loose integrity under dry conditions (di Pietro *et al.* 2007). On the other hand, *C. geophilum* with highly melanised cell walls and a suite of proteins that alleviate drought stress is known to tolerate even very low soil water contents (di Pietro *et al.* 2007; Kerner *et al.* 2012; Fernandez & Koide 2013).

Regarding the abundance of contact exploration types, results from experiments reported in articles I and IV are contradictory. Abundances of contact type ectomycorrhizae of tall trees decreased upon drought (article IV), whereas those of small trees slightly increased (article I). Bakker *et al.* (2006) argue that contact types decline upon drought because of shrinking soils leading to loss of contact with substrate. So, probably the different types of substrates present in the two experiments made the difference as soil water contents in drought treatments of both experiments were comparable. Intriguingly, the relative abundance of long distance types increased in both experiments articles I and IV are based on, even though these types are most carbon costly to maintain. This is shown by highest C allocation from recent assimilates to long-distance ectomycorrhizae under normal soil moisture conditions in the present study and underpinned by up to ten times higher biomass of their extramatrical mycelium compared to short-distance types (Weigt *et al.* 2012). This means that carbon costs of long-distance types do not seem to be limiting plant-fungal interactions in times of soil water shortage, but rather fungal drought resistance may be positively related to the presence of rhizomorphs. The results presented here suggest that the latter is the case, because under drought short-distance types received most of recent assimilates. Yet, the short-distance types did not seem to be very drought tolerant as their relative abundance decreased in both experiments, but generalisation is difficult given the numerous phylogenetic origins of ECM fungi (Hibbett & Matheny 2009; Tedersoo *et al.* 2012; Burke *et al.* 2014) that most probably influence their interaction with host trees as well as their drought resistance.

Most long-distance types have hydrophobic surface properties (Unestam & Sun 1995; Agerer 2001). There is not much work available on the concepts of hydrophobic and hydrophilic ECM fungi. However, Lehto & Zwiazek (2011) stress the probability that hydrophobic ECM fungi are more drought resistant and more able to transport water from drying soil, making them important for plant nutrition in drought conditions. Further, a group of proteins called hydrophobins has been shown to coat hyphal surfaces of filamentous fungi during aerial growth (Wösten & de Vocht 2000). These proteins render fungal surfaces hydrophobic, thus preventing desiccation (Linder *et al.* 2005). As mentioned above, C allocation from recent photosynthesis is positively correlated with mycelial biomass of ECM fungi under adequately watered conditions. However, under drought conditions short-distance types received most of the few assimilation products. Thus, according to the microbial market theory short-distance types should provide the highest benefit to the European beech plants studied in article I. Possible benefits for the plant may be the compromise between relatively low biomass of the ECM fungi that have to be maintained, combined with a voluminous envelope of emanating hyphae (Agerer 2001). This combination can enable the respective fine roots to access pore water of the soil, yet only over short distances. However, as carbon did not seem to be limiting, additional benefits to the plant such as increased ability to mobilise nutrients may have led to the observed preferential C allocation.

Given that C transfer from recent assimilates to roots is strongly reduced during drought, the remaining ECM fungi have to be supported with C from tree reserves like starch. This effect should be reflected by shifts in the natural abundances of ^{13}C. When stomatal conductance decreases, CO_2 concentration in the gaseous spaces within the plant decreases, too thereby reducing the ^{13}C isotope discrimination of RuBisCO (Barbour & Farquhar 2000). Additionally, starch which is used as storage compound by plants and may be used to support ectomycorrhizae (Loewe *et al.* 2000) during drought-impaired photosynthesis (Luo *et al.* 2009) shows $\delta\ ^{13}C$ values that are ca. 2‰ higher than in overall plant biomass (Wanek *et al.* 2001). This leads to higher $\delta\ ^{13}C$ values in ECM root tips of drought stressed plants, which was also found in the experiments communicated in articles I and IV. In the naturally dry year 2003, Nikolova *et al.* (2010) also found an enrichment of ^{13}C due to drought at the same site the experiment reported in article IV was conducted. There are several other studies confirming this finding and stressing a correlation of stomatal conductance and ^{13}C abundance (Peuke *et al.* 2006; Ruehr *et al.* 2009). Another reason for higher ^{13}C values in ectomycorrhizae is their

mode of nutrition. Saprotrophic fungi are enriched in ^{13}C compared to ECM fungi (Hobbie *et al.* 2001; Taylor *et al.* 2003). However, the effect size of both phenomena (stomatal closure during drought and using SOM as C source) is about the same making it difficult to determine if the observed enrichment in ^{13}C of ectomycorrhizae derived from altered nutrition mode of the ECM fungi or from ^{13}C enriched C supply from the tree. Moreover, the saprotrophic potential of ECM fungi is highly debated (Koide *et al.* 2008; Cullings & Courty 2009). In a recent review Lindahl & Tunlid (2015) advocate decomposition of organic matter by ECM fungi to primarily grant access to mineral nutrients rather than to the carbon contained therein. This is in line with findings of Hupperts *et al.* (2017) who reported highest β-glucuronidase activities during full leaf expansion of *Populus tremuloides* and no strong response to leaf abscission, supporting the nutrient acquisition model. Altogether, this points strongly towards C supply of ectomycorrhizae during drought from tree C reserves.

Even EAs do not provide clear evidence of the nutritional mode of ECM fungi, because as pointed out above ECM fungi can degrade organic materials, but without using the C contained therein (Lindahl & Tunlid 2015). Yet, EA profiles are indicative of the interaction of ectomycorrhizae with their environment. The results presented in article IV unequivocally show that even though abundances of ectomycorrhizae strongly declined, the functionality of the ECM community was maintained with respect to EAs on a qualitative basis. The relative stability of ECM EAs upon environmental disturbance has often been observed (Diedhiou *et al.* 2010; Jones *et al.* 2010). Yet, data presented in article IV show an increase of leucine aminopeptidase activity at the ECM root tip level after three years of severe summer drought. This probably constitutes a compensation mechanism for the overall decline in EAs at soil volume level, as also suggested by Herzog *et al.* (2013) who found leucine aminopeptidase activity negatively correlated with the abundance of *C. geophilum*. Repeated summer drought further increased extracellular cellulolytic potential at the ECM root tip level. This again raises the question of the nutritional mode of the ectomycorrhizae. And again, evidence suggests rather breakdown of cellulose to gain access to nutrients contained in dead plant material (Hupperts *et al.* 2017) than saprotrophic carbon acquisition (Courty *et al.* 2007; Bréda *et al.* 2013) as the relative increase of long-distance exploration types suggests that carbon was not limiting, especially given that before drought, long distance types were least abundant (Goisser *et al.* 2016; Hagedorn *et al.* 2016). The present results thus indicate an overall qualitative preservation of functionality regarding EAs at ECM root tip level. However, the

strong decline in numbers of vital ectomycorrhizae led to a quantitative functional loss at ecosystem level. This was made particularly clear by the loss of laccase activity caused by disappearance of laccase active ECM fungal species.

Taken together, the present findings indicate that ECM community structure shifted towards drought tolerant species. The relative increase in long-distance types—be it because of enhanced drought survival due to hydrophobicity or because of promotion by the host plant—probably increased water availability for ECM fungi and plants. At low C support by the plant, short-distance exploration types may take over this function. Regarding EAs, the ECM communities studied here reacted to drought by maintaining or even increasing their EAs qualitatively, but were not able to compensate for quantitative losses, leading to reduced nutrient cycling within the ECM community.

4.2 How does tree species mixture influence ECM fungal communities under drought?

In the present work tree species mixture was studied in a mature Norway spruce-European beech mixed forest. The results presented in article II show that European beech and Norway spruce exhibited similar distribution of fine roots when growing intraspecifically. However, when growing next to each other in mixture, European beech shifted its fine root production to deeper soil layers whereas Norway spruce shifted its fine roots to shallower soil layers. These findings confirm the work of Bolte & Villanueva (2006). Yet, most ectomycorrhizae of both tree species were found in the upper soil, which is the region where nutrients are abundant and where precipitated water can be absorbed. There is not only spatial divergence between European beech and Norway spruce in fine root production, but also temporal divergence in water consumptions: while evergreen Norway spruce consumes soil water from temperatures above 7 °C (Lagergren & Lindroth 2002) European beech begins to transpire considerable amounts of water not before bud break.

Regarding ECM fungal species diversity, tree mixture had a beneficial effect by reducing the decline of ECM fungal diversity due to drought compared to single tree species zones. This result supports the stress gradient hypothesis of increased facilitation among plant species with increasing stress levels (Bertness & Callaway 1994). Among ECM fungi there are species with a very narrow host spectrum down to single tree species and generalist species, such as *C. geophilum* forming symbioses with numerous tree species. Even though most ECM fungal

species found at the plots in Kranzberger Forst were associated with both, European beech and Norway spruce, there were some that have been found exclusively associated with only one tree species. This finding confirms results from a survey in a mixed stand of *Pseudotsuga menziesii* and *Pinus muricata* by Horton & Bruns (1998). However, there is only few data available on the drought tolerance of single ECM fungal species. But it is likely that reduced competition for non-limiting resources due to different soil exploration of the two tree species (Bolte & Villanueva 2006) led to the observed higher ECM fungal species diversity in tree species mixture zones compared to single tree species zones. Another reason could be the different strategies of European beech and Norway spruce to overcome drought. While European beech trees produce new fine roots even at low water potentials, Norway spruce trees stop fine root growth and maintain standing fine root biomass (Mainiero *et al.* 2010; Nikolova *et al.* 2010). This could enable ECM fungal species that colonise fine roots of both trees to occupy not only spatial, but also temporal niches, depending on the fine root activity of their respective host tree, which would facilitate recolonization of fine roots after dry spells. This indicates mixture to increase resilience of forest ecosystems after drought. The theory of recolonization is also supported by the fact that drought-tolerant ECM fungi were found among the four most frequent morphotypes shared by European beech and Norway spruce. Further, ECM fungi colonising both tree species may contribute to resource partitioning between fungi and different trees by forming functional connections (so called "wood wide web") which could facilitate stress resistance (Beiler *et al.* 2010). Another temporal effect is water availability. Before bud break of European beech, soil water consumption of evergreen Norway spruce lowered soil water content more in pure Norway spruce than in mixed zones due to less abundant fine roots of Norway spruce in mix zones and probably also because of the capacity of European beech for hydraulic redistribution (Caldwell *et al.* 1998). This may positively influence recovery of the ECM fungal community during the dormant season of European beech despite the higher transpiration capacity of European beech leaves compared to Norway spruce needles. Further, input of European beech litter may be beneficial for neighbouring Norway spruce, as it can significantly alter the topsoil properties (i.e. reduced humus accumulation and acidity) and hence nutrient release from litter decomposition and nutrient cation mobility (Göttlein *et al.* 2012). This is supported by the higher share of Norway spruce ectomycorrhizae without rhizomorphs in mixture compared to pure Norway spruce which is outlined in detail in the discussion section of article II.

The overall abundance of ectomycorrhizae of all exploration types was lower in tree species mixture zones compared to monospecific zones. This can be explained by the higher distance between individual trees in the mixture zones, resulting in an overall lower fine root density. The lack of a tree species mixture effect is underpinned by annual variation in the results that were transient and did not allow to deduce clear patterns (see also chapter 3.2.3).

Regarding qualitative EAs, the few significant interactions of throughfall exclusion × zone indicated that tree species and mixture situation per se had only minor effects on the outcome of EA in the drought treatment. However, tree species mixture decreased the rise of cellulolytic potentials due to drought and this effect was more pronounced in the topsoil. This could be interpreted as a response to altered litter quality in mixture zones with lower amounts of easily degradable European beech litter requiring higher efficiency in breakdown potential. However, these mixture effects only became apparent after three years of experimental summer drought. This can probably be attributed to changes in niche occupation by roots of the two tree species, as indicated by the reduction in some enzyme activities (leucine aminopeptidase and phosphatase) in Norway spruce from mixture but not in European beech from mixture in the topsoil, and their increase in the deep layers. Thus, tree species mixture seems to have minor influence on qualitative EAs, at least after the time period of three drought years analysed and reported in article IV. The fact that tree species in combination with drought had minor effect on quantitative EAs of their ECM fungal community further supports the assumption that C is not a limiting factor. Given that Norway spruce closes its stomata earlier than European beech during drought establishment, it could be hypothesised that EAs of Norway spruce ectomycorrhizae increase earlier as an increase of ECM EAs as a response reduced C allocation has been shown previously (Cullings *et al.* 2008). According to the microbial market theory (Werner *et al.* 2014) and the assumption that ectomycorrhizae degrade SOM primarily to gain access to nutrients locked therein (Lindahl & Tunlid 2015; Hupperts *et al.* 2017), this increase in EAs upon reduced C allocation could be interpreted as a response to higher "prices" of a scarce resource.

Taken together, mixture of European beech and Norway spruce mitigated negative impacts of recurrent summer drought on ECM fungal diversity, indicating mixture to increase resilience of forest ecosystems after drought. It took three years of experimental summer drought to generate significant mixture effects.

4.3 How does recovery from drought change sink strength and the capacity to mobilise nutrients of ectomycorrhizae and how is this influenced by different N:P stoichiometries in the substrate?

The results reported in article I clearly showed that during drought net photosynthesis and soil respiration were reduced. Using $^{13}CO_2$ as a tracer it could be shown that recent assimilates were translocated to fine roots even during very low water availability. Yet, soil respiration showed a dilution effect caused either by heterotrophic processes or C supply of roots from tree reserves like starch (Loewe *et al.* 2000; Klein & Hoch 2015). Drought further reduced the velocity of C transport from leaves to roots more than previously reported (Ruehr *et al.* 2009). This indicates sink control and not source limitation as outlined in article I.

In both experiments communicated in articles I and III, recovery after severe drought was examined by rewatering the respective systems. Results of both experiments showed increased C allocation from recent assimilates to ectomycorrhizae at similar photosynthetic capacity in previously drought treated setups compared to control treatments. These results also support the concept of sink control, as source activity (photosynthesis) in recovering and control plants was at similar levels. Further, the concept of an ecological stress memory was supported by the imprint of a drought effect on plant source and sink tissues, the underlying mechanisms of which are still poorly understood (Ogle *et al.* 2015). The greater allocation and use of recent assimilates in below-ground sinks after rewatering showed that trees give high priority to investing into their roots for recovery from drought. The likely reason for this response is the metabolic need for root and mycorrhizal restoration to re-establish capability of trees to acquire water and nutrients after an extended drought (Lehto & Zwiazek 2011; Volkmann *et al.* 2016).

Results reported in article III add that fertilisation can influence sink strength of ectomycorrhizae. Only treatments that were not N-fertilised showed significantly higher C allocation to ectomycorrhizae recovering from drought compared to regularly irrigated treatments. When high amounts of N are available there might be less need to restore root and ectomycorrhizal metabolism as N can be used to synthesise amino acids, many of which– especially proline–can also act as osmoprotectants (Handa *et al.* 1986; McNeil *et al.* 1999). P fertilisation also reduced C allocation to ectomycorrhizae recovering from drought compared to non-fertilised treatments, but to a lesser extent than N fertilisation. The fact that fertilisation

reduced sink strength of ectomycorrhizae also supports the microbial market theory (Werner *et al.* 2014). When nutrients are abundant and accessible for the plant, there is no need to "pay" C for ECM foraging. However, in continuously irrigated treatments added nutrients were already taken up at the end of the experiment. Therefore, this hypothesis could not be tested directly under control conditions. But fertilisation enhanced plant growth leading to bigger plants in especially N-fertilised and continuously irrigated treatments with higher nutrient demands. Although not significantly, these bigger plants also had higher C allocation to ectomycorrhizae compared to smaller plants. As N contents in the substrate of all continuously irrigated plants were similar, the bigger and therefore more demanding plants may have invested more C in ECM symbiosis foraging for nutrients which also supports microbial market theory.

Regarding ECM capacity to mobilise nutrients the results presented in article III show that fertilisation had no direct effect on enzyme activities. This is an intriguing result, as downregulation of ECM EAs upon fertilisation has been reported as outlined in the discussion section of article III. A reason may be that these particular ectomycorrhizae were adapted to high nutrient contents in agricultural soils. Exoenzyme activities of ectomycorrhizae can be very stable upon disturbance (Diedhiou *et al.* 2010; Jones *et al.* 2010). Yet, ectomycorrhizae of recovering plants showed higher potentials to degrade plant and fungal cell walls. This would be beneficial in order to obtain N- and P-rich substrates contained in dead tissues from roots and microorganisms that did not survive the drought treatment. Enhanced phosphatase activity during recovery from drought may help to mobilise P from dead cells and tissues, which were made accessible by the chitinolytic and cellulolytic activities of ectomycorrhizae. After severe drought, there might be an increased need for P to restore metabolism.

5 Conclusions and outlook

Using functional traits that serve as effect and response traits at the same time it was possible to predict the indirect effects of environmental change on ecosystem function via ECM fungi. Regarding effects of drought on (several years to long-term) established ECM fungal communities under experimental water exclusion, articles I (drought experiment in open-top chambers) and IV (throughfall exclusion experiment at Kranzberger Forst) have shown that during drought species composition shifted towards drought tolerant species. Soil exploration by ectomycorrhizae shifted towards long-distance exploration types which can transport water and solutes over a distance of several cm. This supports the hypothesis that those ECM exploration types are beneficial for the plant under conditions of low water availability, given the high C cost for maintaining their vast extramatrical mycelium. However, there was no indication that C was limiting. Stable isotope analysis of natural ^{13}C abundance revealed a reduction in allocation of recent assimilates to ectomycorrhizae under drought, but the experimental approach did not allow to determine if this loss was balanced by C from tree reserves or by C from SOM (ECM saprotrophic potential). Drought experiments using ^{13}C labelled plant litter would help to quantify how much C is derived from SOM. However, care has to be taken that decomposition of labelled plant litter and thereby $^{13}CO_2$ from soil respiration does not enter the assimilation process.

Following a $^{13}CO_2$ pulse labelling confirmed reduced C allocation from recent assimilates to ectomycorrhizae under drought. Qualitatively, EAs of ectomycorrhizae under drought showed a remarkable stability. However, qualitative stability could not compensate for quantitative losses as many ECM root tips succumbed to drought. Thus, under drought conditions the surviving drought tolerant ECM fungi, most of which being able to transport water over long distances, received less recent assimilates but C did not seem to be limiting. On an individual level, ectomycorrhizae maintained their EAs, but were not able to compensate for losses at ecosystem level. This may lead to a reduction in nutrient cycling processes.

The interactive platform computing correlation matrices of all EAs and ECM fungal species abundances at sample type level as described in chapter 3.2.3 is envisaged to be used for future studies. Detailed comparisons enable to follow regulation of EA profiles of different single ECM fungal species in selected soil depths, associated with fine roots of selected host

trees. With increasing data sets, it may also be possible to compute co-occurrence and niche partitioning among ECM fungal species instead of the labour intensive method of micro mapping (Agerer *et al.* 2002). Further, the interrelationships that are presented may guide the design of future experiments investigating effects of drought on EAs of certain ECM fungal species.

Regarding the influence of tree species mixture on ECM fungal communities during drought, articles II and IV have shown that mixture *per se* shifted fine root distribution of Norway spruce upwards and fine root distribution of European beech downwards. This effect was enhanced by drought. Tree species mixture alleviated the negative effects of recurrent summer drought on ECM fungal community diversity, thereby increasing resilience of the studied ecosystem after drought. Reasons for this may be the selection for drought tolerant generalist species being able to colonise new grown roots after drought release. Tree species identity and mixture during drought had only minor effects on EAs and soil exploration properties of ectomycorrhizae in the system studied. Thus, from an ECM fungal community point of view, tree species mixture has the potential to increase resilience of the ecosystem after drought.

Regarding the phase of recovery from drought, articles I and III showed that C allocation from recent assimilates was greatly enhanced after one week of recovery from drought compared to continuously irrigated treatments at similar photosynthetic capacity indicating sink control of C fluxes. Trees were found to increase below-ground C allocation thus apparently prioritising to restore root and thereby ECM functions. Fertilisation with mineral N and P reduced this effect with greater influence of N fertiliser compared to P fertiliser. The capacity of ectomycorrhizae to mobilise nutrients from their substrate was more influenced by previous drought with some indirect effects of fertilisation mediated by enhanced plant growth. Thus, C fluxes in trees after drought release seem to be sink controlled with alleviating effects of P and especially N fertilisation regarding impacts of drought stress on ecosystems.

Different levels of experimental control showed results on sink control of C allocation and potential nutrient mobilisation obtained in a highly controlled greenhouse experiment (article III) were transferable to larger scales. Sink control of C allocation from recent assimilates has also been found in the open top chamber experiment (article I) and an increase in cellulolytical potential of ECM root tips was confirmed in the near natural ecosystem setup (article IV).

Facing climate change, forest management should promote mixed forests in order to increase their resistance and resilience to repeated summer drought. Ironically, as fertilisation seems to mitigate negative effects of drought stress on ectomycorrhizae, anthropogenic N deposition may moderate drought impact on forest ecosystems. However, it is questionable if this effect could outweigh the negative effects of anthropogenic N deposition on ECM species diversity. Due to fertilisation of adjacent field crops, agroforestry systems may be rather resilient against short-term drought. Thus, poplars and potentially also other tree species in agroforestry systems may be a promising tool to sequester C, apart from their other ecological and agricultural benefits.

Finally, it would be important to conduct experiments refining hydrophobicity concepts of ECM fungal surfaces regarding drought resistance. To build on the work presented in article III, the next step should be to carry out field experiments to validate the present findings in agricultural practice. The experimental system in Kranzberg forest reported in articles II and IV has shown that the below-ground system is very stable facing drought. However, after three years of throughfall exclusion some Norway spruce trees succumbed to drought showing vulnerability of Norway spruce to drought. To determine whether emerging facilitation effects of tree species mixture will continue, prolongation of the experiment with increasing stress levels and further sampling is required. Thereafter, drought stress release by rewatering as envisaged in the experimental concept of this long-term experiment is ultimately required to determine resilience of the system.

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7 Articles

7.1 I: Recovery of trees from drought depends on belowground sink control

Nature plants, 2, 16111. doi:10.1038/nplants.2016.111.

Year: 2016

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The following article reports results from the open top chamber experiment and an additional pot experiment for analysis of metabolites. Recent photosynthates were traced from source to sink tissues until they were finally released into the soil matrix. This was done during and after drought because climate projections predict higher precipitation variability with more frequent dry extremes. During drought, photosynthetic activity of forest trees decreases, either by stomatal closure or by direct environmental control of sink tissue activities. In the end, drought effects on forests depend on the ability of forest trees to recover. However, the mechanisms controlling ecosystem resilience are largely uncertain. Here, the effects of drought and drought release—as experienced by short but intense summer droughts—on the carbon balances in European beech trees were investigated. This was achieved by combining CO_2 flux measurements, metabolomics and $^{13}CO_2$ pulse labelling. During drought treatment in the open top chambers, net photosynthesis, soil respiration and the allocation of recent assimilates below-ground were reduced. The pot experiment showed that carbohydrates accumulated in metabolically resting roots but not in leaves. These findings indicate sink control of the tree carbon balance. After drought release in the open top chambers, soil respiration recovered faster than net photosynthesis. Further, CO_2 fluxes exceeded those in continuously watered trees for months. This stimulation was related to greater assimilate allocation to the roots and metabolisation of these assimilates in the rhizosphere. These findings show that trees prioritise the investment of assimilates below-ground, probably to regain root functionality after drought. It is proposed that root restoration may play a key role in ecosystem resilience to drought, in so far as the increased sink activity controls the recovery of carbon balances.

nature plants

LETTERS

PUBLISHED: 18 JULY 2016 | ARTICLE NUMBER: 16111 | DOI: 10.1038/NPLANTS.2016.111

Recovery of trees from drought depends on belowground sink control

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Climate projections predict higher precipitation variability with more frequent dry extremes[1]. CO_2 assimilation of forests decreases during drought, either by stomatal closure[2] or by direct environmental control of sink tissue activities[3]. Ultimately, drought effects on forests depend on the ability of forests to recover, but the mechanisms controlling ecosystem resilience are uncertain[4]. Here, we have investigated the effects of drought and drought release on the carbon balances in beech trees by combining CO_2 flux measurements, metabolomics and $^{13}CO_2$ pulse labelling. During drought, net photosynthesis (A_N), soil respiration (R_S) and the allocation of recent assimilates below ground were reduced. Carbohydrates accumulated in metabolically resting roots but not in leaves, indicating sink control of the tree carbon balance. After drought release, R_S recovered faster than A_N and CO_2 fluxes exceeded those in continuously watered trees for months. This stimulation was related to greater assimilate allocation to and metabolization in the rhizosphere. These findings show that trees prioritize the investment of assimilates below ground, probably to regain root functions after drought. We propose that root restoration plays a key role in ecosystem resilience to drought, in that the increased sink activity controls the recovery of carbon balances.

Forests play a crucial role in the global carbon cycle because they hold a large fraction of the global carbon stock and act as a major sink for atmospheric CO_2 (ref. 5). However, drought reduces primary productivity, thereby turning forests from carbon sinks into carbon sources[6]. It has generally been assumed that plant and ecosystem carbon balances under drought are controlled by restricted photosynthetic source activity rather than by changes in the sink activity of plant tissues[7,8]. Recently, direct environmental control of sink activity with feedbacks to CO_2 assimilation has been proposed[9], but no unequivocal evidence has been obtained yet. Of similar importance, but even less understood, are the mechanisms controlling plant and ecosystem carbon balances after drought release, although the ability of plants to restore CO_2 assimilation and other functions determines the resilience of trees and forest ecosystems. Further, limited knowledge on the principles that control carbon allocation in trees prevents us from predicting carbon balances of forests under future environmental conditions characterized by greater variability of precipitation and thus alternating drought and recovery periods.

Using two experimental setups, growing either in model ecosystems in open-top chambers (Supplementary Fig. 1) or in pots, we studied tree and ecosystem carbon fluxes during drought and after drought release. By combining measurements of net photosynthesis (A_N) and soil respiration (R_S) as indicators of source and sink activity, respectively, with $^{13}CO_2$ pulse labelling and metabolomic analyses, we followed seasonal carbon dynamics and tracked assimilate transport through the plant–soil system. Based on a hypothetical framework (Fig. 1), we aimed to test if changes in A_N and R_S, as well as shifts in carbohydrate allocation, indicate source or sink control of carbon balances. If source activity controls carbon balances under drought, we expected an initial decrease in A_N and leaf carbohydrate concentrations and a delayed depletion of carbohydrates in roots, leading to a reduction in R_S (Fig. 1a). A similar response would occur on drought release, with an initial recovery of A_N and leaf carbohydrate concentrations followed by a delayed increase of R_S (Fig. 1b). If, however, the carbon balance is sink controlled, drought would directly reduce R_S, leading to an accumulation of carbohydrates in roots because of reduced carbon demand. In this case, A_N would acclimate to the reduced sink demand after a delay and leaf carbohydrate concentrations consequently would not change (Fig. 1c). On drought release, R_S would increase and, with a delay, the increased belowground carbon demand would positively feed back on A_N (Fig. 1d).

In our model ecosystems, A_N and R_S decreased by 44% and 28%, respectively, throughout the entire drought season (Fig. 2a–c and Supplementary Fig. 2a). At the end of the drought, a $^{13}CO_2$ pulse label was applied to the canopies to trace the fate of recent assimilates in the plant–soil system. Under drought, the uptake of $^{13}CO_2$ decreased by 81% and assimilate translocation to belowground sinks was reduced, as shown by lower ^{13}C signals in mycorrhizal roots and soil microbial biomass and reduced $^{13}CO_2$ soil efflux (Fig. 3a,c,e,g). The reduction in $^{13}CO_2$ soil efflux was 83% and thus similar to that in $^{13}CO_2$ uptake. However, the $^{13}CO_2$ soil efflux showed a stronger reduction (83%) than that observed for R_S (≈50%), indicating that other carbon sources, either related to heterotrophic soil respiration or tree internal carbon storages[10],

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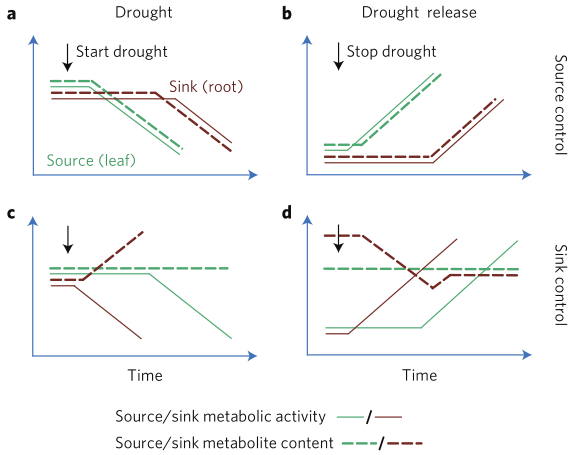

Figure 1 | Hypothetical trajectories of metabolic activity and metabolite concentration in leaves and roots as a consequence of drought onset and drought release. a–d, For both drought (**a**,**c**) and drought release (**b**,**d**), the scenarios for full source (**a**,**b**) and full sink (**c**,**d**) control of the tree carbon balance after the change in soil moisture conditions are shown. Effects in source (green, leaves) and sink tissues (brown, root) are provided for each scenario. We refer to net photosynthesis (A_N) as a source metabolic activity and to soil respiration (R_S) as an integrator of sink metabolic activity in the roots. Metabolite content refers to the most abundant carbohydrates (the NSCs glucose, fructose, sucrose and starch; see also Fig. 4b). Under source control (**a**,**b**), source metabolic activity in leaves (A_N) reacts first to changing conditions and induces changes in assimilate (sugar) availability for sinks and thus affects sink metabolic activity in roots (R_S). Under sink control (**c**,**d**), sink metabolic activity is directly affected by the environmental conditions, leading to changes in sink metabolite levels. After a delay, source metabolic activity is impacted in response to the altered sink demand.

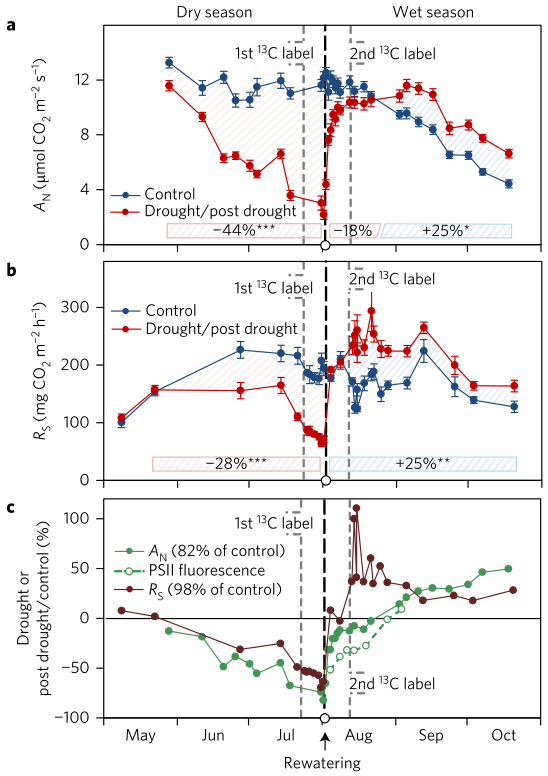

Figure 2 | Reduction of net photosynthesis (A_N) and soil respiration (R_S) in the model ecosystem experiment during drought (dry season), and during recovery and stimulation after drought release (wet season). **a**,**b**, Effects on A_N and R_S, respectively, are shown. Numerical values provide quantitative measures of the drought limitation and the stimulation after full recovery (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; means ± s.e.m.; A_N, $n = 8$; R_S, $n = 3$–8). **c**, The development of drought and post-drought effects on A_N (green) and R_S (brown) are shown, together with the release of metabolic limitation of A_N after rewatering (PSII chlorophyll fluorescence; $n = 8$). Responses are shown as relative deviations from control values.

contributed to soil respiration but were less sensitive to drought. Under drought, the ^{13}C peak in continuously monitored soil CO_2 (Supplementary Fig. 3a) was delayed by one day and mean residence times (MRTs) of assimilated ^{13}C in the plant–soil system increased (drought 76 h, control 30 h), indicating slower assimilate transport to belowground sinks, as previously reported[11]. The reduced and delayed assimilate transport might have been the result of either source limitation or sink control. However, non-structural carbohydrates (NSCs) were not depleted in source leaves in drought-treated model ecosystems (Supplementary Table 1) and thus source limitation was unlikely, as recently proposed[9].

To explore the mechanisms leading to reduced carbon fluxes to belowground sinks, we studied the dynamics of metabolites with progressing drought in a pot experiment. Reductions in soil moisture and A_N in drought-treated pots were similar to those in the model ecosystems (Fig. 4a and Supplementary Fig. 2b). In roots, the NSCs fructose, glucose, sucrose and starch, as well as the osmoprotectant proline, increased under drought, and sucrose accumulated in the release phloem (Fig. 4b). The increase in the concentration of NSCs by up to 700% was very strong but still in the range reported in previous studies with trees[12,13]. In leaves, no NSC increase was observed except for a delayed accumulation of starch and proline as drought progressed. Although NSCs accumulated in roots, sink control of such an increase can only be inferred when the size of carbohydrate pools depends directly on the balance between supply through photosynthesis and demand for growth and respiration[14]. Alternatively, accumulation of NSCs might serve as osmotic adjustment[15], which is not directly related to changes in carbon supply and demand. In our study, however, accumulation of NSCs was only observed in roots, and proline, an indicator of osmotic regulation[16], was enriched in both roots and leaves. It is thus unlikely that osmotic adjustment via an increase in NSC content was achieved only in roots and not in leaves. Instead, active storage of carbohydrates in roots at the expense of metabolic processes might have occurred, as previously suggested[17]. Thus, the strong accumulation of NSCs in roots and the lack thereof in leaves reflect metabolic activity in sink and source tissues. Transferring this information to our model ecosystems indicates that the reduced carbon flux to belowground sinks under drought (Fig. 3c,e,g) was a consequence of decreased sink activity (Fig. 1c). Owing to the rather slow build up of drought over time in our model ecosystems, a clear order of the response of source (A_N) versus sink (R_S) activities could not be derived directly, especially since changes in R_S might have been only partially because of changes in autotrophic root–rhizosphere respiration.

The recovery of plant and ecosystem carbon fluxes after prolonged drought was examined by rewatering the model ecosystems. R_S responded rapidly, reaching control values within the first 3 days and exceeding thereafter values in controls until the end of the growing season (Fig. 2b,c and Supplementary Table 3). This stimulation of R_S nearly compensated for the previous drought reduction, with the flux integrated over the entire growing season amounting to 98% of that in controls. A_N responded similarly but the recovery

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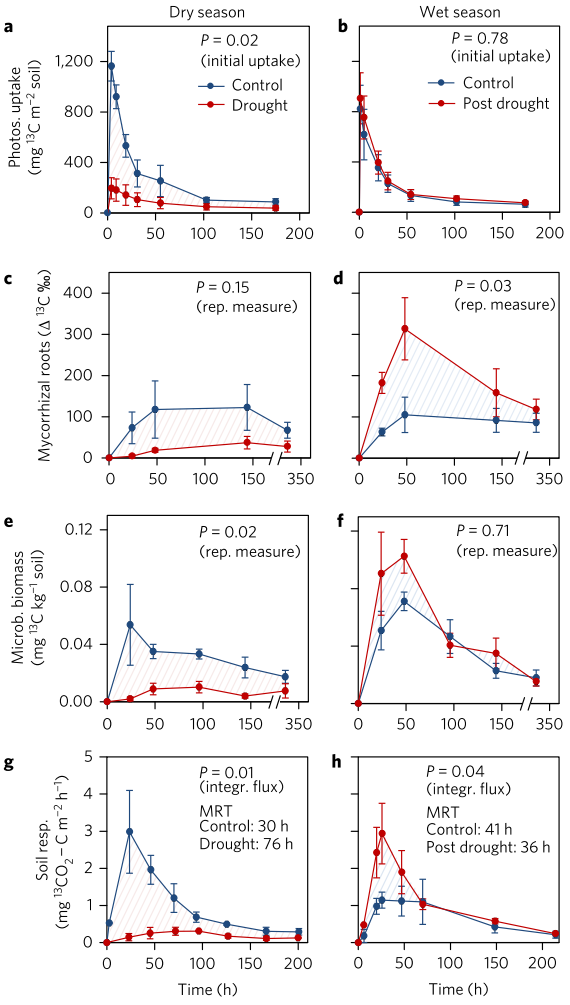

Figure 3 | Suppressed uptake and allocation of ^{13}C assimilates in the model ecosystem experiment under drought (dry season) and increased transfer to and metabolization in the belowground compartment after drought release (wet season). a,b, Leaf photosynthetic uptake of ^{13}C. **c,d**, Incorporation of ^{13}C into mycorrhizal root tips. **e,f**, Transfer of ^{13}C to soil microbial biomass. **g,h**, Respiratory ^{13}C release from the soil including mean residence times of recent assimilates calculated from the $\delta^{13}C$ of continuously measured soil CO_2 (Supplementary Fig. 3). $P<0.05$ indicates statistically significant treatment effects (means ± s.e.m.; $n = 3$ for dry season and $n = 4$ for wet season).

