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

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CHAPTER 6

Non-destructive detection of *Frankia alni* in *Alnus glutinosa* with NIR spectroscopy

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Abstract

Nitrogen is essential for plant growth, yet excessive fertilizer use contributes to environmental degradation. Actinorhizal trees like *Alnus glutinosa* form symbiotic relationships with *Frankia alni*, fixing atmospheric nitrogen and reducing reliance on synthetic fertilizers. However, distinguishing between soil-derived and symbiotically fixed nitrogen remains a challenge. This study investigates the potential of NIR spectroscopy as a non-destructive tool for differentiating N sources in *A. glutinosa*. Seedlings were grown under controlled conditions across a gradient of soil N fertilization, with and without *F. alni* inoculation. Leaf chlorophyll content, biomass, and spectral reflectance were measured to assess plant performance and spectral differentiation. Principal Component Analysis (PCA) and Partial Least Squares (PLS) regression revealed significant spectral differences between *F. alni*-inoculated and uninoculated but fertilized plants, particularly in the visible spectral region. While plants that were fertilized with high N mimicked the growth and chlorophyll effects of those that fixed N with *F. alni*, NIR spectroscopy detected spectral differences that could not be detected by the SPAD measurements. These findings highlight the potential of NIR spectroscopy for rapid, in vivo and in vitro assessment of symbiotic N-fixation in trees, offering a novel and more precise approach than leaf chlorophyll measurements.

Keywords: *Alnus glutinosa, Frankia alni*, nitrogen fixation, near-infrared spectroscopy, spectral differentiation, plant performance

1. Introduction

Nitrogen (N) is a fundamental nutrient for tree growth due to its vital role in the synthesis of amino acids, proteins and chlorophyll, directly influencing photosynthesis and plant growth (Gong et al., 2020; Huang et al., 2021). Although plants can acquire nitrogen directly from the soil, some plants, such as black alder (*Alnus glutinosa* (L.) Gaertn.), are able to fix atmospheric nitrogen in their roots through their symbiotic relationship with N-fixing bacteria like *Frankia alni* (Orfanoudakis et al., 2004, 2009; Pujic et al., 2022). Despite these symbiotic relationships being relatively well documented (Berry et al., 1986; Carney and Matson 2005; Gentili et al., 2006; Van der Heiden et al., 2008), it remains unclear whether N acquired from the soil or from the atmosphere via symbiotic N-fixation can be easily and differentially detected in the foliage of the plant. Near-Infrared Spectroscopy (NIR), which has been used in the past to detect plant infections by microbes via differences in wavelengths (Lim et al., 2017), could provide a promising, nondestructive method of differentiating between soil- and *F. alni*- derived N in *A. glutinosa*.

The mutualistic association between *A. glutinosa* and the actinobacterium *F. alni* enables the formation of root nodules where atmospheric nitrogen 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). In return, the plant provides *Frankia* with photosynthetically derived carbon compounds, primarily dicarboxylates, to support the bacterium's energy needs (Chapin et al., 1987; Orfanoudakis et al., 2010). This nitrogen-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 such environments (Benson and Silvester, 1993). However, past studies have shown that high levels of available N in the soil can lead to similar plant performance as when the plant establishes a symbiosis with *F. alni* (Ballhorn et al., 2017). Therefore, it becomes challenging to disentangle the N source (through symbiont or soil) and therefore quantify the relative contribution of N sources to tree growth.

Plant spectral data encapsulate a wide range of information about both the structure and physiological processes of plants (Kothari et al., 2022). By analyzing reflectance spectra from leaves, scientists can deduce various plant characteristics (Ustin et al., 2020) such as leaf chlorophyll content (Danh et al., 2021) and detect plant stress

(Asner et al., 2016), natural enemies (Sapes et al., 2022) and microbial infections (Lim et al., 2017). N taken up directly from the soil and N derived from the atmosphere via *F. alni* N-fixation are two different pathways for N to enter the plant and as such, they could reflect differently in the visible or infrared light spectra. Although conventional inspection of *Frankia* symbiosis is carried out by directly scavenging the roots of actinorhizal plants for nodules, NIR methods could provide a non-destructive alternative that has not been explored before.

In this study we assess whether NIR spectroscopy can be used to differentiate between N provided through fertilization and N fixed symbiotically by *F. alni*. To investigate whether soil- or *F. alni*- derived N is reflected differently in the NIR spectra, trees were grown in sterilized soil, across gradients of increasing available N (NH_4NO_3) and in the absence or presence of *F. alni*. NIR spectra were obtained from the leaves of trees. We hypothesize that *F. alni*-inoculated plants perform similarly, in regards to plant biomass, to uninoculated plants that receive enough N fertilizer, and that the N derived from *F. alni* will be reflected via unique spectral peaks in the NIR wavelengths compared to soil-derived N.

2. Materials and methods

This study aimed to distinguish between N supplied through fertilization and N symbiotically fixed by *F. alni* using NIR spectroscopy. Black alder seeds were sourced from the Dutch State Forestry Department, Staatsbosbeheer. Root nodules for inoculum preparation in all experiments were collected from a single population of *A. glutinosa* trees growing naturally in a semi-forested area in Leiden, Netherlands. Fresh nodules were collected on the same day as each experiment was set up, ensuring that only healthy, intact nodules of mature *A. glutinosa* trees were used for inoculum preparation.

