The research outline should comprise approximately five pages in total (including references).

Intro:

The current state of research should first be briefly described and supported by approximately five relevant publications from the research area (one page max).

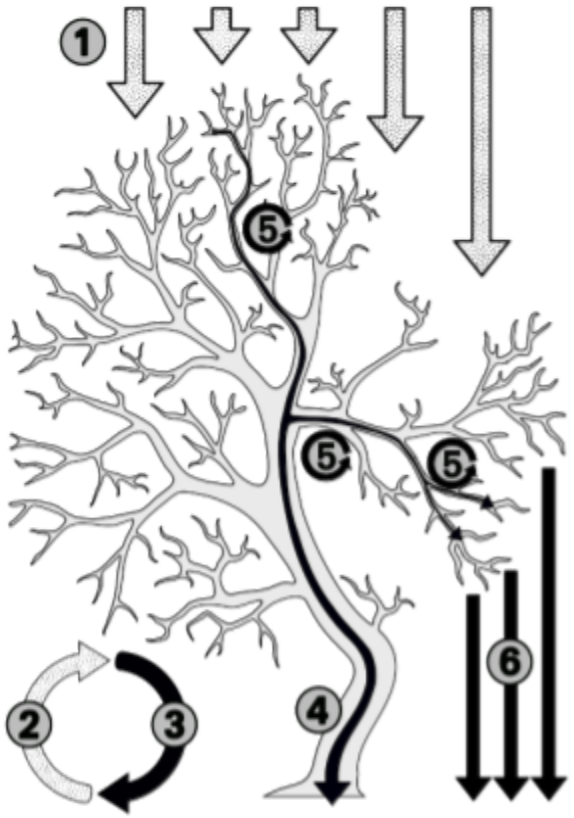
Plants face difficult times. With climate change, the historical rhythms and intensities of many types of plant stressors have been upturned, creating unpredictable new disturbance regimes, and many sources of plant stress are predicted to increase further[1]. Plant genomes contain many answers to these challenges, especially to environmental challenges which have acted as long-term selective pressures on plants[2]. However, plant genomes alone may not contain sufficient existing genetic variation or plasticity to ensure the success of ecologically or socially important plants in the face of decreased climatic stability and large-scale habitat changes[3]. Some have proposed that the plant microbiome, with its collective abundance of microbial genetic resources, may represent an important and adaptive addition to the host plant genome. Together, it is proposed, the microbiome and the plant sum to make up the plant "hologenome", which is more extensive and more dynamic than the plant genome alone[4,5]. The correctness and utility of the hologenome model of the microbiome is actively debated[6–9], but evidence has mounted rapidly that microbiomes in general are important determinants in plant distributions and thus ecosystem stability[10–14]. Microbiomes may become a point of leverage in the near future, wherein humans could increase resilience in plants of interest to environmental and biotic stressors by experimental manipulations of plant-associated microbial communities[15–17]. However, microbiome engineering has yet to achieve widespread success as an agricultural method, most probably due to an underestimation of the ecological complexity that maintains microbiomes[18]. Detailed studies of the dynamics of microbial communities and life-cycle studies of individual microbial species are more necessary now than ever to move microbiome research forward. Here we propose to examine the relative importance of stemflow to intra-host movement of microbes and spatial patterns of functional and taxonomic microbiome traits, in deciduous trees a forested riparian zone in the city of Leipzig, Germany, in central Europe. We hypothesize that stemflow may be an important vector of establishing the plant functional microbiome every season.

The functional microbiome

Many microbes appear to have overlapping or redundant ecosystem functions, probably due lateral gene transfer and hyper-diversity of evolutionary lineages[5]. Thus, while catalogues of associated fungi and bacteria are of great biological interest, they are made much more relevant to questions of microbiome engineering and ecological process models if they are accompanied by concurrent surveys of the functional microbiome. Here we propose to sample the functional microbiome through the use of marker gene surveys of two broad families of enzymes with great importance to secondary metabolism.