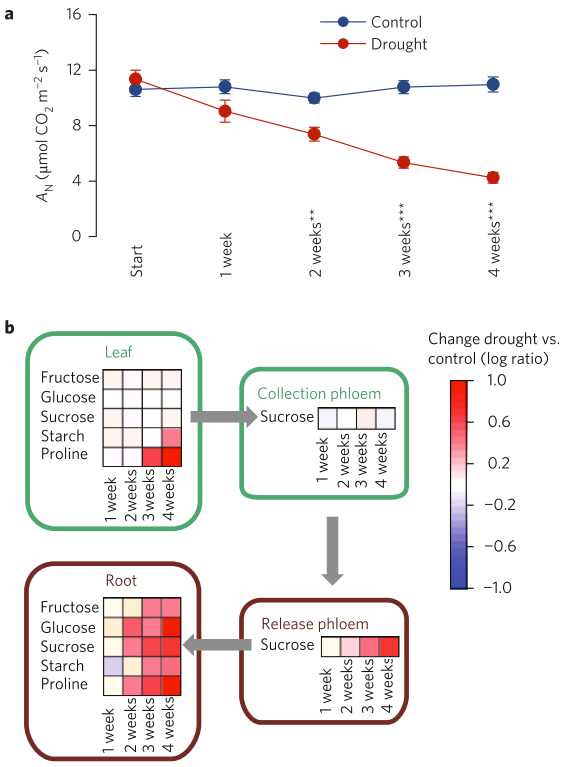

Figure 4 | Decreased net photosynthesis (A_N) in the pot experiment during drought but unchanged metabolite concentrations in leaves and increased concentrations in roots. a, Changes in A_N during the course of drought development (*$P<0.05$, **$P<0.01$, ***$P<0.001$; means ± s.e.m.; $n = 5$) **b**, Changes in metabolite concentrations. Effects on metabolites are shown as $\log_{10}$ ratio of the drought treatment to the control treatment 1, 2, 3 and 4 weeks after the onset of drought. Analysed metabolites comprise the most abundant carbon compounds, as well as proline as an osmoprotectant, occurring in leaves and roots, and sucrose as the main transport sugar in the collection and release phloem[38]. Data shown are means of five replicates.

was delayed by approx. one week because of metabolic limitation, as shown by impaired PSII photochemistry (Fig. 2a,c and Supplementary Table 3). Further, the stimulation of A_N occurred later and compensated for only 82% of the previous drought reduction. A second $^{13}CO_2$ pulse label was applied to the tree canopies when R_S exceeded the values in controls but A_N was still slightly below that of controls. The previous drought exposure increased the translocation of recent assimilates to belowground sinks compared to controls, as shown by higher ^{13}C signals in mycorrhizal roots and soil microbial biomass and by an enhanced $^{13}CO_2$ soil efflux (Fig. 3b,d,f,h). The latter signal was enhanced by 50% and thus increased in relation to photosynthetic ^{13}C uptake, which was not affected. This increase represents exclusively autotrophic respiration, and the comparable increase in R_S (68% R_S vs. 50% $^{13}CO_2$ soil efflux) shows that the plant-driven carbon flux was primarily responsible for the observed stimulation of R_S. Heterotrophic soil respiration was small in our model ecosystems containing low soil organic content and can therefore be excluded as the cause of stimulated R_S because it only responds transiently to rewetting of dry soils by the so-called 'Birch Effect'[18,19]. Our results thus clearly show that a drought effect is imprinted on plant source and sink tissues, supporting the concept of an ecological stress memory of which the underlying mechanisms are still poorly understood[20].

The increased carbon demand of belowground sinks resulted in only a slight feedback on the velocity of carbon transport. Whereas the peak time of $^{13}CO_2$ soil efflux was similar, the MRT of ^{13}C in the plant–soil system was somewhat lower for the previously drought-exposed trees, as calculated from continuously monitored soil CO_2 (post drought 36 h, control 41 h; Supplementary Fig. 3b). However, the mass flow of assimilates to belowground sinks can additionally be increased if less carbon is unloaded from the transport pathway for storage or growth in aboveground tree organs[21]. Indeed, the carbon allocation to growth in twigs, stem and roots did not fully recover after rewatering, indicating that growth-related sink activity along the transport pathway was still reduced (Supplementary Table 4). The greater allocation and use of recent assimilates in below ground sinks after rewatering shows

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that trees give high priority to investing into their roots for recovery from drought. The likely reason for this response is the metabolic need for root and mycorrhizal restoration to restore trees' capability to acquire water and nutrients after an extended drought[22,23]. Effects on root growth can be excluded, as demographic root characteristics were not affected during or after drought (Supplementary Fig. 4a–c). Thus, root and mycorrhizal restoration relied mainly on increased metabolic activity, which explains the fast recovery and stimulation of R_S. Since A_N showed a delayed recovery and later stimulation than R_S, the latter was clearly not source driven and instead reflects the metabolic need for root and mycorrhizal restoration. On the contrary, we postulate that increased belowground sink activity on drought release feeds back on A_N, triggering the delayed recovery and stimulation of CO_2 assimilation (cf. Fig. 1d). Furthermore, our findings support sink control of the carbon balance under previous drought conditions, as a drought-induced depletion of belowground carbon reserves should delay the recovery of R_S compared to A_N if the recovery is source controlled.

There is increasing evidence that drought not only influences ecosystem carbon balances concurrently but also triggers delayed responses that involve multiple mechanisms operating at different scales of time, plant function and ecosystem organization[4,24]. To date, such mechanisms are poorly understood and thus constitute a large uncertainty in projections of ecosystem carbon balances and resilience. Here, we show that tree carbon fluxes not only recover but even increase after drought to compensate for previous stress impacts. This compensation is sink driven, leading to a greater belowground allocation of recent assimilates on drought release. The observed response has important consequences for ecosystem carbon cycling, as it increases the input of plant-derived labile carbon into soils, thereby fuelling soil microbial communities[25]. We suggest that the ability of trees to reactivate root metabolism is vital for ecosystem resilience to drought. However, the extent of this effect very likely depends on the severity and duration of drought and may vary with tree age, as adult trees have larger carbon storage compartments. Taken together, our findings suggest a resilience mechanism that attenuates drought disturbances of seasonal tree carbon balances and needs to be considered when estimating the impact of climate change on the carbon balances of forest ecosystems.

Methods

Plant material and growth conditions. The model ecosystem experiment was conducted in 16 field-based open-top chambers. In each chamber, a model ecosystem was established with young beech trees (*Fagus sylvatica* L.) growing on lysimeters filled with forest soil of low soil organic carbon content (Supplementary Fig. 1; Supplementary Methods and Supplementary Table 2). A summer drought was simulated by reducing the water supply from 22 May to 1 August by 78%. After the trees had developed the critical water deficit for leaf physiological functioning (predawn water potentials below −2 MPa[26,27]), the lysimeters were intensely rewatered and afterwards regularly irrigated until the end of the vegetation season (Supplementary Fig. 2a).

The pot experiment was carried out with beech saplings (*F. sylvatica* L.) in a greenhouse environment (Supplementary Methods). During the drought treatment lasting 4 weeks, the control pots were watered to field capacity whereas pots with the drought treatment received no water at all. Fine root, phloem and leaf samples were taken weekly.

Measurements of net photosynthesis and soil respiration. Net photosynthesis (A_N) was measured on three or four trees per lysimeter between 11:00 and 16:00 central European time using a photosynthesis system (LI-COR 6400) equipped with a broadleaf cuvette. The conditions inside the cuvette were kept constant at 400 ppm CO_2 and a photon flux of 1,000 µmol m^{-2} s^{-1}. Metabolic constraints on A_N were tested by chlorophyll fluorescence analysis using the performance index PI_{total} of PSII[28]. Soil respiration (R_S) was measured with a custom-made static chamber[29] equipped with a diffusion-aspirated non-dispersive infrared analyser connected to a humidity/temperature sensor (GMP343 CO_2 probe, HMP75 rH/T probe; Vaisala). The increase in CO_2 concentrations in the chambers was measured in permanently installed PVC collars (5 cm height, two per lysimeter).

^{13}C pulse labelling. Allocation of assimilates was followed by ^{13}C pulse labelling in six randomly selected lysimeters ($n = 3$ per treatment) at the end of the drought and in eight lysimeters ($n = 4$ per treatment) 2 weeks after rewatering. Before labelling, the soil was covered with plastic foil to minimize diffusion of $^{13}CO_2$ into the soil. All trees in a given lysimeter were covered with a tall tent made of transparent plastic foil. The CO_2 concentration inside was reduced to 200 ppm by flushing the tent with CO_2-free air. The labelling lasted 2 h, during which time we added 100% CO_2 with a 50:50 ratio of $^{13}CO_2$ and $^{12}CO_2$. The CO_2 concentration was kept constant at about 1,500 ppm, which is above the saturation point for CO_2 uptake.

^{13}C analysis in leaves, mycorrhizal root tips, soil microbial biomass and soil-respired CO_2. Leaves from three or four trees per lysimeter were oven dried at 60 °C, milled and weighed into tin capsules for ^{13}C analyses. Mycorrhizal root tips and soil microbial biomass were randomly sampled in each lysimeter in the upper 10 cm soil depth by taking three soil cores with a diameter of 2 cm. Additional roots were taken directly from three or four trees. Vital mycorrhizal root tips were immediately collected under a stereomicroscope and kept at −70 °C until processing. They were pooled per lysimeter, oven dried at 80 °C, milled and weighed into tin capsules for ^{13}C analyses. Soil microbial biomass was determined using the chloroform fumigation extraction method, whereby the concentration and isotopic signature of extracted organic carbon from non-fumigated and fumigated samples were determined by oxidizing extractable carbon to CO_2[31] (ref. 30). The ^{13}C of microbial biomass was calculated as described previously[31]. The ^{13}C signature of soil-respired CO_2 was determined by the closed chamber method[32]. For each sample, the collars were closed with 7 cm tall PVC lids with cellular rubber and gas samples were taken after 15 min. In addition, ambient air close to the soil surface was collected at each sampling occasion.

In gas samples, the $\delta^{13}C$ values and the CO_2 concentration were analysed with a GasBench II coupled to a Delta V Plus mass spectrometer (ThermoFinnigan). The ^{13}C signatures in solid samples were measured with an Elemental Analyser (Euro EA, Eurovector) coupled to the mass spectrometer. The $\delta^{13}C$ value of soil-respired CO_2 was calculated as a mixture of ambient and soil-respired CO_2 sampled in the chamber[33]. The ^{13}C signal ($\Delta^{13}C$) in mycorrhizal roots was the difference between $\delta^{13}C$ values during and before labelling. The amount of ^{13}C assimilated by plants, in soil microbial biomass and in soil-respired CO_2 was estimated by first expressing the δ notations in atom% and then calculating the excess ^{13}C values considering each pool and flux size[11] (Supplementary Methods). The MRT for the ^{13}C soil efflux was calculated as described previously[11].

Analysis of metabolites. Metabolites were analysed according to previous studies[34,35]. In brief, frozen tissue was homogenized and extracted with 87% methanol. Phloem exudates were obtained as previously described[36], dried and redissolved in 87% methanol. Aliquots were derivatized and injected into a gas chromatography (GC)-quadrupole mass spectrometry (MS) system (GC, 7890A; MS, 5975C; Agilent Technologies). GCMS data were then deconvoluted, peak areas quantified and mass spectra identified according to ref. 35. Relative concentration changes were calculated as $\log_{10}$ ratios between drought and control treatments.

Statistical analysis. Data were analysed by fitting linear mixed effects models using maximum likelihood (lme function; nlme package, R version 3.1.2.)[37] (Supplementary Table 3). For the entire measurement period, season (dry, 22 May to 1 Aug, vs. wet, 2 Aug to 31 Oct), treatment (drought/post drought vs. control) and date of measurement were used as fixed effects and lysimeter and individual tree were included as random effects. The corAR1 function was included in the model to account for repeated measurements with a first-order autoregressive covariate structure. Treatment effects were additionally analysed for dry and wet season. To account for the varying ^{13}C signal in the consecutively labelled lysimeters, we included a co-variate as a fixed effect, thereby normalizing the ^{13}C tree uptake in each lysimeter to the treatment mean of the wet and dry season, respectively, which allowed us to consider the treatment-specific ^{13}C uptake by trees. In all final models, normality and homoscedasticity of the residuals were verified with diagnostic plots and the dependent variables were all log or square root transformed.

Received 22 February 2016; accepted 20 June 2016; published 18 July 2016

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Acknowledgements

We thank P. Bleuler for technical assistance and for maintaining the model ecosystem facility. We also thank B. Meier, R. Appenzeller, Y. Bicker, D. Brödlin, D. Christen, N. Hajjar, R. Köchli, F. Schreyer and A. Zürcher for their help with sampling and measurements, and F. Buegger, L. Schmidt and A. Schlumpf for completing the isotope analysis. We are grateful to M. Dawes for critically reading and providing helpful comments on the manuscript. This work was supported by funding from the Swiss Federal Institute for Forest, Snow and Landscape Research WSL and the Swiss Federal Office for the Environment FOEN.

Author contributions

M.A., R.S., F.H., M.S. and A.G. designed the experiments; R.S., F.H., J.J. and M.A. performed the ^{13}C pulse labelling; F.H., K.S. and M.A. measured seasonal CO_2 fluxes and chlorophyll fluorescence; M.A., M.P., K.P., U.G., R.K., V.M., S.E. J.L., J.J., M.W., R.S. and F.H. analysed ^{13}C allocation patterns; A.G., J-F.L. and M.L. analysed metabolites; J.J. and F.H. performed statistical analysis; A.G., J.J., F.H. and M.A. wrote the manuscript.

Additional information

Supplementary information is available online. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.A.

Competing interests

The authors declare no competing financial interests.

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SUPPLEMENTARY INFORMATION

DOI: 10.1038/NPLANTS.2016.111

Supplementary Figure 1: Field-based open top chamber/lysimeter with beech model ecosystems during the ^{13}C pulse labelling. The chambers have a height of 3.5 m and a plantable area of 3 m^2 for each of two lysimeters inside the chamber (only one lysimeter used for the experiments). The beech trees were up to 2.5 m tall at the time of the experiments.

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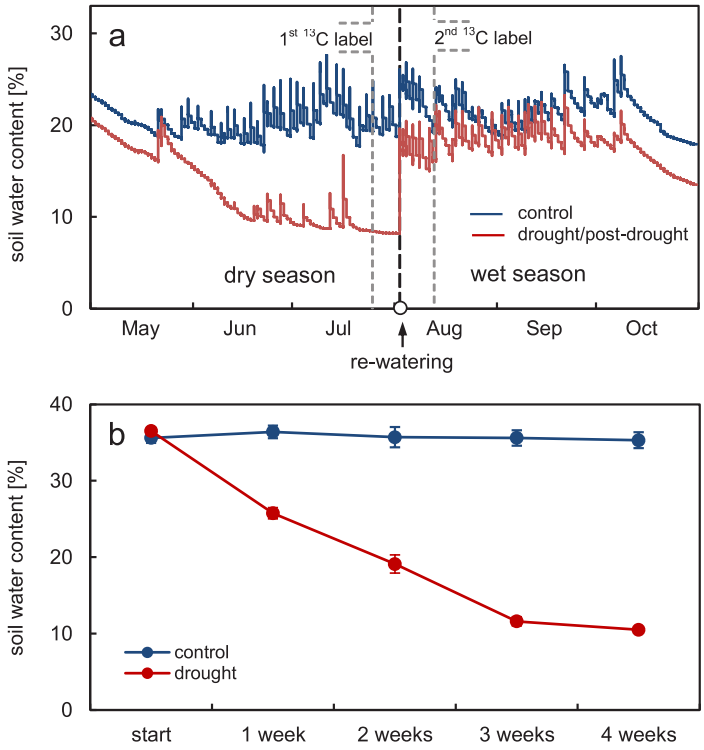

Supplementary Figure 2: Drought development in the model ecosystem and in the pot experiment. **a,** soil moisture in the model ecosystems measured volumetrically with soil moisture probes (5TM, Decagon, USA) at 10 cm soil depth in drought and re-watered ecosystems compared with the regularly watered controls (means ± SE, n = 8). Soil moisture data are taken from[39]. **b,** soil moisture measured gravimetrically in the pot experiment (means ± SE, n = 5).

39 Arend, M., Sever, K., Pflug, E., Gessler, A. & Schaub, M. Seasonal photosynthetic response of European beech to severe summer drought: Limitation, recovery and post-drought stimulation. *Agr. Forest Meteorol.* **220,** 83-89 (2016).

DOI: 10.1038/NPLANTS.2016.111

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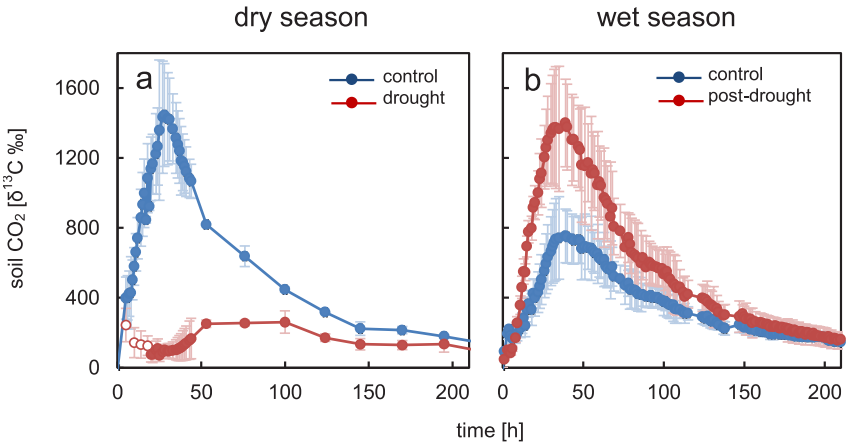
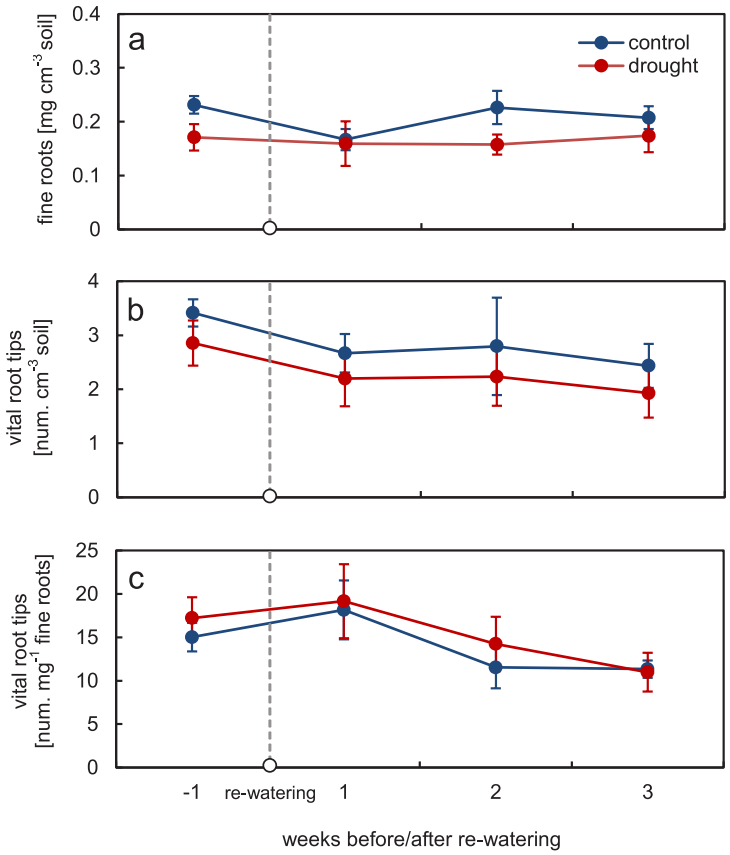


Supplementary Figure 3: High resolution measurements of soil $^{13}CO_2$. The soil CO_2 $\delta^{13}C$ signature at 10 cm soil depth was measured continuously *in situ* at a frequency of 1Hz CO_2 in soil air using an Off-Axis Integrated Cavity Output Spectrometer OA-ICOS (LGR-CCIA 36-d, LosGatos Research Ltd). Data were recorded during the two $^{13}CO_2$-pulse labelling experiments at **a,** the end of the drought period and **b,** after drought release (means ± SE, n = 3 for dry season and n = 4 for wet season). Soil gas was drawn into the OA-ICOS from gas permeable and hydrophobic membrane tubes (Accurel® tubings, 8mm OD and 40 cm length) placed horizontally in the soil at 10 cm depth. The mean residence time of ^{13}C was calculated as previously described[11]. The high resolution measurements show that the sealing of the labelling tent against the soil with plastic foil was effective, as no or only very small initial $^{13}CO_2$ peaks, which would indicate diffusion of $^{13}CO_2$ directly into the soil air, were observed.

SUPPLEMENTARY INFORMATION DOI: 10.1038/NPLANTS.2016.111



Supplementary Figure 4: Quantity/quality of fine roots before and after re-watering. Fine roots were collected from soil cores taken in the upper soil layer (0-10 cm) of drought/re-watered and control model ecosystems. **a,** the mass of fine roots per soil volume; **b,** the frequency of vital mycorrhizal root tips per soil volume; and **c,** the number of vital mycorrhizal root tips per fine root mass. Data analysis yielded no statistically significant differences between treatments (means ± SE, n = 4-8 lysimeters).

DOI: 10.1038/NPLANTS.2016.111

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Supplementary Table 1: Concentrations of main carbohydrates in leaves under drought and after drought release. Sugars, starch and non-structural carbohydrates (sum of soluble sugars and starch) in beech leaves were analysed as described in[40] (% leaf dry weight; mean ± SE, n = 8).

40 Li, M.-H., *et al.* Responses of leaf nitrogen and mobile carbohydrates in different *Quercus* species/provenances to moderate climate changes. *Plant Biol.* **15**, 177-184 (2013).

	dry season (Jul 31th)		wet season (Sep 19th)	
	control	drought	control	post-drought
sugars	11.65 ± 0.39	14.59 ± 0.33	13.27 ± 0.19	13.00 ± 0.31
starch	4.93 ± 0.34	3.64 ± 0.32	2.65 ± 0.21	3.00 ± 0.39
NSC	16.57 ± 0.46	18.23 ± 0.43	15.89 ± 0.25	16.00 ± 0.45

SUPPLEMENTARY INFORMATION DOI: 10.1038/NPLANTS.2016.111

Supplementary Table 2: Physical and chemical characteristics of the acidic soil type. Data are taken from a previous study[38] (with permission of the publisher Wiley-Blackwell).

41 Kuster, T.M., Arend, M., Bleuler, P., Günthardt-Goerg, M.S., Schulin, R. Water regime and growth of young oak stands subjected to air warming and drought on two different forest soils in a model ecosystem experiment. *Plant Biol.* **15**, 138-147 (2013).

texture (% sand, silt, clay)	87, 8, 5
pH (0.01 M $CaCl_2$)	4.0
C_{tot} (%)	0.48
N_{tot} (%)	0.03
P_{tot} (mg kg^{-1})	469
$Ca_{exch.}$ (mg kg^{-1})	142
$Mg_{exch.}$ (mg kg^{-1})	9.5
$K_{exch.}$ (mg kg^{-1})	19.0
$Mn_{exch.}$ (mg kg^{-1})	18.6
CEC (mmol kg^{-1})	24.1
base saturation	36.7

DOI: 10.1038/NPLANTS.2016.111

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Supplementary Table 3: Statistical analysis of seasonal flux and ^{13}C pulse labelling data. The applied linear mixed effects models were adapted to the experimental design and data structure of each measurement.

	seasonal flux		^{13}C pulse labelling			
	A_N	R_S	leaf uptake	mycorrhizal roots	soil microbial biomass	soil respiration
treatment x season	F = 228.6 [(a)] P < 0.001	F = 82.7 [(a)] P < 0.001	F = 8.1 [(b)] P = 0.017	F = 14.5 [(c)] P = 0.004	F = 22.4 [(c)] P = 0.001	F = 26.5 [(d)] P < 0.001
treatment effect dry season	F = 160.1 [(a)] P < 0.001	F = 160.1 [(a)] P < 0.001	F = 13.3 [(b)] P = 0.021	F = 3.78 [(c)] P = 0.147	F = 23.6 [(c)] P = 0.017	F = 30.6 [(d)] P = 0.012
treatment effect wet season	F = 6.57 [(a)] P = 0.026 recovery period ≥ 7 days [(e)]	F = 11.8 [(a)] P = 0.009 recovery period < 3 days [(e)]	F = 0.08 [(b)] P = 0.78	F = 8.73 [(c)] P = 0.032	F = 0.15 [(c)] P = 0.71	F = 8.2 [(d)] P = 0.036

(a) repeated measurements, all data from dry season (drought *vs*. control) and/or wet season (post-drought *vs*. control) after full recovery
(b) initial plant uptake after ^{13}C pulse labelling
(c) repeated measurements during ^{13}C pulse labelling
(d) total fluxes integrated during ^{13}C pulse labelling
(e) time period after re-watering with significant differences between control and treatment (Student's t-test with P < 0.05 after FDR correction for multiple comparisons)

SUPPLEMENTARY INFORMATION DOI: 10.1038/NPLANTS.2016.111

Supplementary Table 4: Allocation of C to tree growth in the drought-treated and control model ecosystems. The amount of carbon allocated to tree growth (g C per tree) was derived from measurements of the stem diameter (10 cm above ground) before the start of the dry season, end of the dry season and end of the wet season (means ± SE, n = 8). The corresponding tree biomass was estimated using a biomass/diameter correlation with the equation $y = -0.036x^3+2.695x^2-28.114x+102.61$ and then converted to C using the molar ratio of 12/30 for C/CH_2OH[10].

10 Klein, T. & Hoch, G. Tree carbon allocation dynamics determined using a carbon mass balance approach. *New Phytol*. **205,** 147-159 (2015).

	dry season	wet season	total
control	32.2 ± 1.1	13.3 ± 0.7	45.5 ± 1.9
drought / post-drought	16.9 ± 0.7	9.7 ± 1.1	26.6 ± 1.8
rel. reduction by drought	-47.6 %	-27.0 %	-41.6 %

DOI: 10.1038/NPLANTS.2016.111

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Supplementary Methods:

Design of the model ecosystem and pot experiment

The model ecosystem experiment was conducted in the model ecosystem facility of the Swiss Federal Institute for Forest, Snow and Landscape Research (WSL). The facility consists of 16 large model ecosystems in field-based open top chambers, each with a height of 3.5 m. The systems are equipped with automated irrigation systems and sliding roofs closing automatically at the onset of rainfall. Below ground, each system is split into two lysimeters with an area of 3 m^2 and a depth of 150 cm, one filled with an acidic soil and one filled with a calcareous forest soil (calcareous soil not considered for this experiment). In spring 2011, 24 saplings with a height of about 20 cm were transplanted to each lysimeter. From May to October, natural precipitation was excluded and the systems were irrigated every second or third day with 67 l m^{-2} deionized water enriched with nutrients to simulate the average composition of ambient rainfall. During hot summer periods, the irrigation frequency was increased to counterbalance higher rates of evapotranspiration and hold the soil moisture at 10 cm soil depth above 20%. In 2014, when the trees had reached a height of up to 2.5 m, a summer drought was implemented in half of the systems by withholding irrigation from 22 May to 1 August. As evapotranspirational water loss was particularly high on hot days, a few intermediate irrigation pulses were applied to prevent the soil from drying too rapidly or intensely and to avoid irreversible drought damage of the trees. The water supply during the drought period was reduced by 78% compared to controls. After the first saplings reached predawn water potentials below -2.0 MPa, the systems were re-watered for 1 day with 200 l m^{-2} and afterwards regularly irrigated as described above.

The pot experiment was carried out with 2-year-old beech saplings in 5.5 l pots (one plant per pot). The trees were on average 56 cm tall and average total dry weight at the time of harvest was 25 g. The seeds originated from the Black Forest growth region in SW-Germany, which is close to the origin of the beech trees in the model ecosystem experiment. Plants were grown in a greenhouse with temperatures of 20°C/17°C (day/night). The photosynthetic photon flux density was kept at 600 µmol m^{-2} s^{-1} or greater at the upper level of the canopy by supplemental illumination and the light period was adjusted to 16 h. During the drought treatment lasting 4 weeks, the control pots were watered to field capacity while pots with the drought treatment received no water at all. Five plants per treatment were harvested before drought and 1, 2, 3 and 4 weeks after the onset of drought. Fine root, leaf and phloem samples were taken.

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Calculations of ^{13}C fluxes with the closed chamber method

The $\delta^{13}C$ values of soil respired CO_2 ($\delta^{13}C_{respired}$) were calculated as a mixture of ambient and soil-respired CO_2 sampled in the chamber:

$$\delta^{13}C_{respired} = \frac{(\delta^{13}C_{chamber} \times CO_{2-chamber} - \delta^{13}C_{ambient} \times CO_{2-ambient})}{(CO_{2-chamber} - CO_{2-ambient})} \quad (1)$$

where $\delta^{13}C_{chamber}$ and $\delta^{13}C_{ambient}$ are the measured isotopic ratios of CO_2 in the soil chamber and in ambient air, respectively, and $CO_{2\text{-}chamber}$ and $CO_{2\text{-}ambient}$ are the corresponding CO_2-concentrations.

The amount of ^{13}C assimilated by plants, in soil microbial biomass and in soil-respired CO_2 during pulse labelling was estimated by first expressing the δ notations in atom% as follows:

$$\text{atom\%} = \frac{100 \cdot 0.0111802 \cdot \left(\frac{\delta}{1000}+1\right)}{1+0.0111802 \cdot \left(\frac{\delta}{1000}+1\right)} \quad (2)$$

(0.0111802 is the standard value for the isotope ratio of the Vienna Pee Dee Belemnite, V-PDB).

Finally, we calculated excess ^{13}C values using the equation:

$$\text{excess}^{13}\text{C} = \frac{(\text{atom\%}_{tx} - \text{atom\%}_{t0})}{100} \cdot \text{B} \quad (3)$$

with excess ^{13}C being the total amount of ^{13}C in each plant compartment, in microbial biomass (in mg ^{13}C m^{-2}) or in soil CO_2 efflux (mg $^{13}CO_2$-C $m^{-2}h^{-1}$) originating from the pulse-labelling; $atom\%_{tx}$ is the atom% of the sample taken at time x; $atom\%_{t0}$ is the atom% in each chamber before the labelling; B is the pool size (g C m^{-2}) or the CO_2 efflux (mg CO_2-C $m^{-2}h^{-1}$).

7.2 II: Does belowground interaction with *Fagus sylvatica* increase drought susceptibility of photosynthesis and stem growth in *Picea abies*?

Forest Ecology and Management, 375, 268-278. doi: 10.1016/j.foreco.2016.05.032.

Year: 2016

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The following article reports results from the experimental site Kranzberger Forst one year before the first throughfall exclusion treatment (2013) investigating drought susceptibility of Norway spruce when neighbouring other Norway spruce or European beech trees. It hast been frequently observed that mixed stands of European beech and Norway spruce over-yield, when compared to respective monospecific stands. Over-yielding is attributed to enhanced resource uptake efficiency through niche complementarity alleviating species competition, for example through enhanced root stratification in mixture. Mixture effects at the organ (leaf, fine root), tree and stand scale were analysed in a mature forest with European beech-Norway spruce group mixture. Under inter-specific conditions fine-root production and depth of water uptake of Norway spruce shifted to shallow, drought-prone soil horizons. Overall, lowered fine root production and ramification along with a reduction in long-distance ectomycorrhizal exploration types resulted in decreased soil exploitation in Norway spruce when growing together with European beech. Drought sensitivity of Norway spruce was exemplified by a distinct decrease in stomatal conductance, net CO_2 uptake rate and stem growth during periods of natural water limitation. Nevertheless, species interaction effects were absent in leaf gas exchange and stem diameter growth, during a natural six-week summer drought period in 2013 as well as in the extremely dry year of 2003. These findings may result from seasonal shifts between positive and negative effects of European beech neighbourhood on soil water availability for spruce and the group-wise mixture pattern, where spruce is exposed to competition with beech only along group edges. The results suggest, compared to single tree mixture, group-wise mixture of beech and spruce to be a favourable silvicultural option in the face of climate change.

Forest Ecology and Management 375 (2016) 268–278

Contents lists available at ScienceDirect

Forest Ecology and Management

journal homepage: www.elsevier.com/locate/foreco

Does belowground interaction with *Fagus sylvatica* increase drought susceptibility of photosynthesis and stem growth in *Picea abies*?

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ARTICLE INFO

Article history:
Received 24 March 2016
Received in revised form 20 May 2016
Accepted 21 May 2016

We would like to dedicate this work to Prof. Dr. Ulrich Lüttge on the occasion of his 80th birthday.

Keywords:
European beech
Norway spruce
Mixed forest
Competition
Drought
Growth

ABSTRACT

Mixed stands of European beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) Karst.) frequently over-yield, when compared to respective monospecific stands. Over-yielding is attributed to enhanced resource uptake efficiency through niche complementarity alleviating species competition, for example through enhanced root stratification in mixture. Under severe and frequent summer drought, however, water limitation may become crucial in modifying the prevailing competitive interaction in mixed beech-spruce forests. We hypothesize, therefore, that under drought (H I) inter-specific interaction with beech reduces water accessibility for spruce more than intra-specific conditions, thus (H II) exacerbating drought susceptibility of spruce in terms of reduced photosynthesis and stem growth. Reactions at the organ (leaf, fine root), tree and stand scale were analysed in a mature forest with beech-spruce group mixture. Under inter-specific conditions spruce's fine-root production and depth of water uptake (assessed via $\delta^{18}O$ of xylem water) shifted to shallow, drought-prone soil horizons, in agreement with H I. Overall, lowered fine root production and ramification along with a reduction in long-distance explorative ectomycorrhizal types resulted in decreased soil exploitation in spruce when growing together with beech. Spruce's drought sensitivity was exemplified by a distinct decrease in stomatal conductance, net CO_2 uptake rate and stem growth during periods of water limitation. Notwithstanding, species interaction effects were absent in leaf gas exchange and stem diameter growth, during a six-week summer drought period in 2013 as well as in the extremely dry year of 2003, hence rejecting H II. Based on results from soil moisture measurements and water uptake depth, we interpret the conflicting findings for H I and H II to result from: (i) seasonal shifts between positive (during spring drought) and negative (during summer drought) effects of beech neighbourhood on soil water availability for spruce, possibly overriding each other in their effect on annual stem diameter growth and (ii) the group-wise mixture pattern, where spruce is exposed to competition with beech only along group edges, i.e. laterally only, so that the putatively adverse beech effect on water accessibility stays limited. Our results suggest, compared to single tree mixture, group-wise mixture of beech and spruce to be a favourable silvicultural option in the face of climate change.

1. Introduction

Mono-specific Norway spruce plantations (*Picea abies* [L.] KARST.), widely promoted outside their natural distribution in Central Europe (Löf and Oleskog, 2005), have proven to be highly susceptible to biotic and abiotic stresses (Albrecht et al., 2010; Neuner et al., 2015; Rouault et al., 2006). Conversely, mixed-stands that include European beech (*Fagus sylvatica* L.) appear to warrant ecological and socio-economic services to extents similar, or even higher, than monocultures of either species (Ammer et al., 2008; Knoke et al., 2008, 2005; Pretzsch and Schütze, 2009; Pretzsch et al., 2010). The mean periodic stand growth of mixed-stands of Norway spruce and European beech and neighbouring monocultures of both species can be used for quantifying the mixing effects on growth. If the growth of the mixed-species stands equals the weighted mean of the two monocultures this indicates an additive mixing effect, i.e., the behaviour

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http://dx.doi.org/10.1016/j.foreco.2016.05.032
0378-1127/

of the mixed stand can simply be derived from the respective monocultures (Forrester and Pretzsch, 2015). In case the mixed stand's productivity exceeds the weighted mean of the monocultures this indicates a multiplicative mixing effect, i.e., species interactions result in an over-yielding of mixed versus mono-specific stands. Frequently found over-yielding of mixed beech-spruce stands may be attributed to niche complementarity of the two species, fostering resource capture efficiency rather than competition (Pretzsch and Schütze, 2009; Pretzsch, 2014; Pretzsch et al., 2012). Consistently, over-yielding in mixed beech-spruce stands is found in particular on nutrient-poor sites (Pretzsch et al., 2010). Over-yielding per se can increase temporal stability of stand-level growth rate (Jucker et al., 2014a). Such growth responses are in line with broad evidence on the positive effects of species richness on ecosystem functioning in natural species communities (Cardinale et al., 2012; Gamfeldt et al., 2013; Lehman and Tilman, 2000). Nevertheless, despite stabilizing effects of species richness on aggregate community properties, e.g. whole stand productivity, inter-specific competition may destabilize individual species populations (Lehman and Tilman, 2000; Loreau and de Mazancourt, 2013). In mixed spruce-beech forests, climate warming will likely modify competition through increasing water limitation (cf. Pretzsch et al., 2012). Most likely are substantial changes in precipitation and temperature, on global but also on a regional scale (e.g. IPCC, 2013, 2007; KLIWA, 2006). Along with distinctly differing temporal variation in annual precipitation (KLIWA, 2006) both, lengths and frequency of climate extremes such as drought may increase severely (Easterling et al., 2000; Jonas et al., 2005; Meehl et al., 2000) and hence strongly influence growth, stability of forests (Fuhrer et al., 2006). For example, reduced rain interception and enhanced stem run-off in beech as compared with spruce, positively affects soil water recharge in mixed beech-spruce systems as compared to pure spruce stands (Augusto et al., 2002; Schume et al., 2004), however, such effects become less important during prolonged periods without precipitation. In fact, higher productivity of mixed forest systems may be linked to an overall higher water demand (Forrester, 2015), resulting in increased drought stress during dry periods (Forrester, 2015; Gebauer et al., 2012; Grossiord et al., 2014a,b), hence endangering drought sensitive tree species within the community (Gebauer et al., 2012; Grossiord et al., 2014b; Jucker et al., 2014b; Maestre et al., 2009). Consistently, Schume et al. (2004) demonstrated faster and more intense (e.g. reaching deeper depths) soil water depletion during summer drought under mixed beech-spruce than under pure beech or spruce stands. In response to belowground interaction with beech, spruce's root system growth shifted vertically towards more shallow soil depths (Bolte and Villanueva, 2006; Schmid and Kazda, 2001; Schume et al., 2004). In addition, the rather conservative strategy of spruce regarding only limited adjustments of fine root morphology (maintaining or even increasing specific fine root length; Bolte and Villanueva, 2006; Grams et al., 2002) in response to drought in belowground competition with beech, implies disadvantages in water exploitation when competing with beech (Bolte and Villanueva, 2006; Schmid, 2002).

In addition to roots, mycorrhizae function in water uptake. Fine roots of both tree species are associated with ectomycorrhizal (ECM) fungi. With respect to their potential to take up water and nutrients by their external mycelium, ectomycorrhizae have been categorized as exploration types (contact, short- and medium-distance and long-distance types, cf. Agerer, 2001). Long-distance types have the potential to retrieve and transport water via distinct rhizomorphs thus may be effective in mitigating drought stress (Lehto and Zwiazek, 2011). However, under drought there may be a trade-off between carbon-costs for building and maintaining long-distance types (Weigt et al., 2011) and reduced carbon supply from drought stressed trees which would lead to a relatively lower abundance of long-distance exploration types in carbon limited spruce compared to beech. Thus under drought, resource availability as determined by soil water content (root distribution), and carbon supply via photosynthesis (anisohydric, isohydric strategy) may influence ECM exploration types differently in mixed interspecific vs. intraspecific situations, respectively.

