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

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CHAPTER 1

General Introduction

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In an age marked by pressing challenges such as climate change, biodiversity loss, land use change and environmental degradation, afforestation stands out as a vital strategy among the solutions at humanity's disposal. Simply defined as the establishment of forests in areas where none previously existed (FAO, 2018), afforestation offers the potential to sequester carbon, restore ecosystems, prevent biodiversity loss and improve the earth's overall soil and environmental health (Banning et al., 2008; Liu et al., 2018). The success of afforestation on former arable land is challenged by the legacy effects that arise from intensive arable farming such as reduced soil diversity, increases in soil borne enemies of crop plants as well as soil conditions (e.g. high pH and bulk density) that do not favor the establishment of trees (Gomes et al., 2025).

In an effort to recover from the legacies that the human footprint leaves on nature, considerable research focus and financial investment are being allocated and new techniques, like soil inoculation (Wubs et al., 2016; Han et al., 2022), are being utilized to restore degraded former agricultural ecosystems to a level that resembles their natural state (Palmer et al., 2016). After tree planting, both the physiochemical properties of the soil, as well as the communities that are present in the soil, undergo gradual changes (Fig 1.1) and interact with one another, progressively adapting and ultimately resembling the conditions found in natural forest ecosystems (Liu et al., 2020; De Marco et al., 2021). The loss or failure to restore certain soil biota groups during the process of afforestation can disrupt key ecosystem functions (Wagg et al., 2014), including plant growth and carbon sequestration, and can influence key characteristics of the developing ecosystem such as foliar herbivory and greenhouse gas exchange (Philippot et al., 2013; Bárcena et al., 2014; Kos et al., 2015). As afforestation starts seeing broader use, the effects of changing soil abiotic and biotic characteristics on trees and their root associated communities are gaining considerable attention in the field of restoration ecology (Preem et al., 2012; De Marco et al., 2021; Guo et al., 2024). Despite this, knowledge is still limited regarding the specific contributions of these characteristics to tree performance and ecosystem functions.

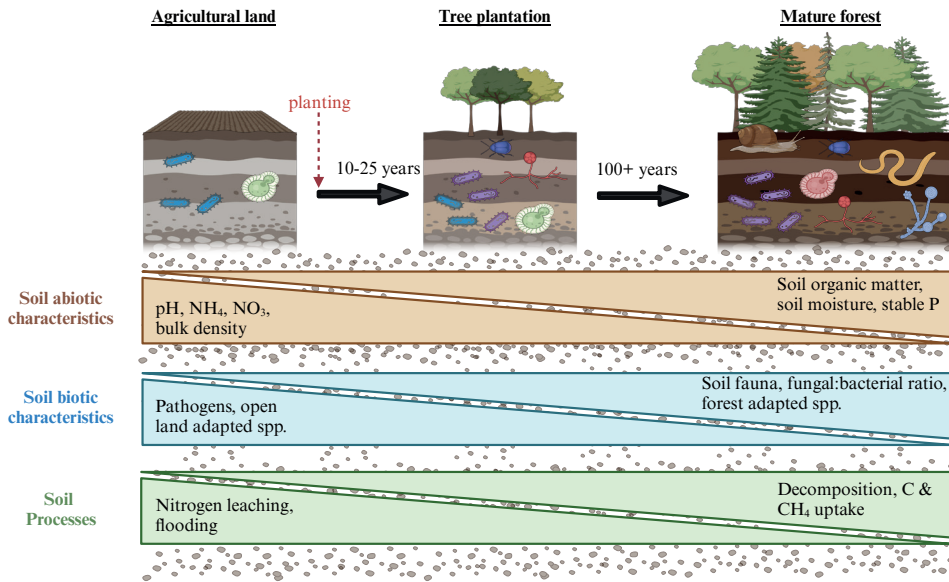


Figure 1.1 | Conceptual schematic of an afforestation chronosequence including temporal changes in soil abiotic and biotic characteristics as well as soil processes inspired by Gomes et al., 2025. Figure created using biorender (<https://www.biorender.com/>)

1.1 Succession and the use of chronosequences in studying ecological change

Succession is a central ecological process describing the temporal development of ecosystems following disturbance which caused the emergence of new substrates and soil legacies (primary succession) or part of the vegetation to be removed (secondary succession) (Cowles, 1899; Miles and Walton, 1993). Over the past century, multiple theories have emerged to explain the mechanisms and trajectories of successional change (Poorter et al., 2023). Early successional theory was largely shaped by the deterministic “relay floristics” model of Clements (1916), who viewed succession as an orderly, linear process leading to a predictable climax community. In contrast, Gleason (1926) proposed a more individualistic model, where species respond independently to environmental gradients and chance events such as dispersal and establishment, producing variable and often unpredictable successional pathways. These foundational perspectives continue to underpin modern discussions of community assembly and ecosystem development.

