Symbiotic Nitrogen Fixation in Faba Bean, Soybean and Lupin Across Swedish Field Sites

Symbiotisk kvävefixering hos åkerböna, sojaböna och lupin på svenska fältplatser

Lisa Sundström

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Swedish University of Agricultural Sciences, SLU
Faculty of Natural Resources and Agricultural Sciences •
Department of crop production ecology
Agriculture programme – Soil and Plant Sciences
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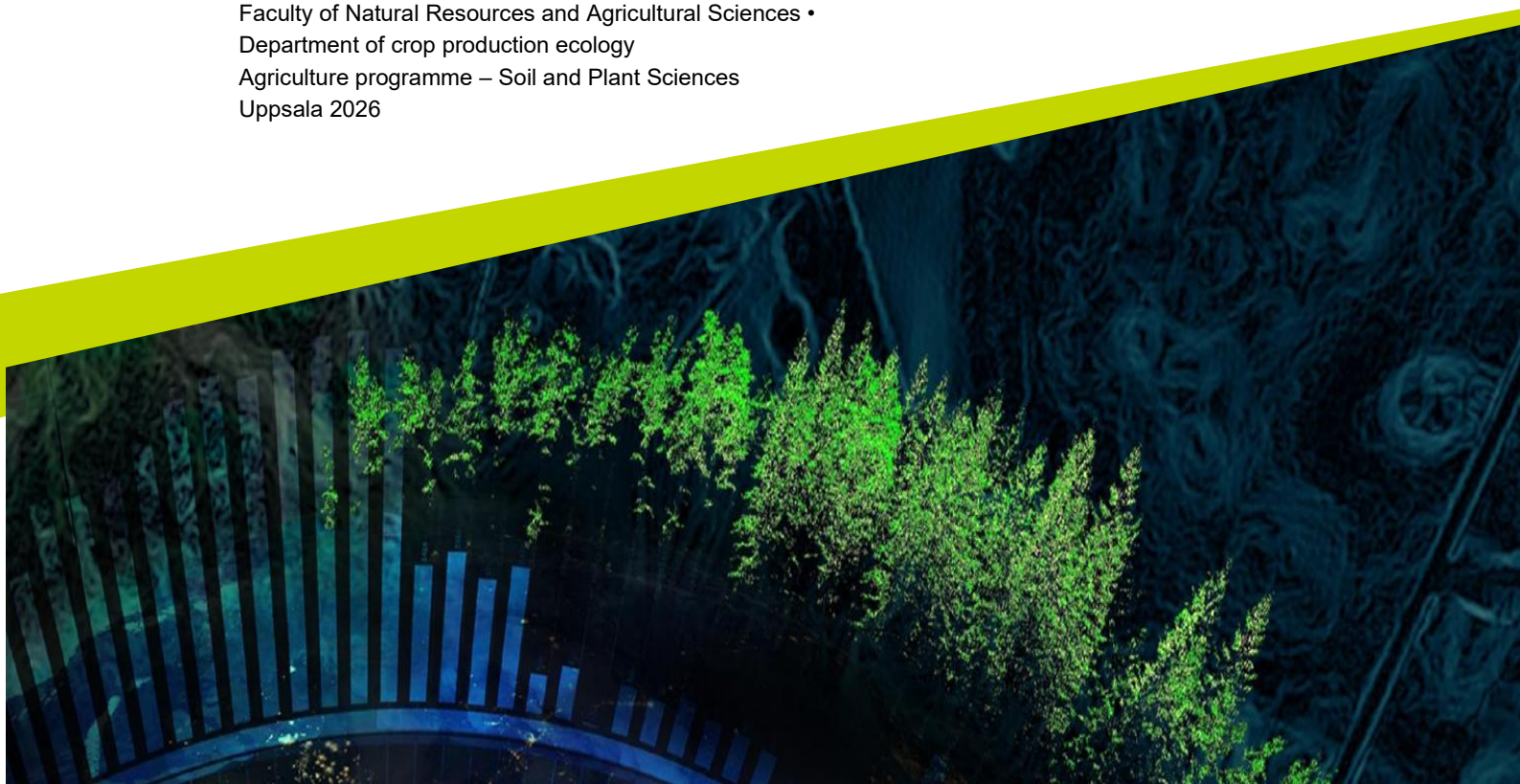

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Lisa Sundström

Supervisor: **Fede Berckx, SLU, Department of crop production ecology**
Examiner: **Jonathan Cope, SLU, Department of crop production ecology**

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Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Crop Production Ecology

Abstract

Grain legumes can contribute to nitrogen self-sufficiency in northern cropping systems through symbiotic nitrogen fixation (SNF), yet knowledge of how nodulation and fixation respond to field conditions in Nordic environments remains limited. This study, conducted within the IMPULSE project, investigated nodulation and SNF in faba bean, soybean and narrow-leaved lupin grown under organic management at three Swedish field sites. Total nodule weight and the proportion of plant nitrogen derived from atmospheric fixation (%Ndfa) were assessed to evaluate species-specific responses to site conditions and weed treatment. Plant nitrogen status was additionally characterised through measurements of mass fraction N (%).