2.1 Seed germination

To minimize external contamination and ensure that *A. glutinosa* seedlings were only inoculated with *F. alni*, we germinated seedlings under sterile conditions. To achieve this, *A. glutinosa* seeds were surface-sterilized by shaking for 20 minutes in a 14% bleach solution and placed on 0.5 Murashige and Skoog (MS) medium (Kahrizi et al., 2018) agar plates for germination. The plates were sealed with parafilm, and incubated vertically in a controlled growth cabinet with a 16:8 light-to-dark cycle

at 20°C during the light phase and 17°C during the dark phase. Seedlings were allowed to germinate and develop for four weeks until they had at least two mature leaves before being used in experiments. To maintain seed surface sterility, plates were inspected daily for signs of bacterial or fungal contamination near the seeds. If contamination was detected, uncontaminated seedlings were carefully transferred to new sterile agar plates under a flow hood, while contaminated seedlings and plates were discarded. Additionally, this method allowed for the selection of seedlings with consistent shoot and root sizes, providing greater uniformity for experimental setups.

2.2 Fertilization vs *F. alni* derived N experiment

An experiment was established to investigate the hypothesis that N derived from *F. alni* will be reflected via unique spectral peaks in the NIR spectra compared to soil derived N. Four weeks after germination, as previously described, seedlings were transferred into 1 L pots (dimensions: 11x11x12 cm) filled with gamma-sterilized grassland soil for which soil properties were analyzed (see Georgopoulos et al. 2025 for details). To create a gradient of N availability, pots were fertilized with solutions containing 1.25, 2.5, 5, 7.5, 10, or 20 mM NH_4NO_3 (Sigma Aldrich). Each pot received 15 mL of the designated solution twice weekly for a period of 12 weeks. For each nutrient level, 20 pots were prepared, half of which were inoculated with *F. alni* while the remaining half served as uninoculated controls (n = 10 replicates). The *F. alni* inoculum, prepared fresh on the day of the experiment, was made by crushing surface sterilized *F. alni* nodules and homogenizing nodule tissue in autoclaved miliQ H_2O (100 mg / 1 ml; Ballhorn et al., 2017). After fertilization, 1 mL of the homogenate was pipetted into a small indentation near the roots of the inoculated seedlings. Due to space limitations, the experiment was performed in two sets, one set including the 1.25 - 5 mM fertilizers and the other set including the 7.5 – 20 mM fertilizers. The two sets were performed using the exact space in the same growth chamber and the exact light and humidity conditions, but 12 weeks apart, and included their own sets of controls. As such, per experiment set, ten pots with sterilized soil were left untreated (neither inoculated nor fertilized, which we call control pure), while another ten pots received only the *F. alni* inoculum, bringing the total number of pots to 160. For both sets, plants were grown in a climate-controlled room with a relative humidity of 70%, a 16-hour light and 8-hour dark cycle, and temperatures of 20°C during the light phase (LED lights; Valoya LightDNA BX120, NS1+FR, 2-channel) and 17°C during the dark phase. Light intensity (%) changed

according to a timed schedule (7:00, 0 %, 8:00, 30 %, 11:00, 80 %, 13:00-17:00, 100 %, 19:00, 80 %, 22:00, 30 %, 23:00, 0%). Pots were watered three times per week to soil saturation. Over the course of 12 weeks, plant growth metrics, including stem height and leaf count were recorded weekly. Leaf chlorophyll was recorded from the fourth until the final week of the experiments using a Chlorophyll Meter SPAD-502Plus (Konica Minolta Sensing Europe B.V.). Prior to unpotting, on the day of the harvest, NIR spectra were measured for the third leaf from the top of each plant (the same leaf used for each chlorophyll measurement) using a Inventech Benelux NIR spectrometer (Oosterhout, Netherlands) with a spectral detection limit of ~330 - 1100 nm. Prior to measuring the first leaf and for every 40 measurements after that, a blank measurement was taken. NIR data were initially corrected based on the blank measurements by removing spectra lower than the blank.

At harvest, plants were carefully unpotted, and roots were rinsed under running tap water. Root nodules were counted, excised using a razor blade, and oven-dried at 40°C to determine the total root nodule biomass. The remaining plant biomass was divided into stems, leaves, and roots. All components were oven-dried under the same conditions to determine aboveground, belowground, and nodule biomass. For leaf nitrogen content analysis, oven-dried leaf samples were ground using a QIAGEN TissueLyser II Bead Mill (Hilden, Germany) at 370 rpm for 5 minutes. Leaf nitrogen percentages were quantified using the dry combustion method (Matejovic, 1997) with a Thermo Scientific FLASH 2000 CN analyzer (Milan, Italy).

Soil NH_4^+-N and NO_3^--N concentrations were determined using a standard 1M KCl extraction and spectrophotometric analysis (Kachurina et al., 2008), while PO_4^{3-}-P concentrations were measured using an extraction method with 0.01M $CaCl_2$ (Houba et al., 2008). Nutrient concentrations were expressed as mg of NH_4^+-N, NO_3^--N, and PO_4^{3-}-P per kg of soil (SI, Table S1).

2.3 Statistical analysis

2.3.1 Fertilization vs *F. alni*-derived N experiment

To compare the two experimental sets and assess chlorophyll levels relative to the uninoculated, unfertilized control, we adjusted the chlorophyll measurements by subtracting the average chlorophyll value of the uninoculated control for each experimental set and week from the corresponding measurements. For each week,

a linear model (LM) was used to evaluate the effects of *F. alni* inoculation (No *Frankia*, *Frankia*) and fertilizer concentration (0, 1.25, 2.5, 5, 10, and 20 mM) on leaf chlorophyll (see SI, Tables S2 and S3), using the *lme4* R package (v1.1-35.1; Bates et al., 2015). Model residuals were assessed for normality through the Shapiro-Wilk test, a QQ plot, and a histogram to visually inspect skewness.

