Afforesting with microbes: disentangling the effects of soil biotic and abiotic characteristics on trees using soil inoculation

Georgopoulos, K.

Summary

Transforming previously unforested land into forest through afforestation sets in motion a series of changes in the soil environment. These changes affect not just the chemical and physical makeup of the soil, but also its living components. As soils shift, so too do the microbial communities around tree roots, which can influence tree health, interactions with herbivores above ground, and vital ecosystem processes like nutrient cycling and gas exchange. With afforestation now a central strategy in restoration work—and with growing use of methods like soil inoculation to restore degraded ecosystems—it's becoming increasingly important to understand how these belowground changes ripple through the system. However, much of the research to date has examined soil change as a whole, without separating the roles of living organisms and abiotic soil properties. This thesis addresses that gap by experimentally isolating biotic and abiotic soil components under controlled conditions, to better understand how each influences tree growth, microbial associations, and broader ecological outcomes.

In **Chapter 2,** I examine how soil abiotic and biotic characteristics of forests change along the course of an afforestation chronosequence. Specifically, I examine which abiotic characteristics, characteristic of each forest age, explain the community composition of certain fungal, soil animal and bacterial functional groups. We sampled soil and roots from three crop fields and 12 afforested stands (10, 15, 25 and 100+ years post afforestation) and recorded the canopy cover. The soils were brought back to the lab where their properties and nutrient content were analyzed and DNA extracted from the soil was sequenced to examine the soil community composition. As expected, afforestation altered soil conditions, with increased soil organic matter (SOM), reduced bulk density, and lower pH in older forests. Surprisingly, NO_3 concentrations were higher in mature forests than in croplands. Fungal communities shifted from pathogen- and saprotroph-dominated in croplands to ectomycorrhizal-dominated in older stands. Soil animal composition also changed, with earthworms increasing in older forests while nematodes and arthropods declined. Among bacteria, *Frankiales* increased in abundance with forest age, potentially explaining the unexpected rise in NO_3. Community composition was largely shaped by soil pH for fungi, SOM for soil animals, and a combination of canopy cover, root traits, and nutrient availability for bacteria. Contrary to expectations, fungal and soil animal richness declined in mature forests. Overall, the soil communities showed parallel

and gradual shifts in composition from cropland to reference forests instead of the anticipated slower trajectory of fungi and soil animals.

In **Chapter 3,** I investigate how biotic and abiotic characteristics of forest soil influence *A. glutinosa* performance and subsequent herbivory during early afforestation. Soils were collected from forests of different ages (10, 15, and 25 years old) that were planted in former agricultural land. Two experiments were conducted to assess the effects of the soil microbiome (live vs. sterilized soil, and inoculated vs. non-inoculated soil) and forest age on tree growth, root-associated microbial communities, and leaf herbivory by a caterpillar (*Mammestra brassicae*). Contrary to my expectation, trees in live soils did not perform better than in sterilized soils likely due to nutrient release and absence of microbial competition from the sterilization. Trees in 10-year-old soils had thinner stems when grown in sterilized soil than in live soil, whereas in older soils, trees had thicker stems and lower fine root percentages in sterilized conditions, suggesting an age-dependent response. Despite no significant differences in root microbial community composition across forest ages, *Streptomyces sp*. and *Rokubacteriales* were more abundant in 15-year-old soils and positively correlated with aboveground biomass. The herbivory assay with *Mamestra brassicae* larvae showed a positive correlation between leaf nitrogen and herbivory in the sterilized soil experiment. Trees in 10-year-old soils experienced the highest herbivore performance, suggesting greater susceptibility in early afforestation, but unlike what I expected this did not differ between live and sterilized treatments. Surprisingly, *F. alni* nodulation was reduced with increasing age.