In view of predicted, exacerbating summer droughts (IPCC, 2013, 2007), basic knowledge about competitive versus facilitative interactions in mature mixed beech-spruce forests is scarce, impeding silvicultural mitigation strategies. In the present study, we therefore hypothesized that under drought (H I) inter-specific interaction with beech reduces water accessibility for spruce more than intra-specific conditions, thus (H II) exacerbating drought susceptibility of spruce in terms of reduced photosynthesis and stem growth. The hypotheses are evaluated based on growth and physiological parameters indicative for stress reactivity in both tree species ranging from ectomycorrhizal exploration types to leaf gas exchange and whole-tree growth dynamics in a mature, group-wise mixed beech-spruce forest. To this end, data originating from a summer drought during 2013 and a retrospective analysis on effects of the distinct drought year 2003 are employed.

2. Materials and methods

2.1. Site description and climatic conditions

The study was conducted in a maturing mixed stand of European beech (*F. sylvatica* L.) and Norway spruce (*P. abies* [L.] KARST.) within Kranzberg Forest (FRE 813/1), located in southern Germany/Bavaria (11°39′42″E, 48°25′12″N; 490 m a.s.l.), approximately 35 km north-east of Munich. The mixed stand consists of large groups of beech (4 groups with 150–200 m^2 each) surrounded by spruce (in 2013: spruce 62 ± 2, beech 82 ± 4 years old). For the age series FRE 813 which includes the Kranzberg Forest experiment (FRE 813/1) the long-term over-yielding at the stand level amounts to 1.18 (Pretzsch et al., 2010). Under normal conditions the mixed stand is by 18% more productive than the weighted mean of the two monocultures; both Norway spruce and European beech contribute approximately the same to this over-yielding. In 2010 twelve plots were established with a total area of 1730 m^2 with a mean stocking density of 659 trees per ha and mean basal area of 52 m^2 per ha. The plots include 63 beech with a mean height of 26.1 m and a mean diameter of 28.9 cm at breast height and 53 spruce trees with a mean height of 29 m and a mean diameter of 34.3 cm at breast height. The detailed stand characteristics of the 12 plots are summarized in the supplementary material, Table S 1.

All measurements were carried out within the central area of each plot, comprising the transition between intra-specific spruce (S) and intra-specific beech (B) forming an inter-specific contact zone (MIX). Trees in the intra-specific zones are referred as SS and BB and trees in the inter-specific zone as SB and BS for spruce and beech respectively (cf. Fig. 1). For the present study, all measurements were carried out within the central area of each plot. Soil is a luvisol developed from loess over Tertiary sediments (eutric cambisols, FAO classification). The average annual precipitation (1971–2000) is 785 mm yr^{-1}, with 497 mm during the growing season. The annual mean temperature is 7.8 °C, with 13.8 °C on average during the growing season (for details see Pretzsch et al., 2012). The present study focused on three climatically different years: (i) 2003 with extraordinarily low precipitation and high air temperatures during the growing season throughout Central Europe (Ciais et al., 2005), (ii) 2012 represented by a warm growing season with average precipitation and

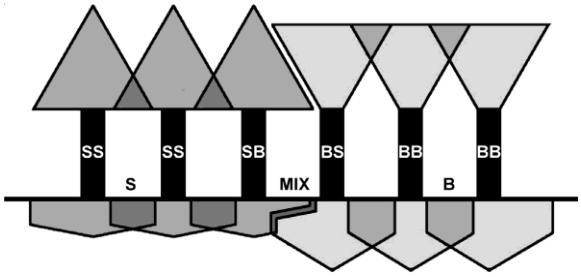

Fig. 1. Schematic illustration of trees with intra- (SS and BB) and inter-specific (SB and BS) competition in the different zones of species interaction (S, MIX and B).

(iii) 2013 with a distinct dry spell in mid/late summer paralleled by high air temperatures (Table 1).

2.2. Soil moisture

Soil moisture (i.e. volumetric soil water content, SWC) was measured via time domain reflectometry (TDR 100, Campbell Scientific, Inc., Logan, Utah, USA). Depending on installation, probe signal integrated SWC either over a soil depth of 0–7 cm or 10–30 cm. At each depth, one TDR probe was installed within each of the three interaction zones of beech and spruce (B, S, and MIX) on each of the twelve plots (n = 12; $n_{total} = 2 \times 3 \times 12 = 72$). Sensor signals of all probes were assessed either monthly during November – April, or weekly during May – October.

2.3. Fine root observation

In 2010, clear acrylic minirhizotron tubes (70 cm long, 6 cm outside diameter) were installed at an angle of 60° from the horizontal to a depth of 60 cm (51 vertical cm). A vertical depth of approximately 50 cm was chosen based on previous research at Kranzberg Forest, as >90% of beech and spruce roots being between 0 and 50 cm (Häberle et al., 2012). Each plot contained six minirhizotron tubes: two in each S and B region, and two within MIX. Each tube was located a minimum distance of one meter from the plot boundaries, and in the case of inter-specific regions, tubes were installed equidistantly from both species. Before installation, minirhizotron tubes were capped at the base with plastic plugs lined with silicon caulk to reduce water infiltration. Tubes, when not in use, were covered with large plastic caps to prevent aboveground water infiltration and light penetration. Beginning in May 2011 and ending in October 2013, contiguous images were taken across the length of each tube using a specialized laparoscopic camera (BTC100X Camera, Bartz Technology, Carpinteria, California). Just prior to leaf emergence (April) and until leaf senescence (November), images were taken every 10–15 days. During the winter months, images were taken monthly. Images were approximately 15 mm in height, and 18 mm wide. All images were analysed for the depth of fine root production and morphology using *WinRHIZO Tron MF* (Regent Inc., Quebec, Canada). Roots that transected more than one observation window were noted and only counted once. Differences between species' roots were determined by visual inspection of epidermal coloration (spruce: brown, beech: reddish white), along with root tip branching patterns (spruce: alternate branching, beech: herringbone and often opposite branching). Root production was calculated on a per plot basis as the total number of root tips produced per square meter of viewing window.

Table 1
Precipitation (P) and air temperature (Tair) in 2003, 2012 and 2013 in comparison with the long-term average of 1971–2000. Indexes indicate sums/means based on different periods of the respective year, A: annual, GS: growing season April – September, S: mid/late summer July – August. Bold numbers indicate significant differences from long-term average: sum/mean is below, –, or above, +, the 99% confidence interval of the respective long-term average. Data from Deutscher Wetterdienst (DWD) station "Weihenstephan-Dürnast" (station ID 5404, at about 3 km distance to the study site, 477 m a.s.l.).

	1971–2000	2003	2012	2013
P_a	785 (±88)	524 (**–261**)	786 (+1)	766 (–17)
T_a	7.9 (±0.7)	8.7 (**+0.8**)	8.8 (**+0.9**)	8.4 (**+0.5**)
P_{vp} (Apr-Sept)	497 (±72)	293 (**–204**)	509 (+12)	495 (–2)
T_{vp} (Apr-Sept)	13.5 (±0.7)	16.0 (**+2.5**)	14.9 (**+1.4**)	14.5 (**+1.0**)
P_s (Jul-Sept)	194 (±51)	107 (**–87**)	202 (+8)	97 (**–97**)
T_s (Jul-Sept)	16.8 (±0.9)	19.9 (**+3.1**)	18.5 (**+1.7**)	19.0 (**+2.2**)

2.4. Root and mycorrhiza sampling

The sampling campaign was carried out on the 7th and 8th of October 2013. Soil was sampled with a corer of 4 cm diameter to a depth of 25 cm. In each plot two soil cores were retrieved for S and B, but four at the MIX position. The uppermost litter layer, consisting of recently fallen leaves was removed before sampling. Each soil core was separated into an upper organic soil part ($O_{f+h}A_h$, average depth of 0–8.6 cm), in the following referred to as upper soil (UP) and a lower mineral soil part (A_lB_v, average depth of 8.6–25 cm), in the following referred to as lower soil (LO). Within each plot, two samples from each interaction zone and depth were pooled in a plastic bag and immediately stored on ice resulting in a total of eight composite soil samples per plot. Root and soil samples were stored for not longer than 4 weeks in the laboratory at 4 °C until further process. Root samples were manually separated from soil, cleaned in tap water and sorted under a microscope into beech and spruce roots. Fine roots (<1 mm diameter) were cut into pieces of 2 cm length and representative subsamples were taken for analysis of ectomycorrhizal morphotype abundances. Vital mycorrhizal tips were assigned to morphotypes based on similarities of colour and surface properties of the mycorrhizal mantle. We further used the concept of exploration types to categorize the morphotypes according to the extent to which hyphae emanating from the ECM surface exploit the soil as contact, short distance, medium distance smooth, long-distance types according to Agerer (2001).

After morphotyping the fine roots were spread on an acrylic glass trough, filled with water to submerge the roots. A flexible plastic slide of the same format was put on top of the thin water film to fix the roots at the same level by adhesion, taking care of avoiding air bubbles. Roots were then scanned (Epson Perfection 4990 Photo) with a resolution of 1200 dpi at 8 bit greyscale in TIF format. Analysis of the scans was done with the software WinRHIZO (Regent Instruments Inc., Canada). Scaled paper was used to calibrate the Software, background distinction and debris removal was performed manually.

2.5. Depth of water uptake: sampling and ^{18}O-analysis of soil and xylem water

Beech and spruce xylem as well as soil cores were sampled on the same day in late July 2012 from plots 1–8 (accessible through canopy crane), to interpolate the mean depth of water uptake of tree individuals from the instantaneous $\delta^{18}O$ gradient in soil water and $\delta^{18}O$ of xylem water (cf. Allison et al., 1983; Craig, 1961; Dansgaard, 1964; Dawson, 1993; White et al., 1985). At the time of sampling SWC was close to field capacity. Three soil cores were taken per plot to a depth of 60 cm with a hand soil probe (core diameter 2 cm). In each of the eight sampled plots one soil core was collected at B, S and MIX (n = 8; $n_{total} = 3 \times 8 = 24$). From each soil core, 3 cm sub-samples were taken from four different depths (5 cm, 10 cm, 20 cm, 50 cm). On each plot, twig xylem (twig

sections of approx. 10 cm length and 0.5 cm diameter, bark removed during sampling) from the upper crown was sampled on one intra-specific and one inter-specific beech and spruce tree respectively. Soil and xylem samples were stored in air tight tubes immediately after sampling to prevent evaporation. Samples were stored at −20 °C until further processing. Subsequently, soil matrix water and xylem water was extracted via cryogenic vacuum distillation (cf. Ehleringer and Osmond, 1998) and analysed for their $\delta^{18}O$ signature with an isotope-ratio mass spectrometer (IsoPrime, GV Instruments Ltd., Manchester, UK; MultiFlow 222XL, Gilson Inc., Middleton, USA). Mean depth of water uptake was inferred from the intersection of $\delta^{18}O$ of xylem water and the spline interpolation of the vertical $\delta^{18}O$ profile of soil water.

2.6. Leaf gas exchange

Leaf gas exchange was assessed on one intra-specific and one inter-specific tree per species on each plot accessible by the canopy crane (n = 8), during three campaigns in 2012 (June, July/August and September) and two campaigns in 2013 (July/August and September). Measurements were carried out on sunny days between 9:00 and 14:00 CET within the crown, using sun-exposed, fully developed leaves of beech and one-year-old needles of spruce, by means of a portable infrared gas analyser (LICOR 6400, LI-COR Inc., USA) using appropriate chambers for the respective leaf types (broad leaves: 6400-02B LED light source; conifer needles: 6400-05 Conifer Chamber, LI-COR Inc., USA). Measurements were conducted at saturating photosynthetic active photon flux density ($PPFD_{SAT}$) >1300 µmol photons m^{-2} s^{-1}, stable CO_2 concentration of 400 ppm, ambient air temperature and humidity. At each time, measurements were repeated on three pre-selected positions within the sun-exposed crown of the respective tree individual. Gas exchange of spruce was measured on 5 cm long twig sections, averaging approximately 115 needles. In the course of the measurement campaigns in late summer, needles of each measured twig section were harvested and immediately scanned (Epson Perfection 4990 Photo, Epson Deutschland GmbH, Meerbusch, Deutschland) to determine the projected needle surface area. Considering the stomatal distribution in the leaf epidermis (spruce polydirectional, beech unidirectional), gas exchange parameters are expressed on a total needle surface area basis for spruce (conversion factor projected leaf area to total surface area: 3.2; cf. Niinemets and Kull, 1995; Perterer and Körner, 1990) and on a projected leaf area basis in beech.

2.7. Carbon stable isotope composition of leaf bulk material

In parallel with gas exchange measurements, samples of adjacent leaves and one-year-old needles were taken for stable isotope analysis, immediately stored in a cooling box at <4 °C and subsequently dried and ball-milled to a homogenous fine powder. $\delta^{13}C$ of organic leaf matter ($\delta^{13}C_{LOM}$) was determined by mass spectrometry (GVI-Isoprime, Elementar, Hanau, Germany coupled to the elemental analyser EA 3000, Euro Vector, Milan, Italy).

2.8. Tree growth performance

For the analysis of tree growth performance was conducted at the basis of long time series of stem diameter measurements (Astralon D1-K permanent tree girth tapes with Pi-units and vernier scale). Stem diameter data are recorded at the Kranzberg Forest up to 10 times per year since 1997. However, only at 9 out of the 12 plots girth tapes have been installed. Based on these measurements annual basal increments (BAI, cm^2 yr^{-1}) were calculated. To eliminate the influence of tree age on the annual BAI values 7-year moving averages were applied. The mean diameter of the spruce trees in 2014 was 37.7 cm (min: 23.2 cm; max: 48.8 cm) while for the beech trees the average was 32.7 cm (min: 20.5 cm; max: 52.3 cm). Indices for resistance, recovery, and resilience, i.e. Rt, Rc, and Rs, respectively, were assessed as detailed by Lloret et al. (2011) and calculated individually for one intra-specific and one inter-specific tree per species on 9 plots (n = 9 for each SS, SB, BS and BB) based on the basal area increment (BAI, cm^2 yr^{-1}). 7-year moving averages were employed for:

$$Rt = Dr/PreDr \quad (1)$$

$$Rc = PostDr/Dr \quad (2)$$

$$Rs = PostDr/PreDr \quad (3)$$

PreDr is the index of BAI during 2001/2002 before drought, Dr of BAI during the 2003 drought, and PostDr of BAI during after-drought in 2004/2005. Rt quantifies the decrease from pre-drought to drought, with Rt = 1 denoting unrestricted resistance (otherwise Rt < 1). Rc covers post-drought, with Rc = 1 indicating persistence at low growth (otherwise Rc < 1 denoting decline, but Rc > 1 recovery from drought). Rs represents the ratio between post-drought and pre-drought increment, with ⩾1 for recovery,

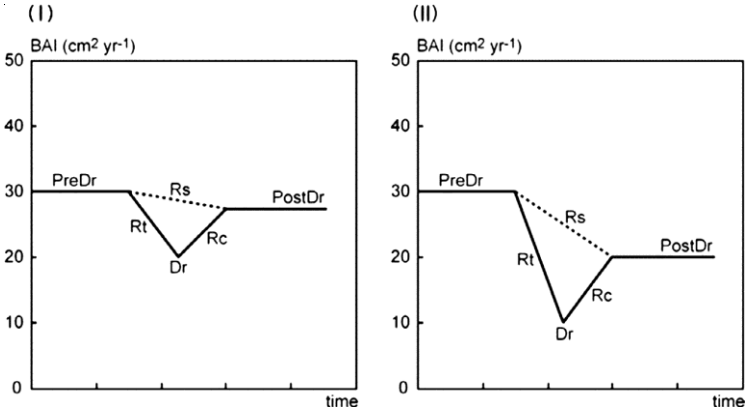

Fig. 2. Course of growth in two different stress events characterized by the growth in the period before drought, PreDr, growth during the drought period, Dr, and growth after the drought period, PostDr (modified after Lloret et al. (2011)). (I) Tree with limited growth decline in response to drought indicated by high resistance and resilience, and medium recovery. (II) Tree with strong growth decline indicated by low resistance, considerable recovery which results in a medium resilience. In the graphs Rt, Rc, and Rs are represented by the gradient of decline from PreDr to Dr, the increase from Dr to PostDr, and the difference in level of PreDr and PostDr, respectively.

but <1 for decline and low resilience. Fig. 2 exemplifies Rt, Rc, and Rs for moderate response to episodic drought (I) and strong growth reduction (II).

2.9. Statistical data analysis

The effect of intra- and inter-specific competition on temporal dynamics of soil water depletion during the 2013 drought was assessed via regression analysis. Three-parametric exponential decay functions were fitted through the overall means (12 plots) of soil water content (SWC) of the respective soil depth under S, B and M conditions (Sigmaplot, release Version 12.5.0.38, Systat Software Inc., 2011) to derive half-time ($T_{1/2}$) of SWC. Significant differences of $T_{1/2}$ were deduced from the 95% confidence intervals (CI-95%) of the respective exponential parameter λ. SWC during spring was analysed for significant differences ($\alpha = 0.05$) between measurement campaigns and intra- versus inter-specific competition using the GLM repeated measures procedure (IBM SPSS Statistics, release Version 21.0.0.0; IBM Corporation, 2012).

$\delta^{18}O$ of soil matrix and xylem water were examined for significant differences ($\alpha = 0.05$) between intra- and inter-specific competition via analysis of variance (ANOVA).

The effect of tree species (beech, spruce), interaction (B and S vs MIX), and year (2012–2013) on mean rooting depth weighted by cumulative root tip production was analysed through three-way ANOVA. Significant predictors were post-hoc analysed via Tukey HSD ($\alpha = 0.05$).

Measures of fine root morphology, assessed by the fine root samples from soil cores, were tested for significant differences by two-way ANOVA using R (R-Development-Core-Team, 2014). Values were log or square root transformed, if necessary, to ensure normality of error and homogeneity of variance. Significant predictors were also post-hoc analysed by means of Tukey HSD ($\alpha = 0.05$). To account for differing amounts of beech and spruce roots in MIX, roots from soil cores of MIX were regarded as one sample without separating species.

Effects on the abundances of ECM exploration types were evaluated using R (R-Development-Core-Team, 2014). The relative abundance of ectomycorrhizae was calculated as the percentage of tips from each exploration type within the total number of mycorrhizal tips in each sample. Differences between the B, S and MIX, soil depths and tree species were examined with the Wilcoxon Rank Sum Test on subsetted data.

Leaf gas exchange and $\delta^{13}C$ were analysed for significant differences ($\alpha = 0.05$) between measurement campaigns and intra- versus inter-specific competition using the GLM repeated measures procedure (IBM SPSS Statistics, release Version 21.0.0.0; IBM Corporation, 2012). Residuals of the calculated models were tested positively for normal distribution (KS-test). For each measurement campaign, the diurnal drift in leaf gas exchange (cf. Zweifel et al., 2002) of spruce and beech was corrected and standardized to 12:00 CET via linear regression (Sigmaplot, release Version 12.5.0.38, Systat Software Inc., 2011).

The group differences between the resistance, recovery, and resilience of Norway spruce and European beech in inter-specific versus inter-specific environment (see Table 2) were scrutinized with the two-sided *t*-test, using SPSS Statistics, Version 21.

3. Results

3.1. Accessibility of soil water for beech and spruce under inter- and intra-specific growth conditions

3.1.1. Annual course of soil water content

Average soil water contents (SWC) of 31.3 ± 2.0 SE%, 31.0 ± 2.2 SE% and 33.3 ± 2.0 SE% in 0–7 cm soil depth and 35.8 ± 1.3 SE%, 34.6 ± 1.7 SE% and 35.7 ± 1.2 SE% in 10–30 cm soil were reached upon saturating precipitation events in 2012 and 2013 for B, S and MIX respectively (data not shown), indicating similar field capacities irrespective of species interaction. Along with rising T_{air} during early spring, soil water under evergreen spruce was gradually depleted, whereas SWC remained near field capacity

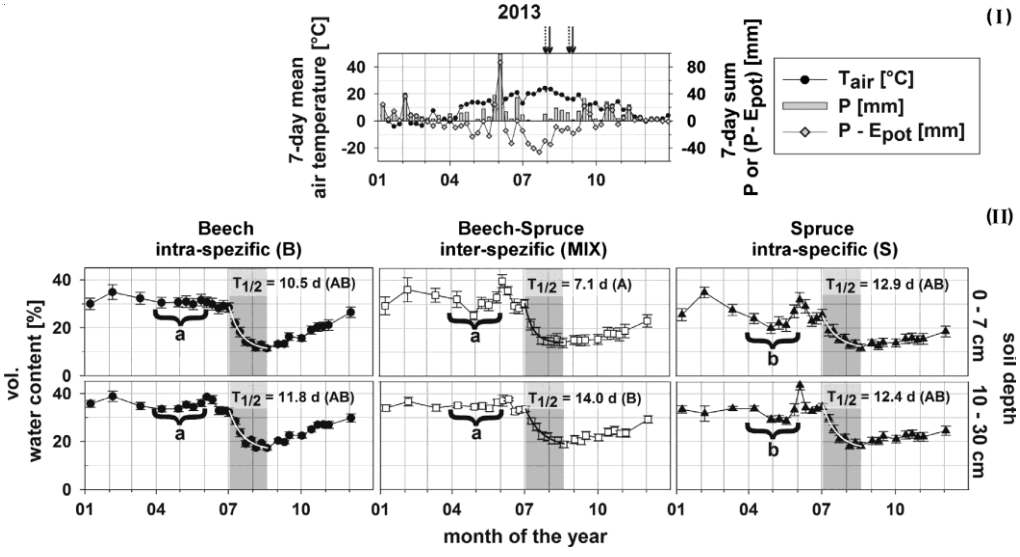

Fig. 3. (I) Climate conditions in 2013 (DWD station 5404): seven-day means and sums of field air temperature (T_{air}, closed circles) and field precipitation (P, grey bars), respectively. Difference between field precipitation and potential evapotranspiration (Penman, 1948) is given by grey diamonds. Arrows indicate dates of leaf gas exchange measurements for beech (broken) and spruce (solid) respectively. (II) Volumetric soil water contents (SWC) in the course of 2013: mean SWC (±SE) at two different depths (0–7 cm; 10–30 cm) under intra- (B; S) and inter-specific (M) growth conditions. Different lower case letters indicate significant differences ($\alpha = 0.05$) in SWC between species interactions during mid/late spring (8th of April through 28th of May). Half-lifes of SWC ($T_{1/2}$) in different soil depths during the dry period in mid/late summer (2nd of July through 19th of August), derived from regression analysis of the overall mean values at the respective depth of SWC in S, B and M (range of adjusted r^2: 0.98–0.93). Different capital letters indicate significant differences in λ (no overlap of CI-95% of λ) and hence in $T_{1/2}$ between species interactions and soil depths during the dry period.

under still leafless beech (for 2013 see Fig. 3 I, II; data for 2012 is not shown). In the soil layers of 0–7 and 10–30 cm, SWC was significantly lowered, therefore, in S relative to MIX and B. High precipitation at the beginning of June restored SWC almost to field capacity. During the subsequent seven-week dry spell (July 2nd through August 19th, 2013) SWC decreased monotonously. Irrespective of species interaction type, mean SWC reached between 11.4% and 13.9% at 0–7 cm and 17.3–18.8% at 10–30 cm depth towards the end of the dry period. During this period similar amounts of soil water per ground area were consumed from 0 to 30 cm soil depth in B, S and MIX ranging from 48.3 L m^{-2} to 45.2 L m^{-2} respectively. However, half-life analysis of SWC indicated faster depletion at 0–7 cm depth in MIX ($T_{1/2}$ = 7.1 days, corresponding to $\lambda = 0.098 \pm 0.013$ SE) as compared to S ($T_{1/2}$ = 12.9 days, corresponding to $\lambda = 0.054 \pm 0.016$ SE) and B ($T_{1/2}$ = 10.5 days, corresponding to $\lambda = 0.066 \pm 0.009$ SE). In MIX, SWC was depleted significantly faster at 0–7 cm than at 10–30 cm depth ($T_{1/2}$ = 14.0 days, corresponding to $\lambda = 0.049 \pm 0.007$ SE)

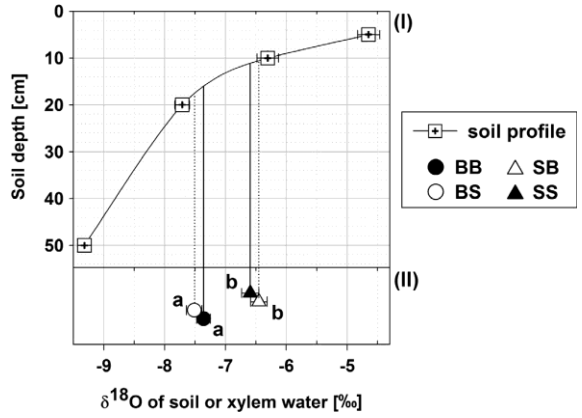

Fig. 4. Vertical profile of the $\delta^{18}O$-signature of soil matrix water (I) and the ^{18}O-signature xylem water (II) of beech (circles) and spruce (triangles) under intra-specific (BB and SS; filled symbols) and inter-specific (BS and SB; open symbols) growth conditions in July 2012. Data of all $\delta^{18}O$ soil water profiles was pooled (no significant differences between of S, B, and M). Data points represent mean values ± SE. Different letters indicate significant ($p < 0.05$) differences in $\delta^{18}O$-signature of xylem water.

3.1.2. Depth of water uptake

No significant differences in the vertical $\delta^{18}O$ profile of the soil matrix water were found between B, S and MIX. Averaging the data of all 24 soil cores, the vertical $\delta^{18}O$ gradient ranged from -4.6 ± 0.2 SE ‰ at 5 cm depth to -9.3 ± 0.1 SE ‰ at 50 cm depth (Fig. 4 I). $\delta^{18}O$ of the xylem water of beech was significantly lower (overall mean, BB & BS: -7.4 ± 0.1 SE ‰) than $\delta^{18}O$ of the xylem water of spruce (overall mean SS & SB: -6.5 ± 0.1 SE ‰; Fig. 4 II), corresponding to a lower average depth of water uptake in beech of 16.9 cm compared to spruce of 10.8 cm. $\delta^{18}O$ in the xylem water of BS was slightly lower and that of SB slightly higher as compared to the respective intra-specific situation BB and SS. This indicates that, by trend, BS trees take up water from deeper and SB trees from shallower soil horizons as compared to their monospecific counterparts.

3.1.3. Vertical distribution of fine root growth

Significant predictors of average rooting depth (average weighted by cumulative fine root production at the respective soil depth) were species ($p < 0.0001$) and species interaction ($p < 0.005$), but not year ($p > 0.1$). When grown intra-specifically beech produced a greater proportion of roots within deeper soils (54% were deeper than 30 cm) compared to intra-specific spruce root production, which concentrated its roots closer to the soil surface (34% ⩽ 10 cm). In B and S, growing root tips of both tree species were, on average, produced deeper than their inter-specific counterparts in MIX (25.6 vs. 18.9 cm). There was no significant interaction between species and species interaction (species × species interaction, $p = 0.1208$). This indicates that, integrated over the whole depth profile, both species did respond similar to the mixture and decreased their average rooting depths within mixed soil regions. Additional significant two or three-way interactions between the tested predictors were not found. Integrated over the whole depth profile, fine root production of spruce was strongly reduced in MIX when compared to S. In beech, by contrast, overall fine root production was similar in MIX and B. When analysed in 10 cm depth increments, significant inter- vs. intra-specific differences in fine root production were observed within 0–10 and 11–20 cm depth increments only (Tukey's HSD test, $\alpha = 0.05$; Fig. 5). In both B and S, a higher abundance of intra-specifically growing root tips were found within 0–10 cm depths when compared to inter-specifically growing root tips. This trend was also observed within 11–20 cm depths in S, but not B (Fig. 5). In B, there

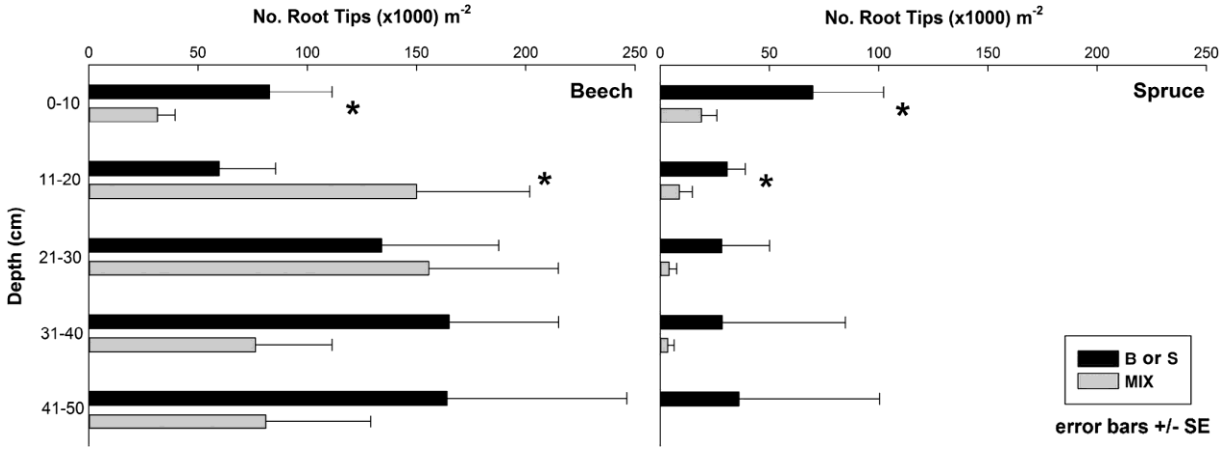

Fig. 5. Cumulative root tip production of *Fagus sylvatica* (beech) and *Picea abies* (spruce) as a function of soil depth (bars represent mean value ± SE). Species interactions: B and S black bars; MIX grey bars. Tukey's HSD test was used to evaluate inter- vs. intra-specific differences in cumulative root tip production within each 10 cm depth increment ($\alpha = 0.05$). Significant inter- vs. intra-specific differences are denoted by ∗.

was a higher abundance of inter-specifically growing root tips within 11–20 depths, which points to inter-specific complementarity in the vertical distribution of both species' roots.

3.1.4. Fine root surface area and branching intensity in the upper soil layers

Irrespective of the species, fine root surface area (FRSA) was higher in the upper soil (UP; overall mean: 3.41 m^2/m^3 ± 0.22 SE) than in the lower soil (LO; overall mean: 1.32 m^2/m^3 ± 0.12 SE; $p < 0.001$). In UP fine root surface area was highest in B (4.31 m^2/m^3 ± 0.43 SE) and lowest in S (2.63 m^2/m^3 ± 0.28 SE; $p < 0.01$). Intermediate values were found in MIX (3.29 m^2/m^3 ± 0.23 SE; $p_{Mix\ vs.\ B} = 0.10$, $p_{Mix\ vs.\ S} = 0.36$). In LO no significant differences of fine root surface area were found between B (1.37 m^2/m^3 ± 0.23 SE), S (0.94 m^2/m^3 ± 0.15 SE) and MIX (1.34 m^2/m^3 ± 0.20 SE). Irrespective of soil depth highest fine root surface area was found in B (3.0 m^2/m^3 ± 0.36 SE) and lowest in S (1.78 m^2/m^3 ± 0.24 SE; $p = 0.001$). Fine root surface area in MIX (2.27 m^2/m^3 ± 0.26 SE) did not differ significantly from that in B but was in tendency higher than in S ($p = 0.067$). In S and B, spruce and beech showed similar UP/LO-ratios of fine root surface area of 2.57 and 2.80, respectively. In MIX, by contrast the UP/LO-ratio was almost three times higher in spruce (4.44) as compared to beech (1.54), indicating a vertical stratification between beech and spruce in response to belowground interaction (Fig. 6).

Fine root branching intensity (data not shown) in spruce was significantly ($p < 0.05$) lower in MIX (821 tips/m; ±26 SE) as compared to S (984 tips/m; ±54 SE). For beech no significant effect of mixing on fine root ramification intensity was found.

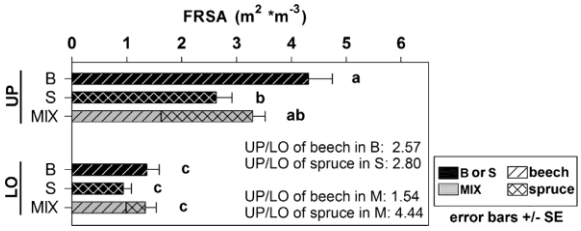

Fig. 6. Fine root surface area (FRSA), extrapolated from soil core data taken in autumn 2013: beech (simple-ruled), spruce (cross-ruled) in the B, S (black) and beech + spruce in MIX (grey) partitioned in upper soil (UP: 0–8.6 cm) and lower soil (LO: 8.6–25 cm). Bars represent the mean values (±SE). Lower case letters indicate significant differences between B, S and MIX in UP and LO ($\alpha = 0.05$; tow-way ANOVA and the Tukey-HSD test).

3.1.5. Exploration types of ecto-mycorrhiza

In total 19,103 vital ECM tips were counted and categorized into exploration types. Contact-, short- and long-distance types were found in beech and spruce, whereas medium-distance smooth types were only found in beech. For analysis, exploration types were categorized into two functional groups, concerning their ability to transport water: first, exploration types with distinct rhizomorphs (r+, long distance types) and second, exploration types without rhizomorphs (r−, all other exploration types, for detailed numbers of each exploration type c.f. supplementary material Table S 2). In each tree species and soil depth, ca. 5 times more ECM without rhizomorphs were found ($p < 0.001$; Fig. 7). For both tree species, relative abundances of ECM groups were not significantly different between upper and lower soil. ECM without rhizomorphs were relatively more abundant in spruce in the upper

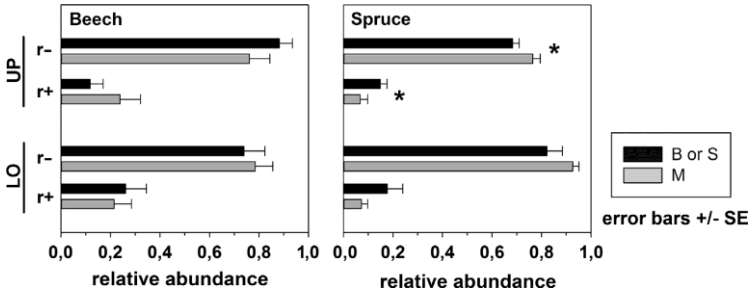

Fig. 7. Relative abundance of ectomycorrhizal exploration types observed in the B, S (black bars) and MIX (grey bars), shown for the upper soil (UP: 0–8.6 cm) and lower soil (LO: 8.6–25 cm). Relative abundance of exploration types with distinct rhizomorphs (r+) and exploration types without rhizomorphs (r−) was calculated as the percentage of tips from each exploration type within the total mycorrhizal tips in every sample. Significant differences ($p \leq 0.05$, similarity percentage test) are indicated by asterisks.

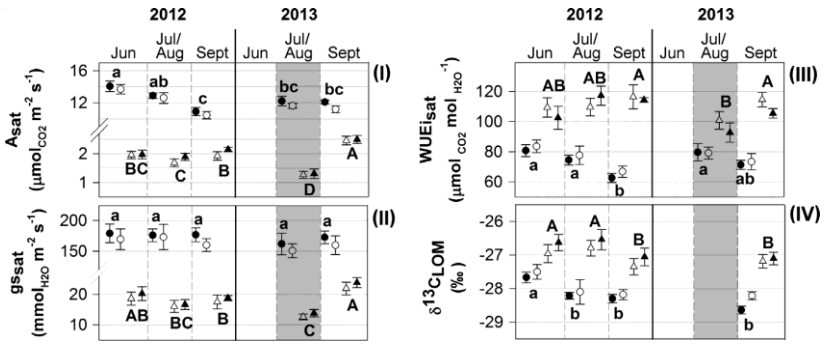

Fig. 8. Leaf gas exchange under saturating light conditions (sat) and ^{13}C-signature of leaves of beech (circles) and spruce (triangles) under intra- (BB and SS, filled symbols) and inter-specific growth conditions (BS and SB, open symbols) in June, July/August and September of 2012 and 2013. The measurements during the dry spell in 2013 are shaded in grey. Mean values (±SE) are shown of (I) assimilation rate (A_{sat}), (II) stomatal conductance (gs_{sat}), (III) intrinsic water use efficiency ($WUEi_{sat}$) and (IV) $\delta\ ^{13}C$ in leaf organic matter (n = 8 each). Different letters indicate significant differences ($p < 0.05$) between estimated marginal means (GLM repeated measures) of the different measurement campaigns for beech (lowercase letters) and spruce (capital letters).

soil in MIX compared to S (92% ± 4 SE) vs. 82% ± 3 SE; $p < 0.05$), while ECM with distinct rhizomorphs were relatively less abundant (8% ± 4 SE vs. 18% ± 3 SE; $p < 0.05$). There were no significant differences among ectomycorrhizal groups of beech in the upper, and in beech as well as spruce in the lower soil.

3.2. Drought response of spruce and beech under intra- and inter-specific growth conditions

3.2.1. Leaf gas exchange and carbon isotope composition

Light-saturated assimilation rate (A_{sat}) and stomatal conductance (gs_{sat}) yielded higher intrinsic water-use-efficiency ($WUEi_{sat}$) and $\delta^{13}C$ of leaf organic matter ($\delta^{13}C_{LOM}$) in spruce (mean $WUEi_{sat}$: 108 ± 2 SE $\mu mol_{CO2}/mol_{H2O}$; mean $\delta^{13}C_{LOM}$: 28.1 ± 0.1 SE ‰) than in beech (mean $WUEi_{sat}$: 75 ± 2 SE $\mu mol_{CO2}/mol_{H2O}$; mean $\delta^{13}C_{LOM}$: 26.9 ± 0.1 SE ‰; Fig. 8). In beech, A_{sat} was lower in late summer (11.2 ± 0.2 SE $\mu mol\ CO_2\ m^{-2}\ s^{-1}$) proceeding leaf-senescence, whereas gs_{sat} remained stable at 168 ± 4 SE $mmol\ m^{-2}\ s^{-1}$ throughout the growing season. Consistently, in beech $WUEi_{sat}$ and $\delta^{13}C_{LOM}$ were lowest in late summer. During the drought in July 2013, beech did not appear to be water-limited, whereas spruce reached its lowest A_{sat} (1.3 ± 0.09 SE μmol_{CO2}) and gs_{sat} (13.2 ± 0.71 SE $mmol_{H2O}$) compared to growing season means (A_{sat}: 2.1 ± 0.06 SE μmol_{CO2}; gs_{sat}: 19.2 ± 0.70 SE $mmol_{H2O}$). Counterintuitively, $WUEi_{sat}$ of spruce was also lowest during the summer drought 2013. Fig. 9 shows that during the intense dry spell in midsummer 2013, the most drought stressed trees (lowest gs_{sat}) diverged from the usual negative correlation between gs_{sat} and $WUEi_{sat}$, indicating photoinhibition perhaps due to the concurrently high insolation. Significant species interaction effects (BB vs BS and SS vs SB) did not emerge from leaf gas exchange and $\delta^{13}C_{LOM}$ analyses.

