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Forum

Endogenous rhythmic growth, a trait suitable for the study of interplays between multitrophic interactions and tree development

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ABSTRACT

As long-lived organisms, trees use resources to support both growth and below- and aboveground trophic interactions. Resources fluctuate in relation to periods of growth cease that are regulated by internal and external factors, and these fluctuations feed backs to trophic partners. Some major forest trees display an endogenous growth rhythm, and related pulses of variation in allocation of resources have been detected. As this trait makes it possible to separate growth into defined phases, it offers an opportunity to disentangle the intermingled complex regulation of growth and multitrophic interactions in trees. We present "TrophinOak", a platform using microcuttings of pedunculated oak, a tree that displays endogenous rhythmic growth characterized by alternating shoot and root growth flushes. We select seven beneficial or detrimental above- and belowground partners including animals (*Lymantria dispar*, *Pratylenchus penetrans*, *Protaphorura armata*), fungi (*Piloderma croceum, Microsphaera alphitoides, Phytophthora quercina*) and bacteria (*Streptomyces* sp.), to synthesize bi- and tripartite trophic interactions, including ectomycorrhizal symbioses, and monitor fluctuations of carbon and nitrogen allocation as well as plant gene expression at distinct phases of oak growth. We use this model to identify and resolve the experimental challenges inherent in synthesizing diverse types of associations in a common microcosm system, in labeling plants with stable N and C isotopes and in analyzing transcripts in a non-model plant, a process which requires generating a specific contig library. We develop hypotheses and experimental design to test them in order to identify core mechanisms that help trees to modulate their own development and their multitrophic interactions for optimizing their long term performance in their environment. First results constitute a proof of concept that the platform works and enables us to test the hypotheses.

1. Introduction

This article highlights the need for experimental platforms in order to unravel the interplay between growth and multitrophic interactions in forest trees, and describes an example of such a platform which enables resource allocation and gene regulation to be coupled in a single study. As perennials, trees undergo phases in which meristem activity ceases; these control tree architecture and ability to survive through unfavorable seasons (Rohde et al., 2007). This episodic growth is linked to variations in allocation of the

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resources that are mobilized below- and aboveground (Cooke et al., 2003). These variations affect the numerous multitrophic associations typical of trees (Schultz et al., 2013), and have feedback effects on growth and plant resource economy (Matyssek et al., 2005). Such integrated networks of interactions govern the functioning of plant communities (De Deyn and Van der Putten, 2005). However, our knowledge about the relevant mechanisms at the level of individual trees, which are pivotal partners in such networks, is scanty.

Trees are classified into several architectural models, depending on whether their growth is continuous or rhythmic, and determinate or indeterminate (Barthelemy and Caraglio, 2007). The continuous and rhythmic growth traits are determined endogenously, but they are modulated by external factors such as temperature, drought or photoperiod (Saxe et al., 2001). In tropical trees, endogenous rhythmic growth is characterized by successive periodic shoot flushes separated by resting phases (Barthelemy and Caraglio, 2007). Endogenous rhythmic growth is also expressed in some major temperate tree species that produce several shoots within each growing season (Späth, 1912). Rhythmic growth has also been shown in roots, where it is controlled endogenously (Reich et al., 1980). As a consequence of rhythmicity, C allocation to roots fluctuates (Willaume and Pages, 2006, 2011), but little is known about N allocation.

Trophic interactions alter resource acquisition and allocation in trees. Mycorrhizal symbioses are sinks for photoassimilates, but they increase nitrogen uptake and photosynthesis by the host plants (Smith and Read, 2008). Haustoria of leaf pathogenic fungi are C-sinks that draw off the products of photoassimilation and affect the plant source-sink system (Hewitt and Ayres, 1976). Herbivory directly impairs resource acquisition by the organs attacked, but also provokes cascades of changes such as N-resorption coupled with modified photosynthetic efficiency, allocation away from the organ affected, compensatory regrowth, or synthesis of defense compounds (Schultz et al., 2013). Rhizophere microorganisms modify the root/shoot ratio and affect photosynthesis (Lehr et al., 2007). Decomposer invertebrates alter root morphology, plant growth and susceptibility to herbivore attack (Bonkowski and Scheu, 2004).

How the processes of growth and multitrophy interact in shaping tree performance, and how their regulatory pathways harmonize, is largely unknown. Trees displaying rhythmic growth offer good experimental models with which to tackle this interplay, because they represent a system in which there are pulses of resources enabling growth to be separated into defined phases. Unfortunately, handling mature trees in the laboratory is difficult due to their large size. Working on young trees is easier, but because seed resources modify the pattern of rhythmic growth (Alaoui-Sossé et al., 1994), seedlings are not an ideal system. Older trees are entities made of numerous shoot and root modules connected by branches, trunks and main roots via phloem and xylem tissues (Barthelemy and Caraglio, 2007; Schultz et al., 2013), which complicates the tracking of variations in resource allocation during growth and multitrophic interactions. In contrast, microcuttings produced *in vitro* consist of a limited number of shoot and root modules with reduced amount of connective tissues. Unfortunately, trees displaying strong rhythmicity (*e.g.*, spruce, fir, pine, walnut, and oak) are more difficult to propagate *in vitro* than species that show continuous growth such as poplar, birch, eucalypt or elm (McCown, 2000). Nonetheless, a miniaturized *in vitro* system has been established for pedunculate oak (*Quercus robur* L.) (Herrmann et al., 1998), one "*foundation species*" in temperate hardwood forests (Plomion and Fievet, 2013). Oaks display a typical endogenous rhythmic growth pattern (Lavarenne, 1968) with alternating shoot flushes (SF) and root flushes (RF) (Reich et al., 1980), and they are among the trees that have the broadest range of trophic interactions (Brändle and Brandl, 2001). Micropropagated oaks have been used to study the impact of rhythmic growth on mycorrhizal symbioses (Herrmann et al., 1998, 2004).