One powerful method to investigate succession, especially over long timescales that exceed human observation, is the chronosequence approach. Chronosequences use a “space-for-time” substitution, where sites of differing ages are assumed to represent

sequential stages of ecosystem development (Walker et al., 2010). This approach has been widely used to study both plant community succession and soil development over decadal to millennial timescales (Walker et al., 2010). Chronosequences are particularly useful in systems where direct long-term monitoring is challenging and can provide critical insights into general patterns of plant succession and soil biotic and abiotic characteristics over time (Fukami and Wardle, 2005). However, the chronosequence approach is not without limitations. A key assumption is that sites of different ages share similar initial conditions and follow a comparable successional trajectory. When this assumption is violated—such as in systems with high spatial heterogeneity, frequent disturbance, or strong stochastic influences—chronosequence interpretations may be misleading (Walker et al., 2010). Despite its constraints, the chronosequence method remains a valuable tool when applied judiciously and has seen widespread use in forestry to study changes in soil biotic and abiotic characteristics post afforestation (Bárcena et al., 2014; Qiu et al., 2015; Liu et al., 2018; Yang et al., 2023).

1.2 Changes in soil abiotic characteristics during afforestation

Naturally, as afforested croplands develop into mature forests, the abiotic characteristics of the soil slowly start to change and in time they start to resemble those of a forest ecosystem (Guo et al., 2016). Within the first 20 years after tree planting, the canopy closes, shaping a unique forest microclimate and as forest stands continue to mature, soil organic matter (SOM) and the carbon-to-nitrogen (C:N) ratio in the soil are expected to increase (Fig. 1; Knops & Tilman, 2000; Zeller et al., 2001; Wan et al., 2021). The increased input of resistant organic matter contributes to the accumulation of C both above ground and in the upper soil layers while the cessation of agricultural practices lead to higher C stabilization and retention as soil disturbance is minimized (Wei et al., 2012). On the other hand, soil pH slowly declines (Fig. 1) and eventually becomes acidic (Ritter et al., 2003; Zhang et al., 2018). Moreover, available N drops significantly within the first 10 years after tree establishment as a result of the increased N uptake by young trees and understory vegetation (Fig. 1; Hansen et al., 2007). Given the close relationship between abiotic and biotic soil properties, these changes are likely to influence the composition and dynamics of soil microbial communities and soil fauna (Zhao et al., 2018).

1.3 Development of soil biota during afforestation

Soils provide habitat for a myriad of microbes and fauna and serve as one of the largest hubs for biological interactions on earth (Brussaard, 1997; Bardgett and Van der Putten, 2014). As soil abiotic characteristics change within the first few decades of afforestation, so does the soil community which increasingly shifts towards a community that consists of forest adapted species (Barsotti, 2024). In general, microbial diversity and microbial species richness tend to rise as forests mature (Cao et al., 2010; Fu et al., 2015). However, some studies have reported contrasting findings, suggesting that microbial diversity may also decline under certain conditions (Pandey & Palni, 1996; Fu & Huang, 2014).

The trajectory of soil community development following afforestation unfolds at different rates for bacteria, fungi, and soil animals. While bacteria dominate in the initial stages, mirroring the conditions typically found in agricultural soils (Siles et al., 2023), fungi progressively increase in abundance and ecological significance as forests mature (Fig. 1; Bailey et al., 2002; Kang et al., 2018). Specifically, changes in the C:N ratio and pH acidification are reported to create conditions that support fungal proliferation and ectomycorrhiza fungi, which typically associate with trees, thrive in forested ecosystems (Zeller et al., 2001; Van der Wal et al., 2006). Additionally, intensive tillage and fertilization in croplands negatively affect mycorrhizal root colonization (Helgason et al., 1998; Kahiluoto et al., 2001) and physically break fungal hyphae thereby reducing fungal biomass (Frey et al., 1999; Stahl et al., 1999). Consequently, croplands tend to have lower fungal-to-bacterial abundance ratios compared to mature forests, where fungi have been argued to play a more prominent role (Fig. 1; Bailey et al., 2002; Kang et al., 2018).