To determine which uninoculated fertilizer treatments most closely resembled the *F. alni*-inoculated control, we subtracted the average aboveground biomass, leaf chlorophyll, and leaf N (%) of the inoculated control from the corresponding values of the uninoculated treatments in each experimental set. One-sample t-tests were then performed to compare the means of each uninoculated, fertilized treatment to zero (inoculated control performance) using the *dplyr* R package (v2.5.0). To account for multiple comparisons, p-values were adjusted using the False Discovery Rate (FDR) with the Benjamini-Hochberg method (SI, Table S4).

2.3.2 NIR spectra

All individual NIR spectra were preprocessed and normalized using standard normal variate (SNV) correction using the *mdatools* package (v.0.14.2; Kucheryavskiy, 2020). To examine dissimilarities between the spectra of each treatment, a principal component analysis (PCA) was conducted using the *factoextra* package (v.1.0.7; Kassambara and Mundt, 2017) based on Euclidean distances in order to account for the negative values after normalizing. Differences between treatments were evaluated through Permanova using the *vegan* package v2.6-4 (Oksanen, 2015). Pairwise comparison tests were conducted with the *ecole* package v.0.9-2021 when significant differences were observed in the Permanova. Finally, partial least square regression (PLS) analysis was used to examine whether the presence of *Frankia* or N-fertilization could be explained by the NIR spectra using the *mdatools* package.

3. Results

3.1 Plant performance

F. alni inoculation had a significant effect on leaf chlorophyll levels from the 9th until the 12th week of plant growth (Fig.1; SI, Table S2). From the 4th until the 8th week of plant growth, chlorophyll levels were indistinguishable between inoculated and uninoculated plants (Fig. 1; SI, Table S2). When excluding the *F. alni* inoculated

treatments, fertilizer concentration had a significant effect on leaf chlorophyll levels at every week of measurement (SI, Table S3). However, only plants that were fertilized with 10 and 20 mM NH_4NO_3 exhibited significantly higher leaf chlorophyll levels than the control pure (uninoculated and unfertilized) after week 6 and 7.5 mM after week 9 (Fig. 1).

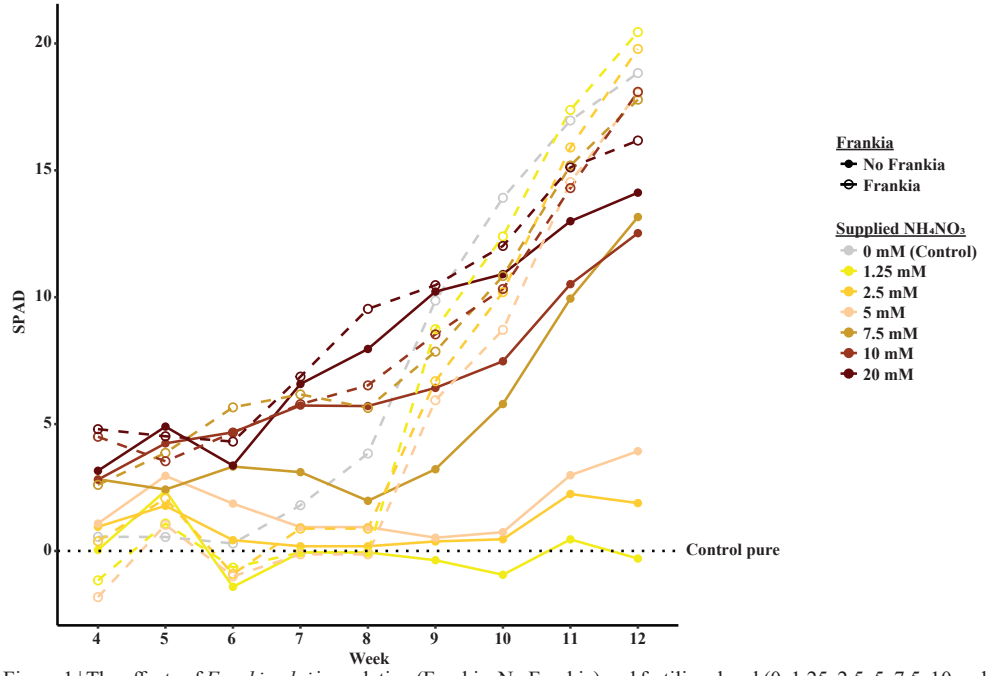

Figure 1 | The effects of *Frankia alni* inoculation (Frankia, No Frankia) and fertilizer level (0, 1.25, 2.5, 5, 7.5, 10 and 20mM) on leaf chlorophyll levels per week. Measurements begin at week 4 as the leaves were too small to measure non-destructively during the first 3 weeks. To make the two experiments comparable, the average chlorophyll value of the uninoculated control (control pure) for each experiment and each week was subtracted from the corresponding chlorophyll measurements of that week. As such, everything above or below the zero-line reflects a better or worse performance than the control pure, respectively. The values were calculated from n= 10 replicates. Error bars have been omitted to enhance visual clarity but the means and standard errors can be found in SI, Table S5.

At the time of the harvest, trees fertilized with 10 mM NH_4NO_3 exhibited similar aboveground biomass to the *F. alni*-inoculated control but had significantly lower leaf chlorophyll and nitrogen content (SI, Table S4; Fig. S1). In contrast, trees receiving 20 mM NH_4NO_3 produced 47% more biomass, maintained comparable leaf chlorophyll levels, and exhibited only a 19% reduction in leaf N relative to the control. Fertilizer treatments below 10 mM NH_4NO_3 resulted in significantly lower biomass, chlorophyll, and leaf N content (SI, Table S4; Fig. S1). Since subsequent NIR measurements were conducted on leaves, the 20 mM NH_4NO_3 treatment was considered functionally equivalent to *F. alni* inoculation in terms of plant

performance, and used for direct comparison between the NIR spectra to disentangle the effect of fertilizer addition and *F. alni* fixed N.