This article describes the optimization of the experimental platform "TrophinOak" (www.trophinoak.de) using microcuttings as a model to study the interplay between rhythmic growth, resource allocation and gene expression in trees engaged in different types of below- and aboveground trophic interactions (Fig. 1). We present the organisms selected, our five central hypotheses and two experimental setups designed to allow us answering the questions raised. We also describe the technical challenges posed by the optimization of this kind of model system and the solutions adopted in TrophinOak. Finally we present first results that validate the concept of TrophinOak.

2. Choice of trophic interactions

2.1. The oak clone DF159

Q. robur was chosen as the model for TrophinOak, because of long experience in handling the micropropagated pedunculate oak clone DF159 (Herrmann et al., 1998, 2004; Herrmann and Buscot, 2007). Microcuttings of DF159 display the full rhythmic growth pattern typical of developed oaks with alternating SF and RF (Reich et al., 1980), while in seedlings less than two years old, only SF develop rhythmically (Alaoui-Sossé et al., 1994). DF159 enabled us to monitor rhythmic growth through four developmental stages characteristic of the apical bud: rest phase (stage A), swelling (B), outburst (C), and leaf expansion (D) (Herrmann et al., 1998). Stage B, which corresponds to maximal root elongation, represents the RF. Stage D represents the SF (Fig. 2).

2.2. Trophic interactions partners

One prerequisite for multitrophic experiments on a single model plant is the selection of compatible partners that affect plant performance, encompass below- and aboveground associations and have effects ranging from the beneficial to the detrimental. Here we introduce the trophic interactions selected for TrophinOak.

Ectomycorrhizas (EM) enhance not only N-uptake and N-allocation toward leaves, but also photosynthesis and transport of assimilates toward roots (Smith and Read, 2008). Thousands of plant genes involved in signaling, defense, development and resource partitioning have been found to be differentially regulated in EM roots (Tarkka et al., 2013). Most studies on DF159 have used a strain of the EM basidiomycote *Piloderma croceum* J. Erikss. & Hjortst, that is deposited in several type collections (TUMY F1598, ATCC Number MYA-4870, DSMZ 4824). This strain stimulates growth and photosynthesis (Herrmann et al., 2004) and affects expression of the oak genome (Herrmann and Buscot, 2007; Tarkka et al., 2013). The genome of this *P. croceum* strain has been sequenced recently (Kohler et al., 2015).

Mycorrhiza helper bacteria (MHB) promote EM formation, but their direct effects on woody plants are largely unexplored (Frey-Klett and Tarkka, 2007). In Norway spruce, the MHB *Streptomyces* strain AcH 505 suppressed plant defense and stimulated photosynthesis and root formation (Lehr et al., 2007), suggesting that it has a direct effect on plant growth and resource acquisition. AcH 505 elicits resistance against powdery mildew by increasing the expression of genes related to systemic defense and photosynthetic activity (Kurth et al., 2014). Moreover, AcH 505 has been shown to counteract the damage by root-feeding nematodes by altering the microbial communities in the oak rhizosphere of DF159 (Caravaca et al., 2015).