Species differed in nodulation characteristics and in the relationship between nodulation and nitrogen fixation. Faba bean did not show statistically significant site effects on total nodule weight or %Ndfa, although numerical variation among locations was observed. Soybean exhibited limited site-related variation in nodulation, but %Ndfa was assessed at only one site, restricting interpretation of site effects. Lupin displayed significant site-dependent variation in total nodule weight, whereas %Ndfa data were available from one site only. The presence or absence of weeds did not significantly influence nodulation or %Ndfa in any species. Across species, increases in total nodule weight were not consistently associated with higher %Ndfa.

Overall, species identity exerted a stronger influence than weed management on nodulation and SNF under the studied field conditions. Structural nodulation traits alone were not reliable predictors of fixation performance, underscoring the importance of species selection and environmental adaptation when integrating grain legumes into Nordic cropping systems.

Sammanfattning

Baljväxter kan bidra till ökad kvävesjälvförsörjning i nordiska odlingssystem genom symbiotisk kvävefixering (SNF), men kunskapen om hur nodulering och kvävefixering påverkas av fältförhållanden i nordiskt klimat är fortfarande begränsad. Studien genomfördes inom IMPULSE-projektet och undersökte nodulering och SNF i ekologiskt odlade åkerböna, sojaböna och smalbladig lupin vid tre svenska försöksplatser. Total nodulvikt och andelen växtkväve som härrörde från atmosfärisk fixering (%Ndfa) analyserades för att bedöma artspecifika responser på lokala förhållanden och ogräsbehandling. Växternas kvävestatus beskrevs även genom analys av massfraktion kväve (% N).

Arterna skilde sig åt i noduleringsmönster och i sambandet mellan nodulering och kvävefixering. För åkerböna påvisades inga statistiskt signifikanta platseffekter på total nodulvikt eller %Ndfa, även om numeriska skillnader mellan platser förekom. Sojaböna uppvisade begränsad variation i nodulering mellan platserna, men eftersom %Ndfa endast analyserades vid en plats var möjligheten att bedöma platseffekter begränsad. Lupin uppvisade signifikant variation i total nodulvikt mellan försöksplatserna, medan %Ndfa-data även här var begränsade till en plats. Förekomst eller frånvaro av ogräs påverkade varken nodulering eller %Ndfa i någon av arterna. En ökad total nodulvikt var inte konsekvent kopplad till högre %Ndfa.

Resultaten visar att artidentitet hade större betydelse än ogräsbehandling för variationen i nodulering och SNF under de studerade fältförhållandena. Strukturella noduleringsmått visade sig inte vara tillförlitliga indikatorer på funktionell kvävefixeringsförmåga, vilket understryker vikten av artval och anpassning till lokala miljöförhållanden vid integrering av baljväxter i nordiska odlingssystem.

Table of contents

1. Introduction **5**

1.1 Background *5*
1.2 Symbiotic nitrogen fixation in legumes *6*
1.3 Nodule types and functional characteristics *8*
1.4 The studied legume species and weed competition *9*
1.4.1 Faba bean (Vicia faba) 9
1.4.2 Soybean (Glycine max) 10
1.4.3 Narrow-leaved lupin (Lupinus angustifolius) 12
2. Aim and objectives **13**

2.1 Research Questions *13*
2.2 Hypotheses *13*
2.3 Summary of expectations *15*
3. Material and method **16**

3.1 Growing conditions and field trials *16*
3.1.1 Experimental design 16
3.1.2 Species, seed material and inoculation 18
3.2 Analysis of root samples *19*
3.3 %Ndfa and mass fraction N (%) *19*
3.4 Statistical analysis and visualisation *21*
3.5 Limitations *22*
3.6 Organisation of the results *22*
4. Result **23**

4.1 Effects of site and weed treatment on nodulation and %Ndfa *23*
4.1.1 Faba bean 23
4.1.2 Soybean 24
4.1.3 Lupin 25
4.2 Species-specific relationships between nodulation traits and nitrogen fixation *26*
4.2.1 Structural patterns in nodulation: nodule number and biomass 26
4.2.2 Plant nitrogen status and fixation efficiency 27
4.2.3 Nodule biomass as a predictor of nitrogen fixation 28
4.2.4 Nitrogen concentration and nodulation investment 29
4.3 Summary of results *30*
5. Discussion **31**

5.1 Relationship between nodule development and nitrogen fixation *31*
5.2 Effect of weed competition on nodulation and nitrogen fixation *32*
5.3 How do the field sites (Uppsala, Alnarp, Gotland) affect nodulation and nitrogen fixation capacity? *34*
5.4 Species-specific regulatory responses *35*
6. Conclusions **37**

References **38**

Popular science summary **43**

Appendix 1 ..... **44**

Faba bean *44*
Soybean *44*
Lupin *45*

Tables

Table 1. Field trial locations in 2021 17
Table 2. Design of the field trial, Replicate I 18
Table 3. Effects of site and weed treatment on total nodule weight in faba bean 23
Table 4. Effects of site, weed treatment and their interaction on %Ndfa in faba bean 23
Table 5. Effects of site and weed treatment on total nodule weight in soybean 24
Table 6. Effect of weed treatment on %Ndfa in soybean at Alnarp 24
Table 7. Effects of site and weed treatment on total nodule weight in lupin 25
Table 8. Effects of site and weed treatment on %Ndfa in lupin 26