3.2 Spectral characteristics of *F. alni*

In total, 121440 raw reflectance spectra were measured from 341 nm to 1100 nm from the leaves of 160 plants. Rising peaks were observed at 500 nm, reaching the highest average reflectance where differences between treatments are visible for that peak range at 555 nm and decreasing henceforth until 673 nm. Reflectance of *A. glutinosa* leaves for this range starts to plateau at 750 nm and reaches its maximum at 1100 nm (Fig. 2B). At 555 nm, *F. alni* inoculation had a significant effect on the reflectance (ANOVA: Df = 1, F = 4.40, p = 0.04) and the reflectance of the 20 mM uninoculated treatment was significantly higher than the *F. alni* inoculated control, suggesting a lower N concentration.

There were significant differences between spectra of the different individual treatments (Permanova: pseudoF = 26.16, R^2 = 0.69, p = 0.001) as observed in the PCA plot (Fig. 2A). Specifically, pairwise comparisons revealed the NIR spectra of the *F. alni*-inoculated treatments were significantly different from all the uninoculated treatments except for those fertilized with 10 and 20 mM (Fig. 2A). A significant dissimilarity was also observed when grouping the plants based on *F. alni* inoculation (SI, Fig. S2; Permanova: pseudoF = 133.54, R^2 = 0.46, p = 0.001). When explicitly comparing the *F. alni*-inoculated control with the similarly performing 20 mM fertilized and uninoculated treatment, these were more similar but still significantly different (SI, Fig. S3; Permanova: pseudoF = 6.68, R^2 = 0.18, p = 0.003), showing that despite the similar plant performance, differences between treatments could still be detected in the NIR spectra.

PLS analysis on the whole dataset (1 component based on lowest RMSEP; SI, Fig. S4A) showed that the presence of *Frankia* could explain 54.8% of the observed variance while PLS analysis on the NIR spectra of only the *F. alni* – inoculated control against the similarly performing 20 mM fertilized and uninoculated treatment (1 component based on lowest RMSEP; SI, Fig. S4B) showed that the presence of *Frankia* could explain 31.2% of the observed variance. No visual differences were observed in the spectral peaks between the two datasets (Fig. 2C; SI, Fig. S5A).

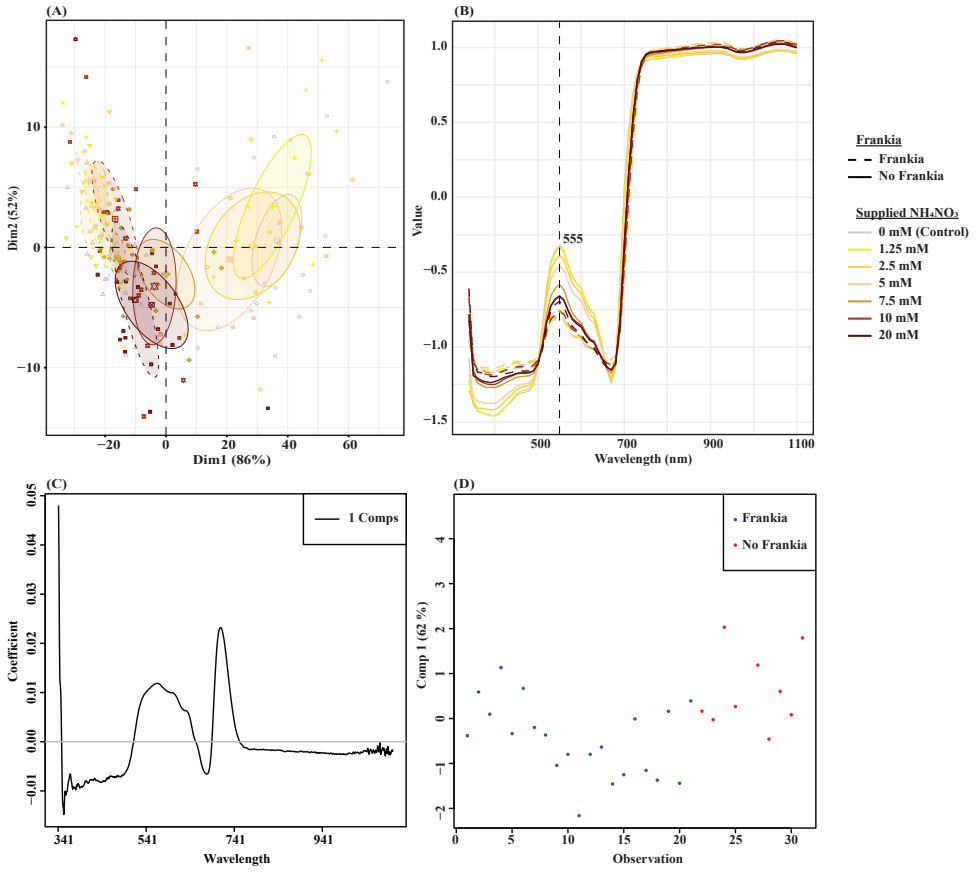

Figure 2 | (A) Principal components analysis showing the dissimilarity between the NIR spectra of the whole dataset after SNV correction. Ellipses represent Euclidean distances at the 95% confidence level. (B) The aggregated NIR spectra of the whole dataset after SNV correction ranging from 341 nm to 1100 nm with visible maximum absorbance peaks linked to the purpose of the study at 555 nm. The y-axis is the normalized reflectance after SNV correction. (C) The coefficient plot and (D) score plot of the PLS model including the NIR spectra of only the F. alni-inoculated control against the similarly performing 20 mM fertilized and uninoculated treatment using 1 component.