Hydrologic highways

General patterns of flux by microbes in the canopy were well-predicted by initial work of Carroll et al.[19,20], and these predictions have been generally born out, (reviewed by Levia and Germer[21] and Ponette-González et al.[22]), including in bacterial systems[23] and small arthropods[24]. Additionally, fungi once known only as highly-adapted aquatic fungi are repeatedly observed as a prevalent and common subset of canopy fungi with spores highly adapted to aquatic dispersal[25–27]. The predictions by Carroll and others can be generalized to other microbial life and a general picture of the fluxes of the forest tree microbiome emerges (fig. 1):



1. The surface of the forest, consisting of the exposed tops of individual trees and their boundary air layer receive microbial propagules from the atmosphere. Due to this and to environmental filtering, the community of upper canopies are usually observed as distinct from lower canopies.
2. Microbes from the forest floor sporulate and (re)populate canopy layers - especially lower canopy layers - with locally abundant microbes from soil and other substrates beneath.
3. Falling leaves and other litter act as vectors directly for microorganisms to other sites in the canopy and to the forest floor.
4. Stemflow enriches rainfall due to both initial washing of leaf surfaces and leaching from other biologically active sites, accumulating numerous microbes along the way.
5. Biologically active sites such as deep bark fissures, treeholes, rot pockets, epiphyte clusters, and lodged litter, all accept microbes and nutrients along the way, and may amplify and diversify microbial populations and nutrient-cycling.
6. Throughfall also enriches rainfall with microbial propagules washed from leaf surfaces and other biologically active sites.

With this schema, stems are therefore potentially fundamentally important "hydrologic highways"[22] for small organisms and nutrients via stemflow.

Questions, Hypothesis, and significance

The outline should focus on a clear description of the questions you intend to address in your research, their originality and significance for the advancement of the research field (approx. two pages).

Questions

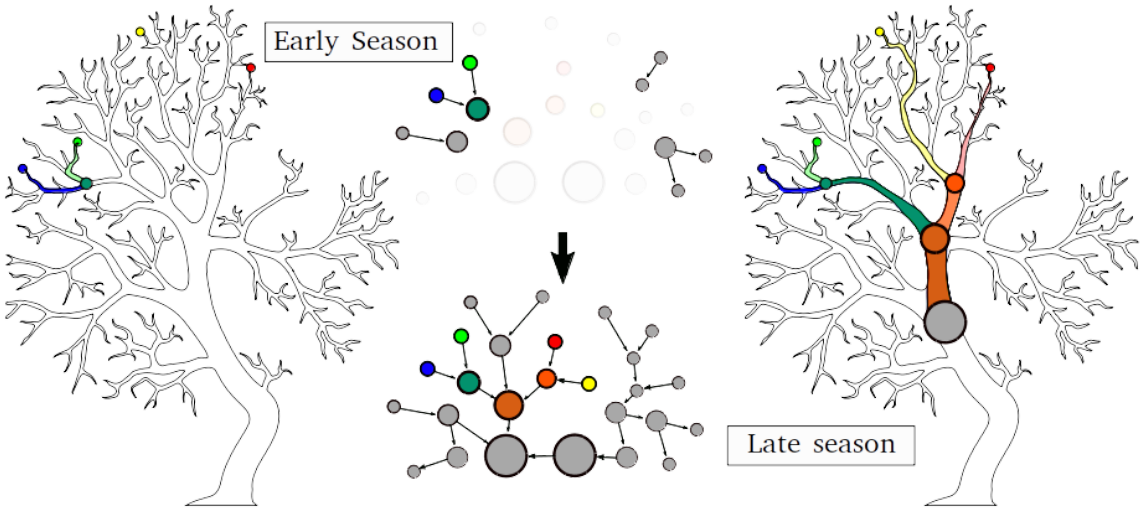
- The following questions motivate this project:
- How important is stemflow to the dispersal of canopy microbes and to tree microbiome assembly?
- Are patterns of establishment of taxonomic and functional microbiomes distinct?
- Does plant microbiome function respond to environmental challenges to the host?
- Do such changes benefit the plant host, representing an adaptive plant hologenome?

Hypotheses and predictions:

H1 - Canopy microbes are subject to dispersal limitation and seasonal variation.
H2 - Stemflow connects microbial communities of the canopy, especially the subset of microbes that are adapted to an aquatic life history phase.

P1 - Some significant portion of the microbial community in a tree canopy can be modeled as an acyclic, directed graph (figure 2). As the growing season progresses, this will be evidenced by the following:

1. Unidirectional increase in microbial diversity at sample sites along lines of stemflow
2. This increasing diversity will be due to shared taxa from less-diverse "upstream sites" but not necessarily other unlinked sites.
3. Individual infections in leaves by rare or unique taxa will show spatial signatures ("streaks") that parallel stemflow paths.



P2 - Leaves of axenic seedlings placed in the path of stemflow will acquire a significant subset of their new microbiome from microbes present in water samples of stemflow, microbes that will not be observed in leaves of adjacent seedlings not subject to stemflow.