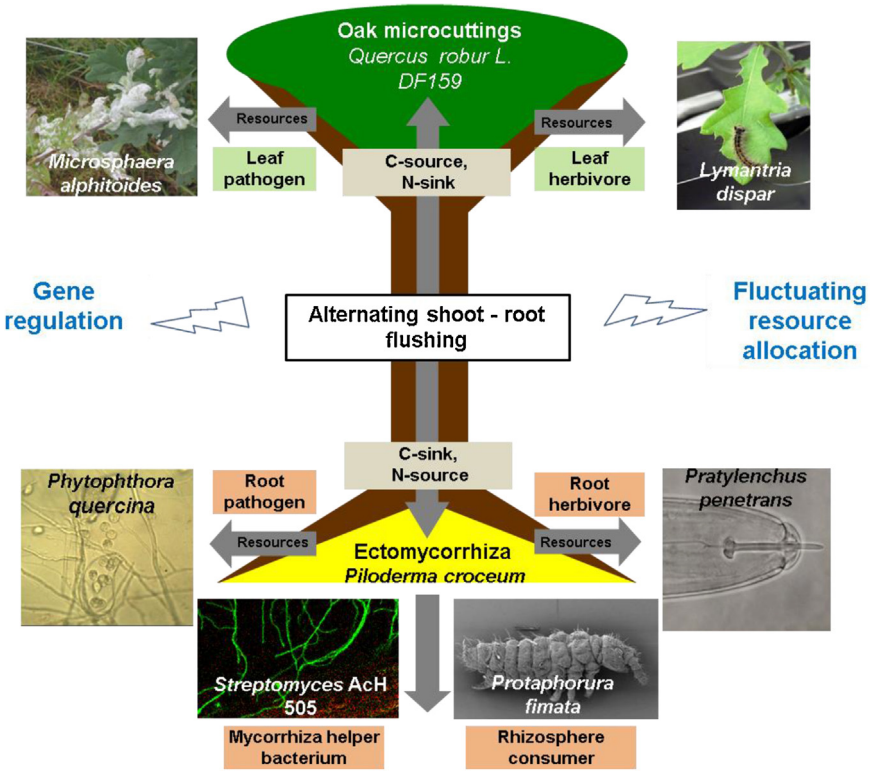


Fig. 1. Schematic representation of the TrophinOak concept. The trees are considered as a double C and N pump fluctuating during the phases of the endogenous rhythmic growth. In addition above- and belowground biotic interactors demand and influence the resource shifts. A genetic regulation of these interplays is postulated.

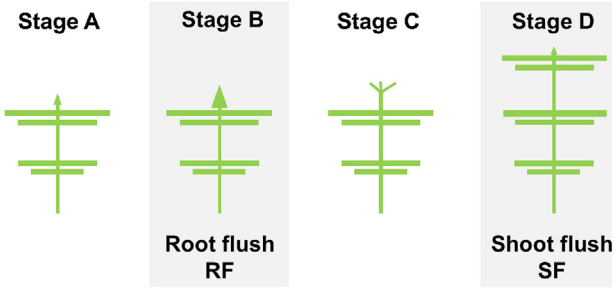


Fig. 2. The four development stages of the apical bud during one endogenous rhythmic growth cycle in pedunculate oak (*Quercus robur*). Rest stage A, swelling stage B corresponding to the root flushing (RF), outburst stage C, leaf expansion stage D corresponding to the shoot flushing (SF).

Leaf chewing caterpillars. Oak is the European tree with the highest number of aboveground herbivore species (Brändle and Brandl, 2001). Insect herbivores such as leaf chewing caterpillars cause defoliation and decrease of photoassimilation (Babst et al., 2008), and impair the allocation of C to roots and EM (Gehring and Whitham, 2002). However, nutrient delivery to the host by EM depends on plant C-flow to the fungus (Smith and Read, 2008). In turn, the leaf nitrogen content affects the palatability of plant tissues (Schädler et al., 2003). Ultimately, interactions between trees and herbivores depend on the regulation of hundreds of genes that are directly related not only to defense but also to the control of photosynthesis and C and N allocation (Porth et al., 2011). The gypsy moth, *Lymantria dispar* L., is a widespread polyphagous species with a strong preference for oaks; it is known to undergo mass outbreaks, which can cause severe damage in oak-dominated forests (Alalouni et al., 2013).

Nematodes are the most important and ubiquitous root feeders in soil, and they can have a severe impact on crop performance in arable systems (Cohn et al., 2002). They also adversely affect forest trees, *e.g.* the genus *Pratylenchus* was reported to have harmful effects on ectomycorrhiza (Marks et al., 1987). Besides damaging plants directly by breaking down cell structure or removing cell contents, nematodes function as strong terminal sinks for nutrients and induce major up-regulation of transport processes by modifying plant gene expression (Hammes et al., 2005). Apart from this transport stimulation, the virulence of *Pratylenchus penetrans* (Cobb) Philip & Stek was shown to depend on resource availability in oak roots (Caravaca et al., 2015).

Collembolans are abundant animals present in virtually all soils. They feed on a wide range of resources (Chahartaghi et al., 2005), and despite living predominantly as detritivores, they may also feed on roots if other resources are scarce (Endlweber et al., 2009). Collembolans have been shown to modify plant growth via a number of direct and indirect mechanisms (Bonkowski and Scheu, 2004). The extent and nature of plant gene expression in response to collembolans is virtually unknown: the only published study investigated a single collembolan species (the euedaphic *Protaphorura fimata* Gisin) and focused on the short-lived model plant *Arabidopsis thaliana* L. (Endlweber et al., 2011). TrophinOak selected *Protaphorura armata* Tullberg, another collembolan often used for experiments with plants (Sabais et al., 2012).

Phytophthora, a member of the Peronosporomycetes, is an important pathogen of herbaceous and woody plants. Some invasive *Phytophthora* species cause tremendous damage in forests, while species that are naturally resident in soils have little impact. In Central European oak stands, *Phytophthora quercina* T. Jung, one factor in the oak decline syndrome, can frequently be isolated among another 11 *Phytophthora* species (Jung et al., 2000). While this hemi-biotrophic pathogen causes significant dieback of roots, it has little impact on aboveground growth *e.g.* of pedunculate oak (Jönsson, 2004). A conceptual model on *Q. robur* suggests that *Phytophthora* damage is counteracted by large amounts of

C-resources in roots, which may increase synthesis of defense compounds (Jönsson, 2006). As shown in Section 5 we could recently challenge this model (Angay et al., 2014). In beech, *Phytophthora* down-regulates genes involved in defense and resource allocation (Schlink, 2010).