In **Chapter 4**, I examine how different size fractions of soil biota from young and mature forests influence *A. glutinosa* performance, root-associated microbial communities, and GHG fluxes. A mesocosm experiment was conducted using soil fractions separated by wet sieving (250, 20, 11, and 3 μm) as inocula from the young and mature forests. The composition of root-associated communities was shaped by forest age but surprisingly not by the size fraction of the inoculum. Although I expected that a reduction in the diversity of soil biota groups to negatively impact tree performance, inoculation with the largest size fraction, that contained all groups of soil organisms from mature forests negatively affected tree growth instead, likely due to increased competition between plants and soil biota. To my surprise, GHG fluxes were not significantly influenced by either the forest stage or the soil biota fraction,

despite differences in microbial community composition. These findings suggest that *A. glutinosa* selectively assembles its root-associated microbiome regardless of the initial inoculum, but this selection is influenced by forest developmental stage. While these microbial interactions impact tree performance, they do not appear to drive changes in GHG fluxes, possibly due to functionally redundant microbes. Much like the previous chapter, *F. alni* nodulation was reduced in the mature forest treatments.

In **Chapter 5,** following the surprising results of **Chapters 3 and 4,** I investigate how microbial communities from the young and mature forests of **Chapter 4**, as well as N and P availability, influence *F. alni* nodulation and *A. glutinosa* performance. In a mesocosm experiment, seedlings were inoculated with bacteria, fungi, or both, cultured from the forest soils, in the presence or absence of *F. alni*. Additionally, controlled nutrient manipulations were used to assess the impact of N and P availability on nodulation. Fungal communities from mature forests suppressed the growth-promoting effects of *F. alni*, although nodule biomass itself remained unaffected. Further, specific bacteria and fungi contributing to this inhibitory effect were isolated and identified. Increased N availability significantly reduced nodule biomass, revealing a threshold beyond which reliance on symbiosis diminished. In contrast, P addition enhanced both nodulation and tree growth. These results suggest that soil microbial communities, particularly in mature forests, can influence the benefits conferred by *F. alni*, and that nutrient availability plays a critical role in shaping the symbiotic relationship.

In **Chapter 6** I explore the use of NIR spectroscopy to differentiate between symbiotically-fixed and soil-derived N in *A. glutinosa* using the N-fertilizer experimental setup of **Chapter 5**. Seedlings were grown under controlled conditions across a gradient of soil N fertilization, with and without *F. alni* inoculation. Plant performance was assessed through leaf chlorophyll content, biomass measurements, and spectral reflectance analysis. Principal Component Analysis (PCA) and Partial Least Squares (PLS) regression revealed distinct spectral differences between *F. alni*-inoculated and fertilized plants, particularly in the visible spectral region. Remarkably, while high soil N fertilization mimicked the growth effects of symbiotic N fixation, NIR spectroscopy identified lower reflectance in *F. alni* inoculated plants which was not shown in the SPAD chlorophyll measurements. These findings demonstrate the potential of NIR spectroscopy as a non-destructive tool for rapid assessment of N fixation in trees, offering a more precise alternative to traditional

leaf chlorophyll measurements.

In conclusion, this thesis demonstrates that both abiotic soil conditions and microbial communities play important roles in influencing tree growth, nitrogen-fixing symbioses, and ecosystem processes during afforestation. Rather than assuming that greater microbial diversity universally benefits restoration, the findings highlight the importance of tailoring soil inoculation strategies to specific contexts. Particularly for nitrogen-fixing species like *Alnus glutinosa*, the composition and origin of microbial communities can make the difference between enhanced growth and suppressed symbiosis. Although the effects on herbivory and greenhouse gas emissions were limited, subtle belowground interactions—especially those involving nitrogen fixation—may still exert meaningful influence aboveground. These insights reinforce the value of integrating microbial ecology into afforestation planning, not as a one-size-fits-all solution, but as a targeted tool for fostering resilient, multifunctional ecosystems. Looking ahead, long-term field trials, cross-site comparisons, and studies involving a broader range of tree species will be essential to translating these findings into effective, scalable restoration practices.