Beyond microbial communities, afforestation also impacts soil fauna, though their initial establishment often lags due to various ecological constraints. In agricultural fields, intensive land management practices, including pesticide application and deep tillage, severely reduce soil fauna and in particular earthworm and other invertebrate populations (Briones & Schmidt, 2017). Generally, after tree planting, soil fauna recover at a slower pace, and are constrained by dispersal limitations, habitat availability, and sensitivity to nutrients (Osburn, Aylward, & Barrett, 2021; Malica et al., 2024).

Although past studies have documented the changes in abiotic and biotic soil

characteristics during afforestation, relatively less is known about these changes affect tree performance, herbivory, GHG emissions and symbiotic relationships between roots and soil microbes, and these effects are explored in this thesis.

1.4 Cascading effects of changing soils

As soil communities change post afforestation, they influence plant performance in various ways, including through mutualistic relationships, pathogenic interactions, and competition for nutrients (Philippot et al., 2013). For example, bacteria and fungi can enhance nutrient availability by decomposing organic matter, thereby benefiting plant growth (Petersen and Luxton, 1982; Bradford et al., 2002). However, these same processes can also drive emissions of methane (CH₄) and nitrous oxide (N₂O), two potent GHGs. Mutualistic interactions between plants and soil microbes are key to nutrient acquisition. Certain bacteria, such as N-fixing species, form symbiotic relationships with plants, enabling them to utilize atmospheric nitrogen (Cocking, 2003; Menge et al., 2023). Mycorrhizal fungi further enhance plant nutrient uptake by extending root systems and improving access to essential resources (Brundrett and Tedersoo, 2018; Mortier et al., 2020).

Beyond their role in plant nutrition, soil biotic communities significantly influence GHG fluxes. Bacteria mediate critical biogeochemical processes such as methane oxidation, nitrification, and denitrification, all of which impact atmospheric gas concentrations (Nielsen et al., 2015; Chen et al., 2018). Methanotrophic bacteria in forest soils, in particular, serve as a major global sink for atmospheric methane (Bárcena et al., 2014). Conversely, shifts in microbial composition, such as the loss of N₂O-reducing bacteria, can lead to elevated N₂O emissions (Domeignoz-Horta et al., 2018).

The interplay between soil properties and plant physiology extends beyond nutrient cycling, and can influence foliar chemical composition and nutritional quality (Bélanger et al., 2011; Ballhorn et al., 2017). These changes, in turn, have direct consequences for herbivore interactions, as changes in leaf chemistry can alter plant susceptibility to herbivory (Kos et al., 2015; Heinen et al., 2018; Huberty et al., 2020). Increased soil N availability, for instance, often raises leaf N concentrations, enhancing their palatability to herbivorous insects (Vandegheuchte et al., 2010). Soil microbial communities further mediate these effects by modulating nitrogen uptake, which can indirectly influence herbivore feeding patterns (Van Dijk et al., 2022).

Root colonization by plant-growth-promoting bacteria, for instance, has been shown to elevate leaf protein content, leading to increased herbivory rates (Ballhorn et al., 2017).

Since most studies focus on only a few broad time periods of ecosystem development and particular parts of the soil community, a deeper understanding of how changes in the abiotic and biotic soil characteristics in the context of afforestation affect tree performance and by extent root associated communities, herbivory and GHG emissions is crucial for future afforestation projects.

1.5 Using inoculation techniques to disentangle the effects of soil abiotic and biotic characteristics

Although soil biota are key drivers of important ecosystem services, they typically receive less attention in afforestation studies than plants as it is often assumed that they will colonize independently after a successful restoration of target plant species (Strickland et al. 2017). However, even after agricultural activities have stopped, soil characteristics from previous land use can remain, potentially obstructing the establishment of soil species adapted to forest conditions (Gomes et al., 2025). Inoculation techniques in the form of either soil transplantation or microbial inoculations have been continuously suggested as a means of helping the process of ecosystem restoration by promoting the establishment of target plant and soil communities (Waterhouse et al., 2014; Wubs et al., 2016). Inoculating with specific organisms or microbial communities to bypass natural dispersal limitations is becoming increasingly popular in afforestation (Lance et al., 2019, 2020; Benetkova et al., 2020; Robinson et al., 2023). Despite this, most studies that focus on inoculation have focused on grasslands, heathlands and meadows (Emam, 2016; van der Bij et al., 2018; Radujković et al., 2019; Contos et al., 2021; Han et al., 2021).