4. Discussion

In this study we investigated the potential use of NIR spectroscopy for non-destructive detection of N fixed by *F. alni* in *A. glutinosa*. The spectral differences observed between the two N sources indicate that biological N fixation has a measurable impact on leaf reflectance, even when plants exhibit similar performance (i.e. growth, chlorophyll levels and leaf N) when supplied with high levels of soil N.

A key finding from our study is that despite the functional equivalency between plants inoculated with *F. alni* and plants fertilized with 20 mM – NH_4NO_3, reflected by their chlorophyll content and their similar concentration of leaf N, their spectral signatures were distinguishable. This suggests that symbiotic N-fixation influences leaf biochemistry and structure in a way that is detectable using NIR spectroscopy. Specifically, the reflectance values at 555 nm differed significantly between the *F. alni*-inoculated control and the plants fertilized with 20 mM NH_4NO_3. It is important to mention that the 555 nm peak falls within the common visible spectral region of chlorophyll absorption for green leaves (400-720 nm; Gitelson et al., 2022; Raddi et al., 2022) and thus it is surprising that two treatments with indistinguishable chlorophyll levels had significantly different reflectance. As such, these differences may be attributed to the higher leaf N of the *F. alni* inoculated treatments, or subtle structural changes in leaf tissue and pigment following symbiotic N-fixation, although the latter is unlikely considering the lack of detectable differences in physical leaf properties in this and other studies that used *F. alni* (Orfanoudakis et al., 2010; Ballhorn et al., 2017; Vincent et al., 2025). It could also simply mean that the NIR meter can more precisely detect slight differences in N that are not detected by the SPAD meter which measures the spectral absorbance of chlorophyll only in the red (600-700 nm) and near-infra-red region, accounting for only part of the 500-670 nm range where we observe rising peaks for our treatments. This is in partial agreement to our initial hypothesis as we were able to detect significant differences between the spectral signatures and reflectance of inoculated and non-inoculated but similarly performing plants, even though these were not owed to any unique spectral peaks in the measured region (341-1100 nm). Past studies have shown that infection by microbes (e.g. *Fusarium graminearum* and *Fusarium asiaticum*) was able to be detected using NIR methods based on differences in the reflectivity intensity despite a lack of unique spectral peaks for infected plants (Lim et al., 2017). To our knowledge this is the first study that has attempted to differentiate between *A. glutinosa* plants inoculated with *F. alni* and uninoculated plants using NIR techniques.

While our study demonstrates the feasibility of using NIR spectroscopy to detect *F. alni*-derived N by more accurately detecting differences in leaf N, several limitations must be considered. First, the spectral differences between *F. alni*-inoculated and high-N fertilized plants, although significant, did not reveal different peaks, characteristic of the presence of *F. alni* within this spectral region but only a difference in the reflectivity. Additionally, the high percentage of variation explained

by latent variables suggests that additional physiological and biochemical factors, beyond nitrogen source alone, contribute to spectral variation. Future research should focus including higher spectral regions (e.g. 1100-2500 nm) and integrating complementary analytical techniques, such as isotope labeling (e.g., $\delta^{15}N$ analysis) or metabolomics, to further validate and refine NIR-based N source differentiation.

5. Conclusion

Our findings highlight the potential of NIR spectroscopy as a rapid and non-destructive method for distinguishing between soil- and symbiotically fixed N in *A. glutinosa*, which is also more reliable than chlorophyll measurements with a SPAD meter. Despite no apparent characteristic peaks emerging from the spectra of *F. alni* treatments, NIR spectroscopy provides a more precise method to detect slight differences in N that are not detected by conventional chlorophyll measurements using a SPAD meter.

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Author contributions

TMB, KG and SIFG designed the mesocosm experiments. KG, and LDN established the mesocosm experiments. KG, LV, KL and LDN collected the data. KG performed the data analyses and visualization with input from TMB and SIFG. KG and SIFG drafted the original manuscript with contributions from TMB. All authors read, edited and approved the final manuscript.

Supplementary information

Tables

Table S1 | Available soil nutrients of the gamma sterilized soil that is used in all the pots of the experiments expressed in mg/kg soil.

NH_4^+	NO_{3-}	PO_4^{3-}
37.82±1.61	14.41±0.57	3.86±0.41

Table S2 | Results from a linear mixed effects model (LMM) testing the effects of F. alni inoculation on leaf chlorophyll levels at each week of plant growth starting from week 4. To make the two experiments comparable, the average chlorophyll value of the uninoculated control for each experiment and each week was subtracted from the corresponding chlorophyll measurements of that week. Presented in the table are the degrees of freedom (DF), the F-statistic and the p-value (p). In cases of significant results ($p < 0.05$), the p-values are bolded.

Effect	DF	F	p
Chlorophyll – Week 4			
Frankia	1	0.814	0.368
Chlorophyll – Week 5			
Frankia	1	4.434	**0.037**
Chlorophyll – Week 6			
Frankia	1	0.586	0.445
Chlorophyll – Week 7			
Frankia	1	0.046	0.829
Chlorophyll – Week 8			
Frankia	1	1.608	0.206
Chlorophyll – Week 9			
Frankia	1	39.724	**<0.001**
Chlorophyll – Week 10			
Frankia	1	73.961	**<0.001**
Chlorophyll – Week 11			
Frankia	1	110.000	**<0.001**
Chlorophyll – Week 12			
Frankia	1	123.910	**<0.001**

Table S3 | Results from a linear mixed effects model (LMM) testing the effects of fertilizer concentration on leaf chlorophyll levels at each week of plant growth starting from week 4. To make the two experiments comparable, the average chlorophyll value of the uninoculated control for each experiment and each week was subtracted from the corresponding chlorophyll measurements of that week. Presented in the table are the degrees of freedom (DF), the F-statistic and the p-value (p). In cases of significant results ($p < 0.05$), the p-values are bolded.