Equation

Equation 1 20

Figures

Figure 1. Soybean nodules before and after alteration 19
Figure 2. Total nodule weight and %Ndfa in faba bean across sites and weed treatments. 24
Figure 3. Total nodule weight and %Ndfa in soybean across sites and weed treatments 25
Figure 4. Total nodule weight and %Ndfa in lupin across sites and weed treatments 26
Figure 5. Nodule number and total nodule weight across species 27
Figure 6. Mass fraction N (%) and %Ndfa across species 28
Figure 7. %Ndfa and total nodule weight across species 29
Figure 8. Mass fraction N (%) and total nodule weight across species 30

Abbreviations

ANCOVA	An analysis of covariance
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (Growth stage scale)
LMM	Linear mixed model
SNF	Symbiotic nitrogen fixation
%Ndfa	Percentage of nitrogen derived from the atmosphere

Glossary

Bacteroid	Differentiated nitrogen-fixing form of rhizobia inside legume root nodules
Calcareous soil	Soil with high calcium carbonate content, typically with elevated pH.
Determinate nodules	Round nodules that lack a persistent meristem, develop uniformly, and have a shorter functional lifespan (e.g., in soybean).
Diazotrophs	Free-living and symbiotic bacteria and archaea capable of fixing atmospheric dinitrogen (N_2) into biologically available forms, such as ammonia.
Dinitrogen (N_2)	Atmospheric nitrogen gas that is reduced to ammonia (NH_3) by the enzyme nitrogenase during biological nitrogen fixation.
Faba bean	*Vicia faba L.*
Indeterminate nodules	Nodules with persistent meristems and elongated structure (e.g., faba bean, lupin).
Lupin	Narrow-leaved lupin, *Lupinus angustifolius L.*
Mass fraction N (%)	Percentage of nitrogen per dry weight; also referred to as tissue nitrogen concentration.
Nitrogenase	The enzyme complex responsible for reducing atmospheric dinitrogen (N_2) to ammonia (NH_3) in nodules.
Nodule biomass	Dry weight or fresh weight of nodules per plant or per sample.
Nodule number	Number of nodules counted per sample.
Rhizobia	A collective group of N_2-fixing bacteria in symbioses with legumes.
Soybean	*Glycine max* (L.) Merr.
Total nodule weight per sample (g)	Total weight of all nodules from a ten-plant sample.

1. Introduction

1.1 Background

Legume species play important roles in agricultural systems by increasing nitrogen self-sufficiency through symbiotic nitrogen fixation (SNF) (Peoples et al., 2009). The ability of legumes to fix atmospheric nitrogen is particularly relevant in northern regions such as Sweden**,** where arable cropping is dominated by cereal-based rotations and grain legumes are cultivated on a comparatively limited area (Jordbruksverket, 2022). Legumes capacity to fix atmospheric dinitrogen (N_2) through SNF makes them particularly valuable in regions with short growing seasons, where soil mineralisation is often slow in early spring and mineral nitrogen fertilisers remain less effective until soils have warmed (Peltonen-Sainio et al., 2009; Haugaard et al., 2012). Legumes can obtain nitrogen both from soil-derived mineral sources and from atmospheric N_2 via SNF, but the relative contribution of these pathways varies among species and environmental conditions (Peoples et al., 2009).

Despite the recognised agronomic benefits of legumes, a recent bibliometric review indicates that research on organic and low-input systems has primarily emphasised management and yield outcomes, whereas integrated field-based assessments of underlying biological processes have received comparatively less attention (Choppin et al., 2023). This underscores the need for field studies examining the functioning of SNF under northern growing conditions, where improved understanding may support more resource-efficient nitrogen management.

Northern climates are characterised by cool spring temperatures and delayed soil warming, conditions that can restrict early root growth, nodule initiation and the establishment of effective SNF (Fogelberg & Mårtensson, 2021; Schmidt et al., 2015). Such climatic constraints, together with agronomic challenges, are often cited as contributing factors to the comparatively limited cultivation of grain legumes in Sweden (Jordbruksverket, 2022; Fogelfors, 2015). However, it remains unclear to what extent these constraints affect nodulation and SNF under field conditions in northern environments, and whether different grain legume species exhibit similar or divergent responses in their capacity to establish effective symbiosis.

On this basis, the present study investigates nodule biomass and SNF in faba bean (*Vicia faba* L.), soybean (*Glycine max* L.) and narrow-leaved lupin *(Lupinus angustifolius* L.) grown at three Swedish field sites, with the aim of assessing species-specific patterns in nodulation and nitrogen fixation under contrasting environmental conditions.

1.2 Symbiotic nitrogen fixation in legumes

Biological nitrogen fixation refers to the microbial reduction of atmospheric dinitrogen (N_2) to ammonia (NH_3) (Udvardi & Poole, 2013; Sickerman et al., 2019). This process is carried out by free-living and symbiotic bacteria and archaea that are collectively referred to as diazotrophs (Unkovich et al., 2008). In legumes, biological nitrogen fixation predominantly occurs as SNF through specialised associations with rhizobia. This symbiosis takes place in root nodules, which are the structures in which rhizobial cells differentiate into bacteroids, a specialised physiological state adapted for nitrogen fixation, and where N_2 is reduced to NH_3 by the nitrogenase enzyme system (Oldroyd et al., 2011; Udvardi & Poole, 2013). In this state, bacteroids rely on plant-derived carbon and regulated oxygen conditions to sustain nitrogenase activity (Udvardi & Poole, 2013).