Powdery mildew (*Microsphaera alphitoides* Griffon & Maubl.) was only detected on European oaks at the beginning of the 20th century, but it is now a major pathogen (Marcais and Desprez-Loustau, 2012). Infection of oak leaves with powdery mildew greatly reduces photosynthesis and C-translocation. Synchrony between tree growth and fungal ascospore production suggests that resource allocation in the two partners is coordinated (Desprez-Loustau et al., 2010). Investigations on powdery mildew have concentrated on agriculturally-important plants, and tackled recognition, defense and haustoria formation rather than resource allocation (Eichmann and Huckelhoven, 2008; Pessina et al., 2014).

3. Central hypotheses and design of experiments

TrophinOak aims to analyze and determine the relationship between resource allocation and regulation of genome expression in oak microcuttings that display a typical rhythmic growth pattern while participating in seven types of trophic interaction (Fig. 1). The primary goal is not to tackle the mechanisms behind the establishment, development and functioning of each interaction, but to compare how trees at different stages in their rhythmic growth cope with distinct types of interacting organisms. We expect to find out how trees with determined growth balance their own development and their interactions with beneficial and detrimental above- and belowground partners, as the basis for understanding the performance of trees in their natural environment. In the following section we introduce the five central hypotheses of TrophinOak and the design of two experimental setups.

3.1. Hypotheses

Hypothesis 1. The rhythmic growth of oak results in reduced downward allocation of C and enhanced upwards nitrogen allocation during SF, with this pattern being reversed during RF.

Initial studies on rhythmic growth in oaks focused on the shoot, and only on the role of carbohydrate shifts (Alaoui-Sossé et al., 1994). Le Hir et al. (2005a) described a link between SF and variations in sucrose synthase expression that were related to changes in sucrose concentration in apical tissues (Le Hir et al., 2005b). Willaume and Pages (2011) ablated source leaves and cotyledons of seedlings of *Quercus pubescens* in order to relate downward export of soluble sugar during RF to fluctuations in root growth. In young apple tree microcuttings, the rhythmic growth of secondary roots alternates with the SF and corresponds to downward carbohydrate transfer (Costes et al., 2006). To our knowledge, shifts in upward N-allocation were never considered in relation to rhythmic growth, although such shifts have been considered theoretically (Costes et al., 2006).

Hypothesis 2. Trophic interactions are stimulated during growth flushes and concomitant increase of resource availability in the plant part they target.

For oaks, this hypothesis is supported by two findings: (i) Buscot and Herrmann (2004) reported that rhythmic growth influenced the dynamics and intensity of EM formationand (ii) powdery mildew is especially virulent during SF in oaks (Marcais and Desprez-Loustau, 2012). As detailed in Section 5, it was reinforced by our recent *Phytophthora* infection experiments on roots (Angay et al., 2014). In the case of herbivore interactions with *Melaleuca quinquenervia*, an invasive tree in Florida, it was reported that feeding took place exclusively on the seasonal flushes of developing foliage at branch apices (Pratt et al., 2005), a finding which also supports Hypothesis 2. In contrast, no similar studies exist neither for root herbivores nor for interactions with the fauna and bacteria of the oak rhizosphere. Hypothesis 2 will form the basis for assessing how sensitivity to multitrophic interactions is modulated by the rhythmic growth.

Hypothesis 3. During growth flushing, numerous genes with different functions (resource metabolism and transport, signaling, growth regulation, morphogenesis) are differentially expressed, and their respective regulation patterns in shoots during SF and in roots during RF present similarities.

More than 500 transcripts associated with energy, protein synthesis and cellular components for development and growth were found to be differentially expressed during dormancy breakout in buds of sessile oak trees (Ueno et al., 2013), but this work did not include roots. Zawaski and Busov (2014) investigated gene regulation during shoot and root growth in poplar, but poplar does not display a rhythmic growth, and the study only targeted functions related to sugar allocation and wood formation. To date, there has been no holistic study on transcriptome differences between SF and RF in relation to endogenous rhythmic growth.

Hypothesis 4. Trophic interactions modify plant gene expression both in interactor-specific ways and in more general patterns that are common to different interactors.

Gene expression specific to trophic interactions in trees is known to occur. For example, herbivory triggers expression of defense related genes (Porth et al., 2011), while *Phytophthora* escapes the recognition system in beech, leading to down-regulation of defense genes (Schlink, 2010). However, the priming effect that MHB exert on the expression of plant defense genes involved in the resistance of oak DF159 to powdery mildew argues for the existence of regulatory pathways common to different biotic interactions (Kurth et al., 2014). Schenk et al. (2012) postulated substantial overlap between pathways; this may reflect similarity of functions and indicate core mechanisms that trees use to cope with the challenges of various biotic interactions in a complex environment.

Hypothesis 5. Genes related to growth flushing which maintain their expression profiles during different trophic interactions are core genes involved in endogenic rhythmic growth.