In addition to their role in afforestation, inoculation techniques can also be used to help scientists gain a mechanistic understanding of plant-soil interactions by disentangling the effects of the soil communities from abiotic characteristics (St-Denis et al., 2017; Georgopoulos et al., 2024) or even separating biotic groups to study their individual versus interactive effects on trees (Georgopoulos et al., 2025). Past studies have utilized both commercial inocula (Lance et al., 2019) as well as whole community inocula (Emam, 2016; Han et al., 2021) to steer communities while others have separated soil community groups based on size to study their

effects on plants separately (Wang., 2019). Additionally, some studies even compared sterilized soils with inoculated ones to distinguish between the effects of biotic and abiotic characteristics (St-Denis et al., 2017). This thesis contributes to the existing body of literature by utilizing several different inoculation techniques to disentangle the effects of abiotic and biotic soil characteristics on trees and their associated root microbiomes in the context of afforestation.

1.6 Focal tree species: *Alnus glutinosa*

Alnus glutinosa, (L.) Gaertn, commonly known as the black alder, is a fast-growing, deciduous pioneer species capable of thriving in diverse ecosystems, notably in harsh and N-deficient conditions (McVean, 1956). Alders are recognized as major contributors to global N inputs. Their presence has been reported to enrich soil N levels, primarily through the decomposition of leaf litter and roots, with a smaller contribution from root exudates (Roy et al., 2007). Beyond their association with beneficial (particularly N-fixing) bacteria, black alders also form symbiotic relationships with many fungi, such as both endomycorrhizal and ectomycorrhizal fungi, enhancing their ability to absorb essential macronutrients like phosphorus from the soil (Orfanoudakis et al., 2010). The ability of these actinorhizal trees to establish complex symbioses with both bacteria and fungi makes them valuable for restoring vegetation in degraded soils (Roy et al., 2007) and an ideal species to use when studying tree-microbe interactions.

1.7 *Alnus glutinosa* and *Frankia alni* in afforestation

Frankia bacteria are widespread in various ecosystems and can fix N either as free-living saprophytes in the soil or as symbionts within root nodules (Bélanger et al., 2011). These bacteria associate with over 200 plant species across 24 genera, forming mutualistic relationships that enhance plant survival in nutrient-deficient environments (Schwencke and Caru, 2001; Normand et al., 2007; Franche et al., 2009). The mutualistic association between *A. glutinosa* (black alder) and the actinobacterium *F. alni* enables the formation of root nodules where atmospheric N is converted into a form usable by plants (Navarro et al., 2003; Pujic et al., 2019). Within the root nodules of *A. glutinosa*, *F. alni* converts atmospheric nitrogen into ammonium (NH_4^+), which is then supplied to the host plant (Carro et al., 2016). Through this symbiosis, *Frankia* can provide 70–90% of the nitrogen required by

its host, allowing plants to thrive in poor soils (Nickel et al., 2001). In return, the plant provides *Frankia* with photosynthetically derived C compounds, to support the bacterium's energy needs (Chapin et al., 1987; Orfanoudakis et al., 2010). This N-fixing capability allows alders and other actinorhizal plants to thrive in soils deficient in nitrogen, such as degraded lands (Diagne et al., 2013), thereby facilitating ecological succession in these environments (Benson and Silvester, 1993).