Effect	DF	F	p
Chlorophyll – Week 4			
Concentration	5	2.470	**0.043**
Chlorophyll – Week 5			
Concentration	5	2.821	**0.024**
Chlorophyll – Week 6			
Concentration	5	7.908	**<0.001**
Chlorophyll – Week 7			
Concentration	5	22.472	**<0.001**
Chlorophyll – Week 8			
Concentration	5	14.117	**<0.001**
Chlorophyll – Week 9			
Concentration	5	15.059	**<0.001**
Chlorophyll – Week 10			
Concentration	5	14.682	**<0.001**
Chlorophyll – Week 11			
Concentration	5	14.500	**<0.001**
Chlorophyll – Week 12			
Concentration	5	18.811	**<0.001**

Table S4 | Results from a one-sample-t-test comparing the aboveground biomass production, leaf chlorophyll and leaf N of trees that received each fertilizer concentration independently against the *F. alni* control. To make the two experiments comparable, the average aboveground biomass, chlorophyll and leaf N of the *F. alni* control for each experiment was subtracted from the respective measurements of each un-inoculated fertilizer treatment, setting the *F. alni* – inoculated control performance for each measured variable as the 0. P values were adjusted using false discovery rate (FDR). Presented in the table are the t-statistic, the p-value (p) and the FDR adjusted p values. In cases of significant results ($p < 0.05$), the p-values are bolded. Significant differences from 0 signify lower/higher performance in relation to the *F. alni* – inoculated control.

Fertilizer concentration	t-statistic	p	FDR adjusted p
Aboveground biomass			
0 mM (Control pure)	-15.23	**4.19e-12**	**2.93e-11**
1.25 mM	-15.23	**9.84e-08**	**2.29e-07**
2.5 mM	-24.46	**1.52e-09**	**5.34e-09**
5 mM	-12.08	**7.25e-07**	**1.27e-06**
7.5 mM	-4.16	**2.44e-03**	**2.85e-03**
10 mM	1.58	0.146	0.146
20 mM	4.31	**1.94e-03**	**2.71e-03**
Chlorophyll			
0 mM (Control pure)	-24.10	**1.04e-15**	**7.31e-15**
1.25 mM	-19.90	**9.48e-09**	**3.31e-08**
2.5 mM	-10.25	**2.88e-06**	**6.74e-06**
5 mM	-9.72	**4.52e-06**	**7.91e-06**
7.5 mM	-3.47	**7.01e-03**	**8.17e-03**
10 mM	-3.80	**4.15e-03**	**5.81e-03**
20 mM	-1.69	0.125	0.125
Leaf N (%)			
0 mM (Control pure)	-14.94	**5.86e-12**	**4.10e-11**
1.25 mM	-8.59	**1.24e-05**	**1.45e-05**
2.5 mM	-34.27	**7.55e-11**	**1.32e-10**
5 mM	-35.53	**5.47e-11**	**1.27e-10**
7.5 mM	-36.26	**4.55e-11**	**1.27e-10**
10 mM	-17.28	**3.26e-08**	**4.57e-08**
20 mM	-4.10	**2.67e-03**	**2.67e-03**

Table S5 | The mean ± standard error (SE) of the measured leaf chlorophyll every week after subtracting the mean of the control pure of each experiment from the values. The 0mM uninoculated cell is missing values as that is the control pure.

Effect	Mean ± SE inoculated	Mean ± SE uninoculated
Chlorophyll – Week 4		
0 mM	0.56±0.99	X
1.25 mM	-1.16±1.17	0.04±0.67
2.5 mM	0.38±0.77	0.95±1.09
5 mM	-1.82±1.08	1.08±0.55
7.5 mM	2.61±0.54	2.82±0.88
10 mM	4.50±0.96	2.81±0.56
20 mM	4.80±0.81	3.16±0.97
Chlorophyll – Week 5		
0 mM	0.55±0.81	X
1.25 mM	1.08±0.88	2.37±0.73
2.5 mM	2.07±0.64	1.78±0.73
5 mM	1.03±0.71	2.96±0.79
7.5 mM	3.86±0.64	2.42±0.48
10 mM	3.53±0.82	4.24±0.73
20 mM	4.52±0.91	4.9±0.78
Chlorophyll – Week 6		
0 mM	0.29±0.94	X
1.25 mM	-0.65±0.90	-1.41±0.51
2.5 mM	-0.91±0.69	0.42±1.18
5 mM	-1±0.82	1.86±1.01
7.5 mM	5.66±0.90	3.32±0.67
10 mM	4.66±0.80	4.67±0.62
20 mM	4.31±0.89	3.36±0.50
Chlorophyll – Week 7		
0 mM	1.8±0.89	X
1.25 mM	-0.06±0.68	-0.06±0.57
2.5 mM	0.88±0.71	0.18±0.75
5 mM	-0.14±0.96	0.94±1.22
7.5 mM	6.17±0.85	1.97±0.81
10 mM	5.78±1.14	5.71±0.96
20 mM	6.87±1.26	7.96±0.78