The perennial life style requires growth cessation phases (Rohde et al., 2007), which are controlled by paradormancy (related to apical dominance), endodormancy (related to internal bud signals) or ecodormancy (related to environmental signals) (Horvath et al., 2003). Studies on spruce (Kayal et al., 2011) and on sessile and evergreen oaks (Ueno et al., 2013) considered genes commonly expressed under variable light and temperature conditions as the core genes controlling endodormancy. In line with these findings, Hypothesis 5 postulates that genes commonly expressed during growth flushes in plants engaged in different trophic interactions and that impact on plant development and resources probably have roles in core mechanisms governing endogenous rhythmic growth.

3.2. Experimental designs

To test the five central hypotheses of TrophinOak, we designed two sets of experiments (Fig. 3). A first experiment was designed to analyze C- and N-allocation and gene regulation in roots and shoots during rhythmic growth, and it therefore includes the four characteristic developmental stages A to D. This design aims to

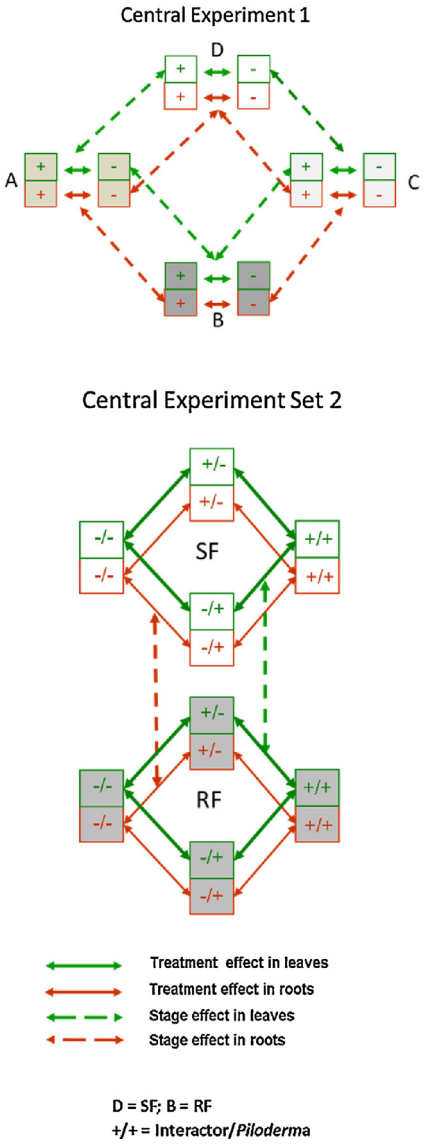


Fig. 3. Design of two series of experiments performed in TrophinOak to analyze variations in C and N resource allocation and in genome regulation in the shoot (green) and root (red) parts of oak microcuttings displaying endogenous rhythmic growth. In *"Experiment 1"* a treatment with the ectomycorrhizal fungus *Piloderma croceum* (+) is tested against a control (−) at all four developmental stages A to D of one rhythmic growth cycle (see explanation in the text and in Fig. 2). In *"Experiment set 2"*, *Piloderma* treatment (−/; +/) is combined in a factorial design with inoculation of one of six different biotrophic partners (/−; /+) during shoot flushing (SF) stage D (white squares) and root flushing (RF) stage B (grey squares). In both experiments the treatment effects are represented as continuous and the growth stage effects as dotted lines. The six partners are *Lymantria dispar*, *Microsphaera alphitoides*, *Pratylenchus penetrans*, *Phytophthora quercina*, *Streptomyces* sp. AcH 505, *Protaphorura armata*.

assess Hypotheses 1 and 3. Since EM influence the C and N acquisition, treatments with *P. croceum* were also applied to tackle Hypothesis 5.

A second set of experiments was designed to determine the impact of rhythmic growth on interactions with the six selected interactors (Fig. 3). For each interactor, the experiment compares, in a full factorial design, plants at RF (stage B) or SF (stage D), which are either inoculated with the EM fungus *P. croceum* or non-mycorrhizal. This setup enables Hypotheses 2 and 4 to be assessed, and the joint inoculations with one interactor and EM test Hypothesis 5.

4. The TrophinOak joint experimental platform (JEP)

Achieving and analyzing sets of multitrophic interactions with oak microcuttings in a microcosm system poses challenges in: (i) establishing different types of interspecific interactions under well-defined and constant experimental conditions; (ii) avoiding drastic consequences such as complete defoliation by herbivory or plant death by parasitism in a miniaturized system; (iii) characterizing below- and aboveground C and N allocation; (iv) performing RNA sequencing from small amounts of plant tissues and carrying transcriptomic analyses without a reference transcript library. The following section details the challenges and the proposed solutions.

4.1. Microcosms in which to synthesize biotrophic associations

The challenge is to optimize a unique type of microcosm to synthesize different types of biotic interactions on single oak microcuttings obtained by micropropagation and rooting (Fig. 4). The agar Petri-dish microcosm of Herrmann et al. (1998) is convenient for associations with EM fungi, MHB, *Phytophthora* and with interactors on leaves, but not for nematodes and collembolans that require a soil-like environment including microorganisms, in order to establish an interaction resembling that under natural conditions. In the TrophinOak Petri-dish microcosms we therefore replace agar with γ-sterilized soil, and a soil microorganism suspension (see Appendix S1 in the Supporting information). The time of inoculation and the procedures used for the six interactors are also specified in Appendix S1. Although all experiments start with plants at stage B, the replicates do not grow synchronously after acclimatization due to the heterogeneous abscission of leaves that had previously developed *in vitro*. After several weeks the biological replicates are distributed across the four stages A, B, C and D, making it possible to harvest at a single time point (eight weeks) plants in all phases of the rhythmic growth.