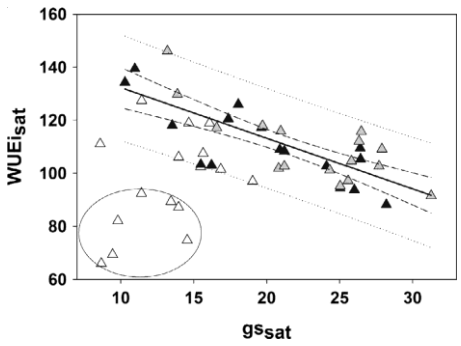

Fig. 9. Intrinsic photosynthetic water-use-efficiency of spruce (calculated from gas exchange data) in relation to stomatal conductance under dry (midsummer 2013: open symbols) and moist conditions (midsummer 2012: filled, black symbols and late summer 2013: filled, grey symbols). Overall regression of $WUEi_{sat}$ vs. gs_{sat} under moist conditions (solid line): $r^2 = 0.59$, $p < 0.01$, 95% confidence band (dashed line) and 95% prediction band (dotted line). Regression of $WUEi_{sat}$ vs. gs_{sat} under dry conditions in 2013: n.s., due to deviant values at very low gs_{sat} (within circle).

3.2.2. Tree growth performance during the drought year 2003

Using tree ring analyses to retrospectively analyse effect of the drought year 2003 on stem diameter growth dynamics, spruce turned out to be less drought-tolerant than beech (Table 2). Resistance and resilience of spruce (SS) were slightly lower compared to spruce when neighbouring beech (SB), and recovery was higher. Resistance and resilience of beech (BB), on the other hand, was slightly increased and recovery decreased when compared to BS. All these comparisons of species-interaction, however, were not significant. Large species-specific differences between spruce and beech led to significantly lower resistance (−11%) and lower resilience (−20%) of SB compared to BS trees. In general, recovery was higher the more pronounced growth depression was during the preceding drought.

4. Discussion

The present study focused on the drought susceptibility of Norway spruce when grown adjacent to European beech, hypothesizing (H I) that water accessibility for spruce is limited by the presence of beech. As a consequence, (H II) mixture with beech may exacerbate drought susceptibility, reducing spruce's photosynthesis and stem growth.

4.1. Soil water accessibility for inter-specific spruce during summer drought

Before bud break in beech, soil water consumption of evergreen spruce significantly lowered the SWC in S when compared to MIX and B (cf. also Schume et al., 2004). During the drought in late summer of 2013, however, soil water depletion was most rapid in 0–7 cm soil depth of MIX. Relative to S, the enhanced total absorptive surface area of beech and spruce fine roots together (Fig. 6) suggest increased belowground competition in MIX and associated exhaustion of soil water in the upper soil. Fast soil moisture depletion exacerbates drought even during a short absence of precipitation, rapidly conveying high doses of drought stress (Goisser et al., 2013; Zang et al., 2014) in the upper soil depths of MIX. Lower production and FR surface area of spruce roots in MIX in comparison to S may be a result of asymmetric competition belowground. Bolte et al. (2013) observed that spruce had a lower root area index, as well as root biomass when growing in mixture with beech. Therefore, the admixture of spruce, with its distinctly different root growth traits, may favour beech in occupying mixed soils. Our results confirm the observation in previous studies that competition with beech shifts spruce fine roots to the upper, more drought-prone soil layers (Bolte and Villanueva, 2006; Schmid and Kazda, 2001; Schume et al., 2004). Corresponding to the observed shift in vertical fine root distribution, $\delta^{18}O$ analysis of xylem water revealed by trend higher, respectively lower, average depths of water uptake in SB and BS trees as compared to their intra-specific counterparts (SS and BB). Spatial and temporal patterns in fine-root growth of spruce appear to be conservative even under water limitation (Gaul et al., 2008; Mainiero et al., 2010), the more so, if constrained by competing beech (cf. Fig. 6). Considering

Table 2
Indices for resistance, recovery and resilience (means ± se) of stem diameter growth according to Lloret et al. (2011) for spruce and beech growing in inter- versus intra-specific neighbourhood based on 9 trees per species in intra- and inter-specific neighbourhood. P-values denote the significance between SS/SB, BB/BS and SB/BS.

Parameter	Spruce				SS/SB	Beech				BB/BS	SB/BS
	SS	se	SB	se	p	BB	se	BS	se	p	p
Resistance	0.43	0.08	0.54	0.04	0.073	0.75	0.08	0.65	0.08	0.139	<0.001
Recovery	1.43	0.32	1.27	0.17	0.251	1.20	0.10	1.35	0.32	0.251	0.144
Resilience	0.62	0.07	0.68	0.09	0.333	0.89	0.08	0.88	0.07	0.689	<0.001

the high drought susceptibility of spruce at Kranzberg Forest (Nikolova et al., 2009) such response seems counter intuitive, as it exposes large parts of the spruce root system to overall drier conditions in the upper soil layers. However, input of beech litter may be beneficial for neighbouring spruce, as it can significantly alter the topsoil properties (i.e. reduced humus accumulation and acidity) and hence nutrient release from litter decomposition and nutrient cation mobility (Goettlein et al., 2012; Rothe et al., 2002). Reduced fine root branching intensity of spruce roots in the presence of beech may result from enhanced nutrient availability (Meyer, 1987), indicating a strategic shift from intense soil exploitation to selective foraging with extending roots (Waisel et al., 2002). In mixture with beech, spruce enhanced its share of ECM without rhizomorphs while lowering the proportion of ECM with distinct rhizomorphs. The latter produces 15 times more biomass, at least, of external mycelia with higher C demand than needed by other types (Rygiewicz and Andersen, 1994; Weigt et al., 2012, 2011). Hence, in mixture with beech, ECM that exploit the nutrients of the beech litter at lower carbon costs may be favoured. Genera with long distance type ECM, conversely, can increase the plant water uptake due to an up to 15-fold extension in hyphal length and a 3-fold increase of the absorbing surface (Lehto and Zwiazek, 2011; Rousseau et al., 1994; Weigt et al., 2012).

In view of the natural distribution of spruce, on nutrient-poor sites and under humid climates (i.e. with only short-term drought, Schmidt-Vogt, 1987; Spiecker, 2000), root dominance in upper soil, high capacity for selective foraging in combination with highly efficient nutrient uptake through fungal partners, appears to be an effective and hence highly competitive strategy for pre-emption of nutrients from litter mineralization and soil water upon drought (Craine and Dybzinski, 2013; Schmid, 2002). During extended periods without precipitation, however, shallow rooting in drought prone upper soil horizons, reduced intensity of soil exploitation and lower water absorbing ECM-surface area of spruce in response to inter-specific competition with beech limits the accessibility to deep soil water (Craine and Dybzinski, 2013) as well as the capacity for water extraction from dry soil. With regard to summer drought, present results hence corroborate H I that the presence of beech roots reduces water accessibility for spruce through (i) a shift of spruce fine roots to shallower, drought prone soil depths and (ii) reduced association with ECM fungi of the long-distance type.

4.2. Drought susceptibility of leaf gas exchange and stem growth in spruce under intra- and inter-specific neighbourhood

Stomatal control of transpiration and water-use-efficiency of carbon assimilation is crucial for plant survival and growth performance, especially under drought (Chaves, 1991). Being one of the earliest responses to water limitation (Flexas and Medrano, 2002), reduced stomatal conductance can serve as indicator for drought stress (Medrano et al., 2002). However, characteristic differences in the drought sensitivity of stomatal response may occur between species with different ecological strategies in controlling internal water relations (i.e. being isohydric versus anisohydric, cf. McDowell et al., 2008). In line with present results regarding stomatal conductance and BAI, spruce has often shown to be more drought-susceptible than beech (Pretzsch et al., 2013; Zang et al., 2011), despite spruce's xeromorphic foliage. Spruce apparently employs an isohydry strategy (Lyr et al., 1992), reducing stomatal conductance at early stages of soil drought. Needle xeromorphism may, hence, be a feature to preserve water in the tree, once the stomata have closed. By contrast, beech may follow an anisohydric strategy, with a less sensitive regulation of stomatal conductance to soil drought during prolonged dry spells compared to spruce (Leuschner, 2009). Consistently, spruce operates at higher WUEi than beech (Fig. 8). However, during midsummer 2013, drought-related reduction of stomatal conductance in spruce resulted in decreased rather than increased WUEi. Stomatal closure under high insolation, exacerbates photo-oxidative (Foyer et al., 1994a, 1994b) as well as temperature stress (Lin et al., 2012). Especially both stresses in combination may exacerbate photoinhibition (Gamon and Pearcy, 1990; Sage and Kubien, 2007), hence being responsible for the reduced WUEi of photosynthesis in drought stressed spruce. Such response illustrates the high susceptibility of spruce to such weather conditions.

Dobbertin (2005) suggests reduced carbon allocation to stem growth as one of the most drought-sensitive responses at the whole tree level. Results of the meta-analysis by Poorter et al. (2012) corroborate such assumption for a wide range of species. Thus, especially the LLoret-indices for resistance, Rt, and resilience, Rs, based on stem diameter growth (Fig. 2) seem appropriate for scrutiny whether water stress and growth reduction of spruce is more severe in mixture with beech than under intra-specific conditions. The group comparison between the indices Rt, Rc, and Rs (Table 2) reveals both whether inter-specific neighbourhood modifies the drought stress reaction compared with intra-specific conditions and to what extend drought stress is modified by different neighbouring tree species. Of special interest are Rt and Rs as they reflect the trees ability to avoid and overcome growth reductions by drought which, in the long term, mean a loss of their fitness and competitiveness within the population.

The comparison reflects the generally higher drought resistance and resilience of European beech compared with Norway spruce (Pretzsch et al., 2013). In contradiction to H II we found no exacerbation of drought stress and growth reduction in mixed compared with monospecific environment. There is even a nearly significant ($p = 0.073$) increase of drought resistance of Norway spruce when growing in neighbourhood of beech (Table 2, first line). Despite supported concerns about negative effects of beech on accessibility of soil water for spruce (H I, supported), we found no evidence of increased drought susceptibility in leaf gas exchange or BAI in spruce when growing in the neighbourhood of beech, thus rejecting H II.

5. Conclusions

Concerns about negative effects of beech on accessibility of soil water for spruce when growing in mixture (H I) were supported. Interacting with beech, spruce produced roots predominately within shallower, drought prone soil horizons. Moreover association with ECM fungi of the long-distance type was distinctly reduced. However at the whole-tree level, our results provided no evidence of increased drought susceptibility of spruce trees grown in a group-wise mixed spruce-beech forest thus rejecting our second hypothesis (H II). The conflicting findings regarding H I and H II are interpreted to result from two aspects: (i) seasonal shifts between positive and negative effects of beech-spruce interaction and (ii) the group-wise mixture pattern of the investigated forest stand. Our results suggests that especially in the case of spring drought, evergreen spruce may benefit from reduced competition for water and hence higher SWC when growing in mixture with deciduous beech (cf. Fig. 3). Carrying forward the results from long-term observations (Del Río et al., 2013) and forest growth modelling (Forrester and Tang, 2016), shifts between positive and negative effects of species-interaction in beech-spruce stands may not only occur inter-annually but also intra-annually, possibly overriding each other in their effect on annual BAI. To disentangle interfering intra-annual mixture effects on tree growth, continuous measurements of stem diameter growth appear to be necessary. In addition, due to the group-wise mixture pattern, inter-specific spruce individuals are only partially exposed to beech competition.

Thus, adverse neighbourhood effects of beech on accessibility of soil water for spruce (cf. Section 4.1) prevail single-sided, which may also explain that only small differences in $\delta^{18}O$ of xylem water and hence estimated average depth of water uptake were observed between SB versus SS and BS versus BB. In line with the results of Pretzsch et al. (2012), we conclude that the group-wise mixture pattern, exposing spruce individuals to beech competition only partially, i.e. single-sided, buffer the putatively adverse neighbourhood effect of beech on drought susceptibility of spruce. In comparison to single tree mixture, group-wise mixture of beech and spruce appears to be the favourable silvicultural option in the face of climate change.

Acknowledgements

We thank C. Blanck, T. Feuerbach, S. Heckmair and P. Kuba for their support during field and lab work. We highly appreciate the funding of the project by the Bavarian State Ministry of the Environment and Consumer Protection, the Bavarian State Ministry of Food, Agriculture and Forestry and the Deutsche Forschungsgemeinschaft.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2016.05.032.

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Supplementary material

Table S 1: Characteristics of the 12 plots at "Kranzberger Forst". N: number of trees per ha; BA: basal area per ha; V: total stem volume per ha; hq: mean height; dq: quadratic mean diameter at 1.3 m breast height.

plot	area (m^2)	spruce					beech					total		
		N	BA (m^2)	V (m^3)	hg (m)	dg (cm)	N	BA (m^2)	V (m^3)	hg (m)	dg (cm)	N	BA (m^2)	V (m^3)
1	131.8	379	33.1	454	28.6	33.4	379	23.4	312	25.9	28.0	758	56.5	766
2	115.2	520	58.2	827	30.0	37.7	520	24.6	316	25.2	24.5	1040	82.8	1143
3	109.8	364	35.8	501	29.3	35.4	545	31.5	421	25.7	27.1	909	67.3	922
4	127.6	235	23.1	322	29.3	35.4	392	26.0	351	26.1	29.0	627	49.1	673
5	142.1	211	19.5	271	28.9	34.3	352	20.5	271	25.8	27.3	563	40.0	542
6	161.7	185	15.9	220	28.5	33.1	247	24.8	355	27.1	35.7	432	40.7	575
7	199.0	352	40.3	571	30.1	38.1	302	18.4	245	25.9	27.9	654	58.7	816
8	156.2	320	29.3	405	28.8	34.1	320	15.6	199	25.3	24.9	640	44.9	604
9	111.3	270	27.2	416	29.4	32.5	359	29.7	416	26.7	32.4	629	56.9	832
10	164.3	305	24.6	336	28.1	32.0	366	27.7	385	26.5	31.1	671	52.3	721
11	174.3	230	22.4	316	29.2	35.3	172	13.7	188	26.6	31.8	402	36.1	504
12	137.1	292	21.6	290	27.6	30.7	292	17.0	223	25.8	27.2	584	38.6	513

Table S 2: Relative abundance, number of replicates and measures of data variation of the distinct morphotypes of beech and spruce in mix and mono zones and both, upper and lower soil parts, respectively.

Soil part	Species interaction	Exploration type	Beech			Spruce		
			Relative abundance	n	SE	Relative abundance	n	SE
UP	Mono	contact	0.63	12	0.09	0.33	12	0.08
UP	Mono	short	0.05	12	0.01	0.49	12	0.07
UP	Mono	medium	0.22	12	0.10	0.00	12	0.00
UP	Mono	long	0.10	12	0.04	0.18	12	0.03
UP	Mix	contact	0.44	12	0.07	0.37	12	0.07
UP	Mix	short	0.15	12	0.06	0.54	12	0.08
UP	Mix	medium	0.18	12	0.07	0.00	12	0.00
UP	Mix	long	0.22	12	0.08	0.09	12	0.04
LO	Mono	contact	0.40	12	0.08	0.23	12	0.07
LO	Mono	short	0.31	12	0.08	0.59	12	0.09
LO	Mono	medium	0.07	12	0.04	0.00	12	0.00
LO	Mono	long	0.22	12	0.08	0.18	12	0.06
LO	Mix	contact	0.51	12	0.08	0.25	12	0.07
LO	Mix	short	0.24	12	0.07	0.60	12	0.10
LO	Mix	medium	0.12	12	0.06	0.00	12	0.00
LO	Mix	long	0.13	12	0.05	0.07	12	0.02

7.3 III: Nitrogen fertilisation reduces sink strength of poplar ectomycorrhizae during recovery after drought more than phosphorus fertilisation

Plant and Soil, 1-18. doi:10.1007/s11104-017-3354-2.

Year: 2017

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The following article reports results from the greenhouse experiment with poplar under drought and different fertilisation regimes. As shown in article I, drought reduced the C flux from leaves (source) to mycorrhizal roots (sink). However, during recovery from drought, C flux exceeded the levels observed in irrigated controls. This process is probably sink-controlled. Here, this source–sink relationship was studied in an agronomically used poplar clone grown at different levels of N and P fertilisation as used in silvoarable agroforestry systems. A fully factorial pot experiment was conducted combining four fertiliser and two drought regimes. Gas exchange and chlorophyll and flavonol indices were regularly monitored. One week after rewatering, a $^{13}CO_2$ pulse labelling was performed. Plants were harvested one week after recovery. At harvest, an excess in C allocation to ectomycorrhizae was observed in non-N-fertilised treatments. However, net photosynthesis only recovered to the level of continuously irrigated controls. Drought increased chitinase, cellulase, phosphatase and peptidase activities, but the latter only in N-fertilised treatments. Higher activities of chitinase and celluluase in ectomycorrhizae of recovering plants suggest the degradation of plant and fungal cell walls and arthropod-derived chitin from individuals that died during drought. Enhanced phosphatase activity during recovery from drought may help to mobilise P from dead cells and tissues, which were made accessible by the chitinolytic and cellulolytic activities of ectomycorrhizae. After severe drought, there might also be a need for phosphorus to restore metabolism. The results further indicate that allocation of recent photosynthates is most likely sink-controlled because, after drought release, source activity recovered to the level in irrigated plants, but ^{13}C allocation to ECM (C-sink) was much higher. However, this was less pronounced in the presence of N and P fertiliser. Overall, this suggests that either (1) sink strength is an indicator of the impact of drought stress and that this sink strength can be reduced especially by fertilisation with N, or (2) that recent photosynthates were partitioned between two sinks (i.e. ECM and above-ground growth).

Plant Soil
DOI 10.1007/s11104-017-3354-2

REGULAR ARTICLE

Nitrogen fertilisation reduces sink strength of poplar ectomycorrhizae during recovery after drought more than phosphorus fertilisation

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Received: 7 March 2017 / Accepted: 20 July 2017

Abstract
Background and aims Drought reduces the carbon (C) flux from leaves (source) to mycorrhizal roots (sink); however, during recovery from drought, C flux exceeds the levels observed in irrigated controls. This process could be source- or sink-controlled. We studied this source–sink relationship in an agronomically used poplar clone grown at different levels of nitrogen (N) and phosphorus (P) fertilisation as used in silvoarable agroforestry systems.
Methods We conducted a fully factorial pot experiment combining four fertiliser and two drought regimes. Gas exchange and chlorophyll and flavonol indices were regularly monitored. One week after rewatering, we performed $^{13}CO_2$ pulse labelling. At harvest, enzyme activities of ectomycorrhizal root tips were determined.
Results After one week of recovery, we observed an excess in C allocation to ectomycorrhizae (ECM) in non-N-fertilised treatments. However, net photosynthesis only recovered to the level of continuously irrigated controls. Drought increased chitinase, cellulase, phosphatase and peptidase activities, but the latter only in N-fertilised treatments.
Conclusions We add evidence that the allocation of recently assimilated C is most likely sink-controlled. Less C allocation to recovering ECM supplied with fertiliser may be either due to better nutritional status and hence higher stress tolerance, or due to partitioning between above and below-ground sinks.

Keywords ^{13}C · Drought stress · Ectomycorrhizae · Poplar · Recovery

Responsible Editor: Juan Barcelo.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-017-3354-2) contains supplementary material, which is available to authorized users.

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Published online: 27 July 2017

Introduction

Future climate models predict warmer and drier summers in the temperate regions and more variability in precipitation (Field 2012). The distribution of assimilated CO_2 in plants is a source–sink relationship, where photosynthesis in the chloroplasts is the source, building up a pool of non-structural carbohydrates (NSC, mostly sugars and starch), which are used in sinks such as growth (building up structural carbohydrates), defence and the maintenance of primary and secondary metabolism (Hartmann and Trumbore 2016). During water

shortage, this C flux from source to sinks can be diminished, whereas during recovery from drought–when there is ample water again–the flux can exceed the rates under optimal water supply (Hagedorn et al. 2016). Several studies have addressed the issue of whether this flux is under source or sink control. As drought reduces primary productivity, it was formerly assumed that C balance under drought is controlled by restricted source activity rather than by changes in sink strength (Guillemot et al. 2015; Sala et al. 2012). This was inferred by the fact that stomata close under water shortage and thereby reduce gas exchange in leaves (Brodribb and McAdam 2011; McDowell et al. 2008). However, owing to loss of turgor during dry periods, meristematic activity (i.e. growth, a C sink) is reduced earlier than CO_2 uptake, leading to an accumulation of NSC in early phases of drought (Muller et al. 2011). Additionally, direct environmental sink control has been proposed (Hagedorn et al. 2016; Körner 2003), although no clear evidence of this has yet been found, probably because of other processes that could be limiting under drought, such as nutrient availability, temperature or cell turgor (Körner 2015).

Even less understood are the mechanisms regulating C fluxes during recovery after drought. Plant C dynamics are often inferred by measuring NSC pools of the plant. Thereby it is assumed that fluctuations in these pools are only due to their utilisation. However, storage of C in plants (NSC pools) may be adaptive and respond to environmental stimuli by basically shifting C from one pool to another (Hartmann and Trumbore 2016). By applying $^{13}CO_2$ pulse labelling to trace recently assimilated CO_2, Hagedorn et al. (2016) showed that beech trees direct recent assimilates preferentially to ectomycorrhizae (ECM) after release from drought. Further, this study observed faster recovery of soil respiration than of photosynthesis and concluded that metabolic activity in the rhizosphere is not source-driven. Moreover, C fluxes to roots and ectomycorrhizal fungi after drought not only recovered, but were actually higher than before the drought and thus compensated for previous effects of stress, leading to the hypothesis that the reactivation of root metabolism is important for ecosystem resilience to drought.

Mycorrhizae are the organs by which most plants including trees acquire nutrients and water. Poplars are among the most commonly planted trees in Central European agroforestry systems (Reisner et al. 2007). They can form both ectomycorrhizal and arbuscular mycorrhizal associations. While arbuscular mycorrhizae (AM) are very common (80% of all plants), ectomycorrhizal symbiosis dominates in forests of the northern hemisphere (Finlay 2008; Smith and Read 2010). Temperate and boreal forest soils contain most nutrients (especially nitrogen and phosphorus) in organically bound form and trees, therefore, depend on nutrient mobilisation by associated or free-living soil microorganisms (LeBauer and Treseder 2008; Maracchi et al. 2005; Smith and Read 2010). This mobilisation is accomplished predominantly by fungal enzymes (Kjøller and Struwe 2002). For the nutrition of forest trees, the enzyme activities of their ECM play a major role (Perez-Moreno and Read 2000; Tibbett and Sanders 2002).

In the present study, we investigated the effect of drought and different levels of nitrogen (N) and phosphorus (P) on the distribution of recently assimilated carbohydrates to ECM of the poplar clone 'Rochester' (*Populus maximowiczii* × *P. nigra var. plantierensis*), which is widely used in short-rotation coppice agroforestry. We used stable isotope labelling to test whether C fluxes during recovery after drought are sink-controlled as in beech (Hagedorn et al. 2016) and if this source–sink relationship is modulated by agriculturally relevant levels of nutrients. We tested the following alternative hypotheses about the source–sink control: (I) If source activity controls the C distribution after drought, an initial excess of photosynthesis is expected, followed later by a C signal in ECM, and (II) if sink activity controls C distribution after drought, an initial C signal in ECM is expected, followed later by a peak of photosynthetic activity. We suggest that C allocation to recovering ECM will be lower in fertilised treatments, as a better nutritional state of the plant may mitigate drought stress. Low N fertilisation has been shown to increase water use efficiency (WUE) of woody plants under drought, thus compensating for drought stress (Wu et al. 2008). By contrast, fertilisation with P has been found to increase drought tolerance mainly of non-woody AM plants (Nelsen and Safir 1982). We further expect enzyme activities of ECM in recovering plants to be generally higher due to the increased nutrient demand needed for regrowth of tissues lost during drought and for rebalancing nutrient stoichiometry (Gessler et al. 2016). Moreover, mineral fertilisation may decrease the enzymatic activity of ECM as there is no need to break down organic compounds in order to take up nutrients. To address these issues, we conducted an experiment to test the effects of N and P fertilisation

on ectomycorrhizal enzyme activities and C source–sink processes of poplar species during recovery from drought via the tracing of labelled photosynthates.

Material and methods

Plant material and growth conditions

We used clonal plants because approximately 50% of the variation in plant traits occurs at the level of individuals (Garnier et al. 2001; Jung et al. 2010). We obtained cuttings of the intersectional poplar variety 'Rochester' (*P. maximowiczii* × *P. nigra var. plantierensis*, institute no. 138/49, registration no. 091960 01001 4), from the Bavarian Office for Forestal Seed and Plant Breeding (ASP) in Teisendorf, Germany. Before planting, the cuttings were first washed in distilled water, dosed with a few drops of the wetting agents Tween 20 and Tween 80. Subsequently, they were surface-sterilised in 30% (*v*/v) H_2O_2 for 5 min and finally rinsed with freshly distilled water. Cuttings were grown in 2.5-L pots (11 × 11 × 21 cm) that had been sterilised with ethanol (80%) and contained a sterile mixture of 1/3 farmland soil and 2/3 vermiculite (Table 1). We chose vermiculite to optimise the growth conditions in the clayey soil and because the ability of vermiculite to sequester phosphorus is rather low (Brix et al. 2001; Hayes et al. 2000; Ramulu et al. 1967). This soil mixture was autoclaved in batches of 15 kg at 134 °C for 120 min to standardise future fungal and microbial growth. The soil had been taken close to an agroforestry plot (silvoarable short-rotation coppice alley cropping) at the research farm Scheyern (Germany, 48°29′46.5″N 11°26′52.3″E) and was characterised as stagnic Cambisol (WRB). The two upper horizons were collected down to ca. 30 cm (Table 2). The surface of the soil mixture was covered with 2 cm of fire-dried and autoclaved quartz sand and a dense polypropylene fabric of 1–2 mm thickness (pond fleece), leaving free only the top bud of the cuttings. Pots were transferred to a greenhouse cabin with a 16 h/25 °C day and 8 h/17 °C night regime at 60% relative humidity. Additional illumination (Philips Son-T Agro 400, ca. 373 μmol m^{-2} s^{-1} PAR at canopy level) was switched on when the light outside was below 15 klux. Plants were watered automatically with deionised (conductivity ≈ 10 μS) water near the maximum holding capacity of the substrate. We defined the maximum water holding capacity as reached when the supplied water began to leak from the bottom of the pots. All leaked water was poured back into the pots to prevent leakage of nutrients. After 11 weeks, established plants were transferred to round 6-L pots (diameter: 24 cm, height: 21.6 cm) containing the same soil mixture with fresh rhizosphere soil (ca. 100 g) added as soil microbial/fungal inoculum. Rhizosphere soil was collected from the top 10 cm at 10 spots at least 9 m distant from each other at a poplar agroforestry plot next to the field the substrate soil had been taken from. Soil crumbs adhering to roots with not more than 1 cm in diameter were shaken off and carefully mixed but not sieved to minimise further disturbance particularly of fungal hyphae. The inoculum was stored overnight at 4 °C and crumbled manually into the planting hole.

As the cuttings sprouted unequally, plants were cut back three weeks after repotting to leave only three buds. The first one that sprouted formed the new shoot. To prevent infestation by thrips during regrowth, the predator mite *Amblyseius cucumeris* was applied. During the experiment, the position of the pots was regularly randomised.

Treatments

Six weeks after the shoots had been cut back, the plants were subjected in a factorial experiment to the eight combinations of two watering treatments (drought and irrigation) and four fertilisation treatments: no fertilisation (0), addition of 700 mg of phosphorus (K_2HPO_4; Carl Roth GmbH & Co. KG, Karlsruhe, Germany) (+P), addition of 290 mg of nitrogen (KNO_3

Table 1 Concentrations [mg kg^{-1} dry matter] of plant available nutrients and total N in fresh soil and the soil mixture used for the experiment

Material	Phosphorus	Ammonium	Nitrate	Nitrite	Total N
fresh soil	8.91	0.27	14.95	0.00	11.27
autoclaved soil	9.34	5.43	14.20	0.00	26.58
autoclaved soil mixture	10.09	14.00	13.21	0.00	34.79

Table 2 Grain-size distribution within the 2 different soil horizons used for the experiment

Horizon	Depth [cm]	Sand [%]	Silt [%]	Clay [%]	Bulk density [g cm^{-3}]
Ap	0–28	18.7	54.6	26.7	1.30
BvSw	28–74	22.6	53.2	24.2	1.68

Ap: ploughed A horizon, BvSw: slack water funnelling brown earth

with 20% ^{15}N; Cambridge Isotope Laboratories Inc., Andover, MA, USA) (+N), and addition of both nutrients (+N + P). The amounts of nutrients added were based on the Bavarian guidelines for farmland fertilisation (Wendland et al. 2014). Each pot received 500 mL of a fertiliser solution, except the non-fertilised treatments, which received 500 mL of deionised water.

Directly after fertilisation, half of the pots were subjected to gradual drought by reducing irrigation each week by 25% of the control amount, until the final level of 25% of control irrigation had been reached after three weeks. The volumetric water content in the drought pots was monitored every 20 min with CMP-2 probes connected to a DL-200 data logger (Umwelt-Geräte-Technik GmbH, Müncheberg, Germany). Additional PF-probes (ecoTech Umwelt-Meßsysteme GmbH, Bonn, Germany) were installed in two drought-treated pots to get an approximation of the soil water potential. The control pots were checked manually with a UMP-1 BT probe (Umwelt-Geräte-Technik GmbH, Müncheberg, Germany). After three weeks, most of the drought-treated pots had a volumetric water content of less than 10% (equal to a pF value of approximately $4 = -10^4$ hPa soil water potential), which was maintained for 24 days. The irrigated pots had a mean volumetric water content of 23 ± 1.5% (Fig. 1).

To initiate the recovery phase, the plants from the drought treatments received the same amount of irrigation as control plants. Rewatering took place one week before labelling with $^{13}CO_2$ on three successive days, 40, 41 and 42 days after beginning of the drought, one for each labelling batch (14–15 pots). At harvest, one day after labelling, there were six plants left for each treatment combination, except for the treatment combinations +N + P + irrigation and +N + P + drought, for which there remained four replicates.

Labelling

Before labelling, the pots were enclosed in autoclaving bags, tightly sealing the opening around the stem base to minimise the dilution of $^{13}CO_2$ by soil respiration. Inside a separate greenhouse chamber (same conditions as in the growth chamber, except air temperature, which was lowered to control warming inside the tent), an airtight transparent plastic tent (ethylene-tetrafluorethylene) with a volume of approximately 7.5 m^3 was erected over the pots. Before labelling, the CO_2 concentration in the tent was decreased to approximately 325 ppm by pumping the air in the tent through soda lime. Labelling started at 09:00 h with 2.6% CO_2 (99 atom% ^{13}C) in N_2 (Westfalen AG, Münster, Germany) supplied at a flow rate of 14 L min^{-1} for 10 min, 0.7 L min^{-1} for 50 min and 0.8 L min^{-1} for 60 min. After closing the gas valve, plants were allowed to assimilate $^{13}CO_2$ for a further 30 min. The use of fans ensured good mixing of the air inside the tent. During the labelling period, air temperature and humidity were measured and the $^{12/13}CO_2$ concentration inside the tent was monitored and its ^{13}C signature was measured with a GC/IRMS-system (Delta Plus; Thermo Fisher, Dreieich, Germany). The mean air temperature was 20.9 °C and the mean relative humidity was 76.3%. During the first 20 min of labelling, the CO_2 concentration inside the tent rose to 957 ± 52 ppm and the ^{13}C signature to 68.2 ± 1 atom% ^{13}C, then increased slowly to 1016 ± 64 ppm and 76.3 ± 0.3 atom% ^{13}C during the following 110 min and thereafter decreased to 951 ± 57 ppm and 76.5 ± 0.4 atom% ^{13}C during the last 30 min without further CO_2 supply.

Leaf gas exchange measurements

Leaf gas exchange was measured weekly on two consecutive days in the greenhouse using a portable gas exchange measuring system equipped with an 8 cm^2 clamp-on cuvette (GFS-3000; H. Waltz GmbH, Effeltrich, Germany). Steady-state measurements were performed each week on leaf #9 (the ninth fully developed leaf counted from the apex; six plants per treatment and four plants in the +N + P treatment), mimicking in the cuvette the growing conditions during daytime [400 ppm CO_2, 25 °C and 300 μmol m^{-2} s^{-1}

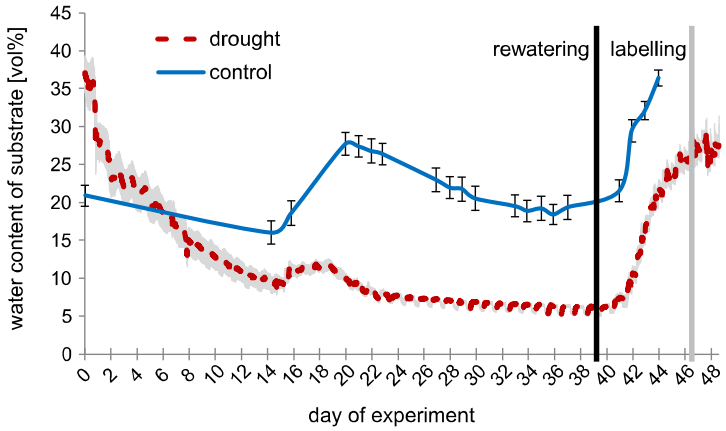


Fig. 1 Water content of drought and control pots during the course of the experiment starting on October 8th, 2014. Water content in drought pots (dashed line) was recorded every 20 min using sensors (n = 20) connected to a data logger; control pots (solid line) have been measured manually. Owing to plant growth, irrigation had to be adjusted on day 15 (October 23rd). Error bars indicate ±1 SE, the vertical lines indicate the start of rewatering at day 40 (black), and the beginning of ^{13}C labelling at day 47 (grey)

photosynthetic photon flux density (PPFD)]. The variables used here are net assimilation rates (A_n) and instantaneous water use efficiency [WUE, ratio of net photosynthetic CO_2 uptake to loss of water by transpiration (Pou et al. 2008)].

Optical non-destructive assessment of leaf chlorophyll, flavonol and nitrogen status

Indices for chlorophyll content (CHL), epidermal flavonols (FLAV) and the nitrogen balance index (NBI), namely, the CHL/FLAV ratio as an indicator of leaf nitrogen content (Cartelat et al. 2005; Cerovic et al. 2012), were monitored in leaves by non-destructive optical measurements with a leaf-clip sensor, Dualex Scientific + ™ (ForceA, Orsay Cedex, France). The indices were measured on leaf #9 (or comparable ±1) once a week. During the first 4 days of recovery from drought, measurements were performed daily. Three measurements were taken on each side (adaxial and abaxial) of the lamina, avoiding veins. The CHL index per leaf was calculated as the mean value of all measurements. As it was shown that the NBI calculated using the sum of FLAV was better correlated to total N in grape leaves than when using only one side (Cerovic et al. 2012), the mean FLAV index per leaf was calculated from the sum of FLAV on the adaxial and abaxial sides of each measuring point. The NBI per measuring point was only calculated when the FLAV index could be measured on both sides per measuring point.

Nutrient analysis of soil mixture

Plants were harvested 24 h after labelling. Three replicate soil mixture samples per pot were taken for the determination of nitrogen and phosphorus content. To determine plant-available nitrogen (N_{min}), 5 g of fresh soil mixture was agitated in an overhead shaker with 20 mL of 0.01 M $CaCl_2$ solution in glass vials for 45 min and filtered through a pleated filter (Whatman 595 ½). Extracts were analysed wet chemically in an automatic analyser with continuous flow air segmented injection (SA20/40, Type 5100; Skalar Analytical, Breda, The Netherlands). Ammonium forms a complex with salicylate, which can be measured at 660 nm, while nitrate is reduced to nitrite in a cadmium column to a diazo complex and measured at 540 nm. Nitrite was measured directly at 540 nm (VDLUFA 1991).

To determine plant-available phosphorus (P_{CAL}), 2.5 g of fresh soil mixture was placed on an overhead shaker with 50 mL of CAL solution (calcium-acetate-lactate) in Falcon tubes for 90 min, filtered and analysed photometrically after colour reaction with molybdenum (VDLUFA 1991). The determination of plant-available P using the CAL method was previously shown to yield results that are more predictive of plant yield than the Pi, Colwell, Bray 1 and Truog tests (Kumar et al. 1994).

Mycorrhiza sampling

Plant roots were washed carefully and samples of fine roots were taken randomly from the root ball.

As not the entire root system was analysed, only relative values were used for statistical analyses and descriptions. Staining of fine roots with ink (Sheaffer black; Sheaffer Pen Corporation, Shelton, CT, USA) and vinegar (Vierheilig et al. 1998) revealed no AM colonisation. Ectomycorrhizal root tips (ECM tips) were separated into morphotypes (Agerer 1991) under a dissecting microscope. Vital ECM tips were collected into tin capsules on dry ice for later stable isotope analyses and on wet filter paper for exoenzyme activity measurement. Additional ECM tips were used for morphotype identification based on their DNA sequence (see method S1). ECM with a crumpled surface, desiccated appearance and no white vascular tissue inside were defined as non-vital. Root tips without a clearly visible hyphal mantle were considered as non-mycorrhizal (no functional ECM) and not sampled. Twenty-one vital ECM tips were selected from each sample for enzyme activity measurements. The number of ECM tips of each morphotype was chosen according to the relative abundance of the respective morphotype in the sample, but was not lower than three. Based on these 21 ECM tips, the weighted mean was calculated and considered as representing the exoenzymatic potential of the ectomycorrhizal community in the sample. Further ECM tips were placed in tin capsules dried at 70 °C until weight constancy and analysed in an Isotope Ratio Mass Spectrometer (IRMS, type delta V Advantage), coupled with an Element Analyser (Euro EA).