H3 - Microbial genes are subject to fewer dispersal constraints than are microbial taxa, due to redundancy in ecological function among microbes.

P3 - Presence of microbial genes of potential importance to plant hosts uncovered in PKS/NRPS metabolome surveys will follow similar trends as taxonomic microbiomes during the early phase of community assembly, but will uncouple in later phases. This will be evidenced in the late season by the consistent presence of shared microbial secondary metabolic pathways even among leaves with taxonomically distinct microbial communities.

H4 - Functional microbiomes of plants are dynamic and adaptive, supplementing functions of the host plant metabolism, changing in response to biotic and abiotic stressors.

P4 - More exposed regions of the upper canopy leaf will host microbiomes with increased genetic pathways for pigmentation, e. g. anthraquinones[28,29] and non-pathogenic melanins[30–33], in comparison to less-exposed regions of the canopy. Similarly, regions of trees showing increased herbivory and pathogen loads will show increased capacity for production of anti-herbivory alkaloids and antifungal compounds.

Methods

Furthermore, the academic methods to be used to achieve these goals should be clearly described and referenced, if appropriate (approx. two pages).

Summary:

Proposed timeline is two years. In summer of the first year, surveys of both the fungal microbiome and the microbial (fungal + bacterial) secondary metabolome will be conducted multiple times throughout the growing season. Timing and location of surveys will be coordinated with planned 16S metabarcoding and stemflow surveys that are to be undertaken by other members of the Leipzig canopy research group. In the second year, both surveys will be repeated, accompanied by placement and monitoring of axenic seedlings to experimentally demonstrate transmission of microbes.

Field Site: Leipzig Canopy Crane Facility

The proposed field site is located in the Leipzig floodplain mixed hardwood forest, located near the city of Leipzig in Germany. Climatic conditions are characterized by warm, wet summers and an annual mean temperature of 8.4 °C with an annual precipitation of 516 mm[34]. A rail-based crane facility (Leipzig Canopy Crane facility, LCC) exists for the investigation of forest tree canopies was established in this floodplain forest in 2001. Combined with a gondala system, the LCC facility allows access to almost to every possible position within the canopy of every tree, in

about 800 tree individuals in up to 33 m height, covering a total area of 1.65 ha. The LCC has a substantial and important history of microbial canopy ecology, including important surveys of fungal patterns in the canopy[35,36], and recent, ongoing sampling of prokaryote sampling in the canopy[34]. This proposed project is intended to update fungal sampling at LCC and complement ongoing prokaryotic research.

DNA extraction and Fungal taxonomic surveys

Epiphytic and endophytic microbial communities will be sampled separately. To sample epiphytes of leaves will be submerged in 20 ml of 0.1% tween / sterile water mixture, and vortex-ed for 5 minutes. This wash fluid will be centrifuged, and the pellet frozen for later DNA extraction. Leaf endophytes sampling will follow Thomas et al.[37], using a bleach-based surface sterilization method, followed by tissue homogenization and DNA extraction. Bulk DNA will be purified from host plant tissue and epiphyte wash-pellets using column kits, and selectively PCR-amplified with ITS region 1 primers, in addition to PKS and NRPS primers (see functional microbiome surveys). Amplicon libraries will be prepped according to Illumina recommendations[38] and will be sequenced using a Illumina MiSeq platform sequencer, with a MiSeq V3 kit (2 x 300 bp).

Functional microbiome surveys

Functional microbiomes will be sampled using two metabarcode surveys of type I polyketide synthases (PKS) and nonribosomal peptide synthases (NRPS). These data will paint a general picture of some broad classes of secondary metabolites that tree microbiomes are predicted to produce differentially in response to environmental challenges. These enzymes encode backbone molecules for two of the broadest families of microbial secondary metabolites (polyketides and nonribosomal peptides)[39,40] and exist in diverse homologs that can be used to infer medium-to-fine grain portrait of the secondary metabolome of the plant microbiome. Primer pairs for this survey will be based on two studies conducted by Charlop-Powers et al.[41,42] with degenerate primers that target the ketosynthase (KS) region of type I polyketide synthases (PKS) and adenylation (AD) domains of nonribosomal peptide synthases (NRPS). Limited, initial exploration of these primers *in-silico* of KS-targeting primer sites has shown that they are present in both fungal and bacterial PKSes (see jupyter notebook), and AD primers are expected to perform as well in fungal NRPS searches. In soil, these primers tended to produce amplicon sizes of 350-500 bp, suitable for paired-end MiSeq platform sequencing. These primers on public databases show very few matches to plant sequences, with the vast majority of observations of NRPS and type I PKSes in public databases originating from microbial sequences (see jupyter notebook). However, the possibility exists that plants are greatly under-sampled for type I PKS gene clusters[43]. Since our hypothesis focuses on the complementary role of the microbiome, we will attempt to enhance primers with plant-blocking oligonucleotides[44]. The sampling depth necessary to capture the full diversity of secondary metabolites is unknown, but estimated numbers of unique PKS and NRPS types in soil samples in both studies by Charlop-Powers et al. range from 2000 to less-than-10,000 enzyme “OTUs”. As both enzyme families (PKS and NRPS) are thought to occur only rarely in higher plants, and