4.2. Stable isotope labeling procedures and equipment

State-of-the-art allocation studies use stable isotope labelling as a non-invasive tool (Epron et al., 2012), avoiding reductionist experimentation by this more holistic approach (Hood-Nowotny and Knols, 2007). Labeling of photoassimilates with ^{13}C is often performed by injecting pure $^{13}CO_2$ into a simple chamber volume, *e.g.* a plastic bag enclosing a branch or a whole plant (Frost and Hunter, 2008). The downside of this approach is that CO_2 concentration within the chamber is not controlled in parallel to unavoidable changes of other environmental influences, such as increases in RH or air temperature (T_{air}) that can induce stress in the labeled plants. As these confounding effects are likely to bias C allocation and, in particular, the highly responsive transcriptome, we rejected this approach. Hence, the challenge was to label the photoassimilates with ^{13}C without affecting the environmental conditions. In addition, experiments required simultaneous labeling of replicates. To meet these challenges a ventilated Plexiglas chamber able to accommodate up to 160 microcuttings and to maintain 23 °C inside temperature during day and night was specially developed (Fig. 5). The photoassimilates are labeled with ^{13}C at a CO_2 concentration of 400 ± 2 μL/L (mean ± SD) during the 16 h day before harvesting (see Appendix S2 in the Supporting information). ^{15}N labelling is performed three days prior to harvest by means of irrigation with double-labelled ammonium-nitrate (98% ^{15}N; 0.1 mg/plant).

4.3. Transcriptomic optimization and analyses

Large scale analyses of gene expression by next generation RNA-Seq is receiving growing attention in plant research (Schenk et al., 2012). Optimization of the RNA extraction step is a challenge in the

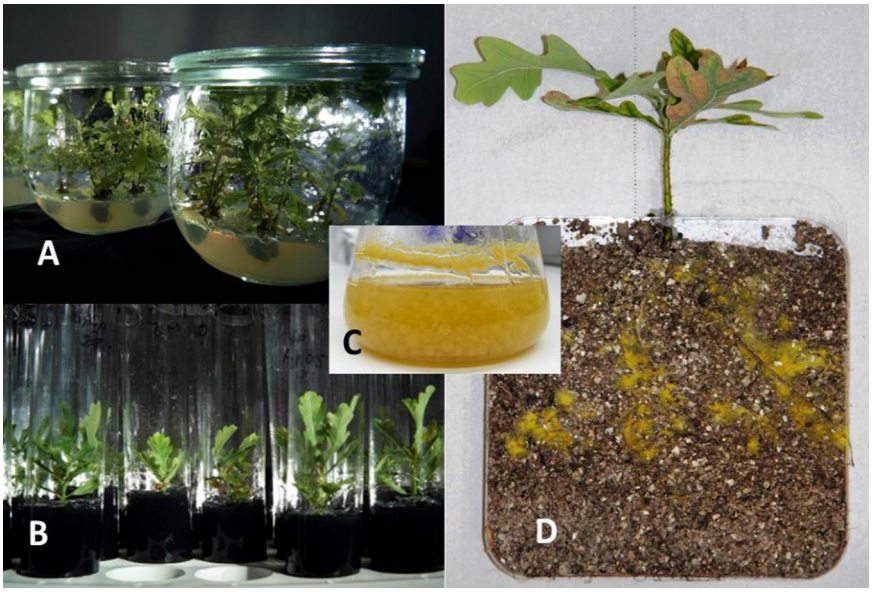


Fig. 4. Main steps in preparing microcosms to synthesize biotic interactions with microcuttings of the oak clone DF159 (*Quercus robur*). (A) Micropropagation of the oak clone; (B) Rooting; (C) Precultivation of the biotic partners, here the ectomycorrhizal fungal partner *Piloderma croceum*; (D) Association synthesis in microcosms made of square Petri Dishes with sterilized soil, here ectomycorrhiza formation on roots by *P. croceum* (see Appendix 1 of Supporting information).

case of woody plants, since the material often contains low quantities of RNA and large amounts of impurities that hamper cDNA synthesis and amplification (Le Provost et al., 2007). The optimization workflow in TrophinOak consisted in firstly selecting an RNA extraction kit, and secondly running pilot studies on a standard tissue samples to test the influence of varying the RNA extraction temperature or quantity of RNA used on the success of a RNA-seq run (see Appendix S3 in Supporting information).

Oaks take an increasingly prominent place in research on tree genomics, and efforts to fully sequence the genomes of trees in the *Fagaceae*, including *Q. robur* are in progress (Petit et al., 2013). However, since no suitable genome sequence is yet available, TrophinOak was faced with the major challenge of generating a reference library, in order to map the differentially regulated genes revealed by RNA Seq. To embrace the whole complex ensemble of interactors and oak development, the TrophinOak consortium merged sixteen 454 libraries from all types of interaction on DF159 with eight Illumina libraries from leaves, roots and EM with *P. croceum*, and constructed the specific "OakContigDF159.1" reference library, which comprises 65,712 contigs with a mean length of 1003 bp. (Tarkka et al., 2013). With this as the basis, a work flow starting with RNA extraction, followed by RNA-Seq and bioinformatics analysis can be initiated. The most important bioinformatics steps consist in checking the quality of the libraries, removing cDNAs from the interactors, normalizing the depth of the sequencing between the treatments, grouping the transcript according to function, measuring the level and significance of gene expression, and running control qRT-PCR for a number of contigs (see Appendix S3 in the Supporting information).