Enzymatic activities were measured in microplates (Pritsch et al. 2011). Seven substrates bound to 4-methylumbelliferone (MU) or aminomethylcoumarin (AMC) and ABTS [diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate)] were used: l-leucine 7-AMC (Leu-AMC) for the detection of leucine aminopeptidase (EC 3.4.11.1), 4-MU β-d-xylopyranoside (MU-X) for xylosidase (EC 3.2.1.37), 4-MU-β-d d-glucuronide hydrate (MU-GU) for glucuronidase (EC 3.2.1.31), 4-MU β-d-cellobioside (MU-C) for cellobiohydrolase (EC 3.2.1.91), 4-MU N-acetyl-β-glucosaminide (MU-NAG) for chitinase (EC 3.2.1.14), 4-MU β-d-glucopyranoside (MU-G) for β-glucosidase (EC 3.2.1.3), 4-MU phosphate-free acid (MU-P) for phosphatase (EC 3.1.3.2) and ABTS for laccase (EC 1.10.3.2) activities. In brief, individual ectomycorrhizal root tips were placed in wells of 96-well filterplates (AcroPrep™ Advance 96 Filter Plate, 30–40 μm PP/PE, 350-μL wells, NTRL; Pall Corporation, Ann Arbor, MI, USA) and incubated with the respective substrate. The solution with the incubated substrate was transferred to a black 96-well plate (FluoroNunc™ F96 MicroWell™ Plate) by applying a vacuum under the filter plate with a vacuum manifold (Pall Corporation, Ann Arbor, MI, USA). From fluorescence and absorbance measurements, the quantity of substrate turned over was calculated and expressed as pmol mm^{-2} min^{-1}.

Leaf sampling

At harvest, leaves (including the petiole) were separated from the shoot and dried in paper bags at 70 °C until weight constancy. After drying, all leaves per plant were pre-ground with a rotor mill (Fritsch Rotor Speed Mill Pulverisette 14; Fritsch GmbH, Idar-Oberstein, Germany) equipped with a 0.5-mm sieve (Fritsch GmbH, Idar-Oberstein, Germany) at 8000 rpm and finally homogenised with the same mill equipped with a 0.08-mm sieve (Fritsch GmbH, Idar-Oberstein, Germany) at 10,000 rpm. From each sample, three technical replicates of ca. 1.5 mg were analysed for ^{13}C and ^{15}N, total N and total C content in an Isotope Ratio Mass Spectrometer (IRMS, type delta V Advantage; Thermo Fisher, Dreieich, Germany), coupled with an Element Analyser (Euro EA; Eurovector, Milan, Italy). Leaf P content was analysed via inductively coupled plasma atomic emission spectrometry (ICP-AES, Ciros Vision; Spectro, Kleve, Germany) after digestion of homogenised leaf material with nitric acid.

Stable isotope analysis

^{15}N and ^{13}C abundances were determined with an IRMS (Delta V Advantage) coupled with an Elemental Analyzer (Euro EA). A laboratory standard (acetanilide) was used at regular intervals of every measurement sequence. It was also used at different weights to determine the isotope linearity of the system. The laboratory standard itself is calibrated against suitable international isotope standards (IAEA; Vienna) for ^{15}N and ^{13}C. Several international isotope standards were also part of every sequence. For ^{13}C, these were IAEA 309 A and IAEA 309 B. For ^{15}N, these were IAEA N2, USGS 41, USGS 26 and USGS 32. For samples with

high ^{15}N abundances, laboratory standards between 6 and 15 atom% were used. These standards were formerly calibrated with standards from Fischer Analysen Instrumente (Leipzig, Germany).

Statistical analysis

Values in this article are given as mean ± standard error (SE) if not indicated differently. All statistical analyses were conducted using R (R Core Team 2016). The effects of the treatments on measures of growth and stable isotope abundance in ECM and leaves of the plants were analysed using three-way analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) test. In the analysis of the influence of the treatments on ^{13}C abundance in ECM, leaf area was used as a covariate to take into account differences in photosynthetic capacity between plants. To test the influence of irrigation on the distinct fertiliser combinations, one-way ANOVA was used on the respective subsets of data. Enzymatic activity patterns were analysed with ANOVA. To ensure normally distributed residuals and homogeneity of variance, the dependent variables were log- or square-root-transformed, if necessary. Data from optical non-destructive assessment of leaf chlorophyll, flavonol and nitrogen status as well as gas exchange measurements and soil moisture probes data were analysed using mixed effect models. The R package nlme (Pinheiro et al. 2014) was used and individual plants were considered as a random factor, namely, we used random intercepts for plants. Random slopes per day were not used because this did not result in a significant improvement of the model. Data were transformed to meet model assumptions, if necessary.

Results

At harvest (49 days after fertilisation and onset of drought), the N content in leaves was dependent on N fertilisation and the irrigation regime. In continuously irrigated plants, leaf N content was rather low (+N: 0.65 ± 0.01%, −N: 0.57 ± 0.01%). In leaves of recovering plants, N content was slightly higher in non-N-fertilised treatments (0.82 ± 0.08%), but much higher in N-fertilised treatments (1.33 ± 0.05%; treatment × N: $p < 0.001$). More ^{15}N from the fertiliser was found in leaves of non-P-fertilised plants (10.38 ± 0.34 atom%) than in P-fertilised plants (9.54 ± 0.42 atom%; $p = 0.140$). Leaf P content of P-fertilised plants (4.03 mg g^{-1}) was 2.2 times higher than in non-P-fertilised plants (1.84 mg g^{-1}; $p < 0.001$). This resulted in low N:P ratios when only P was fertilised (below 1.7) and high N:P ratios when only N was fertilised (over 5.26). Leaves of drought treated plants generally had higher N:P ratios than leaves of continuously irrigated plants (Table 3).

Following nitrogen balance index (NBI) of leaf #9 over time revealed that fertilised N was taken up quickly at the beginning of the experiment in continuously irrigated plants, whereas uptake was retarded in drought treatments. Fertilisation with N increased NBI during two weeks after N application to values 1.6–2 times higher than in non-N-fertilised plants (Fig. 2). While NBI of leaf #9 in irrigated plants declined within the following three weeks to values in the range of plants without N application, NBI and hence N nutrition in drought-treated plants remained significantly higher until the end of the experiment (significant irrigation × N fertilisation interaction during severe drought and during recovery: $p < 0.01$; Table 4).

The low N content in leaves at harvest and the fast decrease of NBI in irrigated plants two weeks after fertilisation reflected the rapid depletion of N from the soil mixture. Respectively, higher N contents and NBI in drought treated recovering plants showed impeded N-uptake during drought. At harvest, the supplied nitrogen was depleted from 18.8 ± 3.8 and 80.7 ± 3.8 mg/kg dry soil mixture in non-N- and N-fertilised treatments, respectively, to 1.6 ± 0.2 mg/kg at harvest in all treatments. Thus, 4.6 times more N was taken up in N-fertilised treatments. Phosphorus content in the substrate at the beginning of the experiment was 53.1 ± 1.2 mg/kg in non-P- and 156.8 ± 1.2 mg/kg in P-fertilised treatments. Thus, 3.4 times more P was taken up in P-fertilised treatments (Table 5). The soil mixture in the pots was entirely penetrated by roots.

Numerous significant irrigation × N interactions ($p < 0.001$, Table 3) showed that the increase in aboveground plant growth and thereby activities of aboveground C sinks due to N fertilisation were much higher in the irrigated treatments. There were also three significant N × P interactions ($p < 0.05$): in the absence of N, P addition reduced leaf biomass and increased shoot water content and leaf area, whereas the opposite was observed in the presence of N (Table 3). However, soil moisture, stomatal conductance and WUE were not significantly altered by fertilisation regime, indicating

Table 3 Mean values ± standard errors for traits of *P. maximowiczii* × nigra clone 'Rochester' at harvest, one week after rewatering. The treatments are irrigation without fertiliser (C0), with nitrogen (CN), with phosphorus (CP) and with both nitrogen and phosphorus fertiliser (CNP); and reduced irrigation without fertiliser (T0) with nitrogen (TN), with phosphorus (TP) and with both nitrogen and phosphorus fertiliser (TNP). Below: Analysis of variance of the influence of irrigation and nitrogen (N) and phosphorus (P) fertilisation on selected traits of *P. maximowiczii* × nigra clone 'Rochester'. Significant results ($p < 0.05$) are in bold

Treatment		Leaf N:P ratio		growth increment [mm]		Shoot biomass [g]		Leaf Biomass [g]		Shoot height [cm]		Leaf area [cm^2]		Shoot water content [%]		Leaf water content [%]		Leaf area [cm^2]	
		mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se
C0		2.86	0.09	7.3	0.4	8.2	0.8	12.6	0.8	79.9	3.1	1320	103.4	51.3	2.4	58	0.7	1320	103
CN		5.26	0.46	9.6	0.2	14.3	0.5	20.5	0.9	112.2	4.5	2063	84.4	57.4	1.7	57.7	0.7	2063	84
CNP		2.4	0.08	8.7	0.2	14.2	0.8	20.5	0.5	120.8	8.9	2014	69.4	56.8	0.6	58.9	0.5	2014	69
CP		1.25	0.06	7.5	0.7	6.7	0.5	10.2	0.5	75.8	3.4	1084	57.8	56.4	0.6	60.1	0.7	1083	58
T0		4.04	0.29	6.6	0.2	5.6	0.4	7.5	0.5	74	3.6	893.8	56.8	57.7	0.6	62.7	0.6	894	57
TN		5.76	0.18	7.4	0.2	7	0.5	7.2	0.7	87.7	1.8	956.3	62	64.7	1.1	66.1	2	956	62
TNP		4.11	0.61	7.3	0.2	7	0.4	8	0.1	83	3.5	1057	29.1	64.5	0.6	66.4	0.3	1057	29
TP		1.68	0.16	6.1	0.3	4.2	0.3	5.4	1.1	68.8	3	666.4	136.5	61.2	1	67.7	3.2	666	137
Source of variation	df	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Irrigation	1	40.71	**<0.001**	24.38	**<0.001**	128.5	**<0.001**	267.8	**<0.001**	34.61	**<0.001**	133.9	**<0.001**	46.89	**<0.001**	11.43	**0**	24.38	**<0.001**
N-fert.	1	172.8	**<0.001**	26.53	**<0.001**	113.6	**<0.001**	94.44	**<0.001**	87.88	**<0.001**	74.57	**<0.001**	20.23	**<0.001**	0.2	0.7	26.53	**<0.001**
P fert.	1	192	**<0.001**	1.25	0.27	5.46	**0.025**	4.14	**0.049**	1.04	0.315	3.63	0.065	5.57	**0.024**	0.04	0.8	1.25	0.27
Irrig x N	1	0.26	0.61	1.49	0.23	19.78	**<0.001**	57.84	**<0.001**	11.07	**0.002**	26.03	**<0.001**	0.95	0.336	0.05	0.8	1.49	0.23
Irrig x P	1	2.28	0.14	0.07	0.797	0.06	0.814	0.3	0.589	1.12	0.298	0.33	0.566	0.13	0.724	0.78	0.4	0.07	0.797
N x P	1	7.77	**0.009**	0.1	0.76	3.61	0.065	6.08	**0.019**	1.23	0.275	4.34	**0.044**	6.19	**0.018**	1.09	0.3	0.1	0.76
Irrig x N x P	1	4.72	**0.037**	0.19	0.19	0.08	0.785	0.07	0.793	0.76	0.389	0.32	0.577	0.27	0.607	0.25	0.6	0.19	0.19
Residual	36																		

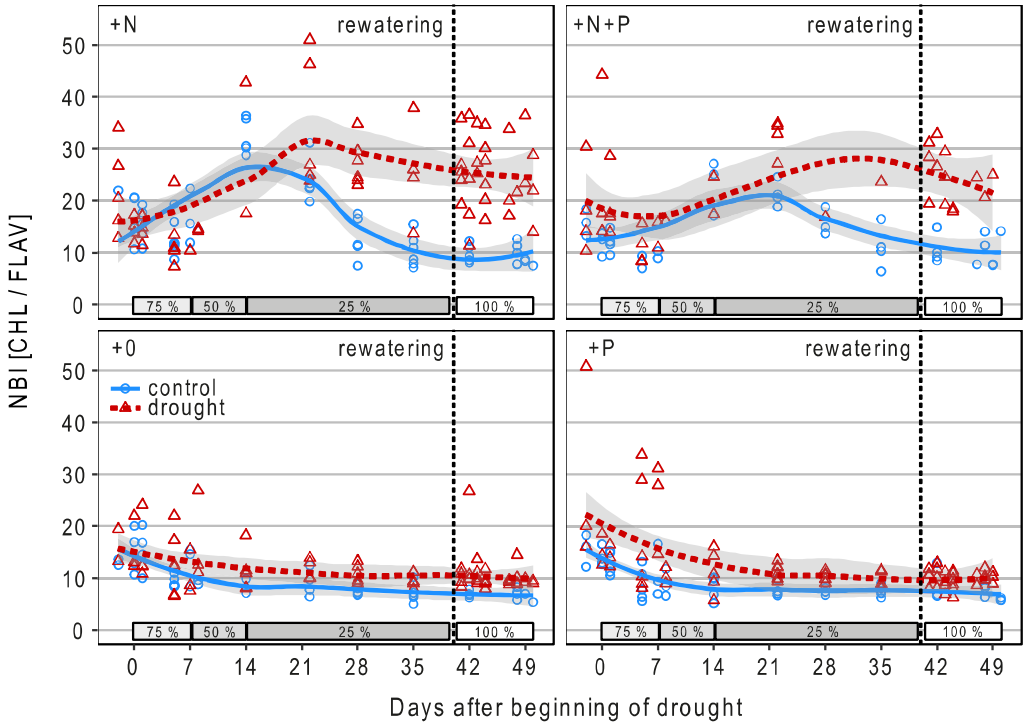


Fig. 2 NBI (nitrogen balance index: CHL/FLAV) indices in leaf #9 from the apex of *P. maximowiczii* × *nigra* clone 'Rochester' during the experiment in irrigated (open circles) and drought-stressed plants (open triangles) separated by fertilisation regime (+0 = no fertilisation, +N = N fertilisation, +P = P fertilisation, +N + P = N and P fertilisation). Curves show LOESS fits with 95% confidence interval (grey shading) of irrigated (solid line) and drought-stressed (dashed line) plants

that bigger plants did not experience differential drought stress. Growth increment, shoot biomass, leaf biomass, shoot height and leaf area were significantly positively correlated among each other. Water content of leaves and shoots was negatively correlated with the above-mentioned variables and among each other they were significantly positively correlated (Table S2).

Despite effects of N fertilisation on leaf nutrition and plant growth, the fertilisation regime did not significantly affect net assimilation (A_n) and WUE. During severe drought with 25% irrigation compared to the control, A_n and hence source activity (Fig. 3)a was significantly reduced by the irrigation regime ($p < 0.05$), while WUE (Fig. 3)b was significantly increased ($p < 0.001$). During recovery (100% irrigation of the control), the WUE of recovering plants was still significantly higher than that of control plants ($p < 0.05$), but there was no significant difference in A_n anymore ($p = 0.350$).

One day after labelling ^{13}C abundance in leaves (C-sources and C-sinks when still growing) was lower than in ECM. ^{13}C abundance was significantly higher in leaves of plants recovering from drought (227.0 ± 16.8‰ V-PDB) than in continuously irrigated plants (178.4 ± 9.5‰ V-PDB; $p < 0.05$). Overall C content was higher in leaves of recovering plants (43.8 ± 0.2% vs. 42.8 ± 0.3%; $p < 0.001$) and in leaves of plants fertilised with N (44.1 ± 0.2% vs. 42.6 ± 0.3%; $p < 0.001$).

The below-ground sinks (ECM tips) of plants recovering from drought showed higher ^{13}C abundances (C allocation) than ECM tips from irrigated treatments ($p < 0.005$). This effect was highest in treatments without N fertilisation (555.6 ± 99.0 vs. 209.5 ± 38.0‰ V-PDB, $p < 0.005$; Fig. 4). ECM tips of recovering plants that were fertilised with nitrogen did not show significant differences in ^{13}C abundances (388.5 ± 70.5‰ V-PDB) compared to ECM tips of irrigated plants (312.0 ± 87.6‰ V-PDB). The non-fertilised treatment showed the greatest difference in ^{13}C abundance between ECM tips of irrigated and recovering plants. A significant ($p < 0.05$) ordinal irrigation × P fertilisation interaction showed that ECM tips of recovering plants received more C when not fertilised with P (Table 6). There was no significant effect of fertilisation on ^{13}C abundance among the ECM tips of recovering and control plants.

Table 4 Effects of irrigation, nitrogen (N) and phosphorus (P) fertilisation on nitrogen balance index (NBI), net photosynthesis (A_n) and instantaneous water use efficiency (WUE) in leaf #9 from the apex of *P. maximowiczii* × *nigra* clone 'Rochester' during severe drought (25% irrigation of control plants for 32 days) and during recovery (100% water for 7 days). *p*-values obtained by linear mixed effects models are shown. Significant results ($p < 0.05$) are in bold

Time	Source of variation	NBI	A_n	WUE
During severe drought (25% irrigation)	Date	**<0.001**	**0.003**	0.247
	Irrigation	**<0.001**	**0.036**	**<0.001**
	N-fertilisation	**<0.001**	0.35	0.389
	P fertilisation	0.359	0.31	0.066
	Irrig. x N-fert.	**0.005**	0.388	0.287
	Irrig. x P fert.	0.543	0.971	0.402
	N-fert. x P fert.	0.663	0.415	0.138
	Irrig. x N x P	0.533	0.876	0.708
During recovery (100% irrigation)	Date	0.157	**<0.001**	**0.008**
	Irrigation	**<0.001**	0.35	**0.011**
	N-fertilisation	**0.015**	0.697	0.436
	P fertilisation	0.658	0.093	0.868
	Irrig. x N-fert.	**<0.003**	0.642	0.624
	Irrig. x P fert.	0.583	0.687	0.587
	N-fert. x P fert.	0.924	0.238	0.911
	Irrig. x N x P	0.966	0.673	0.186

Drought reduced the relative number of belowground sinks. At harvest, double the amount of non-vital ECM tips were found in the drought treatments (40.4 ± 5.5% relative abundance) compared to the irrigated treatments (21.1 ± 3.2% relative abundance; $p < 0.01$). There was no significant difference in the degree of mycorrhization (72.6 ± 2.9% mycorrhizal root tips) or in the abundance of any of the species/morphotypes among the treatments. *Sphaerosporella brunnea* was the dominant ectomycorrhizal species (40.8 ± 3.0% overall relative abundance) identified from three out of five morphotypes. The fourth morphotype turned out to represent two macroscopically similar taxa: *Tomentella ellisii* and *Otidea tuomikoskii* (1.0 ± 0.7% overall relative abundance). The fifth morphotype did not yield an evaluable ITS sequence (0.2 ± 0.1% overall relative abundance).

In ECM tips of plants recovering from drought, activities of xylosidase, glucuronidase and cellobiohydrolase did not show significant differences among treatments. Laccase activity was not detectable and hence omitted from further analyses. Yet, activities of chitinase, β-1,4-glucosidase (both degrading cell walls) and phosphatase (P mobilising) were significantly higher in ECM tips of plants recovering from drought (83.5 ± 6.2, 66.2 ± 5.9 and 31.7 ± 5.3 pmol mm^{-2} min^{-1}, respectively) compared with continuously irrigated treatments (63.7 ± 4.3, 48.9 ± 4.0 and 20.2 ± 2.0 pmol mm^{-2} min^{-1}, respectively). The effect of drought on the activity of the N mobilising enzyme leucine aminopeptidase was significantly different depending on nitrogen fertilisation. In N-fertilised treatments, ECM tips of plants recovering from drought showed significantly higher activity (185.6 ± 47.5 pmol mm^{-2} min^{-1}) than tips from irrigated treatments (69.1 ± 21.7 pmol mm^{-2} min^{-1}), while in treatments without N addition, the activity of recovering ECM tips was decreased (91.9 ± 21.1 pmol mm^{-2} min^{-1}) compared with that in continuously irrigated treatments (146.9 ± 30.7 pmol mm^{-2} min^{-1}; Fig. 5, Table 7). So ectomycorrhizae of recovering plants had a higher potential to release nutrients from soil organic matter. All enzyme activities were positively correlated among each other (Table S3).

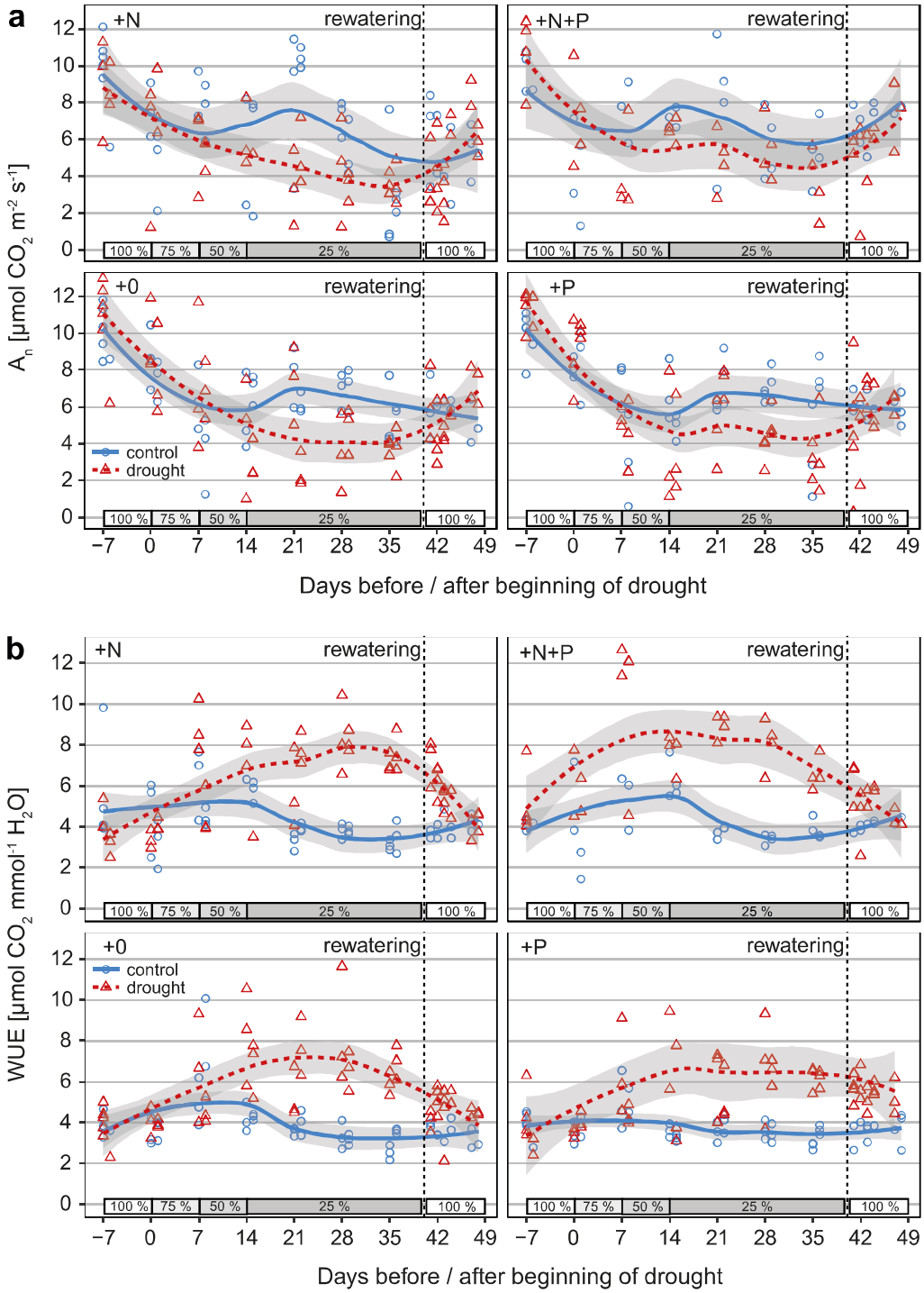
Discussion

Net photosynthesis (A_n, source activity) was reduced by drought and recovered after rewatering to the level of continuously irrigated plants, but did not go to excess. A

Table 5 Mean values [mg/kg dry soil mixture] ± standard error of N and P contents in the soil mixture before and after fertilisation as well as at harvest. Values for nutrient contents after fertilisation result from the addition of respective nutrient amounts to the values in the soil mixture before fertilisation. The treatments are irrigation without fertiliser (C0), with nitrogen (CN), with phosphorus (CP) and with both nitrogen and phosphorus fertiliser (CNP); and reduced irrigation without fertiliser (T0) with nitrogen (TN), with phosphorus (TP) and with both nitrogen and phosphorus fertiliser (TNP). Below: Analysis of variance of the influence of irrigation and nitrogen (N) and phosphorus (P) fertilisation on N and P contents in the soil mixture at harvest of *P. maximowiczii* × nigra clone 'Rochester'. Significant results ($p < 0.05$) are in bold

Treat-ment		before fertilisation				after fertilisation (calculated)				at harvest			
		N		P		N		P		N		P	
		mean	se	mean	se	mean	se	mean	se	mean	se	mean	se
C0		18.80	3.84	53.10	1.20	18.80	3.84	53.10	1.20	1.01	0.10	12.87	1.19
CN						80.66				2.13	0.84	12.40	1.45
CNP								156.84		1.90	0.55	22.16	2.08
CP						18.80				1.73	0.29	12.16	0.97
T0								53.10		1.79	0.63	15.04	0.75
TN						80.66				1.35	0.17	14.46	0.88
TNP								156.84		1.17	0.10	20.90	2.17
TP						18.80				1.73	0.28	24.88	2.49
Source of variation	df									F	p	F	p
Irrigation	1	initial conditions were qual				calculated values				0.97	0.330	1.90	0.177
N-fert.	1									0.01	0.907	3.18	0.083
P fert.	1									1.62	0.210	49.47	**<0.001**
Irrig x N	1									0.33	0.571	0.01	0.911
Irrig x P	1									0.14	0.713	1.63	0.210
N x P	1									0.94	0.337	0.15	0.705
Irrig x N x P	1									1.89	0.177	0.19	0.666
Residual	36												

similar effect was also observed in *Fagus sylvatica* (Hagedorn et al. 2016). Hagedorn et al. (2016) showed that European beech also increased A_n and C allocation to ECM (C-sink) during recovery after drought. They concluded that C allocation is sink-driven because an initial excess of C signal in ECM was observed, followed by a delayed excess of photosynthetic activity, according to the hypotheses on source–sink control tested here. In the present experiment, we obtained similar results: reduced irrigation decreased A_n, rewatering led to recovery but not an excess of A_n, while an excess allocation of recent photosynthates was observed in ECM even though drought reduced the relative number of below-ground sinks. This indicates sink driven C allocation. We additionally tested different nutrient levels in the soil and found significantly higher C allocation to ECM in recovering plants of non-N-fertilised treatments. This C is most probably used to restore roots and functions of ECM (i.e. uptake of water and nutrients) after drought. Effects on root growth are unlikely as increased root growth after rewatering would have resulted in a relative decrease of mycorrhization–which was not observed here–because ECM formation takes longer than one week. The same trend was observed in N-fertilised treatments, but the effect was not significant. By implication, under high N availability, there might be less need to restore root and ectomycorrhizal metabolism as N can be used to synthesise amino acids, many of which–especially proline–can also act as osmoprotectants. These molecules accumulate during water shortage not inhibiting cellular metabolism, but protecting cells from damage (Handa et al. 1986; McNeil et al. 1999; Rhodes et al. 1986; Yancey et al. 1982). In a microcosm experiment, Bidartondo et al. (2001) showed that N uptake increased the C sink strength of ectomycorrhizal fungi due to the high C and energy demands. However, in sufficiently irrigated treatments, C allocation was not significantly higher when N fertiliser had been applied. Therefore, we suggest that, despite the high energy demand of N uptake,


a
+N
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drought
100 %
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100 %
A_n [µmol CO_2 m^-2 s^-1]
Days before / after beginning of drought
b
+N
rewatering
+N+P
rewatering
+0
rewatering
+P
rewatering
control
drought
WUE [µmol CO_2 mmol^-1 H_2O]
Days before / after beginning of drought

◀ **Fig. 3** **a**) Net photosynthesis (A_n) and **b**) instantaneous water use efficiency (WUE) of *P. maximowiczii* × *nigra* clone 'Rochester' during the experiment in irrigated (open circles) and drought-stressed plants (open triangles) separated by fertilisation regime (+0 = no fertilisation, +N = N fertilisation, +P = P fertilisation, +N + P = N and P fertilisation). Curves show LOESS fits with the 95% confidence interval (grey shading) of irrigated (solid line) and drought-stressed (dashed line) plants. Data obtained from steady-state gas exchange measurements that were performed on leaves 9–11 (from apex) measured under average growth conditions (25 °C and 300 μmol m^{-2} s^{-1} PPFD). Shading and numbers above the x-axis indicate water supply of drought treatments relative to continuously irrigated treatments

the better nutritional status of the N-fertilised plants during recovery lowered the impact of stress and thus the C sink strength of ECM. This is also emphasised by the overall significantly higher C allocation to the ECM of recovering plants, when no P was applied as fertiliser. An example for stress induced C allocation is, that the sink strength of poplar leaves was higher after insect wounding and the application of jasmonic acid (Arnold and Schultz 2002). The fertilisation regime had no significant influence on C allocation to ECM under continuously watered conditions. The added N was taken up before harvest under irrigated conditions, as indicated by the results of soil mixture N content and NBI measurements. Therefore, a direct effect of fertilisation might not have been observable.

In our experiment, poplar plants showed increased instantaneous WUE under drought, which was sustained after rewatering in the plants without N fertiliser, similar to the findings of Liu et al. (2005) in *Glycine max* under laboratory conditions. As expected,

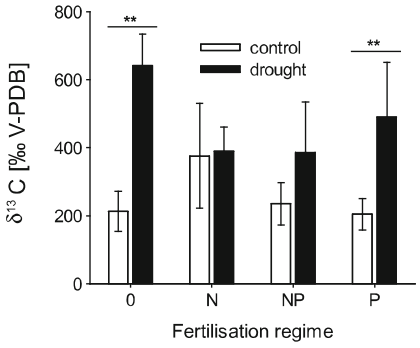

Fig. 4 δ ^{13}C values of ECM tips of *P. maximowiczii* × *nigra* clone 'Rochester' at harvest, from continuously watered plants compared with drought-stressed plants one week after rewatering, one day after labelling. 0 = no fertilisation, N = nitrogen fertilisation, NP = nitrogen and phosphorus fertilisation, P = phosphorus fertilisation. ** = $p < 0.005$

Table 6 Analysis of covariance of the influence of irrigation and nitrogen (N) and phosphorus (P) fertilisation on ^{13}C abundance in ECM of *P. maximowiczii* × *nigra* clone 'Rochester' one day after labelling. Leaf area was used as a covariate to correct for different assimilation areas of the plants. Significant results ($p < 0.05$) are in bold

Source of variation	^{13}C abundance in mycorrhizae		
	df	F	p
Leaf area	1	0.25	0.618
Irrigation	1	8.07	**0.008**
N-fert.	1	5.27	**0.028**
P fert.	1	0.43	0.518
Irrig x N	1	0.71	0.404
Irrig x P	1	4.68	**0.038**
N x P	1	0.03	0.854
Irrig x N x P	1	0.09	0.766
Residual	33		

drought reduced growth while nitrogen fertilisation enhanced it. Similar morphological responses of *Populus* species to drought stress have been reported in previous experiments (Ibrahim et al. 1997; Yin et al. 2005; Zhang et al. 2004). Higher above-ground growth is also a higher above-ground C sink, which could explain the lower ^{13}C abundance in ECM of recovering plants that received N fertiliser alternatively to the better nutritional

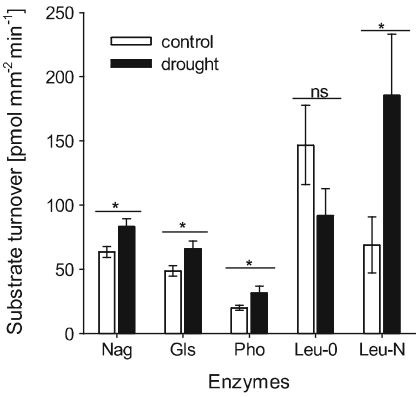

Fig. 5 Enzymatic activities of mycorrhizal root tips of *P. maximowiczii* × *nigra* clone 'Rochester' at harvest, from continuously watered plants compared with drought-stressed plants one week after rewatering. Nag = chitinase, Gls = β-glucosidase, Pho = phosphatase, Leu-0 = leucine aminopeptidase not N-fertilised plants, Leu-N = leucine aminopeptidase N-fertilised plants. Nag, Glu and Pho activities are significantly higher in ECM tips of plants recovering from drought. There is a significant interaction effect of irrigation regime and nitrogen fertilisation on Leu activity. * = $p < 0.05$

Table 7 Mean values and standard errors (n = 4–6) of exoenzyme activities (pmol mm − 2 min − 1) of ECM tips of *P. maximowiczii* × nigra clone 'Rochester' at harvest, one week after rewatering. The treatments are irrigation without fertiliser (C0), with nitrogen (CN), with phosphorus (CP) and with both nitrogen and phosphorus fertiliser (CNP); and reduced irrigation without fertiliser (T0) with nitrogen (TN), with phosphorus (TP) and with both nitrogen and phosphorus fertiliser (TNP). Below: Analysis of variance of the influence of irrigation and nitrogen (N) and phosphorus (P) fertilisation on selected exoenzyme activities of mycorrhizae of *P. maximowiczii* × nigra clone 'Rochester'. Significant results ($p < 0.05$) are in bold

Treatment		Leucine-aminopep-tidase		Xylosidase		Glucuron-idase		Cellobio-hydrolase		Chitinase		β-1,4-gluco-sidase		phosphatase	
		mean	se	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se
C0		115.98	23.84	31.62	5.26	17.94	7.63	26.73	6.86	65.84	9.15	45.71	7.21	21.60	4.52
CN		72.93	34.00	35.56	7.22	13.96	6.96	23.59	6.45	62.18	7.46	41.34	7.38	17.94	2.98
CNP		63.36	24.95	44.12	16.64	19.98	8.42	28.91	9.62	64.99	12.73	52.86	9.02	23.46	6.81
CP		177.74	56.60	50.74	8.32	15.51	7.94	26.21	8.88	62.37	8.65	57.00	8.98	19.00	3.01
T0		108.40	31.64	42.35	11.14	12.82	2.10	25.17	2.47	62.12	3.00	56.30	5.51	21.08	1.09
TN		226.41	74.05	59.80	17.63	21.42	4.71	34.69	2.85	97.93	7.03	84.96	11.14	37.87	5.84
TNP		124.27	33.96	42.66	15.64	18.73	5.98	32.44	9.40	91.51	21.35	64.52	16.87	24.61	3.75
TP		75.50	29.09	45.60	19.36	35.46	20.22	52.81	21.31	85.01	14.44	58.29	12.60	40.74	18.49
Source of variation	df	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Irrigation	1	0.41	0.525	4E-04	0.985	0.77	0.386	3.05	0.089	7.24	**0.011**	4.49	**0.041**	6.41	**0.016**
N-fert.	1	0.09	0.764	0.12	0.733	0.34	0.562	0.01	0.904	1.70	0.201	0.34	0.563	0.33	0.571
P fert.	1	0.31	0.583	0.14	0.712	0.01	0.906	0.13	0.725	0.08	0.778	0.02	0.881	0.01	0.928
Irrig x N	1	6.68	**0.014**	1.36	0.251	0.33	0.566	0.04	0.841	2.00	0.166	1.93	0.173	0.63	0.432
Irrig x P	1	1.26	0.270	1.32	0.258	0.16	0.690	0.07	0.796	0.25	0.623	2.64	0.113	0.01	0.916
N x P	1	0.25	0.617	0.33	0.571	0.38	0.540	0.17	0.683	0.76	0.390	0.24	0.626	0.50	0.486
Irrig x N x P	1	0.11	0.739	0.28	0.597	0.19	0.666	1.21	0.279	1.46	0.235	0.45	0.508	2.72	0.108
Residual	36														

status of the plants. Yet, this would underpin the hypothesis of source-driven allocation of C. However, ^{13}C abundance in leaves of recovering N-fertilised plants was not higher than in not fertilised plants, but this may result from dilution by assimilation of ambient CO_2 as plants were harvested one day after labelling. Thus, we cannot finally clarify if the lower ^{13}C abundance in ECM tips of recovering N-fertilised plants is due to the better nutritional status of the plants or due to the partitioning of recent photosynthates between two sinks.

More non-vital ECM tips were found in the drought treatments and this share was not influenced by the fertilisation regime. AM colonisation was previously reported to be very low on roots of *Populus tremuloides* in a field experiment (Neville et al. 2002). As the authors found evidence that the occurrences of ECM and AM are negatively correlated and linked to soil depth, the absence of AM in our experiment may partly be due to a pot effect.

We found no support for our hypothesis that mineral fertilisation alone reduces the enzyme activities of ECM. In fact, leucine aminopeptidase activity was higher in N-fertilised ECM of recovering plants than in continuously irrigated plants. This is an intriguing result as proteolytic activity of the ectomycorrhizal fungus *Hebeloma crusutliniforme* has been shown to be reduced in the presence of easily available N (Zhu et al. 1994). Another explanation could be higher N demand by N-fertilised plants due to their larger size and because the N content was very low in all treatments at harvest. Our hypothesis that the enzyme activities of ECM increase upon recovery from drought was confirmed by three out of seven enzymes, whereas the activities of the other four enzymes did not respond significantly to the drought treatment. Exoenzyme activities of ECM can be very stable upon disturbance. It has been shown that ectomycorrhizal communities can maintain the level of their enzymatic activities under stress conditions (Diedhiou et al. 2010; Jones et al. 2010). Even within a single ectomycorrhizal species leucine aminopeptidase activity was regulated depending on the abundance of this species to maintain a stable activity (Herzog et al. 2013). Herzog et al. (2013) found neutral responses to drought in the activities of the same seven enzymes we measured in *Cenococcum geophilum* ectomycorrhizae of different *Quercus* species. The authors attributed this result to the generalist lifestyle of *C. geophilum* in contrast to specialist species as for example *Lactarius subdulcis* or *Xerocomus pruinatus*, because these specialist species accounted for most of the effects of liming on the EA profile of an ectomycorrhizal community in a Norway spruce and beech stand (Rineau and Garbaye 2009). In our experiment the dominant ectomycorrhizal species *S. brunnea* is also most probably a generalist as suggested by its wide distribution and host range (Danielson 1984), but with upregulation of activities of three out of seven enzymes which underlines the significance of these alterations. Higher activities of β-glucosidase and chitinase in ECM of recovering plants suggest the degradation of plant and fungal cell walls and arthropod-derived chitin from individuals that died during drought (Pritsch and Garbaye 2011). This supports the need to mobilise N, as reflected in the similar increase in leucine aminopeptidase activity. A high proportion of dead roots and ECM may have stimulated cellulolytic and chitinolytic activities in the remaining vital ECM in order to attain N- and P-rich substrates inside these dead cells and tissues. Furthermore, the uptake of NH_4^+ (and NO_3^-, which is eventually metabolised to NH_4^+) requires C to form the C skeleton for glutamate or glutamine. Although this C is supplied to the fungus by the plant, the breakdown of proteins and enhanced β-glucosidase activity may augment plant C supply (Abuzinadah and Read 1988; Chalot and Brun 1998; Chalot et al. 1994), as up to 40% of the supplied C has been shown to be used for N assimilation in culture experiments with ectomycorrhizal fungi (Martin and Canet 1986; Martin et al. 1988). Enhanced phosphatase activity during recovery from drought may help to mobilise P from dead cells and tissues, which were made accessible by the chitinolytic and cellulolytic activities of ECM. After severe drought, there might also be a need for phosphorus to restore metabolism.