as plant-blocking nucleotides will be sought for primers, it should be possible to sample endophytic PKS and NRPS gene clusters as well as epiphytic. Endophytic and epiphytic microbes will therefore be sampled separately as with the fungal taxonomic surveys above.

We will emphasize the detection of enzymes in pathways of microbial secondary metabolites that could be "useful" to plant hosts in response to stressors of herbivory, pathogen load, and exposure (drought stress and solar UV radiation inundation). Our survey will broadly target all microbial gene clusters secondary metabolites with polyketide or non-ribosomal peptide backbones (and hybrids), which will likely uncover a vast array of pathways, of which only a subset will be applicable to our questions. As such, we will first examine results for:

- Ergot alkaloids (a terpene but with NRPS-catalyzed pathway steps)[45–47] and other alkaloids in response to high herbivory loads,
- Fungal anti-mycotic compounds such as echinocandins (NRPS pathway)[40,48], polyene anti-mycotic including Nystatin and amphotericin (PKS)[49–51], and strobilurins (PKS)[52,53] in response to high pathogen pressure,
- Pigments including anthraquinones[28,29] and melanins[30–33] (PKS pathways) that may potentially act as antioxidants and UV-protectants.

Secondary metabolites often have ambiguous ecological roles in plant interactions, such as when fungal melanins act as virulence factors in plant disease[54], but could also aid their hosts in reducing oxidative or UV-induced damage. Additionally, some metabolites may have differential effects on hosts depending on their position within the host (epiphytic/endophytic). Direct benefits to host can be difficult to ascertain, and candidate secondary metabolites detected by these surveys will need further investigation in future studies to confirm their possible benefit to plant host.

Estimation of plant stressors

We will emphasize the detection of enzymes in pathways of microbial secondary metabolites that could be "useful" to plant hosts in response to stressors of herbivory, pathogen load, and exposure (drought stress and solar UV radiation inundation). Plant pathogen loads and damage from herbivory will be phenotyped non-invasively from photos[55] of leaves from the region of each sampling of the tree. Exposure levels will be estimated from light levels and humidity as gauged by in-place sensors.

Axenic seedlings

In the second year, we propose to augment leaf microbiome surveys with use of the axenic seedlings[56,57], to experimentally establish transmission of stemflow-vectored microbes to new leaves. Seedlings will be germinated from sterilized seedlings of *Quercus robur,* and grown in controlled conditions with filtered air, using growth chambers, generally following Christianson et al.[56], with modifications for growth requirements of *Q. robur,*. A second seedling species (*Tilia*

cordata) will be used for comparison of host-species effects. Seedlings will be positioned adjacent to a subset of stemflow sample sites, with tubing to siphon a portion of stemflow onto seedlings, simulating leaves in a pendant (downstream) branch position. At each site, three seedlings will be positioned - two seedlings of the host tree species, one with contact to stemflow and one protected from stemflow. Additionally, at each sample site, one seedling of *T. cordata* will be placed and subjected to stemflow for comparison of host effects on recruitment of stemflow microbes. Seedlings will be placed several weeks following budbreak, to allow establishment of rich microbiomes in nearby host leaves, and if possible placement will be timed to coincide with significant stemflow-inducing storm events. They will be sampled as soon as possible after the first significant stemflow event to which they are exposed. All seedlings will be placed with watering trays that will be refilled weekly or as necessary to maintain constant soil moisture. Methods for sampling of seedling leaf microbiomes are as per host tree above, with two sample events: at the beginning and end of their stay in the canopy.

Stemflow sampling scheme and timing

Two individuals of three tree species will be sampled (*Quercus robur, Tilia cordata* and *Fraxinus excelsior*), and each will be equipped with triplicate stemflow samplers at three different height levels (top, mid, bottom position). Sampling of fungal community and functional microbiome will occur 4 times per growing season. Separate sample indices will be given to epiphytic and endophytic microbial communities.