The efficiency of SNF varies widely among legume species and is influenced by factors such as temperature, soil nitrogen availability and the effectiveness of the rhizobial strain involved (Dong et al., 2023; Salvagiotti et al., 2008; Udvardi & Poole, 2013). Low soil temperatures during early spring can delay rhizobial colonisation and reduce the establishment of effective nodulation and SNF in field-grown legumes (Schmidt et al., 2015; Fogelberg & Mårtensson, 2021). Host–strain compatibility also plays a central role in SNF, as only certain combinations of legume hosts and rhizobial strains result in effective nodulation and nitrogen fixation (Thies et al., 1991; Mendoza-Suárez et al., 2021). Consequently, the presence of nodules does not necessarily imply high rates of SNF if the host–strain match is suboptimal (Thies et al., 1991; Udvardi & Poole, 2013).

In agricultural systems, this interaction is often influenced by the application of commercial rhizobial inoculants intended to enhance nodulation and nitrogen fixation (Thies et al., 1991; Mendoza-Suárez et al., 2021). Host–strain interactions are shaped by complex signaling processes and immune modulation in the root, meaning that successful nodule formation does not necessarily imply high nitrogen fixation efficiency (Dong et al., 2023; Mathesius, 2022). Rhizobial strains vary in both their competitiveness for nodule formation and their capacity

to fix nitrogen, and these traits do not necessarily coincide, as shown under both controlled and field conditions (Thies et al., 1991; Sickerman et al., 2019). As a result, nodules formed by highly competitive strains may contribute relatively little to overall nitrogen fixation, leading to substantial site-specific variation in SNF (Mendoza-Suárez et al., 2021).These complexities highlight the importance of distinguishing between nodule formation and effective nitrogen fixation when evaluating symbiotic performance under field conditions (Mendoza-Suárez et al., 2021).

Percentage of nitrogen derived from the atmosphere (%Ndfa) reflects the proportion of plant nitrogen derived from SNF and thus the plant's reliance on symbiotic fixation (Unkovich et al., 2008; Lyu et al. 2020). In contrast, the nitrogen concentration of plant tissue is expressed as mass fraction N (%) (Jensen et al. 2010), which reflects total plant nitrogen status irrespective of whether nitrogen originates from SNF or soil uptake (van Ittersum et al., 2025). These two measures therefore describe different aspects of plant nitrogen status: %Ndfa reflects nitrogen source, whereas mass fraction N (%) reflects total nitrogen status. Because they are governed by partly different processes, the two measures may vary independently across species or environments (Unkovich et al., 2008; Salvagiotti et al., 2008). Such situations may arise when a plant may exhibit a high %Ndfa while still having a low tissue nitrogen concentration if total nitrogen uptake is limited, or a low %Ndfa with a high tissue nitrogen concentration when soil nitrogen uptake is high (Salvagiotti et al., 2008).

While %Ndfa enables comparisons across species and growing conditions, the measure is sensitive to incomplete nodulation and variation in nodule functionality (De Notaris et al., 2023). Under field conditions, soil nitrogen availability and temperature can differ substantially between sites and seasons, thereby influencing nodulation and fixation processes (Herdina & Silsbury, 1990; Zahran, 1999). Competition between inoculated and native rhizobial strains can lead to uneven nodulation and reduced fixed efficiency under field conditions (Thies et al., 1991).

Nodulation and SNF are closely related but not identical processes. Nodule formation can occur without high nitrogen fixation activity, as fixation depends on nodule functionality and plant regulation of carbon allocation and oxygen conditions within the nodule (Unkovich et al., 2008; Udvardi & Poole, 2013). Because SNF is energetically costly, legumes may rely more on soil mineral nitrogen when it is readily available, whereas fixation becomes relatively more important under low soil nitrogen conditions (Lyu et al., 2020). Consequently, nodulation traits, %Ndfa and plant nitrogen concentration should not be interpreted as equivalent indicators of symbiotic performance.

1.3 Nodule types and functional characteristics

Root nodules in legumes vary in structure, developmental pattern and functional longevity, and these differences influence both the level and duration of SNF. Nodules are broadly classified as either indeterminate or determinate, based on the structure of their meristematic tissue.

This classification is also associated with differences in bacteroid development and persistence within the nodule, which in turn affect how long nitrogen fixation is maintained during the growing season (Unkovich et al., 2008).

Indeterminate nodules, as found in faba bean and lupin, retain an active apical meristem, resulting in distinct developmental zones within the nodule. This results in the simultaneous presence of young, developing and fully active bacteroids within the same nodule (Udvardi & Poole, 2013), allowing nitrogenase activity to be sustained over an extended period and enabling indeterminate nodules to remain active for a larger part of the growing season under favourable conditions (Minchin & Witty, 1997).