The utilisation of supplied N was much higher in irrigated plants than in drought-treated ones, as shown by their enhanced growth and the rapid change in NBI after fertilisation. However, leaf N content at harvest was higher in recovering plants, probably reflecting retarded N uptake during the early phase of recovery and because low water availability in the rhizosphere may have decreased ion uptake and transport to shoots (Greenway et al. 1969; Pitman 1981). N enrichment may thus be a result of reduced absorption processes in the root and ion movement in the soil during drought (Dunham and Nye 1976; Russell 1973). Even N-fertilised plants were N-limited at harvest as suggested by leaf NBI development and N:P ratios ranging from

ca. 1.5 to 5.5 which is below the optimal value of 9 for *P. nigra* × *P. maximowiczii* (Kelly and Ericsson 2003). The fertiliser design mirrored agricultural practice with one fertilisation application at the beginning of the experiment in contrast to continuous fertilisation. As the soil mixture was entirely rooted at harvest, N-supply calculated by pot surface might have been too low considering the pot volume. Yet, we found strong N-fertilisation effects on C allocation to ECM and—depending on the irrigation regime—on measures of growth. Plants of non-P-fertilised treatments might not have experienced P limitation as reflected by plant growth and leaf P content (Jug et al. 1999; van den Burg 1985). For example, field crops did not show P deficiency, despite reduced fertilisation for 5 years, and no adaptation of the soil microbial community was observable (Browne et al. 2009; Conry and Hogan 2001). In fact, P fertilisation in our experiment resulted in slight growth suppression, which was counteracted by N fertilisation, as shown by the significant N × P interaction for leaf biomass and the same trend for shoot biomass. This indicates slight P toxicity because of the high amounts of P added at the beginning of the experiment. This amount was calculated according to guidelines for farmland. We added the missing amount of P for a rich supply. However, the amount of P present in the substrate before fertilisation might have been underestimated because only plant available P was measured. Probably there was labile P in the substrate which was made available by soil biota during growth of the plant (Frossard et al. 2000). In *Eucalyptus grandis*, a suppressive effect of high, spatially homogeneous P fertilisation on shoot growth has also been reported and attributed to reduced nitrate uptake at high P levels (Costa et al. 2016; Graciano et al. 2009). Trends in ^{15}N contents of leaves of N-fertilised plants in our experiment support these findings, although not significantly.

Conclusion

Our results indicate that allocation of recent photosynthates is most likely sink-controlled because, after drought release, source activity (A_n) recovered to the level in irrigated plants, but ^{13}C allocation to ECM (C-sink) was much higher. However, this was less pronounced in the presence of N and P fertiliser. Overall, this suggests that either (1) sink strength is an indicator of the impact of drought stress and that this sink strength can be reduced especially by fertilisation with N, or (2) that recent photosynthates were partitioned between two sinks (i.e. ECM and above-ground growth). Testing a wider range of nutrient stoichiometries and quantifying above- and below-ground sink strength would reward further studies investigating the interplay of carbon source-sink dynamics in recovering plants and nutrient availability in soils.

Acknowledgements The authors wish to thank two anonymous reviewers, the handling editor and Diethart Matthies (Philipps University of Marburg) for valuable comments to improve the manuscript, Tatjana Gartner for measurements in the greenhouse, Peter Kary for technical support, Peter Grill for analysis of leaf phosphorus content. The plant material was kindly provided by Karolina Faust from the Bavarian Office for Forestal Seed and Plant Breeding (ASP).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Mehtod S1: Identification of morphotypes via Sanger sequencing

Three × ten root tips per morphotype were collected in 2.0-mL microcentrifuge tubes and DNA was extracted with the MoBio-htp 96 Well Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA), in accordance with the manufacturer's manual. DNA yield was measured with a NanoDrop 1000 (Peqlab, Erlangen, Germany). The phylogenetically informative region ITS was amplified via PCR and the primer pair ITS1F [Gardes and Bruns (1993); 5'-CTTGGTCATTTAGAGGAAGTAA-3'] and ITS4 [White et al. (1990); 5'-TCCTCCGCTTATTGATATGC-3'] using the Qiagen Taq PCR Master Mix Kit (Qiagen, Hilden, Germany). PCR mixtures contained 25 μL of Qiagen Taq PCR Master Mix, 1 μL of both primers (10 μ*M*), 20–30 ng of DNA template and water to 50 μL. Following initial denaturation at 94 °C for 4 min, samples were amplified by 35 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 75 s, and then a final extension at 72 °C for 5 min. PCR success was tested via gel electrophoresis (1% agarose gel, 120 V, 30 min). PCR products were cleaned up with ethanol/EDTA/sodium acetate precipitation and subjected to chain termination PCR using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Life Technologies, Carlsbad, CA, USA). For each primer, a separate PCR mixture was prepared containing 2 μL of amplified DNA template, 1 μL of 5x BDT reaction buffer, 1 μL of primer (10 μ*M*) and 1 μL of terminator mix (dNTPs and labelled ddNTPs). After initial denaturation at 96 °C for 1 min, samples were amplified by 50 cycles of 96 °C for 10 s, 50 °C for 10 s and 60 °C for 4 min. Chain termination fragments were cleaned up with ethanol precipitation and analysed on an ABI 3730 48-capillary sequencer. Sequences were checked and assembled with the software codoncode aligner (CodonCode Corporation, Centerville, MA, USA) and contigs were blasted against the UNITE database (Kõljalg et al. 2013).

Table S2 Matrix with Spearman correlation coefficients (ϱ) of physiological data. Significant correlations ($p < 0.05$) are in bold.

	Growth increment	Shoot biomass	Leaf biomass	Shoot height	Leaf area	Shoor water content	Leaf water content
Growth increment	1	**0.84**	**0.76**	**0.68**	**0.82**	-0.17	**-0.35**
Shoot biomass	**0.84**	1	**0.85**	**0.86**	**0.86**	**-0.34**	**-0.52**
Leaf biomass	**0.76**	**0.85**	1	**0.61**	**0.96**	**-0.54**	**-0.74**
Shoot height	**0.68**	**0.86**	**0.61**	1	**0.64**	-0.06	-0.25
Leaf area	**0.82**	**0.86**	**0.96**	**0.64**	1	**-0.39**	**-0.61**
Shoot water content	-0.17	**-0.34**	**-0.54**	-0.06	**-0.39**	1	**0.71**
Leaf water content	**-0.35**	**-0.52**	**-0.74**	-0.25	**-0.61**	**0.71**	1

Table S3 Matrix with Spearman correlation coefficients (ϱ) of enzyme activities. Significant correlations ($p < 0.05$) are in bold.

	Leucine amino-peptidase	Xylosidase	Glucuroni-dase	Cellobio-hydrolase	Chitinase	Glucosi-dase	Phosphat-ase
Leucine aminopeptidase	1	0.28	0.17	0.18	**0.31**	**0.53**	**0.43**
Xylosidase	0.28	1	**0.52**	**0.34**	**0.37**	**0.44**	**0.31**
Glucuronidase	0.17	**0.52**	1	**0.77**	**0.67**	**0.59**	**0.57**
Cellobiohydrolase	0.18	**0.34**	**0.77**	1	**0.90**	**0.76**	**0.67**
Chitinase	**0.31**	**0.37**	**0.67**	**0.90**	1	**0.83**	**0.76**
Glucosidase	**0.53**	**0.44**	**0.59**	**0.76**	**0.83**	1	**0.68**
Phosphatase	**0.43**	**0.31**	**0.57**	**0.67**	**0.76**	**0.68**	1

Axis 1 of the PCA with physiological data explains most of the variability in the dataset and separates samples of continuously irrigated, N-fertilised plants from samples of all other treatments (Fig. S4). The most influential variable and the only one that contributed significantly to the distribution in the plot was leaf area for axis 1. Axis 2 depicts mostly changes in water content of the leaves. All other variables cluster near the origin and seem not to influence the distribution of samples in the ordination graph.

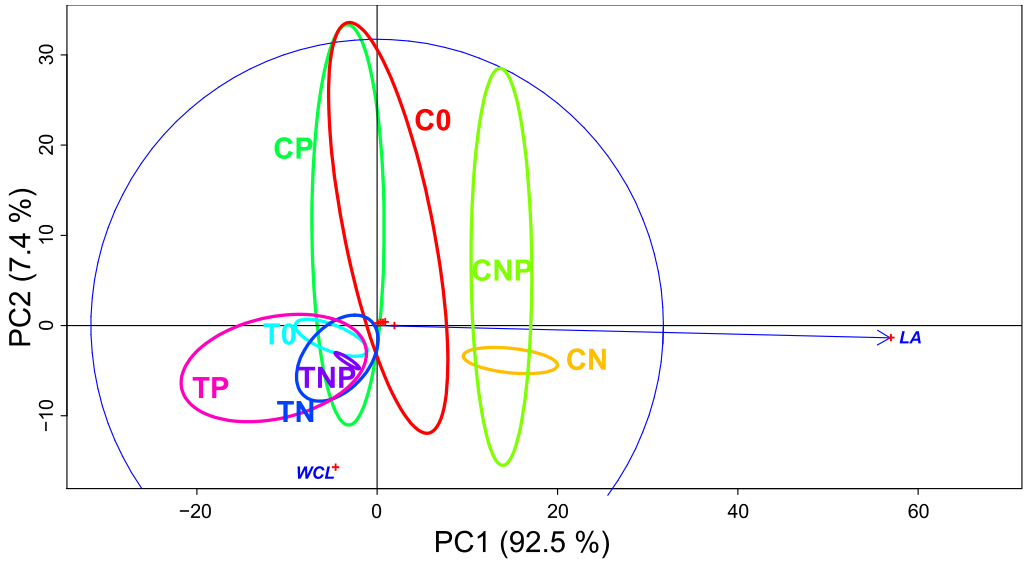

Fig S4 Principal component analysis (PCA) of plant growth parameters of *P. maximowiczii* × *nigra* clone 'Rochester' at harvest, one week after rewatering. LA: leaf area, WCL: water content in leaves. All other measures of growth cluster near the origin as they are highly correlated. Confidence ellipses (90 %) are drawn for each of the treatments: irrigation without fertiliser (C0), with nitrogen (CN), with phosphorus (CP) and with both nitrogen and phosphorus fertiliser (CNP); and reduced irrigation without fertiliser (T0) with nitrogen (TN), with phosphorus (TP) and with both nitrogen and phosphorus fertiliser (TNP). Growth parameters outside the equilibrium circle (blue circle) significantly contributed to the ordination graph.

Although leucine aminopeptidase contributed significantly to the distribution of samples in the ordination graph, enzyme activities did not separate treatments via PCA (Fig. S5).

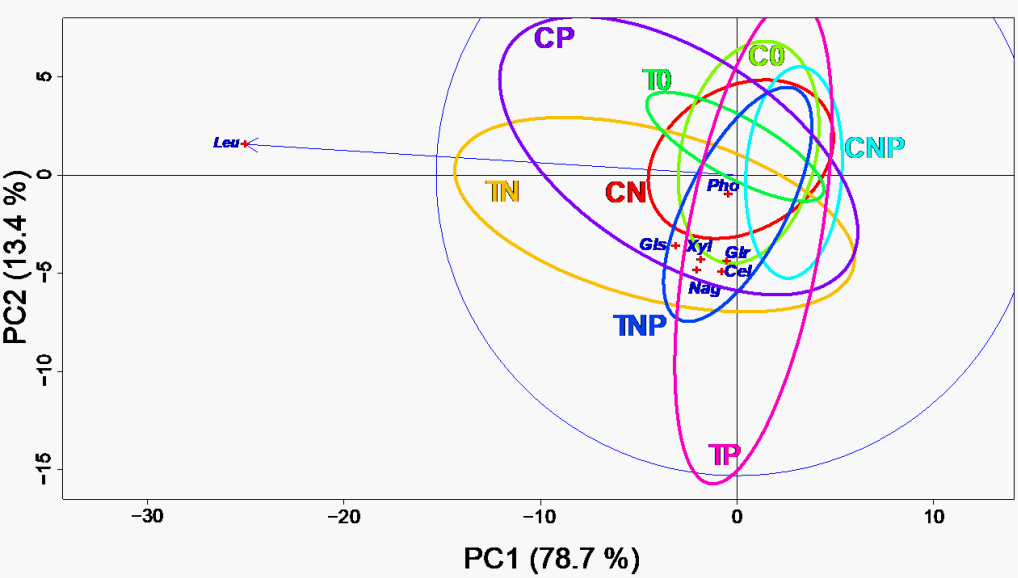

Fig S5 Principal component analysis (PCA) of exoenzyme activities of ECM tips of *P. maximowiczii* × *nigra* clone 'Rochester' at harvest, one week after rewatering. Leu: leucine aminopeptidase, Pho: phosphatase, Gls: glucosidase, Xyl: xylosidase, Glr: glucuronidase, Cel: cellobiohydrolase, Nag: chitinase. Confidence ellipses (90 %) are drawn for each of the treatments: irrigation without fertiliser (C0), with nitrogen (CN), with phosphorus (CP) and with both nitrogen and phosphorus fertiliser (CNP); and reduced irrigation without fertiliser (T0) with nitrogen (TN), with phosphorus (TP) and with both nitrogen and phosphorus fertiliser (TNP). Enzymes outside the equilibrium circle (blue circle) significantly contributed to the ordination graph.

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7.4 IV: Quantitative losses vs. qualitative stability of ectomycorrhizal community responses to 3 years of experimental summer drought in a beech-spruce forest

Global Change Biology. doi: 10.1111/gcb.13957

Year: 2017

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The following article reports results from the first three years of the throughfall exclusion experiment at Kranzberger Forst. Forest ecosystems in central Europe are predicted to face an increasing frequency and severity of summer droughts because of global climate change. European beech and Norway spruce often coexist in these forests with mostly positive effects on their growth. However, their different below-ground responses to drought may lead to differences in ECM fungal community composition and functions. This was examined at the individual root levels and at ecosystem levels. Retractable roofs were installed over plots at the experimental site to impose repeated summer drought conditions. Zones were assigned within each plot where trees neighboured the same or the respective other tree species to study mixed species effects (see also article II). ECM fungal community composition changed and the numbers of vital mycorrhizae decreased for both tree species over 3 drought years (2014–2016), with the ECM fungal community diversity of beech exhibiting a faster and of spruce a stronger decline. Mixed stands had a positive effect on the ECM fungal community diversity of both tree species after the third drought year. Relative abundance of ectomycorrhizae with long rhizomorphs increased in both species under drought, indicating long-distance water transport. However, there was a progressive decline in the number of vital fine roots during the experiment, resulting in a strong reduction in enzyme activity per unit volume of soil. Hydrolytic enzyme activities of the surviving ectomycorrhizae were stable or stimulated upon drought, but there was a large decline in ECM fungal species with laccase activity, indicating a decreased potential to exploit nutrients bound to phenolic compounds. Thus, ectomycorrhizae responded to repeated drought by maintaining or increasing their functionality at the individual root level, but were unable to compensate for quantitative losses at the ecosystem level. These findings demonstrate a strong below-ground impact of recurrent drought events in forests.

Received: 19 June 2017 | Revised: 8 September 2017 | Accepted: 11 October 2017

DOI: 10.1111/gcb.13957

PRIMARY RESEARCH ARTICLE

WILEY Global Change Biology

Quantitative losses vs. qualitative stability of ectomycorrhizal community responses to 3 years of experimental summer drought in a beech–spruce forest

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Funding information
Bayerisches Staatsministerium für Ernährung, Landwirtschaft und Forsten (Bavarian State Ministry of Food, Agriculture and Forestry); Bayerisches Staatsministerium für Umwelt und Verbraucherschutz (Bavarian State Ministry of the Environment and Consumer Protection); Deutsche Forschungsgemeinschaft, Grant/Award Number: MU 831/23-1

Abstract

Forest ecosystems in central Europe are predicted to face an increasing frequency and severity of summer droughts because of global climate change. European beech and Norway spruce often coexist in these forests with mostly positive effects on their growth. However, their different below-ground responses to drought may lead to differences in ectomycorrhizal (ECM) fungal community composition and functions which we examined at the individual root and ecosystem levels. We installed retractable roofs over plots in Kranzberg Forest (11°39′42″E, 48°25′12″N; 490 m a.s.l.) to impose repeated summer drought conditions and assigned zones within each plot where trees neighboured the same or different species to study mixed species effects. We found that ECM fungal community composition changed and the numbers of vital mycorrhizae decreased for both tree species over 3 drought years (2014–2016), with the ECM fungal community diversity of beech exhibiting a faster and of spruce a stronger decline. Mixed stands had a positive effect on the ECM fungal community diversity of both tree species after the third drought year. Ectomycorrhizae with long rhizomorphs increased in both species under drought, indicating long-distance water transport. However, there was a progressive decline in the number of vital fine roots during the experiment, resulting in a strong reduction in enzyme activity per unit volume of soil. Hydrolytic enzyme activities of the surviving ectomycorrhizae were stable or stimulated upon drought, but there was a large decline in ECM fungal species with laccase activity, indicating a decreased potential to exploit nutrients bound to phenolic compounds. Thus, the ectomycorrhizae responded to repeated drought by maintaining or increasing their functionality at the individual root level, but were unable to compensate for quantitative losses at the ecosystem level. These findings demonstrate a strong below-ground impact of recurrent drought events in forests.

KEYWORDS
climate change, ectomycorrhizae, enzyme activities, *Fagus sylvatica*, forest ecosystems, fungal diversity, *Picea abies*, summer drought

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1 | INTRODUCTION

European beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) KARST.) have a wide ecological range and are among the dominant tree species in mesic temperate forest ecosystems across Europe (Ellenberg, 1988; Fang & Lechowicz, 2006). Together with close relatives, these ectomycorrhizal (ECM) tree species are major components of ecosystems throughout the Holarctic realm (Fang & Lechowicz, 2006; Lockwood et al., 2013). However, both species are at risk from the increased frequency and intensity of droughts that are predicted by future climate change scenarios (Geßler et al., 2007; Spiecker, 1995; Young et al., 2017), with spruce being particularly vulnerable because of its shallow roots system and its low drought tolerance (Boden, Kahle, von Wilpert, & Spiecker, 2014).

Both species form monospecific and mixed forest stands, with spruce mostly exhibiting increased overall productivity when growing in mixture (Pretzsch et al., 2014). These positive mixed stand effects have been attributed to improved soil properties and increased overall biodiversity by beech (Ammer, Bickel, & Kölling, 2008). In addition, below-ground resource partitioning is likely to contribute to the positive effects of mixture as beech shifts its fine roots from upper to lower soil depths when growing alongside spruce because of competition (Bolte & Villanueva, 2006; Goisser et al., 2016). Under severe summer drought conditions, spruce can adapt by decreasing its fine-root growth (Puhe, 2003) while maintaining its standing fine-root biomass (Nikolova, Andersen, Blaschke, Matyssek, & Häberle, 2010), whereas beech exhibited slightly increased fine-root growth during the severe summer drought of 2003 (Nikolova et al., 2010). Thus, water limitation evokes different below-ground responses in these species (Schume, Jost, & Hager, 2004), with spruce decreasing water consumption and growth in the early stages of drought (Dobson, Taylor, & Freer-Smith, 1990; Maier-Maercker, 1998) and beech continuing to grow (Burkhardt & Pariyar, 2016). The distinct physiological responses of these tree species to drought suggest that their ectomycorrhizae will be exposed to different conditions under the same drought scenario.

From the thousands of ECM fungal species potentially forming ectomycorrhizae (Tedersoo et al., 2014), at the plot level, fine roots of spruce and beech have been found to form ectomycorrhizae with an estimated 60 species of Basidiomycota and Ascomycota (Pena et al., 2010; Taylor, Martin, & Read, 2000). ECM fungi form a hyphal mantle around the primary roots and hyphal networks outside the roots (extramatrical mycelia), which constitute functional extensions of the plant roots (Finlay & Read, 1986). The ability of ECM fungi to exploit the nutrients and water contained in the surrounding soil gives them the potential to improve the nutritional status of trees associated with ECM fungi and to contribute to tree water uptake from the soil, attenuating drought stress in those trees (Allen, 2007; Lehto & Zwiazek, 2011). ECM fungi mediate plant nutrient uptake either directly in solubilized form or following enzymatic mobilization from organic debris (Abuzinadah, Finlay, & Read, 1986; Pritsch & Garbaye, 2011). Extracellular enzyme activities (EAs) of ectomycorrhizae are considered functional traits that are indicative of changing conditions in plant–soil ecosystems (Koide, Fernandez, & Malcolm, 2014). Although nutrient turnover processes are generally decreased in dry soils (Sardans & Peñuelas, 2005), ECM fungi can overcome a local soil water deficit by transporting water through their mycelia and particularly through their rhizomorphs (Brownlee, Duddridge, Malibari, & Read, 1983; Duddridge, Malibari, & Read, 1980; Lilleskov, Bruns, Dawson, & Camacho, 2009), thereby retaining or even increasing the potential for nutrient mobilization. Besides, a local soil water deficit can be attenuated by hydraulic lift (Caldwell, Dawson, & Richards, 1998): At night, water moves passively through roots of deep rooting trees such as *Fagus sylvatica* from deep soil layers (higher water potential) to shallow soil layers (lower water potential) where nutrients and fine roots are abundant.

On the basis of the organization of their extramatrical mycelia, ECM fungi can be categorized as contact, short-distance, medium-distance or long-distance exploration types (Agerer, 2001). ECM fungi of the long-distance and medium-distance exploration types form rhizomorphs, increasing water transport to the roots (Cairney, 1992; Duddridge et al., 1980). Bakker, Augusto, and Achat (2006) found that moist forest sites contained more contact types, while dry forest sites contained more short-distance and long-distance types, indicating an increase in functionality of ECM fine-root systems with respect to water transport. However, clear evidence for exploration type preferences to local soil moisture conditions is still lacking as the locations examined by Bakker et al. (2006) also differed in tree species, soil type and nutrient status (Lehto & Zwiazek, 2011).

Ectomycorrhizal fungi are exposed to periodic soil drought even in regions with normally adequate amounts of precipitation. Drought tolerance differs among ECM fungal species (reviewed in Lehto & Zwiazek, 2011) and probably also among populations of a species (Lamhamedi, Bernier, & André-Fortin, 1992), resulting in diverse changes in ECM fungal community composition under drought (Cavender-Bares, Izzo, Robinson, & Lovelock, 2009; di Pietro, Churin, & Garbaye, 2007; Richard et al., 2011; Swaty, Deckert, Whitham, & Gehring, 2004). To determine whether altered ECM fungal community composition is critical to ecosystem functioning or is indicative of a plastic functional system with high adaptive potential, it is important to also consider the functional traits of these communities, such as the capacity to transport water through rhizomorphs or the activity of their extracellular enzymes (Dahlberg, 2001; Kipfer, Wohlgemuth, van der Heijden, Ghazoul, & Egli, 2012). ECM fungal community composition ultimately determines the functionality of a fine-root system through the different properties of the ECM fungal species involved (Cairney, 1999; Godbold & Berntson, 1997; Shi, Guttenberger, Kottke, & Hampp, 2002) and so alterations in community composition are likely to alter the function of the fine-root system. If such alterations are driven by a certain stress factor, they may affect functionality in the direction of stress resistance or resilience.

In the present study, we examined the responses of the ECM fungal communities of beech and spruce under repeated summer drought as part of the Kranzberg Roof Experiment (KROOF) project,

which is a throughfall exclusion experiment being carried out in a maturing (age 60–70 years) beech–spruce forest (see Pretzsch et al. [2014] for a detailed description of the experimental site).

We investigated how ECM fungal communities of beech and spruce reacted upon repeated summer drought in terms of ECM fungal diversity and community composition, the potential to transport water through ECM fungal rhizomorphs and the potential activity of extracellular enzymes of vital ectomycorrhizae. We addressed three hypotheses: H1, repeated years of throughfall exclusion influence ECM fungal community composition and functions more strongly in spruce than in beech; H2, repeated drought leads to changes in the functionality of the ECM fine-root system towards traits that are related to drought resistance, irrespective of the tree species; and H3, the negative effects of drought on ECM fungal communities of beech and spruce are attenuated in mixed stands compared with monospecific stands.

2 | MATERIALS AND METHODS

2.1 | Site description and climatic conditions

This study was conducted in Kranzberg Forest, which is a mixed mature forest situated in southern Germany (11°39′42″E, 48°25′12″N; 490 m a.s.l.). The study site had an average annual precipitation rate of 723 ± 27 mm/year between October 2011 and October 2016, of which approximately 500 mm fell during the growing season (April–October), and an annual mean temperature of 8.4 ± 0.4°C, with an average of 13.1 ± 0.5°C during the growing season (Figure 1). Weather conditions at the site differed strongly during study years (2013–2016) including an extremely hot and dry period in 2015 (rainfall sum in summer [June to August] reduced by 56% compared to mean of rainfall sums in summers 2014 and 2016) imposing natural drought on trees also on control plots (Figure 1). This site is dominated by European beech and Norway spruce, with an average age of 82 ± 4 years (beech) and 62 ± 2 years (spruce) in 2013. The soil is a nutrient-rich luvisol developed from loess over tertiary sediments (eutric cambisols; Food and Agriculture Organization [FAO] classification).

In 2010, 12 plots (100–200 m²) were established at the study site by digging trenches to a depth of approximately 1 m, where a water impermeable clay layer prevents water transport from below. Water impermeable canvas was then used to vertically separate plots from adjacent areas, preventing the lateral movement of water. Each of the 12 plots contained zones in which spruce trees neighboured other spruce trees (spruce zone), beech trees neighboured other beech trees (beech zone) and beech trees neighboured spruce trees in an interspecific contact zone (mixture zone; Figure 2). In 2013, six of the plots were assigned to the throughfall exclusion treatment group and equipped with retractable roofs, while the remaining six plots served as controls. The roofs were set to automatically close during rain events from 6 May, 2014 to 9 December, 2014, 10 March, 2015 to 21 November, 2015 and 6 March, 2016 to 4 November, 2016. The roofs remained open at all other times to minimize any changes in temperature and other stand conditions that were not related to precipitation, and remained open throughout winter. Air temperature and precipitation levels were recorded at the Bavarian forest ecosystem monitoring plot ca. 5 km west of Freising.

2.2 | Root, mycorrhiza and soil sampling

Sampling was carried out once per year at the end of the vegetation period in the year before throughfall exclusion (8 October, 2013),

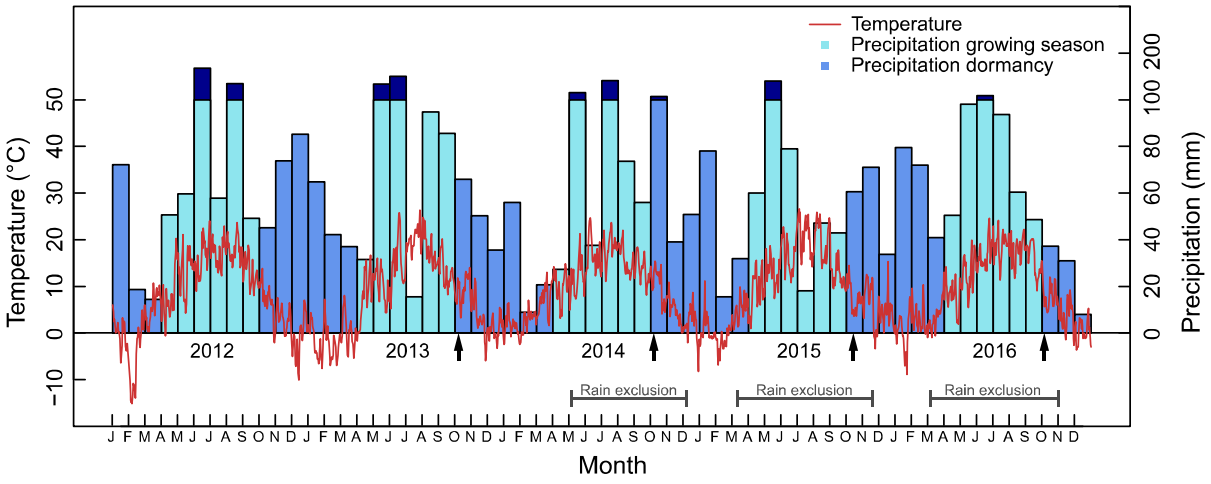

FIGURE 1 Mean temperature and precipitation at the experimental site in Kranzberg Forest from 1 year before the start of the experiment. The *y*-axes are scaled after Walter and Lieth (1960) so that precipitation < temperature indicates an arid month (e.g. July 2013 and 2015) and precipitation > temperature indicates a humid month. Precipitation: light, growing season; medium, dormant season; dark, five-fold compression of the precipitation axis. Arrows indicate sampling dates (8 October, 2013, 6 October, 2014, 12 October, 2015 and 2 November, 2015, and 4 October, 2016)

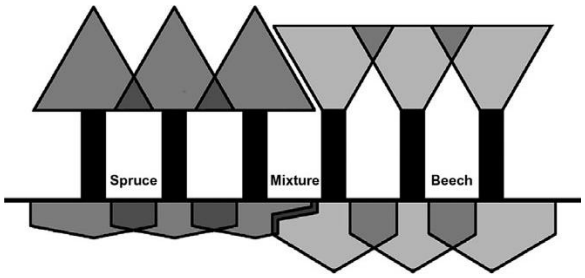

FIGURE 2 Schematic diagram of the sampling zones in the plots. Spruce, zone of spruce neighbouring spruce; mixture, interspecific contact zone between beech and spruce; beech, zone of beech neighbouring beech; modified from Goisser et al. (2016)

and before continuously opening the roofs during winter in the years with throughfall exclusion (6 October, 2014, 12 October, 2015 and 2 November, 2015, and 4 October, 2016). Soil cores of 4-cm diameter were taken to a depth of 25 cm (2013, 2014) or 40 cm (2015, 2016) after removing any loose superficial litter. In each plot, one soil core was obtained from each of beech and spruce zones, respectively, and two were obtained from the mixture zone. Each soil core was separated into an upper part "topsoil" (average thickness = 8.6 cm), which combined the $O_f + {}_hA_h$ horizons, and a lower part "deep layers" (>8.6 cm), which consisted of A_lB_v (KA5 classification; Eckelmann, Sponagel, & Grottenthaler, 2005). Samples from the mixture zone were combined giving a total of six soil samples per plot. Each sample was collected in a plastic bag, cooled immediately in the field and stored for up to 4 weeks at 4°C until further processing. The root material within these samples was used to examine ECM fungal community structure with two different approaches (morphotyping and high-throughput sequencing), and to measure exoenzyme activities.

2.3 | Soil parameters

The volumetric soil water content was measured continuously using a time-domain reflectometer (TDR 100; Campbell Scientific, Logan, UT, USA). With vertical installation, the probe signal integrated the soil water content over a soil depth of 10–30 cm. Therefore, the uppermost probes were installed horizontally, integrating the signal over the top 0–7 cm of mineral topsoil. One TDR probe was installed at both depths within each of the three zones (beech, spruce and mixture; Figure 2) in each of the 12 plots (n_{total} = 72). The sensor signals of all probes were assessed weekly throughout the year.

2.4 | Fine-root parameters

Roots were manually separated from the soil, cleaned in tap water and sorted under a stereomicroscope into beech and spruce roots. Samples were named according to tree species and zone, giving four sample types: spruce roots from spruce zones (SS), spruce roots from mixture zones (SMix), beech roots from beech zones (BB) and beech roots from mixture zones (BMix). Depending on the amount and vitality of fine roots in a respective soil sample, either the entire sample (when few roots were present) or a subsample (in case many roots were present) was used for morphotype assessments and EA as detailed below. Subsampling was used to assure processing of one sample within 1 hr thus assuring comparability between different samples. Subsamples were generated by cutting all fine roots (<1-mm diameter for beech; <2-mm diameter for spruce) of one sample into pieces of 2-cm length and by randomly picking a representative subsample (50%, 33% or 25% of the total sample). In all, 21 ECM tips per sample, respectively, subsample were used up for enzyme activity assays and morphotype identification by Sanger sequencing. All remaining fine roots of each sample were stored below −20°C and subsequently used for DNA extraction and high-throughput sequencing.

2.5 | Ectomycorrhizal morphotype diversity and abundance

Vital mycorrhizal tips were assigned to morphotypes according to the colour and surface properties of the mycorrhizal mantle, and were categorized into exploration types according to Agerer (2001). The number of each morphotype was counted and used to calculate morphotype abundance per unit volume of soil. The ECM tips collected for enzyme activity measurements were frozen at −20°C after finishing the assays for later identification according to their internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequence using polymerase chain reaction (PCR) and Sanger sequencing (see Method S1), resulting in several sequences per morphotype and year. These sequences were checked and assembled with CodonCode Aligner (CodonCode, Centerville, MA, USA) and contigs were submitted to BLAST searches against the UNITE database (Kõljalg et al., 2013) and the International Nucleotide Sequence Database (INSD). We only used the first entry of blast results and defined the following criteria to assign OTUs to species records: (i) sequence similarity ≥95% and (ii) a BLAST e-value $< 2 \times 10^{-31}$. If more than 50% the sequences of one morphotype yielded different species, but same genus, we used the genus information and if more than 50% of the sequences of one morphotype yielded different genera, we kept our internal morphotype numbering (e.g., MT_18).

To distinguish between ECM fungal ability of potential water transport over several cm distance, we assigned the ECM tips to the following three exploration type groups that indicate soil exploration by extramatrical mycelia: (i) contact types (soil exploration radius 0 mm, no emanating hyphae), (ii) short-distance (soil exploration radius up to 5 mm, some emanating hyphae) and medium-distance types (soil exploration radius up to 3 cm; fringe types: fans of emanating hyphae, mat types: undifferentiated rhizomorphs, smooth types: slightly differentiated rhizomorphs) and (iii) long-distance types (soil exploration radius up to several dm, mostly highly differentiated rhizomorphs).

2.6 | Potential extracellular enzyme activities of ectomycorrhizae

In all, 21 vital ECM tips were randomly selected from each sample, placed on wet filter paper and stored at 4°C overnight. The number of tips of each morphotype was chosen according to its relative abundance in the sample, but was not lower than three. This design allowed the direct calculation of a weighted mean of EAs in each sample:

$$\overline{EA}_{\text{per tip}} = \frac{\sum_n EA}{n}, \quad (1)$$

where n is the number of ECM tips assayed per sample. This value was then further normalized to the number of ECM tips that occurred per unit volume of soil:

$$EA_{\text{per vol}} = \frac{\overline{EA}_{\text{per tip}} \times n}{\text{soil volume}_{\text{sample}}}, \quad (2)$$

where n is the number of vital ECM tips in a particular sample, representing the total EA in the sample.

The entire assay followed the procedure of Pritsch et al. (2011). In brief, seven substrates bound to 4-methylumbelliferone (MU) or aminomethylcoumarin (AMC) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; ABTS) were used to detect EAs: L-leucine-7-AMC (Leu-AMC) for the detection of leucine aminopeptidase (EC 3.4.11.1), 4-MU-β-d-xylopyranoside (MU-X) for xylosidase (EC 3.2.1.37), 4-MU-β-d d-glucuronide hydrate (MU-GU) for glucuronidase (EC 3.2.1.31), 4-MU-β-d-cellobioside (MU-C) for cellobiohydrolase (EC 3.2.1.91), 4-MU-N-acetyl-β-glucosaminide (MU-NAG) for N-acetyl-glucosaminidase (EC 3.2.1.14), 4-MU-β-d-glucopyranoside (MU-G) for β-glucosidase (EC 3.2.1.3), 4-MU-phosphate (MU-P) for phosphatase (EC 3.1.3.2) and ABTS for laccase (EC 1.10.3.2). Individual ECM tips were placed in the wells of 96-well filter plates (AcroPrep Advance 96, 30–40 μm PP/PE, 350 μl well, NTRL; Pall, Ann Arbor, MI, USA) and incubated with the respective substrates. Following filtration of the reaction solutions, the fluorescence (AMC and MU substrates) or absorption (ABTS) was measured. All assayed ECM tips were scanned to determine their projection area using the software WINRHIZO (Reg 2013e 32 Bit; Regent Instruments, Canada) and then immediately frozen for later identification according to their ITS rDNA.

2.7 | Sample processing for high-throughput sequencing

The frozen fine roots from each sample were ground separately in liquid nitrogen, giving 384 samples [4 years × 2 soil depths × 12 (6 control, 6 throughfall exclusion) plots × 4 sample types (BB, SS, BMix, SMix)]. Contamination was controlled with extraction and PCR from negative controls. Approximately, 350–450 mg of homogenate (or the total amount when less material was found) was used for DNA extraction with PowerSoil®-htp96 and PowerSoil DNA Isolation Kits (Mo-Bio, Carlsbad, CA, USA) following the manufacturer's instructions, with some modification for the initial bead beating as pre-experiments had shown very different levels of cell disruption, DNA yields and PCR success between samples. Frozen homogenates were transferred to 2-ml screw cap vials containing 5-mm steel beads, 600 μl PowerSoil bead solution and 60 μl PowerSoil C1-buffer from the kit, and were processed with a disruptor (2 × 30 s, 5,000 rpm; Precellys24, Rockville, MD, USA) to separate the root tissues and hyphae into microparticles. The steel beads were then magnetically removed from the vials and replaced by garnets from the kit (1 g per sample), following which 150 μl PowerSoil bead-solution was added and shaken twice for 10 min at 20 Hz in a TissueLyser II (Qiagen, Hilden, Germany) to further disrupt the cells. The PowerSoil manufacturer's protocol was then followed. The resulting DNA was stored below −20°C.