The use of fast-growing N-fixing trees such like *A. glutinosa* and other actinorhizal trees has been suggested as a means to assist in land restoration projects due to their ability to thrive in nutrient poor conditions and mitigate biotic and abiotic stresses (Diagne et al., 2013). This N-fixing partnership plays a crucial role in sustaining forests, and other disturbed landscapes in both tropical and temperate regions (Zahran, 1999). However, changes in soil abiotic characteristics and microbial interactions have been shown to both positively and negatively affect this symbiosis (Gentili et al., 2006; Orfanoudakis et al., 2010). For example, past studies have shown that increased levels of available N can reduce *F. alni* nodulation (Ballhorn et al., 2017) while increased P (Valverde et al., 2002) or mycorrhizal association (Orfanoudakis et al., 2010) can be beneficial for these symbionts. However, soil nutrients mimicking forest conditions, and *Frankia* interactions with non-mycorrhizal fungi have not been adequately explored. As such it becomes very important to study these trees and their associated microbes in the context of afforestation for their potential use in facilitating forest ecosystem succession.

1.8 Thesis outline

In this thesis I examine how soil biotic and abiotic characteristics change and interact throughout the course of afforestation and how they subsequently impact tree performance. Furthermore, I dive deeper into the relationship between *A. glutinosa* and *F. alni* and assess how these characteristics also impact this symbiosis.

A graphical abstract of the research questions of my thesis from research chapters 2-6 is presented in Fig 1.2.

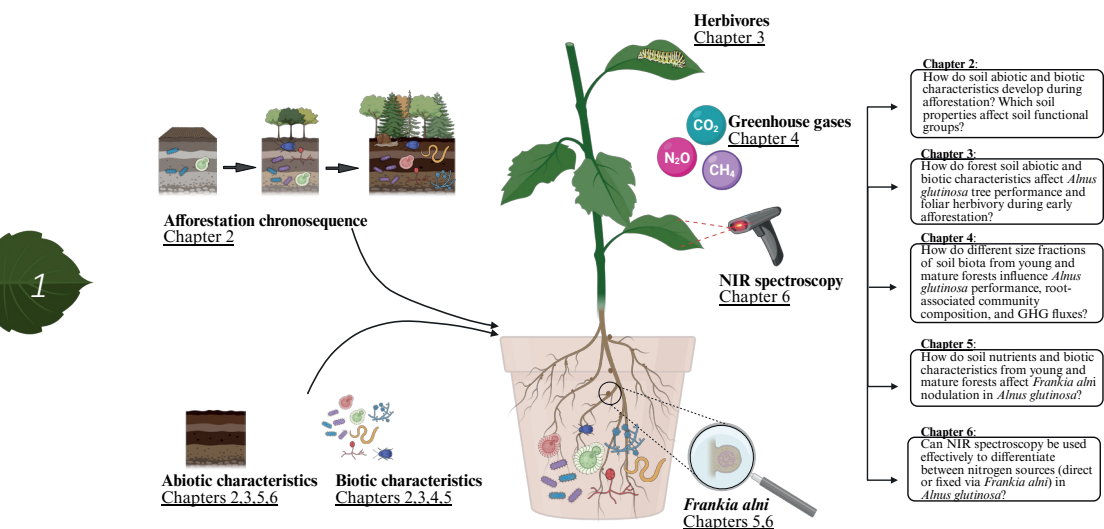


Figure 1.2 |A schematic overview of the experimental chapters of this thesis. 12 Forests and three crop fields were sampled along an afforestation chronosequence in Drenthe. The changes in the abiotic and biotic characteristics in the different soils are examined in chapter 2. In chapter 3 I zoom in on the early stages of afforestation where we examined how abiotic and biotic characteristics during the first 25 years of afforestation affect *A. glutinosa* and foliar insect herbivory. In chapter 4 I focus on the biotic characteristics of young and old forests and assess the effects of size-based filtering of soil biota groups on *A. glutinosa* performance and GHG emissions. Based on the consistent effects on *F. alni* nodulation in previous chapters, in chapter 5 I examine how soil nutrients and soil microbes from young and old forests affect *F. alni* nodulation. In chapter 6 I assess whether NIR spectroscopy can be used to differentiate between N derived from the soil or from *F. alni* N-fixation. Figure created using biorender (<https://www.biorender.com/>).