Determinate nodules, typical of soybean, lack a persistent meristem and instead develop and senesce synchronously (Oldroyd et al., 2011; Udvardi & Poole, 2013). Bacteroids therefore mature and senesce together, resulting in a shorter functional lifespan and a decline in nitrogenase activity as nodules age (Oldroyd et al., 2011). Consequently, total nitrogen fixation in species with determinate nodules depends on the continued initiation of new nodules throughout the growing season. The number and size of nodules can further reflect differences in infection frequency and developmental pattern, as each nodule is initiated through a separate rhizobial infection process (Minchin & Witty, 1997).

Nodule function is further shaped by environmental and physiological constraints. Oxygen regulation, carbon supply from the shoot and internal nodule turnover influence nitrogenase activity (Minchin & Witty, 1997; Udvardi & Poole, 2013). Nitrogenase activity in root nodules is strongly influenced by oxygen availability, as the enzyme is highly sensitive to oxygen while still depending on aerobic respiration to function. Nodules therefore regulate oxygen diffusion to maintain conditions that allow nitrogen fixation to proceed (Minchin & Witty, 1997; Sprent & James, 2007).

Carbon supply from the shoot is also critical, since nitrogen fixation requires a continuous input of photosynthetically derived carbon to support respiration in the bacteroids. When carbon supply to the nodules is reduced, for example under stress or limited photosynthesis, nitrogenase activity declines accordingly

(Salvagiotti et al., 2008; Jensen et al., 2010). In addition, nitrogenase activity changes as nodules develop and age, and the capacity of bacteroids to fix nitrogen decreases as nodules senesce, even if nodules remain present on the roots (Salvagiotti et al., 2008; Peoples et al., 2009). Stress conditions such as drought, nutrient imbalance or low temperature can reduce both the duration and efficiency of SNF by accelerating functional declines in nodule activity (Ding et al., 2019; Zhao et al., 2017).

1.4 The studied legume species and weed competition

The early stages of crop establishment represent a critical phase in legume development, as plants have not yet developed sufficient leaf area or root systems to secure access to light, water and nutrients (Knezevich et al., 2003). Limitations during this period may have lasting effects on biomass accumulation and the functioning of SNF (Kebede, 2021). The ability of a crop to tolerate weed pressure therefore depends strongly on successful early canopy development and root establishment under prevailing field conditions (Bulson et al., 1997; Lithourgidis et al., 2011). Under northern growing conditions such as those examined in this study, low soil temperatures can delay rhizobial colonisation and restrict nitrogenase activity during early establishment (Montañez et al., 1995; Schmidt et al., 2015). Such climatic constraints may reduce early plant vigour and influence nodulation and SNF (Zimmer et al., 2016; Fogelberg & Mårtensson, 2021).
Against this background, the three species examined in this study differ in their rhizobial symbioses, nodulation characteristics and early growth dynamics, factors that may shape their performance across sites and weed treatments (Ren, 2018; Faria et al., 2022). Differences in early biomass development and reliance on SNF are associated with species-specific responses to environmental constraints (Zahran, 1999). Early-season weed competition may interact with these inherent species traits by limiting access to light, water and nutrients during establishment (Lithourgidis et al., 2011; Petraki et al., 2025). When environmental conditions constrain early crop vigour, the relative influence of weed pressure may become more pronounced (Lithourgidis et al., 2011). Consequently, interspecific variation in early establishment may contribute to differential competitive responses among the studied legumes (Petraki et al., 2025).

To contextualise these differences, the following sections provide a brief overview of each species, focusing on traits relevant to nodulation, nitrogen fixation, environmental adaptation and early competitive performance.

1.4.1 Faba bean (Vicia faba)

Faba bean exhibits a high capacity for SNF under cool-temperate conditions (Peoples et al., 2009; Jensen et al., 2010). The species forms large, indeterminate nodules that sustain nitrogenase activity over extended periods (Ren, 2018). In addition, faba bean maintains active nodulation at relatively low soil temperatures compared with many other legumes (Herdina & Silsbury, 1990), contributing to a high reliance on atmospheric nitrogen for growth (Kebede, 2021).

Faba bean is generally regarded as day-neutral, although some genotypes may respond to long-day conditions for flowering, making the species suitable for cultivation under the extended photoperiods typical of northern latitudes (Karkanis et al., 2018). Long-term field studies report high %Ndfa values and substantial nitrogen yields across temperate environments (Jensen et al., 2010; De Notaris et al., 2023).

Although early season weed competition may reduce growth and grain yield (Petraki et al., 2025), faba bean has been reported to exhibit relatively strong competitive ability through rapid canopy development and biomass accumulation This competitive capacity is supported by its robust taproot system and relatively vigorous early growth (Fogelfors, 2015), as well as by its comparatively greater plant height and early canopy closure relative to soybean and lupin (Karkanis et al., 2018). In contrast, soybean often exhibits slower early crop development and greater sensitivity to early-season competition under temperate field conditions (Lyu et al., 2020), while lupin is frequently characterised by more variable early crop establishment and biomass production (Vollmann, 2016). These traits may contribute to stable nodulation under competitive conditions.

While faba bean is frequently reported to derive a substantial proportion of its nitrogen from SNF under temperate conditions (Jensen et al., 2010; De Notaris et al., 2023), soybean demonstrates greater plasticity in nitrogen acquisition, reducing SNF when mineral nitrogen is readily available (Salvagiotti et al., 2008; Zimmer et al., 2016).