Amplification of ITS2 rDNA was performed with PCR primer mixes optimized for maximum phylogenetic recovery (Tedersoo et al., 2014, 2015; Table S2). All primers carried the respective forward or reverse overhang adapter sequences for the Illumina Miseq workflow (protocol Part # 15044223; Illumina, San Diego, CA, USA). Reactions consisted of 1 μl DNA (5 ng), 0.5 μl ITS3 mix (10 pmol equimolar mix of ITS3-Mix1 to -Mix5), 0.5 μl ITS4 mix (10 pmol equimolar mix of ITS4-Mix1 to ITS4-Mix4), 10 μl NEBNext® High-Fidelity 2× PCR Master Mix (New England Biolabs, Frankfurt, Germany) and 8 μl H_2O. PCR conditions were 5 min at 95°C, 28 × [30 s at 95°C, 30 s at 55°C and 60 s at 72°C] and 10 min at 72°C. The quality of all products was checked on agarose gels. Triplicate samples from successful PCRs were pooled and cleaned using Agencourt AMPure XP (Beckman Coulter, Krefeld, Germany) with a bead:DNA ratio of 1. Removal of primer dimers was controlled with the Bioanalyzer DNA1000 Kit (Agilent Technologies, Waldbronn, Germany) and yield was quantified using the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, Paisley, UK).

Indexing for multiplexed sequencing was performed using eight PCR cycles with individual dual-index combinations of Nextera XT Index Kit v2 Sets A–D (Illumina). Reactions consisted of 1 μl DNA (5 ng), 2.5 μl Primer 1 (Nextera i7 series), 2.5 μl Primer 2 (Nextera i5 series), 12.5 μl NEBNext High-Fidelity 2× PCR Master Mix and 6.5 μl H_2O. Indexed amplicons were cleaned, size-checked and quantified as above. The amplicons (4 nM) from each sample were pooled and rechecked with a Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies). The final preparations and sequencing (Miseq v3 chemistry, 600 cycles flow cell, Illumina) followed the manufacturer's recommendations for 16S Metagenomic Sequencing Library Preparation (protocol Part # 15044223 Rev. B).

2.8 | Processing of high-throughput sequencing reads

Data were obtained as demultiplexed FASTQ files and processed using the fungal ITS analysis pipeline PIPITS v1.3.6 (Gweon et al., 2015) on Biolinux v8.0.6 (Field et al., 2006). Sequence processing followed Gweon et al. (2015): read pairs were joined with PEAR v0.9.10 (parameters: -q 30; Q33, p-value of assembly ≤.0001; Zhang,

Kobert, Flouri, & Stamatakis, 2013) and FASTQ_QUALITY_FILTER (parameters: -q 30, -p 80; Q33; FASTX-Toolkit, http://hannonlab.cshl.edu, accessed 12 February, 2017); ITS2 of fungal origin was extracted with ITSX v1.0.11 (Bengtsson-Palme et al., 2013); sequences <100 bp were removed and operational taxonomic units (OTUs) were clustered by 97% sequence identity with VSEARCH v2.1.2 (https://github.com/torognes/vsearch/, accessed 12 February, 2017); chimera were removed using the UNITE UCHIME reference dataset (v01.01.2016; http://unite.ut.ee/repository.php, accessed 12 February, 2017); and reads were mapped onto OTUs, singletons were removed and the taxonomy of OTUs was assigned with RDP Classifier v2.12 (Wang, Garrity, Tiedje, & Cole, 2007) against a reference set of fungal ITS data (UNITE 7.1; Kõljalg et al., 2013). OTU and phylotype abundance tables were then produced, whereby OTUs were defined as "clusters of reads with user-defined thresholds" and phylotypes were defined as "clusters of sequences binned into the same taxonomic assignments" (Gweon et al., 2015). Phylotypes were used for all further analyses of high-throughput sequencing data because phylotypes better resemble data obtained by morphotyping of ECM fungi. Taxonomic assignments with a confidence threshold <0.85 were omitted. Finally, all phylotypes were given a status that reflected whether they were ECM-forming and their exploration type during a manual review guided by Agerer (2001) and Tedersoo and Smith (2013).

2.9 | Statistical analyses

All values are presented as means ± standard errors unless otherwise indicated.

For the morphotyping data, diversity indices were calculated using the package BiodiversityR (Kindt, 2016) in R (R Core Team, 2016). The effects of throughfall exclusion, tree species, competitive situation and soil depth on ECM fungal species abundances, diversity indices and extracellular enzyme activities were analysed with analysis of variance (ANOVA) using the software IBM SPSS Statistics 19 (IBM, Armonk, NY, USA). In this analysis, the effect of the two tree species growing in three different species mixture situations was partitioned into three orthogonal contrasts: (I) BMix and SMix vs. BB and SS; (II) BMix vs. SMix; (III) BB vs. SS. As a measure of effect size, we calculated ω^2 (Hays, 1963). Detailed comparisons between subsets of the data were conducted in R using unpaired two-sample *t* tests where the data were normally distributed (Shapiro test) or Wilcoxon signed-rank tests. To test the correlation between the extracellular enzyme activities of the ECM tips and soil parameters and morphotype abundances, Spearman's rank correlation coefficients were computed in R. The average contribution of each species to the average overall Bray–Curtis dissimilarity was assessed by calculating the similarity percentage (Clarke, 1993) in BiodiversityR. Differences in variation between study years and treatment were tested with mixed effect models that considered plots as a random factor, using the R package nlme (Pinheiro, Bates, DebRoy, & Sarkar, 2014).

Prior to further analysis of the high-throughput sequencing phylotype data, five samples with low sequencing depth (<17,000 sequences per sample) were removed from the dataset, as well as rare non-fungal or unassignable phylotypes as determined during the taxonomic assignment step. The sequence reads were then randomly rarefied 10^4 times using GUniFrac for R (Chen, 2012) and the results were averaged to compare all samples at equivalent sequencing depths (Weiss et al., 2015). Bray–Curtis dissimilarities between the samples (ECM fungal community variation) and Shannon diversity indices (ECM phylotypes only) were calculated using the vegan package (Oksanen et al., 2017). Taxonomic overviews and ordinations were produced with the phyloseq package (McMurdie & Holmes, 2013), and multivariate testing for the effect of environmental characteristics on the ECM fungal community was conducted using Bray–Curtis dissimilarity matrices with Adonis (permutational multivariate ANOVA using distance matrices; 10^5 permutations) in vegan. Statistical analyses that mirrored those for the morphotyping data were performed as described above.

3 | RESULTS

3.1 | Soil moisture

Throughfall exclusion decreased the volumetric soil water content during the vegetation period in the topsoil (0–7-cm depth), from ca. 30% in 2013 to ca. 10% in 2016. There was also a significant reduction of volumetric soil water content in the deep layers (10–30-cm depth) during the second and third throughfall exclusion period in 2015 and 2016, from ca. 35% in 2013 to 20%–25% under beech and to 15%–20% under spruce (Figure 3).

3.2 | ECM fungal community composition

In total, 45,181 vital ECM tips were counted and categorized into 43 morphotypes, from which 25 species were identified by their ITS rDNA. Three morphotypes did not yield evaluable sequences. On average, four ECM morphotypes were found per sample (minimum = 1, maximum = 11; see Table S3 for ECM morphotype abundances and distributions).

High-throughput sequencing yielded 18×10^6 quality-filtered reads, which were assigned to 4,820 OTUs and 1,411 phylotypes. In all, 11 samples were removed (five because of a low sequencing depth and six because of a low number of roots), leaving 373 samples for further analysis. The median abundance of fungal reads was 42,937 sequences per sample (minimum = 17,280, maximum = 103,220). In total, 144 phylotypes were identified as ECM fungi during manual inspection of all phylotypes following normalization to an equal sequencing depth. On average, 11 ECM phylotypes were found per sample (Table S4).

In 2013 (i.e. 1 year before throughfall exclusion), there was no significant difference in the measures of ECM fungal community composition between the control and throughfall exclusion plots. On the basis of morphotypes, drought was a strong predictor for the abundance of the contact and short- and medium-distance exploration type groups. Repeated summer droughts led to a progressive

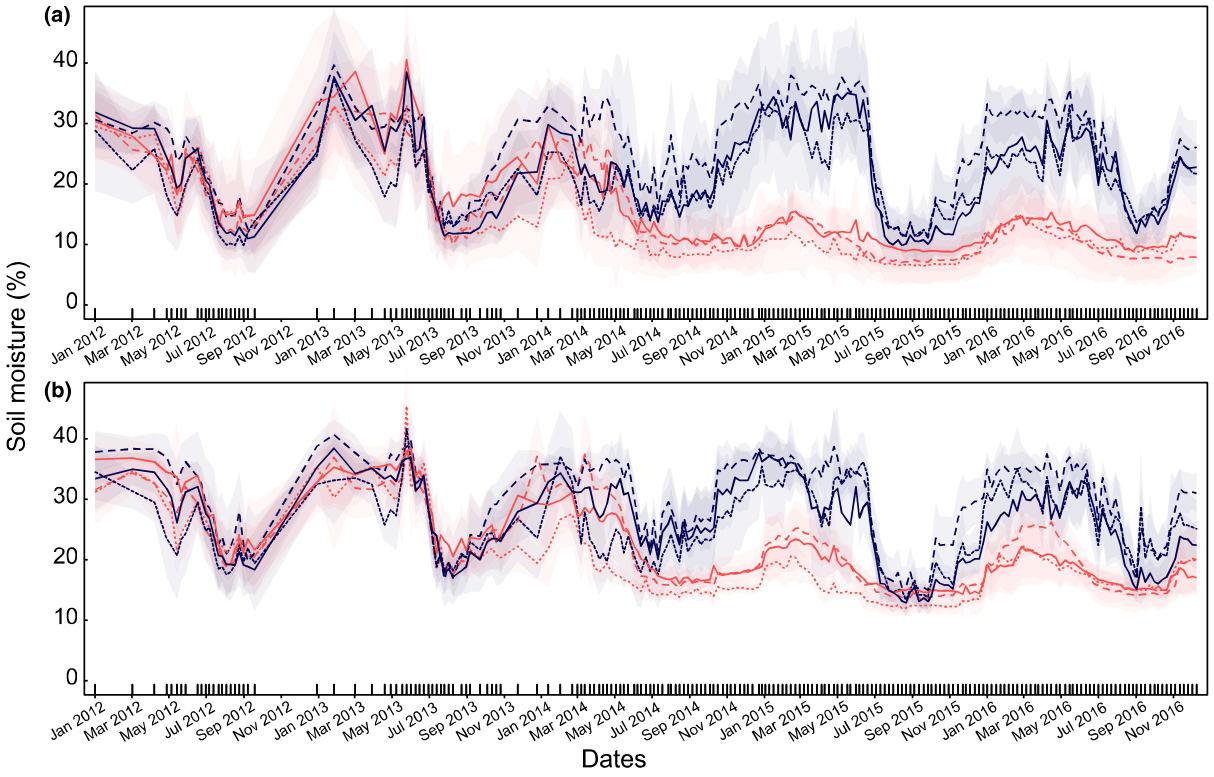

FIGURE 3 Volumetric soil water content at the experimental site in Kranzberg Forest measured at depths of (a) 0–7 cm and (b) 10–30 cm with time-domain reflectometer (TDR) probes. Throughfall exclusion periods were from 6 May, 2014 to 9 December, 2014, 10 March, 2015 to 21 November, 2015 and 6 March, 2016 to 4 November, 2016 (see Figure 1). Values were averaged for throughfall exclusion plots (red) and control plots (dark blue) for mixture zones (solid lines), beech zones (dashed lines) and spruce zones (dotted lines). Shaded areas indicate the standard deviations; marks along the *x*-axis indicate the measurement dates

decline in contact types relative to control (decline by 67 ± 27% in 2014 ($p < .05$), by 64 ± 27% in 2015 ($p < .01$), by 83 ± 21% in 2016 ($p < .01$), and a strong decline relative to control in short-distance and medium-distance types (decline by 54 ± 28% in 2014, by 83 ± 21% in 2015, by 96 ± 11% in 2016; $p < .05$ in all years) causing a strong increase in relative abundance of long-distance types relative to the other types (Fig. S5). By contrast, long-distance types were not affected in the first 2 years of drought, significantly decreasing in abundance relative to control only after 3 years (decline by 88 ± 18% in 2016, $p < .01$). Soil depth was also a major predictor for the abundance of all three exploration type groups, with ca. 90% vital tips occurring in the topsoil. In throughfall exclusion plots, changes in abundance and the proportion of exploration types were stronger and occurred earlier in the topsoil (throughfall exclusion × soil depth interaction: $p < .05$ in all years except for long-distance types in 2014 and 2015). By contrast, on the basis of phylotype data, drought was a weak predictor for the abundance of exploration type groups, with only the abundance of short-distance and medium-distance types being decreased significantly in the final year (decline by 72 ± 25% compared to control in 2016, $p < .01$).

The morphotype and phylotype Shannon diversity indices were quite similar (Figure 4), but the phylotype diversity indices showed a smaller decline following repeated droughts. In the control plots, Shannon diversity indices were generally higher in the topsoil than in the deep layers (morphotype: 1.24 ± 0.04 vs. 0.92 ± 0.06, respectively, $p < .05$ in all years; phylotype: 1.39 ± 0.04 vs. 1.23 ± 0.05, respectively, $p < .05$ in 2014; Table 1). The effect of throughfall exclusion on ECM fungal diversity indices differed between beech and spruce, irrespective of the competitive situation. In the throughfall exclusion plots, the ECM fungal diversity indices remained unchanged in the first 2 years of drought for beech but declined from the second year of drought (2015) onwards for spruce. In 2016, there was a significant difference in the morphotype diversity indices between beech (control: 1.01 ± 0.08; throughfall exclusion: 0.80 ± 0.02) and spruce (control: 0.93 ± 0.21; throughfall exclusion: 0.27 ± 0.11) depending on the treatment (throughfall exclusion × BB vs. SS interaction: $p < .01$), with a much stronger decline being observed in spruce. Phylotype diversity indices responded less to drought, but also exhibited a pronounced decline in the SS samples after the third drought period in 2016. After 3 years of drought, throughfall exclusion had a smaller effect on morphotype diversity indices of ECM fungal communities from the mixture zone (0.82 ± 0.09 to 0.57 ± 0.14) than on those from the spruce and beech zones

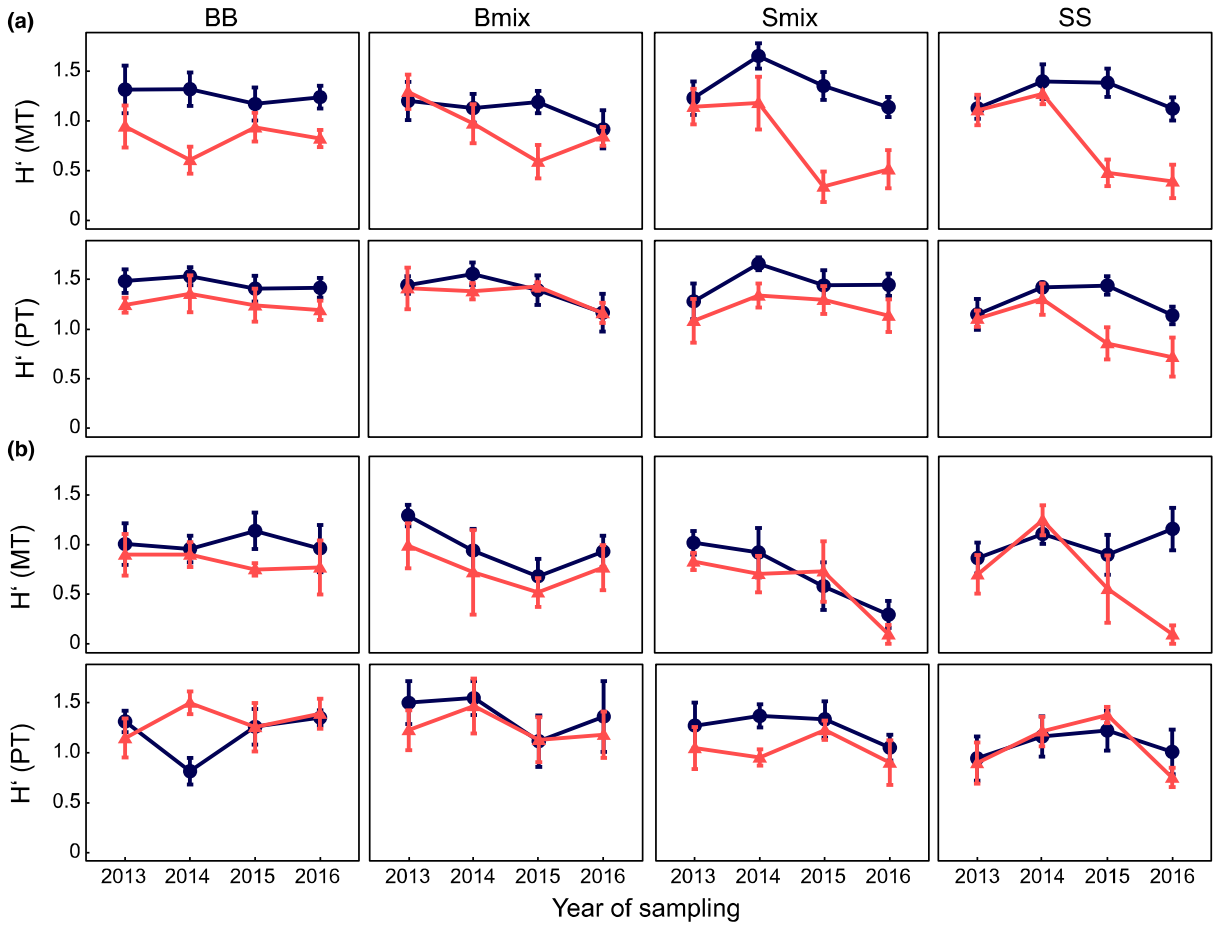

FIGURE 4 Ectomycorrhizal (ECM) fungal diversity (Shannon diversity H′) in the topsoil (a) and the deeper layers (b) before (2013) and during 3 consecutive years of throughfall exclusion during the vegetation period. Plots were subdivided into zones in which beech and spruce were surrounded by the same species (BB, SS) or neighboured the other species (BMix, SMix). Error bars: ±1 *SE*; dark blue lines and circles: control plots ($n = 6$); red lines and triangles: throughfall exclusion plots ($n = 6$); H′ (MT): calculated from data according to morphotypes; H′ (PT): calculated from high-throughput sequencing phylotypes; sampling dates: 8 October, 2013, 6 October, 2014, 12 October, 2015, 2 November, 2015 and 4 October, 2016

(1.11 ± 0.09 to 0.53 ± 0.11; throughfall exclusion × BMix and SMix vs. SS and BB interaction: $p < .05$).

The composition of ECM fungal communities (phylotypes and morphotypes) also changed gradually following throughfall exclusion, with differences becoming apparent after three successive drought years (phylotypes: Figure 5). While there was no significant difference between the ECM fungal communities in the control and throughfall exclusion plots in 2013 before the start of the experiment, they became distinct after 3 consecutive years of throughfall exclusion [Adonis $R^2_{adjusted}$ (phylotypes) = 0.03 (2013), 0.02 (2014), 0.01 (2015) and 0.16** (2016); Adonis $R^2_{adjusted}$ (morphotypes) = −0.02 (2013), 0.09* (2014), 0.08 (2015) and 0.21** (2016) with $*p < .05$, $**p < .01$]. The effects of a species mixture on drought tolerance differed among the four most frequent ECM fungal species that were shared by both tree species. For example, the absolute morphotype abundances of *Cenococcum geophilum* and *Russula ochroleuca* were not affected by drought and not influenced by the competitive situation or tree species, whereas the morphotype abundances of *Lactarius tabidus* and *Xerocomus pruinatus* were negatively affected by drought but this was less pronounced when the tree species grew in mixed situation compared to BB samples (Fig. S6).

3.3 | Potential extracellular enzyme activities

Overall, the most pronounced effect of throughfall exclusion on EAs and differences between the qualitative measure $EA_{per\ tip}$ and the quantitative measure $EA_{per\ vol}$ was observed in the topsoil. As visualized by the regression lines of EA from throughfall exclusion vs. control plots, $EA_{per\ tip}$ (Figure 6a) was remarkably stable in spruce and beech ectomycorrhizae (except for laccase as detailed below) while $EA_{per\ vol}$ (Figure 6b) decreased over repeated drought years, which

TABLE 1 Effect size ω^2 (explained variance) of an analysis of variance (ANOVA) examining the effect of throughfall exclusion (TE), zone [three orthogonal contrasts: roots of beech and spruce from monospecific and mixed stands], soil layer (topsoil vs. deep layers) and their interactions on the Shannon diversity index (H′)

	H′ (morphotypes)				H′ (phylotypes)			
Source of variation	**2013**	**2014**	**2015**	**2016**	**2013**	**2014**	**2015**	**2016**
Throughfall exclusion (TE)	0.080	**0.277**	**0.637**	**0.475**	0.010	−0.047	0.068	0.134
Zone	−0.004	0.084	0.077	**0.263**	**0.205**	0.072	−0.059	**0.236**
BMix and SMix vs. BB and SS	0.018	−0.023	0.084	0.080	0.034	0.071	−0.021	−0.011
BMix vs. SMix	−0.001	0.007	−0.031	**0.121**	**0.101**	0.040	−0.023	−0.013
BB vs. SS	−0.019	**0.096**	0.029	**0.125**	**0.126**	−0.027	−0.019	**0.254**
TE × zone	−0.078	−0.022	−0.002	**0.206**	−0.050	**0.164**	−0.060	−0.035
TE × BMix and SMix vs. BB and SS	−0.029	−0.031	−0.028	**0.119**	−0.025	**0.141**	−0.021	−0.025
TE × BMix vs. SMix	−0.030	−0.020	0.004	−0.007	−0.022	−0.006	−0.026	−0.022
TE × BB vs. SS	−0.023	0.023	0.030	**0.171**	−0.014	0.048	−0.020	0.001
Soil layer	**0.163**	**0.153**	**0.132**	**0.121**	0.037	**0.096**	−0.016	−0.013
Soil layer × TE	−0.007	**0.055**	**0.085**	−0.017	−0.014	**0.058**	0.028	−0.012
Soil layer × zone	−0.024	0.075	−0.039	0.111	−0.056	−0.001	0.008	0.073
Soil layer × BMix and SMix vs. BB and SS	−0.017	0.050	−0.009	−0.008	−0.015	−0.014	0.027	−0.001
Soil layer × BMix vs. SMix	0.001	0.033	−0.020	0.123	−0.022	0.032	−0.009	**0.095**
Soil layer × BB vs. SS	−0.008	−0.012	−0.009	−0.011	−0.020	−0.014	−0.017	−0.013
Soil layer × TE × zone	−0.004	−0.006	0.055	−0.023	−0.050	0.057	0.001	−0.031
Soil layer × TE × BMix and SMix vs. BB and SS	0.009	0.011	0.027	0.000	−0.006	0.030	0.033	−0.006
Soil layer × TE × BMix vs. SMix	−0.015	−0.005	0.009	−0.003	−0.023	−0.013	−0.023	−0.014
Soil layer × TE × BB vs. SS	0.000	−0.002	0.020	−0.011	−0.022	0.041	−0.006	−0.022
$R^2_{adjusted}$	0.346	0.417	0.396	0.368	0.309	0.201	–0.126	0.312

H′ (morphotypes): calculated from morphotype data; H′ (phylotypes): calculated from high-throughput sequencing phylotypes. Values of ω^2 with $p < .05$ are written in bold. Adjusted R^2 values for the respective ANOVA models are given in the last row of the table.

was mainly caused by a decline of vital ectomycorrhizae on throughfall exclusion plots (Figure 6c).

In detail, there was no significant difference in $EA_{per\ tip}$ of the seven tested hydrolytic enzymes between throughfall exclusion and control plots in 2013 (prior to the treatment) and in the first 2 years of throughfall exclusion (2014 and 2015). Even after three drought periods, in 2016 $EA_{per\ tip}$ of only three out of seven hydrolytic enzymes changed significantly: xylosidase had significantly higher levels in the throughfall exclusion plots than in the control plots (4.43 ± 0.74 vs. 2.89 ± 0.48 pmol cm^{-2} min^{-1}, respectively), and the cellulose-degrading enzymes cellobiohydrolase and β-glucosidase exhibited a greater increase following drought in the mixture zone than in the beech and spruce zones (Table S7). By contrast, the $EA_{per\ tip}$ of laccase (which releases nutrients bound to phenolic compounds) was significantly lower in throughfall exclusion plots than in control plots from 2014 onwards, and was also decreased in control plots compared to the other years in the naturally dry year 2015 (2014: 90.53 ± 15.09 vs. 199.17 ± 33.19; 2015: 26.17 ± 4.36 vs. 27.75 ± 4.62; 2016: 94.76 ± 15.80 vs. 151.12 ± 25.19 mmol cm^{-2} min^{-1}, respectively). Laccase activity disappeared in spruce ECM from the topsoil of the spruce zones in 2016 along with laccase-positive morphotypes. Comparison of the influence of the contrasting study years (2014–2016) and the throughfall exclusion treatment on $EA_{per\ tip}$ showed variations between years to be greater than between treatments, except for leucine aminopeptidase. The activity of this enzyme was increased (yet not significantly: $p = .077$) by treatment over the years.

ANOVA (Table S8) revealed that there were few interactions between throughfall exclusion and other factors (zone, soil depth) for the $EA_{per\ tip}$ data from 2015 onwards. There was, however, a significant interaction between throughfall exclusion and soil depth for xylosidase, glucuronidase and chitinase in 2015 and for phosphatase in 2016, reflecting an increase in EA in the deep layers of drought plots but no change in the topsoil (Table S7). Furthermore, significant interactions between throughfall exclusion, soil depth and mixture situation were found for cellobiohydrolase in 2015, and for cellobiohydrolase, β-glucosidase and laccase in 2016 (Table S8), with the $EA_{per\ tip}$ of cellobiohydrolase and β-glucosidase being higher in control plots than in throughfall exclusion plots in the topsoil of the mixture zone. In 2016, the $EA_{per\ tip}$ for laccase in the topsoil increased in the beech and spruce zones but declined in the mixture zone, while that in the deep layers decreased in the beech and spruce zones and exhibited no significant change in the mixture zone in throughfall exclusion plots (Table S7). The interaction between throughfall exclusion and soil depth and species mixtures became significant only in the third year of throughfall exclusion, with

leucine aminopeptidase and phosphatase being decreased in SMix samples and not affected (phosphatase) or stimulated (leucine aminopeptidase) in BMix samples in the topsoil, and exhibiting the opposite response in the deep layers in 2016 (Table S8).

In contrast to qualitative stability of $EA_{per\ tip}$, $EA_{per\ vol}$ showed a progressive decline on throughfall exclusion plots over repeated drought years. Before the onset of treatment (2013), there were no significant differences in $EA_{per\ vol}$ between control and throughfall exclusion plots, but after three consecutive summer droughts, the $EA_{per\ vol}$ of both tree species significantly declined in the throughfall exclusion plots (Table S7). Xylosidase, cellobiohydrolase, β-glucosidase, glucuronidase and phosphatase were significantly altered from 2015 onwards, while N-acetyl-glucosaminidase and leucine aminopeptidase significantly changed in 2016 (Table S8).

In the deep layers, $EA_{per\ tip}$ of beech was also very stable but tended to increase in spruce ECM fungi with progressing drought (Fig. S9a). Soil depth significantly affected almost all $EA_{per\ vol}$ (Fig. S9b) in all years (Table S8) because on average there were 5–10 times fewer roots in the deeper layers than in the topsoil (Fig. S9c). $EA_{per\ vol}$ of beech ECM continuously decreased during repeated drought, whereas $EA_{per\ vol}$ of spruce ECM did not respond with a clear increase or decrease (Fig. S9b). The relative decline in vital ECM tips in throughfall exclusion plots compared to control plots was less pronounced in the deep layers than in the topsoil. Vital ECM tips of spruce only declined in 2016 (Fig. S9c) as reflected in the respective diversity indices (Figure 4).

There were significant interactions between soil depth and throughfall exclusion in 2014 and 2015, indicating the effects of faster drying in shallower soil (Figure 3). Most interactions with soil depth were transient and (with the exception of phosphatase) disappeared in 2016 when the deeper soil had dried more thoroughly (Table S8). The zones within a plot tended to have a larger effect on enzyme activities at the beginning of the experiment, with this effect disappearing with repeated throughfall exclusion (Table S8). Only phosphatase and laccase $EA_{per\ vol}$ showed a zone effect in 2016. Overall, $EA_{per\ vol}$ declined more strongly in SMix samples compared to SS samples (Table S7).

The EAs of some ECM fungi became dominant under throughfall exclusion, that is, *L. tabidus* in both tree species, *Russula fellea* in beech and *C. geophilum* in spruce, mainly because other morphotypes disappeared. These species were also identified as making a high (>10%, SIMPER $p < .05$) contribution to the differences in ECM morphotype community between control and throughfall exclusion plots.

4 | DISCUSSION

4.1 | Do repeated years of throughfall exclusion influence ECM fungal community composition and functions more strongly in spruce than in beech?

While shifts in ECM fungal community composition after drought have repeatedly been reported (Cavender-Bares et al., 2009; Shi et al., 2002), a reduced ECM fungal diversity as in our study has rarely been detected (Swaty et al., 2004). Shannon diversity of ECM fungal communities from the beech and spruce zones reflected the contrasting strategies of beech and spruce to cope with drought. Beech exhibited a decline in ECM fungal diversity after the first year of drought and then maintained a slightly lower level than the control, which supports the previous finding that beech continues to produce new fine roots during drought (Burkhardt & Pariyar, 2016; Nikolova et al., 2010) allowing the surviving ECM fungi to colonize newly growing roots. By contrast, ECM fungal diversity did not exhibit a marked change in spruce following the first drought, but declined dramatically after the second drought year. After one severe summer drought, Nikolova et al. (2010) found that spruce sustained standing fine roots rather than growing new ones, supporting a strategy of decreased growth during drought conditions (Dobson et al., 1990; Maier-Maercker, 1998). Our results of a decline in ECM fungal Shannon diversity in spruce suggest that on the longer term, this strategy would prevent new colonization by ECM fungi and, over several years, lead to a decline in diversity. This indicates that spruce is particularly vulnerable to predicted future climate change scenarios for those areas in the observed climate zone that are prone to repeated summer drought (Zang, Hartl-Meier, Dittmar, Rothe, & Menzel, 2014), supporting our first hypothesis.

The changes in Shannon diversity did not directly translate to losses in qualitative enzymatic potentials of ECM fungal communities which further supports presence of highly complementary and functionally redundant hydrolytic enzyme activities in ECM fungal communities even under severe drought (Buée, Courty, Mignot, & Garbaye, 2007; Courty, Pritsch, Schloter, Hartmann, & Garbaye, 2005; Jones et al., 2010). However, both $EA_{per\ tip}$ and $EA_{per\ vol}$ of laccase were strongly decreased in ECM fungal communities in throughfall exclusion plots already from the first year of throughfall exclusion onwards and in control plots in the naturally dry year 2015, suggesting that ECM fungi expressing laccase activity were drought sensitive at the Kranzberg site. Activity of the oxidative enzyme laccase is very widespread in the fungal kingdom (Iyer & Chattoo, 2003; Junghanns, Moeder, Krauss, Martin, & Schlosser, 2005; Vasconcelos, Barbosa, Dekker, Scarminio, & Rezende, 2000) with several functions in degradation, but also morphogenesis (Baldrian, 2006; Thurston, 1994). Oxidase activities in soil are more dynamic than hydrolytic activities (Sinsabaugh et al., 2008), corresponding to our observations. In ECM fungi, laccase is related to the release of nutrients (particularly N) enclosed in recalcitrant polymers or protein–phenol complexes (Baldrian, 2006). Whether the strong decline in ectomycorrhizae with laccase enzyme activity causes lasting effects on nutrient relations in forest soil will depend on how long it takes for the full functional spectrum in ECM fungal communities to be restored following drought release, and on how other soil fungal groups are affected by drought. In ECM fungi, laccase is only present in some lineages (Luis et al., 2005) and by selecting dominant morphotypes in EA measurements, we likely excluded ECM fungal taxa that became relatively rare as a consequence of the decline in vital fine roots. The decline in vital fine roots leading

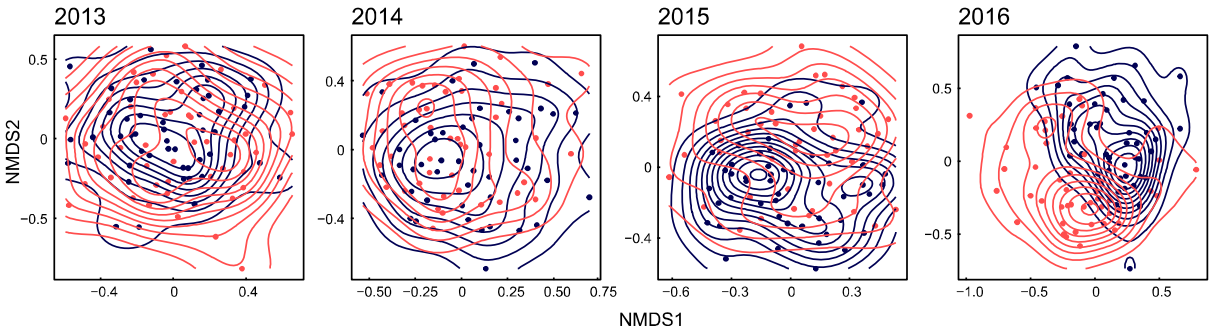

FIGURE 5 Non-metric multidimensional scaling (NMDS) plots showing changes in the ectomycorrhizal (ECM) phylotypes before (2013) and during 3 consecutive years with (red) or without (blue) throughfall exclusion during the vegetation periods (2014–2016). Dots represent single root samples; distances represent differences in ECM fungal community composition based on Bray–Curtis dissimilarities. Density lines were plotted according to the distribution of the samples in the graph using the function geom_density2d() from the package ggplot2 (Wickham, 2016) in R

to a complete loss of formerly dominant ECM fungi with laccase activity again supports our first hypothesis of stronger drought effects on spruce ECM fungal community composition and functions.

4.2 | Does repeated drought lead to changes in the functionality of the ECM fine-root system towards traits that are related to drought resistance, irrespective of the tree species?

We observed a decrease in contact type ectomycorrhizae following drought, confirming the findings of Bakker et al. (2006), who interpreted this as caused by shrinking soils and thus reduced contact with the substrate. In addition, the dominant contact types at our plots were *Lactarius* spp. with thin cell walls prone to loosing cellular integrity under dry conditions (di Pietro et al., 2007) which makes them sensitive to drought. The relative increase in long-distance type mycorrhizae in both tree species following drought suggests that they have higher drought resistance because of their ability to explore and transport water beyond the root surface (Cairney, 1992; Duddridge et al., 1980). The direction of changes in exploration types as functional traits that is, increasing long-distance type and decreasing contact and short-distance type ectomycorrhizae was the same in both tree species, thus supporting our second hypothesis.

Relative stability of extracellular enzyme activities has often been observed in ECM fungal communities upon environmental disturbance (Diedhiou et al., 2010; Jones et al., 2010), which underlines the importance of finding alterations in three enzymes in the present experiment in the third year of throughfall exclusion. One enzyme activity (leucine aminopeptidase) was strongly increased in ECM fungal communities of both tree species under drought (albeit in different mixture situations). Interestingly, a stimulation of this EA under a strong drought was also observed in *C. geophilum* ectomycorrhizae associated with different *Quercus* species (Herzog, Peter, Pritsch, Gunthardt-Goerg, & Egli, 2013). In that study, $EAs_{per\ tip}$ of the other enzymes measured (the same six enzymes as in our study) showed neutral responses. Herzog et al. (2013) found that the decrease in abundance of *C. geophilum* was negatively correlated with leucine aminopeptidase activity and suggested that this EA had to be compensated for by an increased activity of the remaining vital tips of *C. geophilum*. Our results suggest a similar mechanism at the whole ECM fungal community level.

Drought increased the extracellular cellulolytic potential per vital tip irrespective of tree species. Extracellular cellulolytic activity may be stimulated by the presence of dead fine-root material, which accumulated during repeated drought events, to gain access to nutrients contained in these dead tissues (Hupperts, Karst, Pritsch, & Landhäusser, 2017; Lindahl & Tunlid, 2015; Pritsch & Garbaye, 2011). The alternative explanation of saprotrophic carbon acquisition by ECM fungi from organic matter decay rather than the internal carbon supply of the plant (Bréda et al., 2013; Courty, Bréda, & Garbaye, 2007) seems unlikely as the observed increase in long-distance types under drought suggests that carbon was not limiting. These findings indicate an overall qualitative preservation of functionality in ECM fungal communities at the level of vital root tips. However, a decline in the number of vital tips led to quantitative functional losses in ECM fungal communities at the ecosystem level.

Thus, structural diversity supported our second hypothesis that repeated drought leads to changes in the functionality of the ECM fine-root system towards traits that are related to drought resistance, irrespective of the tree species at the vital root tip and ecosystem level, while enzyme activities did not support it at the ecosystem level.

4.3 | Does tree mixture attenuate negative effects of drought on ECM fungal communities of beech and spruce compared with monospecific stands?