Fig. 5. Plexiglas chamber used to label the oak microcosms with ^{13}C (see Appendix 2 of Supporting information).

5. First results

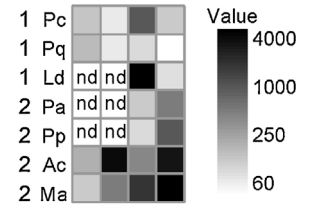
The following section gives a proof of concept of TrophinOak platform to test the above detailed hypotheses. Further, it presents a quantitative synthesis of the gene expression profiles obtained in single interactions with the seven biological partners.

5.1. Proof of concept of the TrophinOak design and hypotheses

In accordance with TrophinOak Hypotheses 1 and 2, three works verify the existence of resource shifts in relation to the endogenous rhythmic growth and of their stimulating impact on biotic interactions. Angay et al. (2014) showed that RF is related to an increase concentration of nonstructural and soluble carbohydrates in the plant root, increasing virulence of the root pathogen *P. quercina*. Enhanced carbohydrate concentrations in plants inoculated with *P. croceum* further triggered pathogen virulence. Similarly, an enhanced parasitic infection of roots by the nematode *P. penetrans* was displayed during bud swelling stage B that corresponds to maximal root elongation rate and C allocation to roots (Caravaca et al., 2015). In the same line, infection by the leaf fungal parasite *M. alphitoides* was higher during shoot flushing and corresponding enhanced upward C allocation (Mailander, 2014).

Addressing Hypothesis 1, the *first experiment* of TrophinOak considered all four developmental stages of the rhythmic growth pattern in parallel to an inoculation treatment with the EM fungus

A



B

Interacting organism	Root or leaf interactor	Type of interaction	ROOTS						LEAVES					
			Number of DEC in RF	Share of all contigs (%)	Up/down-regulated (%)	Number of DEC in SF	Share of all contigs (%)	Up/down-regulated (%)	Number of DEC in RF	Share of all contigs (%)	Up/down-regulated (%)	Number of DEC in SF	Share of all contigs (%)	Up/down-regulated (%)
Pc	Ro	B	124	0.2	54	69	0.1	48	886	1.3	43	121	0.2	27
Pq	Ro	D	150	0.2	51	67	0.1	52	844	1.3	88	44	0.1	66
Ld	Le	D	nd	nd	nd	nd	nd	nd	4447	6.8	76	80	0.1	88
Pa	Ro	B	nd	nd	nd	nd	nd	nd	120	0.2	54	467	0.7	90
Pp	Ro	D	nd	nd	nd	nd	nd	nd	91	0.1	51	895	1.4	32
Ac	Ro	B	182	0.3	62	3490	5.0	50	337	0.5	67	3043	4.6	50
Ma	Le	D	112	0.2	53	458	0.7	44	1626	2.5	72	4182	6.4	67

Fig. 6. Gene expression patterns in leaves and roots of pedunculate oak (*Quercus robur*) during rhythmic shoot and root growth flushes and for seven biotic interactions. (A) Schematic representation of the numbers of differentially expressed contigs (DEC). From left to right, the four columns show the numbers of DEC in pairwise comparisons of lateral roots (Ro) and leaves (Le) under root flush (RF) and shoot flush (SF) between the control treatment without inoculation and in inoculated treatments with *Piloderma croceum* (Pc), *Phytophthora quercina* (Pq), *Lymantria dispar* (Lp), *Protaphorura armata* (Pa), *Pratylenchus penetrans* (Pp), *Streptomyces* sp. AcH 505 (Ac) and *Microsphaera alphitoides* (Ma). Color intensity indicates the numbers of DEC in the treatments. Significant down- and up-regulation was determined by edgeR with a threshold Benjamini–Hochberg adjusted *p*-value 0.01. Root DEC data of Ld, Pa and Pp is not available (nd, no data). (B) Tabular representation of DEC. The letter B indicates beneficial and D detrimental interaction.

P. croceum. As detailed above, allocation of ^{13}C and ^{15}N to shoots and roots were assessed by pulse labelling (Herrmann et al., 2015). Belowground C allocation was increased during stages A and B, whereas allocation to leaves was favored during stages C and D. For N, the pattern was delayed as allocation to shoots was increased only during stage D of SF compared to RF. This experiment also tackled mechanisms ruling the endogenous rhythmic growth pattern. Inoculation with *P. croceum* markedly enhanced the observed C and to a less extent N allocation patterns of the plants. Noteworthy, these effects of *P. croceum* inoculation on C and N allocation had no impact on the rhythmic growth and its period, suggesting that this trait is not driven by resources availability as suggested in several former reports (Le Hir et al., 2005a,b; Costes et al., 2006). Parallel to the allocation studies we analyzed Differentially Expressed Contigs (DEC) in leaves and roots during the transitions between the four growth stages (Herrmann et al., 2015). We detected rhythmic growth and organ specific patterns that exhibited pools of common DEC as postulated in Hypothesis 3. According to Hypothesis 5 we analyzed these pools of DECs in common between growth stage, organ and inoculation treatment with *P. croceum* to detect putative key genes regulating the endogenous rhythm. Confirming the endogenous character of the rhythmic growth pattern we found a number of genes known in relation to circadian clock events (Herrmann et al., 2015).