Chlorophyll – Week 8		
0 mM	3.84±1.56	X
1.25 mM	-0.06±0.68	-0.06±0.57
2.5 mM	0.88±0.71	0.18±0.75
5 mM	-0.14±0.96	0.94±1.22
7.5 mM	5.63±0.98	1.97±0.81
10 mM	6.52±0.81	5.71±0.97
20 mM	9.54±1.57	7.96±0.78
Chlorophyll – Week 9		
0 mM	9.86±1.49	X
1.25 mM	8.74±1.19	-0.36±1.25
2.5 mM	6.69±1.13	0.37±1.12
5 mM	5.94±1.33	0.52±1.34
7.5 mM	7.85±0.83	3.22±0.70
10 mM	8.53±1.13	6.42±0.76
20 mM	10.47±2.11	10.22±1.12
Chlorophyll – Week 10		
0 mM	13.91±1.43	X
1.25 mM	12.39±1.42	-0.94±1.04
2.5 mM	10.20±1.05	0.46±1.66
5 mM	8.71±1.39	0.74±1.55
7.5 mM	10.83±1.40	5.79±0.85
10 mM	10.32±1.32	7.47±0.72
20 mM	12.02±1.58	10.91±1.22
Chlorophyll – Week 11		
0 mM	16.96±1.16	X
1.25 mM	17.38±1.01	0.45±1.12
2.5 mM	15.90±0.91	2.24±1.73
5 mM	14.54±1.42	2.98±1.52
7.5 mM	15.19±1.58	9.94±0.96
10 mM	14.30±1.52	10.51±0.84
20 mM	15.11±1.33	12.99±1.77
Chlorophyll – Week 12		
0 mM	18.83±1.27	X
1.25 mM	20.45±1.65	-0.3±1.05
2.5 mM	19.78±1.26	1.88±1.82
5 mM	18.05±0.89	3.93±1.71
7.5 mM	17.78±1.31	13.16±1.13
10 mM	18.09±1.34	12.52±1.19
20 mM	16.17±1.19	14.12±1.75

Figures

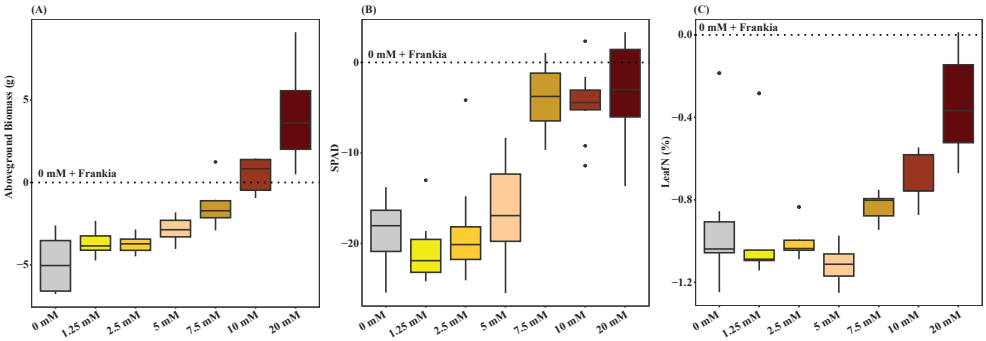

Figure S1 | (A) The aboveground biomass production, (B) leaf chlorophyll and (C) leaf N of uninoculated trees that received each fertilizer concentration against the *F. alni* control (dashed line at 0). To make the two experiments comparable, the average aboveground biomass, chlorophyll and leaf N of the F. alni control for each experiment was subtracted from the respective measurements of each uninoculated fertilizer treatment. Statistical outputs from the one-sample-t-test can be found in Table S5.

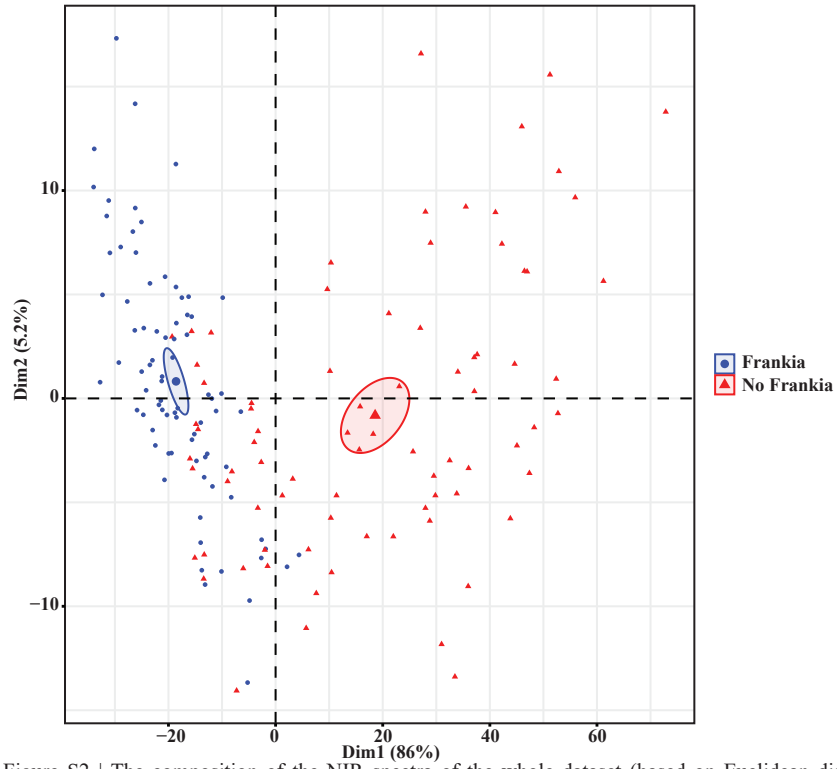

Figure S2 | The composition of the NIR spectra of the whole dataset (based on Euclidean distances), grouped depending on whether they were inoculated with *F. alni* or not. The ellipses are drawn based on 95% confidence interval.