1.4.2 Soybean (Glycine max)

Soybean forms typically spherical, determinate nodules with a comparatively shorter functional lifespan, and nitrogenase activity therefore tends to decline earlier than in legumes with indeterminate nodules (Ferguson et al., 2019; Udvardi & Poole, 2013). In addition to structural differences in nodulation, soybean exhibits strong autoregulation of nodulation and pronounced feedback control of SNF, enabling dynamic adjustment of fixation activity in response to soil mineral nitrogen availability (Jensen et al., 2010; Ciampitti & Salvagiotti, 2018). This regulatory flexibility allows soybean to balance carbon allocation and

nitrogen acquisition by shifting between atmospheric nitrogen fixation and soil nitrogen uptake depending on environmental conditions (Salvagiotti et al., 2008).

Soybean is sensitive to low root-zone temperatures, and suboptimal soil conditions can delay rhizobial infection, nodulation and the onset of SNF (Schmidt et al., 2015; Kempster et al., 2021). Field-based evidence further shows that low early-season soil temperatures delay the establishment of nodulation and SNF in soybean (Fogelberg et al., 2021; Kempster et al., 2021).
Weed competition can reduce early crop growth primarily through competition for light, leading to reduced photosynthetic carbon assimilation and lower biomass accumulation (Knezevic et al., 2003). Because nodulation and nitrogen fixation are metabolically demanding processes, reduced carbon allocation to roots under competitive stress may limit nodule formation and activity (Birchall, 1995; Khalifa & Fakkar, 2020). Such constraints may be particularly relevant under cool soil conditions typical of Nordic environments, where early root development and rhizobial infection are already temperature-limited (Kempster et al., 2021; Fogelberg, 2021). This sensitivity to seasonal temperature and site conditions is reflected in field studies from temperate Europe, which report moderate and variable %Ndfa values in soybean, with SNF strongly influenced by seasonal temperature and site conditions (Zimmer et al., 2016).

1.4.3 Narrow-leaved lupin (Lupinus angustifolius)

Lupin performs poorly on alkaline and calcareous soils, where high soil pH is associated with reduced growth and impaired nodulation (Tang et al., 1995). The species forms indeterminate nodules with a persistent apical meristem (Sprent & James, 2007). Indeterminate nodules maintain continuous meristem activity, allowing sustained nodule development and prolonged nitrogen fixation compared with determinate nodules (Sprent & James, 2007; Ferguson et al., 2019). Sensitivity to alkaline soils becomes apparent early in the growing season, as elevated soil pH can restrict root development during crop establishment (Tang et al., 1995; Ding et al., 2019).

Lupin is a long-day species, and flowering is accelerated under extended photoperiods typical of northern Europe, which may shorten the vegetative phase and influence early biomass accumulation (Sacristán-Bajo et al., 2023; Peltonen-Sainio et al., 2009). As lupin often exhibits relatively slow early growth, weed competition during establishment can reduce early growth and grain yield (Piekarczyk, 2023). Field studies indicate that lupin shows intermediate competitive ability compared with other grain legumes (Piekarczyk, 2023).

Lupin develops a deep taproot system and can form cluster roots that improve nutrient uptake in soils with low nutrient availability, a trait common among *Lupinus* species (Ding et al., 2019). Although these root adaptations enhance nutrient acquisition under limiting soil conditions, early above-ground growth may remain comparatively slow, which can reduce competitive ability under field conditions (Piekarczyk, 2023). Because early growth conditions influence nodulation and nitrogen fixation in legumes (Herdina & Silsbury, 1990), reduced early vigour may also affect subsequent symbiotic performance.

Under favourable conditions, lupin has been reported to rely substantially on atmospheric nitrogen, with significant contributions of SNF to total plant nitrogen in temperate cropping systems (Peoples et al., 2009; Lucas et al., 2015). Despite constraints on early growth and competitive ability, lupin is therefore recognised as an effective nitrogen-fixing grain legume that can contribute substantial amounts of SNF to cropping systems (Peoples et al., 2009).

2. Aim and objectives

This master's thesis was conducted within the IMPULSE project (FORMAS Grant 2019-02402), initiated in 2020 to support the development of legume production under northern growing conditions. Grain legumes have the potential to enhance nitrogen self-sufficiency in northern cropping systems, yet knowledge of species-specific nodulation and symbiotic nitrogen fixation under Nordic field conditions remains limited. It remains unclear how early-season conditions influence nodulation and nitrogen fixation across different grain legume species. To address this, field experiments were conducted at three Swedish locations: Uppsala, Alnarp and Gotland, to examine nodulation and nitrogen fixation in faba bean, soybean and lupin under contrasting site conditions and weed management.

2.1 Research Questions

This study addresses the following research questions:

1. *How are nodule number and total nodule biomass related to the amount of nitrogen fixed in faba bean, soybean and lupin?*
2. *To what extent does weed management influence nodulation and SNF?*
3. *How do site-specific characteristics influence nodulation and nitrogen fixation capacity?*
4. *Do legume species differ in their nodulation and nitrogen fixation responses to weed management and site conditions?*

2.2 Hypotheses

1. Relationship between nodule development and nitrogen fixation

It is hypothesised that species with greater total nodule biomass will exhibit higher %Ndfa, although the strength of this relationship may differ among legume species. Because total nodule biomass reflects both nodule number and size, it represents the amount of potentially active tissue available for nitrogen fixation. In these species, total nodule biomass may therefore correspond more closely to the proportion of functionally active tissue than in soybean. Consequently, the association between nodule biomass and %Ndfa is expected to be stronger and more consistent in faba bean and lupin than in soybean.