Transcriptome analyses in *one experiment of the second set* further confirmed Hypothesis 3, as during interactions with the MHB *Streptomyces* strain AcH 505, hundreds of oak DEC encompassing a wide range of metabolic, development, recognition and defense functions were found with distinct patterns according to the considered plant part and rhythmic growth stage (Kurth et al., 2015). These patterns were highly modified in a development stage and plant part depending manner when *P. croceum* was co-inoculated (Kurth et al., 2015). Similar specific co-inoculation effects were detected in pre-experiments with simple or double inoculation with powdery mildew and the mycorrhization helper bacterium (Kurth et al., 2014).

5.2. TrophinOak in action

In the frame of the *second experimental set up*, we started to compare oak gene expression responses in leaves and roots during SF and RF for the seven interactions of TrophinOak by measuring the number DEC compared to non-inoculated control plants. First results gathered for partners, already showed that the number of DEC varies highly in a specific manner for each partner according to the rhythmic growth stage and the organ (Fig. 6). This fully confirms the overarching hypothesis of TrophinOak that rhythmic growth and trophic interactions interfere. The preliminary results summarized in Fig. 6 suggest that there are two general patterns in the number of DEC. Pattern 1 corresponds to increased levels of DEC upon RF as found for *P. croceum, P. quercina* and *L. dispar*, while in pattern 2 the number of DEC is higher during SF than during RF as observed for *P. penetrans*, AcH 505 and *M. alphitoides*. This suggests that according to the interaction partner, the plant regulatory adjustment is higher in both organ types either during RF or during SF. Noteworthy, as both root and leaf interactors can elicit either pattern 1 or pattern 2, the maximum regulatory adjustment is not necessarily in the organ targeted by the interactor. Ongoing detailed studies of the gene ontology will tackle the functions behind these DEC and help to deeper assess Hypotheses 3–5.

6. Conclusions

Like all plants, trees do not grow alone; they interact with diverse above- and belowground organisms (De Deyn and Van der Putten, 2005), which affects their long term performance in their natural habitats (Hersh et al., 2012). When focusing on resource-driven trophic interactions, it is particularly tempting, rather than using the continuously-growing model tree poplar (Tuskan et al., 2006), to investigate tree systems that follow endogenous rhythmic patterns of growth with well-defined growth phases characterized by shifts in resource allocation (Willaume and Pages, 2011). Even though it implies not to have available extensive genomic resources (Tuskan et al., 2006), the choice of non-model organisms such as oaks is supported by their complex anchorage in ecosystems, which requires a broad repertoire of functions and may at the end even improve annotation of genes without known functions in model species (Tagu et al., 2014). Our first results using the TrophinOak platform exemplify the feasibility of this approach and its potential to reveal new aspects.

TrophinOak integrates resource allocation and transcriptome studies to identify both specific and also central mechanisms

underlying tree development in a multi-interaction environment. For this combined approach, models based on microcuttings offer advantages. They are controlled "mini-ecosystems" with a reduced number of modules, which means that they react rapidly in terms of shifts in resource allocation and genome regulation. They are clonal, which increases the likelihood of more uniform responses to treatments and facilitates gene expression analyses. This strategy, which requires the development of procedures, equipment or contig libraries, as in TrophinOak, is justified for trees such as pedunculate oaks that have a broad geographic distribution not only across Europe. Integrating the insights gained by studying the interplay between plant growth and multitrophic interactions, at the levels of element allocation and gene expression, in trees with and trees without rhythmic growth, should lead to progress, which will expand our understanding of tree ecology.

7. Perspectives

The experiments presented here do not take into account the mechanisms behind the establishment and functioning of each of the interaction studied. However, our experimental platform based on a rhythmically growing plant can also be used for mechanistic studies on the pathogens and herbivores that have important impacts on forestry and agriculture, including those that were selected in TrophinOak. Similarly, the platform allows us to vary abiotic parameters (*e.g.*, temperature, photoperiod) in order to study their influences on episodic tree growth and trophic interactions. The combined inoculation of an EM fungus and one additional organism is a first step towards multitrophic interaction studies. One long term aim of TrophinOak is, by comparing and combining very different functional groups of interactors, to identify common key mechanisms by which oak trees modulate their development according to the multitude of interactors with which they are confronted, and to produce sets of biomarkers of these general processes at the morphological, physiological and gene expression levels. These indicators will be used to monitor individuals of the DF159 clone released as PhytOmeters at sites with different climates, soil types, land use intensities or management regimes. Finally, combining analyses carried out in microcosms and field experiments opens us the possibility of predicting how long-lived organisms like trees adapt to global change, and producing evidence-based recommendations for forest management strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ppees.2016.02.003.

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