Sampling summary:
3 trees species
× 2 individual trees per species
× 3 heights per tree
× 3 triplicate stemflow samplers per height
× 2 compartments (epiphytic/endophytic) per tissue sampled at each stemflow sampler
× 4 times per season
× 2 seasons

Each leaf sampling event will be coordinated with solute sampling and 16S metabarcode surveys of stemflow that are planned by the Leipzig canopy research team for 2021 and 2022. Micro-climatic sensors are already in place at these trees, with data for up to five previous years. Data will be shared collaboratively, but 16S and stemflow solute surveys have separate funding arrangements, and are not included as part of this application.

Bioinformatic and Statistical Analyses

Metabarcoding results will be subject to standard bioinformatic pipelines to reduce sequencing artifacts and group highly similar reads[58]. Following this, amplicon reads will be evaluated and grouped using closest-known biosynthetic gene clusters using the software package eSNaPD[41,59] and public databases. The resulting gene-cluster-by-sample-site matrix will be examined standard numerical ecology-based methods for modeling large matrices as

dependent objects of environmental data: “regression” of metabarcoding results against environmental data, biotic stressors such as pathogen and herbivory scores, and spatial patterns using redundancy analyses, including MEM and variation-partitioning methods)[60–63]. Read libraries will also be mined for specific biosynthetic pathways of interest (alkaloids, antibiotics, pigments, antioxidants, etc), or unknown gene clusters that may appear be acting to control disease, to the level of specificity allowed by the homology of the polyketide and peptide backbones. and these will be treated as point patterns[64], along with the results of any targeted PCR tests that may also be performed. Point patterns of secondary products of interest will be analyzed for co-occurrence with environmental challenges, herbivory, pathogen loads, and proximity and size of conspecifics and cluster size of nearest conspecifics.

Budget

DNA will be purified both from host leaves (for endophytes) and from leaf epiphytes from 6 trees, 4 times per year, from 3 heights, with three replicates at each height (see methods). This amounts to 432 DNA extractions per year, not including seedlings. This DNA will be amplified with 3 different primer sets, equaling 1296 successful PCR reactions per year.

Illumina sequencing costs:

A standard V3 kit MiSeq run will yield ~ 23×10^6 sequences of 300 bp length. As regions amplified by ITS, KS, and AD primers will often exceed 300 bp, alignment of paired-end sequences will be necessary to span regions of interest. With the goal of an average sample depth of 20,000 reads (= 40,000 reads, paired-end), we can include over five hundred samples per run. Thus, we can safely lump all multiplexed PCR product from each primer pair time series for a growing season into its own run: we would therefore have 3 Illumina MiSeq runs of ~432 samples each year (+36 samples from seedlings in second year). Each Illumina sequence event costs approximately €2000, so this amounts to approximately €6000 in sequence costs per year.

Amplicon library preparation

Multiplex primers

We will need enough unique illumina primers to multiplex 432 samples plus controls (24 forward × 20 reverse), in addition to KS/AD/ITS primers with illumina adapter regions: 24 forward primers + 20 reverse primer pairs = 44 oligo sequences (for 480 unique combinations). 44 oligo sequences x 30 bp (est) x $0.50 x BP at 100 nmole = $660 for multiplex primers. This should be a one-time expense for the project.

Misc. PCR consumables

Additional PCR reagents and gel electrophoresis consumables such as primers, plasticware and polymerase will be required, estimated at €3 per PCR reaction:

432 DNA purifications × 3 primer sets (ITS/KS/AD) × €3 = $3888

DNA purification

With an estimated 432 DNA purifications per year for two years. Where possible, high-throughput column kits will be applied, such as N × 96-sample centrifuge kits (see here). Estimated €1000 in column kits per year.

Canopy access

Leipzig Canopy Crane facility requests €150 per day of crane usage. Though some cost- and labor-sharing are possible with other ongoing projects in the canopy, we estimate 10 working days per growing season for collections, positioning of equipment, and seedling placement and maintenance: €1500 per year.

Seedlings

Seedlings will be grown for one species of host tree, *Q. robur.* For this tree, seedlings will be placed on both individuals at every stemflow station in every height zone, and each seedling microbiome will be sampled at the beginning and end of its time in the canopy. Costs of processing seedling samples are largely folded into the general laboratory and canopy access costs mentioned above. Seedlings will incur greenhouse (growth chamber) costs: €300, second year only.

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