Given its adaptation to cool-temperate conditions and vigorous early growth, faba bean is further expected to develop greater total nodule biomass under the field conditions examined in this study than soybean and lupin,

potentially contributing to more consistent nodulation performance under variable environmental conditions.

2. Effect of weed competition on nodulation and nitrogen fixation

Weed competition is expected to reduce both nodulation and nitrogen fixation by limiting light, water and soil nutrient availability. Reduced photosynthesis and carbon allocation under high weed pressure may restrict the supply of photosynthate to the root system, thereby limiting nodulation and nodule activity. As a result, plants growing under weedy conditions are predicted to develop fewer or smaller nodules and show reduced levels of total nitrogen fixation compared with weed-free conditions.

Because nitrogen fixation depends on continuous carbon supply to nodules, species with determinate nodules such as soybean may be particularly sensitive to reductions in early growth and carbon availability. In contrast, species forming indeterminate nodules may better sustain nitrogenase activity within existing nodules, potentially buffering short-term competitive stress.

3. Influence of geographic location and climate

Geographic variation in climate and soil conditions is hypothesised to influence the establishment and efficiency of rhizobial symbiosis. Warmer soil temperatures and longer growing seasons, as expected at more southern sites, may promote nodulation and nitrogen fixation, whereas cooler conditions may restrict early infection, nodule development and subsequent %Ndfa.

In addition to climatic differences, site-specific soil properties such as calcareous conditions may further constrain nodulation in sensitive species. Climatic and soil conditions are therefore expected to interact with species specific physiological traits in shaping nodulation and SNF across sites.

4. Species--specific responses

Based on differences in temperature sensitivity, soil adaptation, early growth characteristics and nitrogen uptake and fixation strategies, species-specific responses in nodulation and %Ndfa are expected.

Faba bean is hypothesised to maintain relatively stable fixation across sites due to its tolerance to cool soil temperatures and vigorous early growth. Soybean may show greater variation depending on soil nitrogen availability and establishment conditions, reflecting its flexible nitrogen uptake and fixation strategy. Lupin is expected to be most sensitive to suboptimal soil and climatic conditions, particularly with respect to nodulation and %Ndfa.

2.3 Summary of expectations

Nitrogen fixation was expected to be positively related to total nodule biomass, with plants forming either numerous or large nodules predicted to exhibit higher %Ndfa. However, because species differ in nodule developmental pattern and functional longevity, the strength of this relationship was expected to vary among species.

Nitrogen fixation was expected to be highest and most stable in faba bean, reflecting its adaptation to cool temperate conditions, vigorous early growth and capacity to maintain nodulation under relatively low soil temperatures. In contrast, soybean and lupin were expected to show lower or more variable nitrogen fixation, as these species are more dependent on favourable soil temperatures and establishment conditions for effective nodulation and SNF.

Weed presence was hypothesised to reduce nodule biomass and nitrogen fixation through limitations in light interception and carbon allocation. Species differences in nodulation strategy were expected to influence sensitivity to weed competition, with soybean potentially exhibiting greater sensitivity to reductions in early growth and carbon availability due to its reliance on continuous nodule initiation.

Geographic location was expected to influence nodulation and SNF through variation in soil temperature, growing season length and soil chemical conditions. Warmer southern sites were predicted to promote nodulation and nitrogen fixation, whereas cooler sites and calcareous soil conditions were expected to constrain these processes, particularly in sensitive species such as lupin. Overall, nodulation and nitrogen fixation were expected to reflect the combined effects of nodule development, weed pressure, climatic conditions and species-specific physiological traits.

3. Material and method

3.1 Growing conditions and field trials

Field trials were conducted in 2020 and 2021 as part of the IMPULSE project (FORMAS grant 2018-02402). However, the analysis presented here is based solely on data from the 2021 growing season. Faba bean and lupin were sown in late May, while soybean was sown in June, with sowing dates varying slightly between field sites. Harvest took place in late summer or early autumn 2021 depending on crop maturity at each location. The crops were managed according to standard organic practices, without the use of synthetic fertilisers or pesticides, and weeds were controlled by mechanical weeding performed twice during the growing season in weed-free plots. Winter wheat served as the pre-crop at all locations. Each plot measured 2 × 12 m (24 m²).

Field trials were located at three sites: Alnarp (Skåne), Roma on Gotland (Gotland county), and Lövsta near Uppsala (Uppland). These sites represent contrasting soil and climate conditions, including maritime moraine soils in Alnarp, calcareous clay soils on Gotland, and heavy inland clay at Uppsala (SGU, 2025).

3.1.1 Experimental design

Each site consisted of three replicate blocks arranged in a randomised incomplete block design (Table 1–3). The same block strip layout was applied across replicates, with plots organised in vertical columns and weed treatments (weed-free and weedy) applied as horizontal strips across the columns. The assignment of weed treatment strips within blocks was fixed, while species were allocated across plots within each block to minimise systematic spatial bias.