In **Chapter 2**, I examine how soil abiotic and biotic properties change along an afforestation chronosequence, with a focus on identifying which abiotic factors that are associated with stand age shape fungal, bacterial, and soil animal community composition. Soil and root samples were collected from croplands, afforested stands (10, 15, and 25 years old), and reference forests (>100 years). We analyzed soil chemistry, canopy cover, and used DNA sequencing to characterize microbial and faunal communities. This chapter establishes the trajectory and environmental drivers of soil community shifts during forest development. Specifically, we address the question: How do soil abiotic and biotic characteristics develop during afforestation, and which soil properties influence the composition and diversity of soil functional groups? We hypothesize that bacterial communities will shift gradually after afforestation, while fungal and soil animal communities will remain more similar to cropland communities in the first decade due to dispersal limitations. Community dissimilarity is expected to be greatest between croplands and mature forests. We further expect fungal composition to be primarily driven by soil pH, and soil fauna

by SOM, with both factors also influencing functional group diversity. Lastly, we anticipate that microbial and soil fauna richness will increase with stand age.

In **Chapter 3**, I assess how soil conditions from forests of different ages (10-15- and 25-year-old) influence the growth of *A. glutinosa* and associated leaf herbivory during early afforestation. Using two experiments, I tested the effects of live versus sterilized soils and microbial inoculation on tree performance, root microbial communities, and herbivore damage by *Mamestra brassicae*. This chapter aimed to disentangle the effects of soil abiotic and biotic characteristics on tree performance and leaf herbivory. Specifically, we address the question: How do forest soil abiotic and biotic characteristics affect *A. glutinosa* tree performance and foliar herbivory during early afforestation? We hypothesize that biotic factors, particularly the presence of plant growth-promoting bacteria, will enhance tree performance more than abiotic factors alone in early stages. Additionally, we expect higher herbivory on trees grown in soils containing soil microbes, especially those with a greater abundance of growth-promoting taxa.

Chapter 4 focuses on the role of different soil biota size fractions in influencing *A. glutinosa* performance, root associated microbiome and GHG emissions. Using wet-sieved soil fractions from young and mature forests, I conducted a mesocosm experiment to test how four different community size fractions (sieved through 250, 20, 11 and 3 μm) influence plant growth, root-associated microbial composition, and greenhouse gas fluxes. This chapter explores the contribution of soil community complexity to plant-microbe interactions and ecosystem functioning. Specifically, we address the question: How do different size fractions of soil biota from young and mature forests influence *A. glutinosa* performance, root-associated community composition, and GHG fluxes? We hypothesize that biota from different forest stages will lead to distinct root-associated communities reflecting their source environments. Specifically, we expect greater variation in fungal communities among size fractions from young forests. Within each stage, we anticipate that smaller size fractions will lead to reduced microbial richness, lower tree performance, and decreased CO₂ and N₂O emissions due to reduced community activity.

In **Chapter 5**, following some surprising results of **Chapters 3 and 4** on root nodulation, I investigate how microbial communities from young and mature forests, along with N and P availability, influence *F. alni* nodulation and *A. glutinosa* growth.

Using cultured bacteria and fungi as inocula, I assess how microbial communities from two forest developmental stages and nutrient levels alter root nodulation and tree performance. This chapter helps disentangle how microbial groups and soil nutrient status shape the tree–symbiont relationship during afforestation. Specifically, we address the question: How do soil nutrients and biotic characteristics from young and mature forests affect *Frankia alni* nodulation in *A. glutinosa*? We hypothesize that microbial communities from mature forests will reduce nodulation and plant performance. We also expect that higher soil N will suppress nodule biomass, while elevated P will enhance nodulation. Lastly, we anticipate that combined high N & P inputs may stimulate nodulation by lowering the soil N/P ratio.

In **Chapter 6**, I evaluate the potential of near-infrared (NIR) spectroscopy to distinguish between symbiotically fixed and soil-derived nitrogen in *A. glutinosa*. Building on the fertilizer and inoculation treatments from **Chapter 5**, I analyze leaf spectral reflectance, chlorophyll content, and biomass to determine whether NIR can serve as a non-destructive indicator of nitrogen acquisition strategy. This chapter contributes a methodological tool for studying N fixation in trees. Specifically, we address the question: Can NIR spectroscopy be used effectively to differentiate between nitrogen sources (direct or fixed via *F. alni*) in *A. glutinosa*? We hypothesize that *Frankia*-inoculated plants will perform similarly in biomass to uninoculated plants receiving sufficient N fertilizer, and that N fixed via *Frankia* will produce distinct spectral signatures in the NIR range compared to soil-derived nitrogen.

Finally, **Chapter 7** integrates the experimental findings, discusses broader implications for afforestation and soil community development, and outlines directions for future research.