Growth in mixed stands had significant positive effects on morphotype diversity indices of the ECM fungal communities of both tree species after 3 years of throughfall exclusion, at which time the low soil water content indicated rather high stress levels (Davidson, Belk,

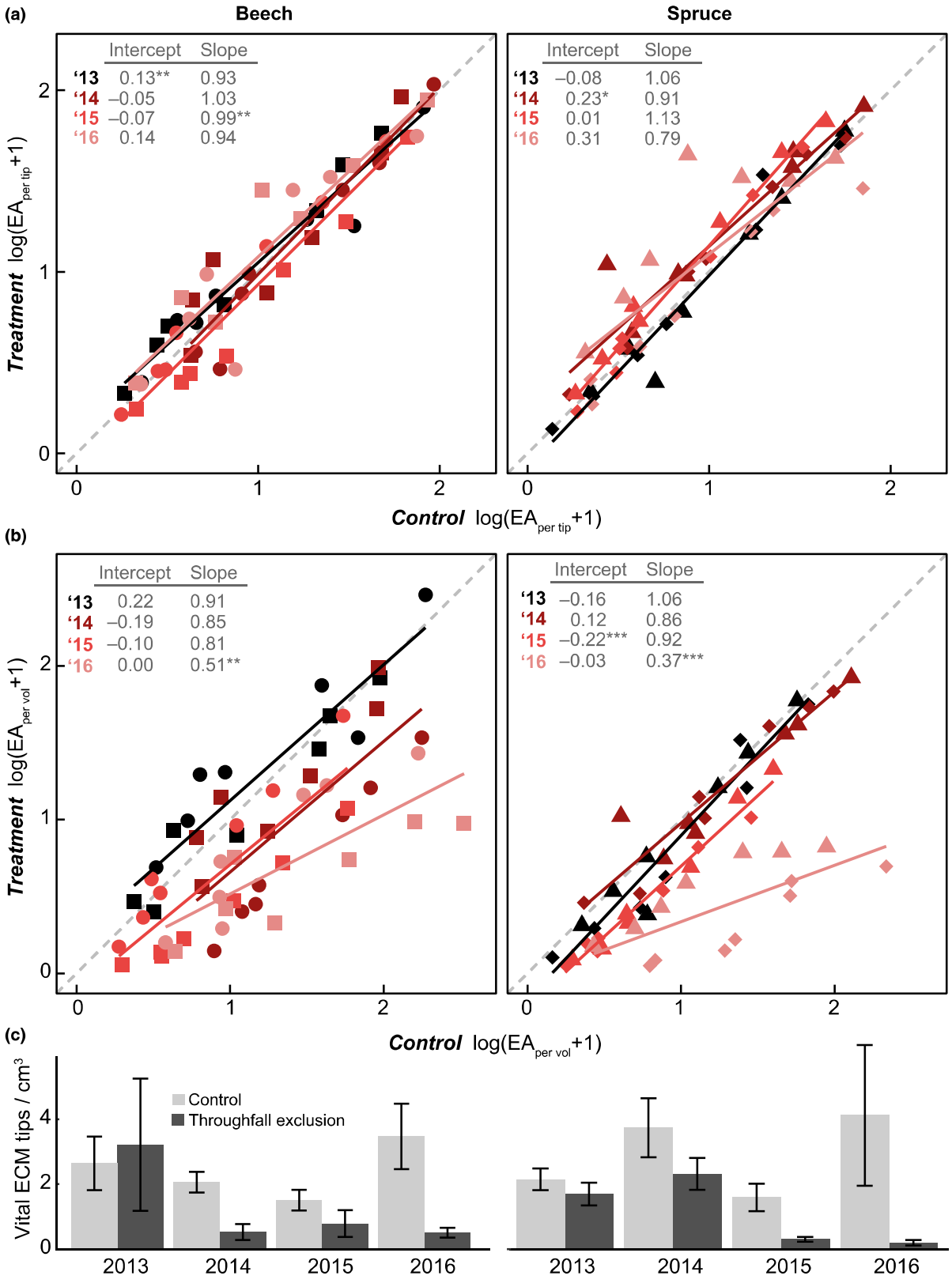
(a)
Beech
Spruce
Intercept Slope
'13 0.13** 0.93
'14 –0.05 1.03
'15 –0.07 0.99**
'16 0.14 0.94
Intercept Slope
'13 –0.08 1.06
'14 0.23* 0.91
'15 0.01 1.13
'16 0.31 0.79
Treatment log(EA per tip +1)
Control log(EA per tip +1)
(b)
Intercept Slope
'13 0.22 0.91
'14 –0.19 0.85
'15 –0.10 0.81
'16 0.00 0.51**
Intercept Slope
'13 –0.16 1.06
'14 0.12 0.86
'15 –0.22*** 0.92
'16 –0.03 0.37***
Treatment log(EA per vol +1)
Control log(EA per vol +1)
(c)
Vital ECM tips / cm3
Control
Throughfall exclusion
2013
2014
2015
2016

FIGURE 6 Potential enzyme activities (EAs): (a) $EA_{per\ tip}$ (pmol cm^{-2} min^{-1}) as weighted mean of EA per ectomycorrhizal (ECM) tip in an ECM community (see materials and methods Equation 1), (b) $EA_{per\ vol}$ (pmol cm^{-2} min^{-1} cm^{-3}) taking into account the number of vital ECM tips per soil volume (see Section 2 Equation 2), and (c) number of vital ectomycorrhizae of spruce and beech in topsoil samples over four study years (2013–2016). EA values of the respective same sample type in control and throughfall exclusion plots (Table S7) were log transformed and plotted against each other for each year separately to illustrate overall effects of throughfall exclusion. Linear regressions were calculated and plotted for these pairs per year with the colour code from darker in 2013 to lighter in 2016. The grey dashed line with a slope of 1 and an intercept of 0 was drawn to indicate when EAs under control is equal to EAs under throughfall exclusion. Deviation of the slope of regression lines from 1 with an intercept remaining close to 0 indicates similar relative degrees and directions of change in all EAs, whereas shift in the intercept indicates that EA values changed to different degrees and/or directions. Values of intercept and slope are given in the top left corner of each panel with asterisks indicating significant differences from a slope of 1 and an intercept of 0 (*$p < .05$, **$p < .01$, ***$p < .001$). For ease of visualization, the different enzymes were not specifically indicated in this representation, and EA values were plotted without standard error (for detailed values see Table S7). Symbols represent sample types (circles BB, squares BMix, triangles SS, diamonds SMix) resulting in four values per enzyme and a total of 28 values per year of seven hydrolytic enzymes (xylosidase, cellobiohydrolase, β-glucosidase, chitinase, leucine aminopeptidase, phosphatase and glucuronidase). From the eight studied EAs, laccase was excluded as it showed a clearly different behaviour compared to the seven hydrolytic enzymes (Fig. S10). Error bars in panel (c) indicate ±1 standard error

& Boone, 1998). This supports the stress gradient hypothesis of increased facilitation among species with increasing stress levels (Bertness & Callaway, 1994). To determine whether these emerging facilitation effects would continue, further sampling is required with increasing stress levels. Thus, our third hypothesis was only preliminarily supported by the morphotype data. A probable reason for the observed higher morphotype diversity in the mixture zones could be reduced competition for non-limiting resources due to different soil exploration of the two tree species (Bolte & Villanueva, 2006).

Ectomycorrhizal fungi colonizing both tree species may contribute to resource partitioning between fungi and different trees, facilitating stress resistance (Beiler, Durall, Simard, Maxwell, & Kretzer, 2010). Among those ECM fungi were a contact and a long-distance type species. The decline of these two abundant species upon drought was reduced in the mixture zones. This suggests that mixture provides vital ectomycorrhizae with different functional attributes as starting material for recolonizing newly grown roots during recovery after drought: in our study, this was indicated by drought-tolerance among the four most frequent morphotypes shared by beech and spruce. This indicates mixture to increase resilience of forest ecosystems after drought.

The hydrolytic $EA_{per\ tip}$ was maintained even after two consecutive summers with prolonged drought periods, with mixture effects only becoming apparent in the third year of throughfall exclusion. This can probably be attributed to changes in niche occupation by roots of the two tree species, as indicated by the reduction in some enzyme activities (leucine aminopeptidase and phosphatase) in spruce from mixture but not in beech from mixture in the topsoil, and their increase in the deep layers. $EA_{per\ vol}$ did not exhibit any mixture effects. Thus, according to the assumption that 3 years of throughfall exclusion evoked strong stress on ECM fungal communities, we do not accept H3 according to enzyme activity data.

4.4 | Overall implications of repeated drought on below-ground functioning of forest ecosystems

Regarding nutrient cycling in forest soils under repeated drought, our results suggest that the potential to forage for nutrients contained in organic materials is retained in surviving ectomycorrhizae. Moreover, preferential carbon allocation of trees to ECM fine roots upon recovery from drought has recently been demonstrated to be an important mechanism for restoring fine-root functionality in forest ecosystems (Hagedorn et al., 2016).

In soils, low moisture leads to low EAs in situ due to impaired diffusion processes and death/inactivity of decomposers, which in turn lead to a retardation of decay processes and thereby to an accumulation of substrate (Brando et al., 2008; van der Molen et al., 2011). Upon rewetting, high amounts of substrate meet a functional ECM fungal community and stimulate the recovery of soil microbial processes (Hagedorn et al., 2016). An increasing amount of dead ectomycorrhizal fine roots may lead to a retardation of decay processes in forest soils and is currently debated to either increase or decrease carbon stocks in forest ecosystems (see Fernandez & Kennedy, 2016 for a review). However, low water availability is likely more growth limiting in these temperate systems than nutrient limitation (Sardans & Peñuelas, 2005) because spring and autumn still provide time and water for mineralizing organic compounds in temperate regions struck by summer drought. Thus, also phases of recovery from drought may be critical to assess when nutrient relations in forest ecosystems are considered after severe drought (Geßler, Schaub, & McDowell, 2017; Hagedorn et al., 2016).

4.5 | Methodological implications

The different numbers and abundances of morphotypes and phylotypes in our study resulted from known methodological constraints of high-throughput sequencing, which overestimates diversity by including the DNA of non-vital ECM fungi, single hyphae and resting stages (Medinger et al., 2010). By contrast, morphotyping is prone to underestimating species richness even when including ITS rDNA information as it cannot distinguish between visually similar ectomycorrhizae (Erland, Jonsson, Mahmood, & Finlay, 1999). However, manual morphotyping allows direct observations of degree of mycorrhization and vitality of ectomycorrhizae and fine roots. Shannon diversity was remarkably similar for morphotypes and phylotypes, indicating that both methods provide similar basic ecological

information on ECM fungal community composition. However, because vital and non-vital ECM tips were not distinguished, high-throughput sequencing results reveal a potential rather than actual community composition. Therefore, RNA-based approaches (Baldrian et al., 2012; van der Linde & Haller, 2013) should be used to assess the active ECM fungal community via high-throughput sequencing.

4.6 | Vulnerability of temperate forests under drought

In this study, we experimentally applied drought stress to a habitat that was not adapted to repeated summer droughts. The combined analysis of ECM fungal community diversity and functional traits suggested that correlations between enzyme activities and ECM fungal species varied depending on the interplay between throughfall exclusion, tree species interaction and soil depth. Such context dependency has also been reported in several previous studies on ECM fungal communities, as reviewed in Bahram, Peay, and Tedersoo (2015). However, by subjecting this mesic forest ecosystem to repeated summer droughts, we were able to detect a strong reduction in enzymatic activities and ECM fungal abundances at the ecosystem level because of fine-root die-back under water shortage. We showed that niche complementarity may be important in attenuating the effects of repeated summer droughts on ECM fungal communities in beech–spruce mixtures. One important mechanism of niche complementarity may be the redistribution of water to shallow soil layers by hydraulic lift.

Our findings underline the vulnerability of temperate forests and similar Holarctic ecosystems to prolonged and frequent summer droughts (Allen et al., 2010). Therefore, we advocate long-term experiments when studying forest ecosystems in the context of drought and support the assertion that mesic forests are endangered by long-term drought (Young et al., 2017). Such experiments would allow us to explore whether ECM fungal communities develop further mechanisms for drought tolerance depending on their habitat, how the same ECM fungal species from dry and moist sites perform under repeated droughts and how they influence host tree performance. This may guide future forest management in areas with predicted alterations in precipitation regimes.

ACKNOWLEDGEMENTS

We are very grateful to Prof. Dr. Reinhard Agerer for reviewing all assignments of exploration types of the high-throughput sequencing data and to Prof. Dr. Diethart Matthies for valuable statistical advice. We gratefully acknowledge the constructive comments of three anonymous reviewers on the manuscript. This work was funded by the Deutsche Forschungsgemeinschaft DFG (MU 831/23-1), the Bavarian State Ministry of the Environment and Consumer Protection, and the Bavarian State Ministry of Food, Agriculture and Forestry. We thank Ahmad Mahmood, Johanna Kössl, Christina Hartung, Benjamin Hafner and Neele Alberding for their skilled assistance during sampling and lab work.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Nickel UT, Weikl F, Kerner R, et al. Quantitative losses vs. qualitative stability of ectomycorrhizal community responses to 3 years of experimental summer drought in a beech–spruce forest. *Glob Change Biol*. 2017;00:1–17. https://doi.org/10.1111/gcb.13957

Method S1: ITS sequence determination for ECM morphotypes (Sanger sequencing)

For Sanger sequencing, one to ten ECM tips per morphotype were collected in 2.0 ml microcentrifuge tubes and DNA was extracted with the MoBio-htp 96 Well Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) or with the Qiagen DNeasy 96 Plant Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's manual. DNA yield was measured with a NanoDrop 1000 spectrophotometer (peqlab, Erlangen, Germany). ITS sequences were PCR-amplified with primer pairs ITS1F (Gardes and Bruns (1993); 5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (White, Bruns, Lee, and Taylor (1990); 5'-TCCTCCGCTTATTGATATGC-3') using the Qiagen Taq PCR Master Mix Kit (Qiagen). PCR mixtures contained 25 µl Qiagen Taq PCR Master Mix, 1 µl of each primer (10 µM), 20–30 ng DNA template and molecular biology grade water to a final volume of 50 µl. An initial denaturation at 94 °C for 4 min was followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 75 s and a final extension at 72 °C for 5 minutes. PCR success was tested via gel electrophoresis (1 % agarose gel, 120 V, 30 min). PCR products were cleaned up with ethanol/EDTA/NA–acetate precipitation and subject to chain termination PCR using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA). The PCR mixture for each primer contained 2 µl amplified DNA template, 1 µl 5x BDT reaction buffer, 1 µl primer (10 µM) and 1 µl terminator mix (dNTPs and labelled ddNTPs). After initial denaturation at 96 °C for 1 minute, samples were amplified by 50 cycles of 96 °C for 10 seconds, 50 °C for 10 seconds and 60 °C for 4 minutes. Chain termination fragments were cleaned with ethanol precipitation and analysed on an ABI 3730 48-capillary sequencer (Applied Biosystems, Foster City, CA, USA).

Supporting Method References:

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. *Molecular ecology, 2*(2), 113-118. doi:10.1111/j.1365-294X.1993.tb00005.x

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Table S2

ITS primers + adaptor sequences (MiSeq) according to Tedersoo et al. (2015)

ITS3-Mix1	(Fungi)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATCGATGAAGAACGCAG
ITS3-Mix2	(Chytridiomycota)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAACGATGAAGAACGCAG
ITS3-Mix3	(Sebacinales)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCACCGATGAAGAACGCAG
ITS3-Mix4	(Glomeromycota)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATCGATGAAGAACGTAG
ITS3-Mix5	(Sordariales)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATCGATGAAGAACGTGG
ITS4-Mix1	(Fungi)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC
ITS4-Mix2	(Chaetothyriales)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTGCGCTTATTGATATGC
ITS4-Mix3	(Archaeorhizomyc.)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCGCCTTATTGATATGC
ITS4-Mix4	(Tulasnellaceae)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTGAWTAATATGC

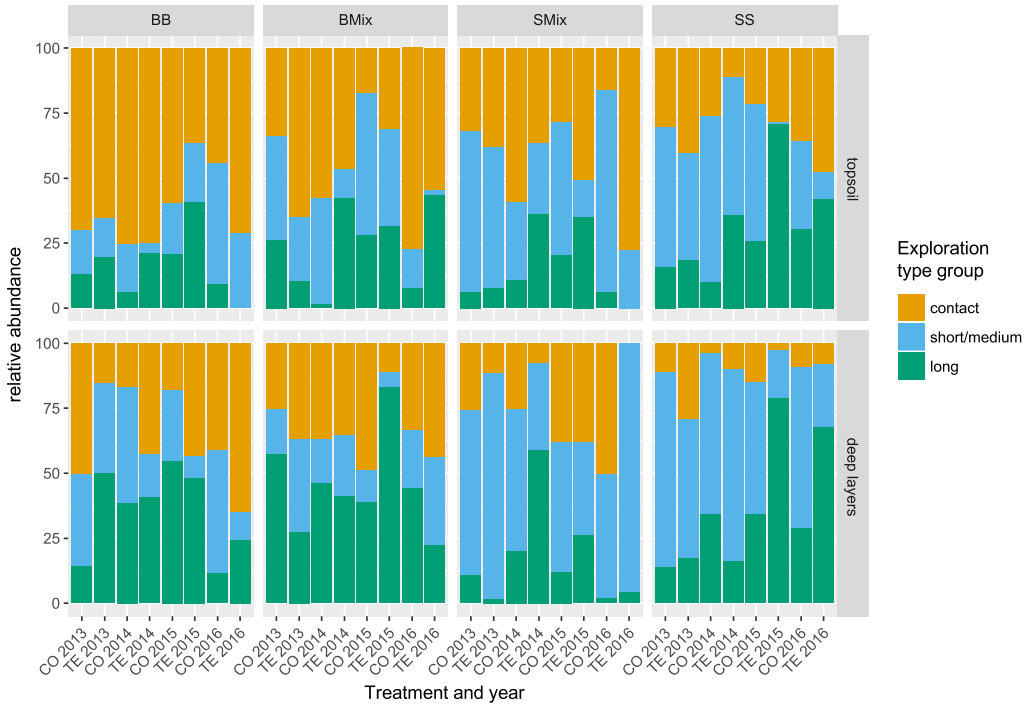
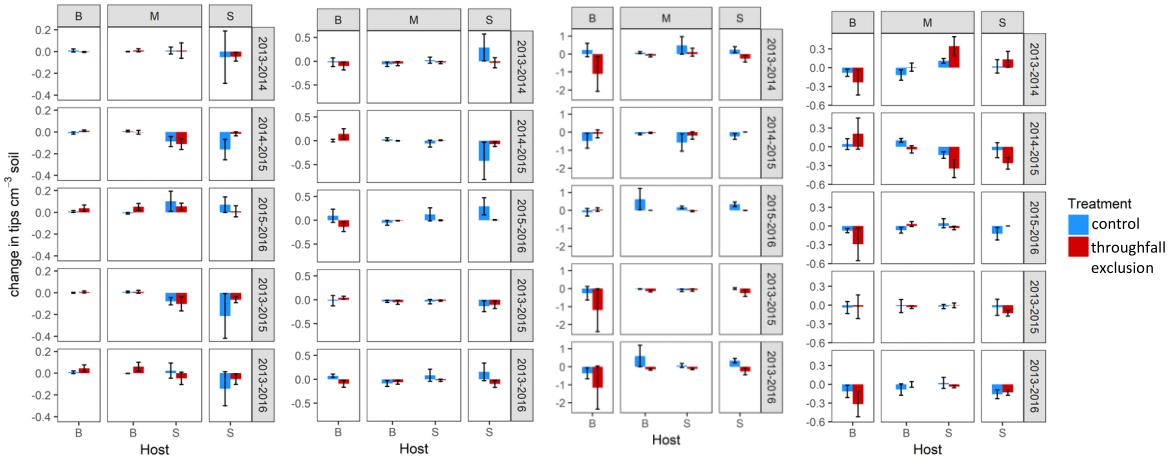


Fig. S5 Relative abundance of each exploration type group. CO: control; TE: throughfall exclusion. contact: contact types (soil exploration radius 0 mm, no emanating hyphae); short/medium: short-distance (soil exploration radius up to 5 mm, some emanating hyphae) and medium-distance types (soil exploration radius up to 3 cm, fringe types: fans of emanating hyphae, mat types: undifferentiated rhizomorphs, smooth types: slightly differentiated rhizomorphs); long: long-distance types (soil exploration radius up to several dm, mostly highly differentiated rhizomorphs). BB: beech roots from beech zones; BMix beech roots from mixture zones; SMix: spruce roots from mixture zones; SS: spruce roots from spruce zones. Topsoil: combined horizons O_f + $_hA_h$ from 0 to 8.6 cm depth; deep layers: horizon A_lB_v, from 8.6 to 25 (2013 and 2014), respectively 40 cm (2015 and 2016) depth.



a) R. ochroleuca *b) C. geophilum* *c) L. tabidus* *d) X. pruinatus*

Fig. S6 Changes in vital ECM tips per cm³ soil between years in abundance of ECM fungal species shared by beech (B) and spruce (S), in control (blue) and in throughfall exclusion (red) plots. Trees grew in zones where they were surrounded by the same species (B zones and S zones) or in mixture with the respective other species (M zones).

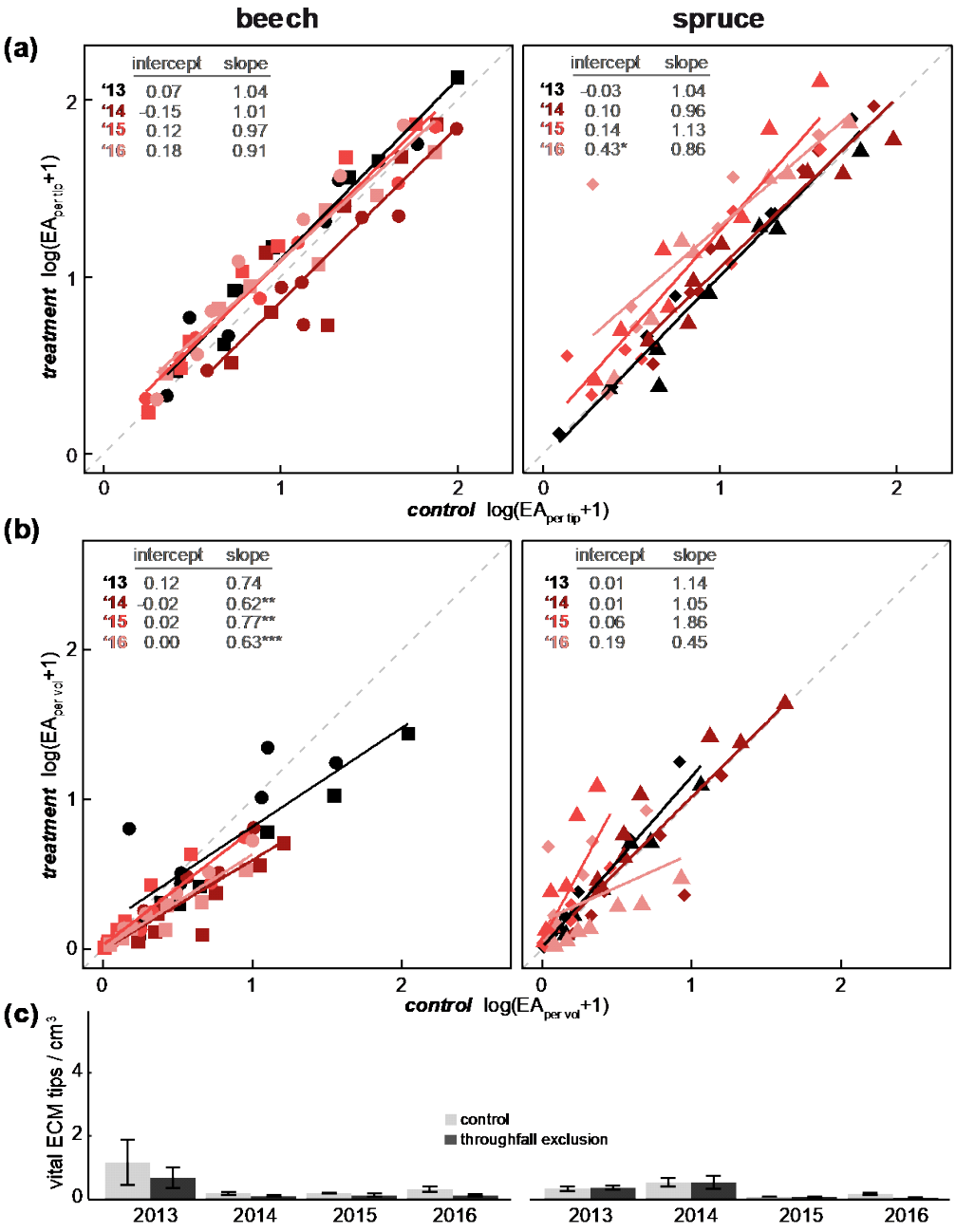


Fig. S9 Potential enzyme activities (EAs): (a) $EA_{per\ tip}$ (pmol cm^{-2} min^{-1}) as weighted mean of EA per ectomycorrhizal (ECM) tip in an ECM community (see materials and methods equation 1), (b) $EA_{per\ vol}$ (pmol cm^{-2} min^{-1} cm^{-3}) taking into account the number of vital ECM tips per soil volume (see materials and methods equation 2), and (c) number of vital ectomycorrhizae of spruce and beech in deep layer samples over four study years (2013-2016). EA values of the respective same sample type in control and throughfall exclusion plots (Table S7) were log transformed and plotted against each other for each year separately to illustrate overall effects of throughfall exclusion. Linear regressions were calculated and plotted for these pairs per year with the colour code from darker in 2013 to lighter in

2016. The grey dashed line with a slope of 1 and an intercept of 0 was drawn to indicate when EAs under control are equal to EAs under throughfall exclusion. Deviation of the slope of regression lines from 1 with an intercept remaining close to 0 indicate similar relative degrees and directions of change in all EAs, whereas shift in the intercept indicates that EA values changed to different degrees and/or directions. Values of intercept and slope are given in the top left corner of each panel with asterisks indicating significant differences from a slope of 1 and an intercept of 0 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). For ease of visualization, the different enzymes were not specifically indicated in this representation, and EA values were plotted without standard error (for detailed values see Table S7). Symbols represent sample types (circles BB, squares BMix, triangles SS, diamonds SMix) resulting in four values per enzyme and a total of 28 values per year of seven hydrolytic enzymes (xylosidase, cellobiohydrolase, β-glucosidase, chitinase, leucine aminopeptidase, phosphatase and glucuronidase). From the eight studied EA, laccase was excluded as it showed a clearly different behaviour compared to the seven hydrolytic enzymes (Fig. S8). Error bars in panel (c) indicate ± 1 standard error.

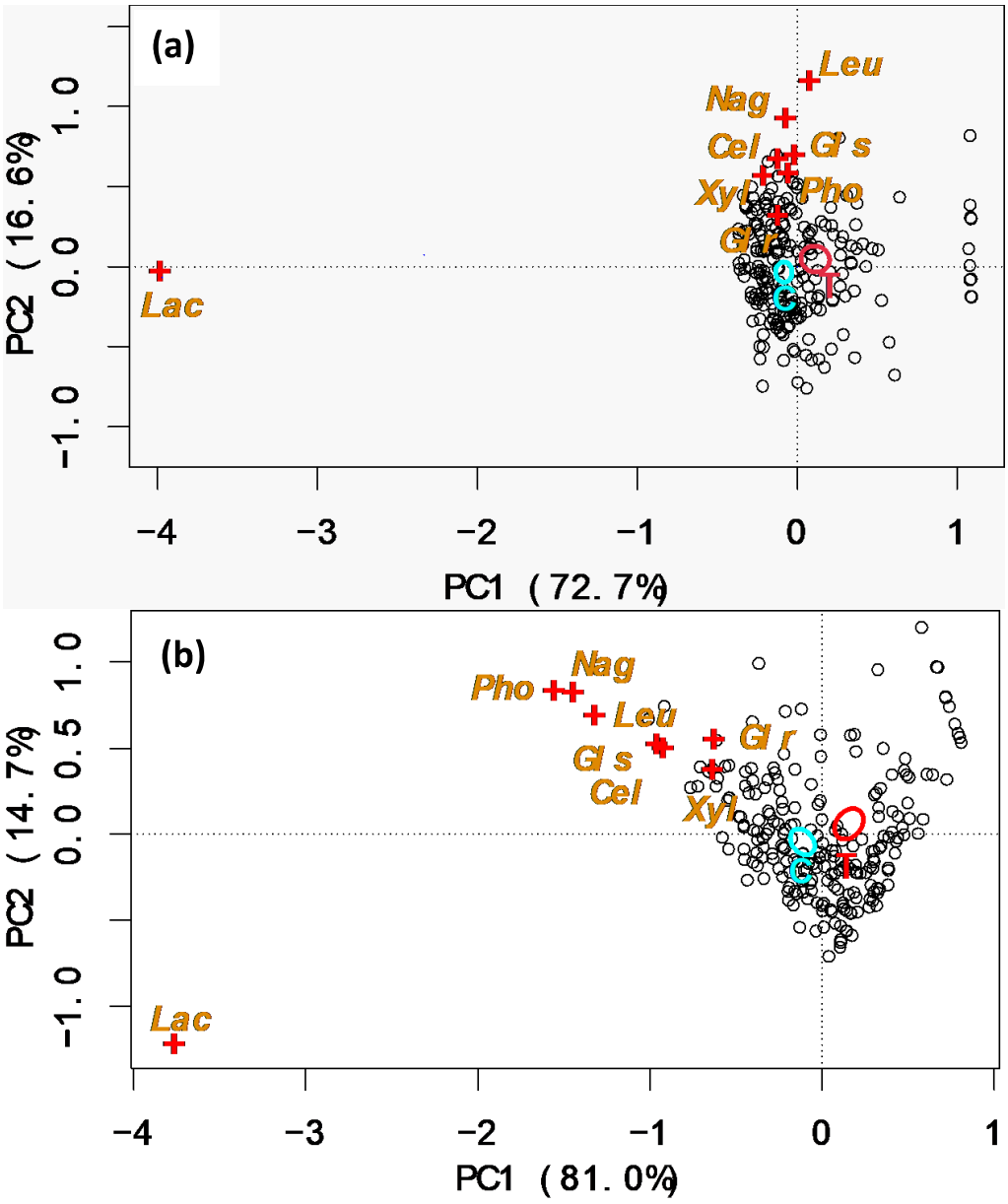


Fig. S10 Principal component analysis (PCA) of exoenzyme activities (EAs) per tip (a) and per vol (b) of ECM tips of beech and spruce in a throughfall exclusion experiment. All samples from 2014, 2015 and 2016 were pooled for this analysis and values of EAs were normalised by log transformation. Leu: leucine aminopeptidase, Pho: phosphatase, Gls: β-glucosidase, Xyl: xylosidase, Glr: glucuronidase, Cel: cellobiohydrolase, Nag: N-acetylglucosaminidase, Lac: laccase. Confidence ellipses (90 %) of the standard errors of the points are drawn around the centroids of each of the treatments: C: control, T: throughfall exclusion. Both PCAs show that activity of the oxidative enzyme laccase responds differently to throughfall exclusion than activities of the other seven hydrolytic enzymes. Further

transformation of laccase activity values (using nmol or µmol scale) did not alter the meaning of the PCA results.

Tables S3, S4, S7 and S8 are not suitable for printing. Therefore, refer to the electronic appendix of the published original article, please.

8 Appendix

8.1 Abbreviations used in figures 2–5

Cel	cellobiohydrolase activity
Glr	gucuronidase activity
Gls	glucosidase activity
Lac	laccase activity
Leu	leucine-aminopeptidase activity
Nag	N-acetyl-glucosaminidase activity
Pho	phosphatase activity
Xyl	xylosidase activity
Cen_geo	abundance of *Cenococcum geophilum* (FR.)
Cla_1	abundance of *Clavulina* (J. SCHRÖT.) sp. 1
Cor_1	abundance of *Cortinarius* [(PERS.) GRAY] sp. 1
Gen_his	abundance of *Genea hispidula* (BERK. ex TUL. & C. TUL)
Ino_umb	abundance of *Inocybe umbrina* (BRES.)
Lac_ame	abundance of *Laccaria amethystine* [(HUDS.) COOKE]
Lac_bic	abundance of *Laccaria bicolor* [(MAIRE) P.D. ORTON]
Lac_ble	abundance of *Lactarius blennius* (FRIES)
Lac_tab	abundance of *Lactarius tabidus* (FR.)
Lact_1	abundance of *Lactarius* (PERS.) sp.
MT_11	abundance of unidentified morphotype 11
MT_18	abundance of unidentified morphotype 18
MT_58	abundance of unidentified morphotype 58
No_myc	abundance of vital, but not mycorrhized root tips
Rus_1	abundance of *Russula* (PERS.) sp. 1
Rus_2	abundance of *Russula* (PERS.) sp. 2
Rus_fel	abundance of *Russula fellea* [(FR.) FR.]
Rus_nig	abundance of *Russula nigricans* (FR.)
Rus_och	abundance of *Russula ochroleuca* (PERS.)
Scl_cit	abundance of *Scleroderma citrinum* (PERS.)
Thel_1	abundance of *Thelephora* (PERS.) sp. 1
Tom_1	abundance of *Tomentella* (PERS.) sp. 1
Tom_2	abundance of *Tomentella* (PERS.) sp. 2
Tom_bot	abundance of *Tomentella botryoides* [(SCHWEIN.) BOURDOT & GALIZIN]
Tub_pub	abundance of *Tuber puberulum* (BERK. & BROOME)
Tyl_ast	abundance of *Tylospora asterophora* [(BONORD.) DONK]
Xer_chr	abundance of *Xerocomus chrysenteron* [(BULL.) ŠUTARA]
Xer_pru	abundance of *Xerocomus pruinatus* [(FR.) ŠUTARA]

8.2 Approval letters from publishers

8.2.1 Article I

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8.2.4 Article IV

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Danksagung

Während meiner Arbeit am Helmholtz Zentrum München und dem Verfassen dieser Dissertation haben mich viele Menschen finanziell, fachlich, seelisch und tatkräftig unterstützt. An dieser Stelle möchte ich meinen Dank für diese Unterstützung aussprechen, ohne die diese Arbeit nicht möglich gewesen wäre.

Zuallererst möchte ich meiner Doktormutter, Chefin und Mentorin PD Dr. Karin Pritsch danken. Sie hat diese Dissertation angeregt, mich sicher über alle Hürden geführt und stand mir stets mit ihrem Wissen und ihrer Unterstützung zur Seite. Sie hat mir wertvolle Dienstreisen zu Kongressen ermöglicht und war eine nie versiegende Quelle der Inspiration.

Danken möchte ich auch Herrn Prof. Dr. Jean-Charles Munch, in dessen Institut meine Reise begann. Er hat mir auf seine unkomplizierte Weise alle Mittel zur Verfügung gestellt, die ich für die Durchführung dieser Arbeit benötigt habe und war immer bereit, sich für mich einzusetzen.

Nach der Emeritierung von Prof. Munch wurde ich von Prof. Dr. Jörg Durner in seinem Institut aufgenommen wo ich meine Arbeit ohne Unterbrechung fortsetzen konnte. Ihm und Prof. Dr. Johannes Kollmann möchte ich auch für ihre Bereitschaft danken, meine Arbeit zu prüfen.

Ich möchte Prof. Dr. Diethart Matthies, der auch schon meine Masterarbeit betreut hat, für seine Bereitschaft danken, meinem Thesis Committee anzugehören und mich auch durch das Doktorat zu begleiten. Er gab mir wertvolle statistische Unterstützung. Von ihm lernte ich die Grundlagen des experimentellen Designs und der Datenanalyse, was mir stets eine große Hilfe war.

Für das Regenausschlussexperiment im Kranzberger Forst war viel Vorbereitung nötig. Ich danke Dr. René Kerner für die Einarbeitung und die Organisation der ersten beiden Jahre, für die vielen lustigen und verrückten Stunden am Binokular und für die Freundschaft, die daraus entstand.

Ein weiterer Pfeiler für das tägliche Überleben war Dr. Fabian Weikl; der beste Büronachbar und anfangs noch Mitdoktorand, den man sich wünschen kann (auch wenn es manchmal recht warm in der guten Stube war). Mit seiner mikrobiologischen Expertise, als Ko-Autor und nicht zuletzt als Freund war er mir eine große Hilfe und ein Vorbild. Danken

möchte ich auch meinem Mitdoktoranden Philipp Koal, der mir die Welt der Bodenkunde nahegebracht und mich den Umgang mit Bodenproben gelehrt hat.

Ohne Franz Buegger und Gudrun Hufnagel wären viele technische Probleme ungelöst, tausende Proben ungemessen und so manche Weinflasche ungeöffnet geblieben. Ihre Kompetenz, Zuverlässigkeit und Freundschaft waren mir eine große Hilfe.

Ein großer Dank geht auch an meine Ko-Autoren/innen und an meine Kollegen/innen, die mich als Ko-Autor und zu ihren Experimenten eingeladen haben, allen voran Dr. Michael Goisser und Dr. Martina Peter. Ich bedanke mich für die tolle Zusammenarbeit und den kulturellen und fachlichen Austausch mit den Kollegen aus der Schweiz (ich vermisse schmerzlich das Znüni mit Kaffee und Ragusa).

Tatkräftige Unterstützung habe ich von unserem Labortechniker Arsin Sabunchi erhalten und von etlichen Praktikanten/innen, Studenten/innen und Auszubildenden. Besonders erwähnen möchte ich hier die Arbeit von Christina Hartung, Carina Meyer, Neele Alberding, Ahmad Mahmood, Elisabeth Koc, Charlotte Völkel, Alexander Wendler, Mareike Bamberger, Jelena Vucic und Johanna Kössl.

Ohne die Unterstützung meiner Familie hätte ich diese große Aufgabe nicht bewältigen können. Hier möchte ich ganz besonders meiner wundervollen Frau Catharina danken. Sie hat immer an mich geglaubt und mir immer Kraft gegeben, hatte immer zwei offene Ohren und hat an all das gedacht, was ich sonst vergessen hätte. Danke für alles; bis zur Unendlichkeit und noch viel weiter!

Ich danke meinen Eltern Gudrun und Peter, meinen Schwiegereltern Christiane und Horst, meinen Schwägerinnen und Schwagern Connie, Tobi, Steffi und Alex und deren Kindern Mathilda, Gregor, Eleonore und Henri für die vielen entspannenden Momente, aufbauenden Gespräche, inspirierenden Erlebnissen und musikalischen Soiréen. Ganz besonders danke ich meiner Großmutter Elfriede, die in ihrem Garten meine Begeisterung für Biologie und Botanik geweckt hat, die immer einen guten Rat zur rechten Zeit hat und die einzige Person ist, mit der ich Akkordeon-Duette spielen kann.

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Publikationen:

- Hagedorn F *et al.* (2016): Recovery of trees from drought depends on belowground sink control. Nature plants, 2, 16111. doi:10.1038/nplants.2016.111.
- Goisser M *et al.* (2016): Does belowground interaction with Fagus sylvatica increase drought susceptibility of photosynthesis and stem growth in Picea abies? Forest Ecology and Management, 375, 268-278. doi: 10.1016/j.foreco.2016.05.032.
- Nickel UT *et al.* (2017): Nitrogen fertilisation reduces sink strength of poplar ectomycorrhizae during recovery after drought more than phosphorus fertilisation. Plant and Soil, 419(1), 405–422. doi:10.1007/s11104-017-3354-2.
- Nickel UT *et al.* (2017): Quantitative losses vs. qualitative stability of ectomycorrhizal community responses to 3 years of experimental summer drought in a beech-spruce forest. Global Change Biology. doi: 10.1111/gcb.13957

Tagungsbeiträge:

- New Phytologist Symposium 2014, CH-Zürich, Posterpräsentation
- Jahrestagung der Arbeitsgemeinschaft Stabile Isotope e.V. 2014, München, Posterpräsentation
- 8th International Conference on Mycorrhiza 2015, US-Flagstaff/Arizona, Posterpräsentation
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