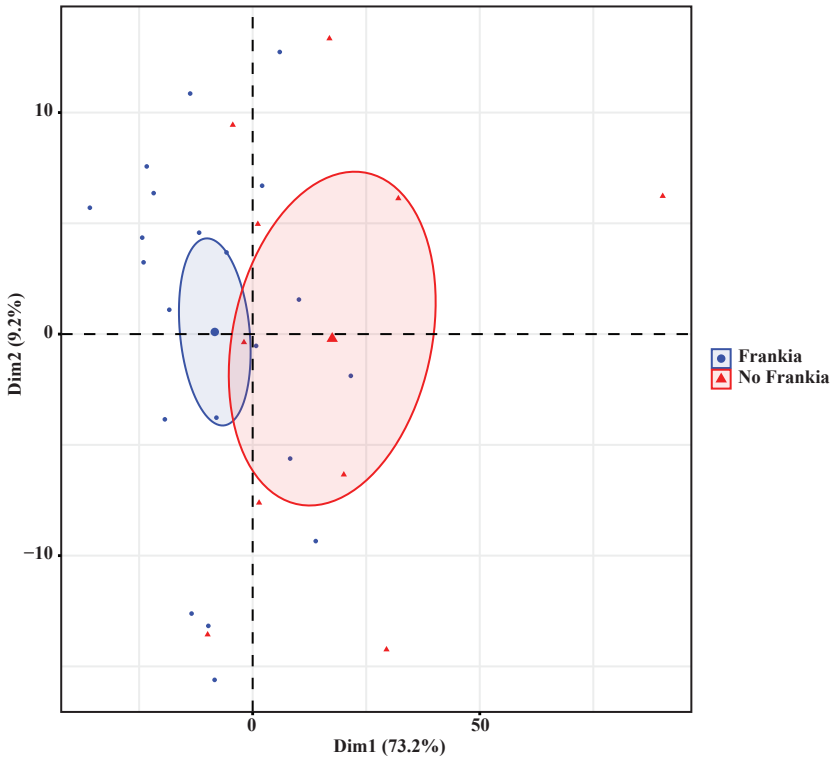

Figure S3 | The composition of the NIR spectra of only the *F. alni* – inoculated control against the similarly performing 20 mM fertilized and un-inoculated treatment (based on Euclidean distances), grouped depending on whether they were inoculated with F. alni or not. The ellipses are drawn based on 95% confidence interval.

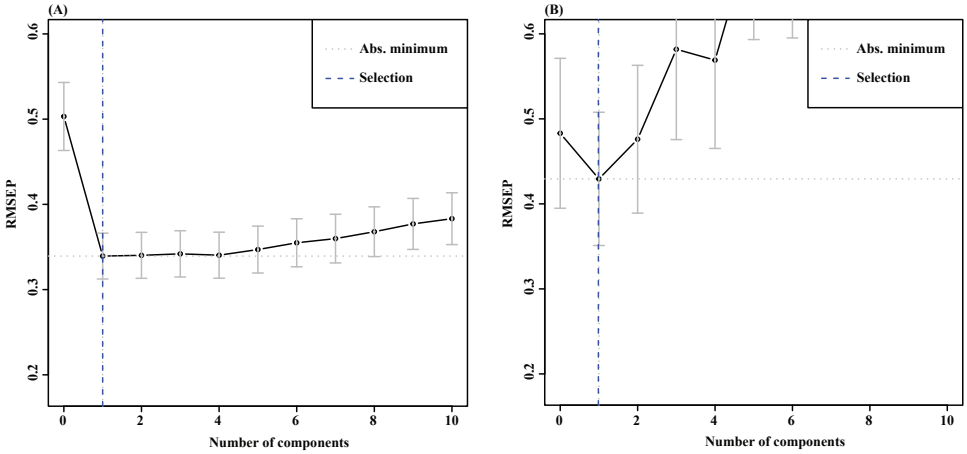

Figure S4 | The component selection based on the lowest root mean square error (RMSEP) of the PLS model of (A) all the NIR spectra and (B) the NIR spectra of only the *F. alni* – inoculated control against the similarly performing 20 mM fertilized and un-inoculated treatment. The blue dashed line reveals the selected number of components.

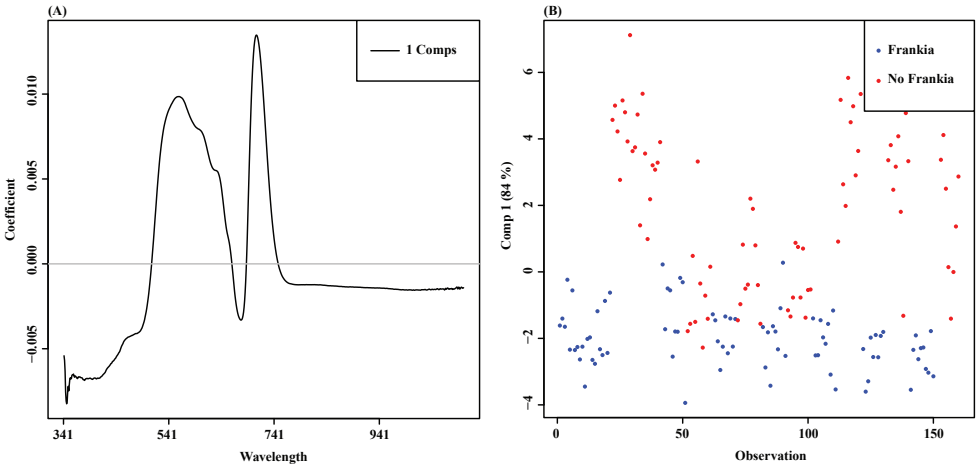

Figure S5 | The coefficient plot and score plot of the PLS model including the whole NIR spectra.