In addition to the grain legume species, non-legume crops; buckwheat (*Fagopyrum esculentum*) and spring wheat (*Triticum aestivum*) were included in the experimental layout. Buckwheat was used as the reference plant for the estimation of the proportion of plant nitrogen derived from atmospheric fixation (%Ndfa) using the ^{15}N natural abundance method (Unkovich et al., 2008). As a non-N_2-fixing species, buckwheat reflects soil-derived nitrogen uptake under field conditions. Its rooting depth and growth pattern are also comparable to those of the grain legume species examined in this study.

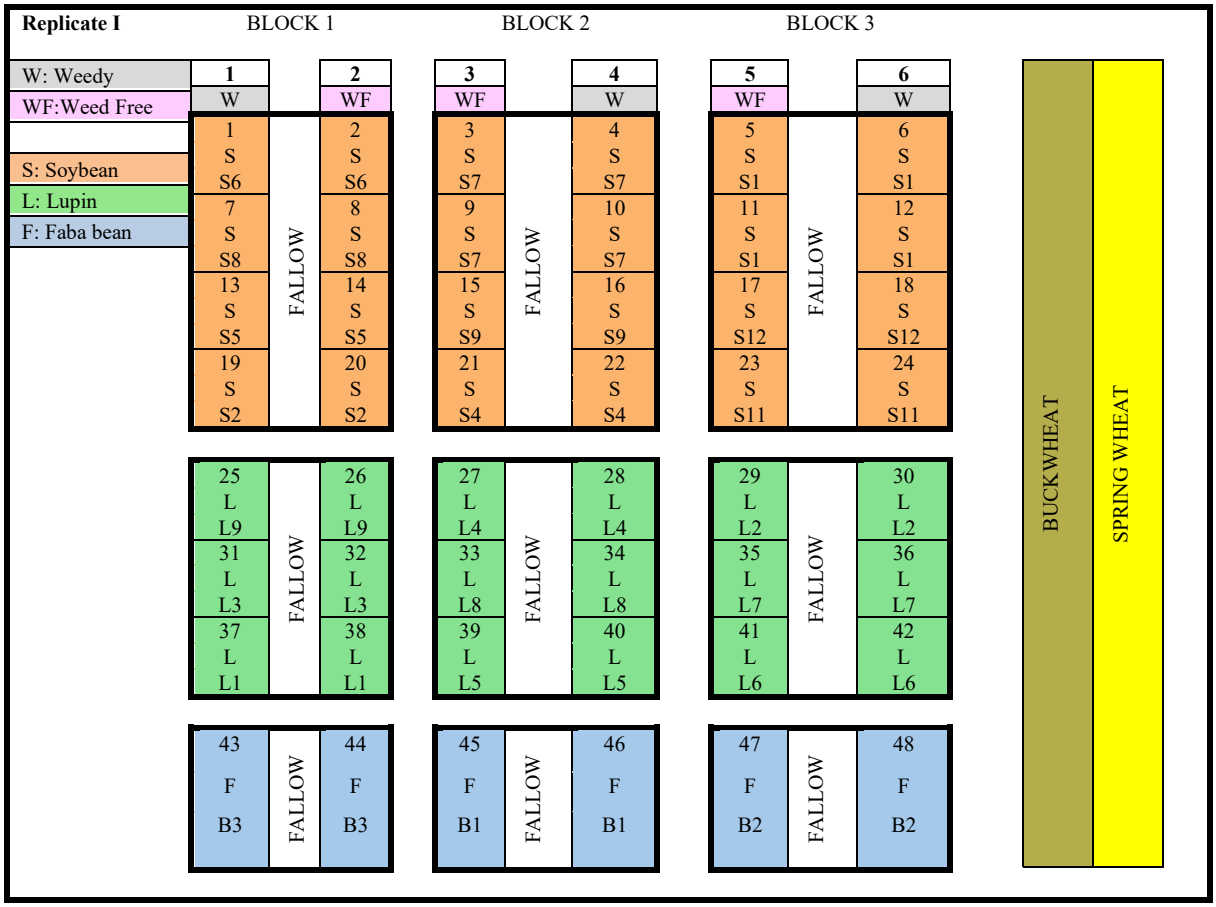
Table 1 summarises the distribution of faba bean, soybean and lupin across the three field sites. Not all species were examined at all sites.

Table 1. Distribution of faba bean (Vicia faba), soybean (Glycine max) and narrow-leaved lupin (Lupinus angustifolius) across the three Swedish field sites (Alnarp, Gotland and Uppsala) included in the 2021 field experiment. X indicates that the species were included at the respective field site.

	Locations		
Species	**Alnarp**	**Gotland**	**Uppsala**
Soybean	X		X
Lupin	X	X	X
Faba bean		X	X

Columns functioned as incomplete blocks to account for spatial heterogeneity across the field. Not all species were represented within every block, which necessitated the use of an incomplete block structure. This design allowed the spatial arrangement of plots to be incorporated into the statistical analysis and was used to define the random-effects structure in the linear mixed models (LMM) described in section 3.4.

Table 2. Experimental field layout for replicate I at Alnarp in the 2021 field trial. The table shows the plot layout, species distribution and weed treatments within replicate I. Species included faba bean (Vicia faba), soybean (Glycine max) and narrow-leaved lupin (Lupinus angustifolius). Weed treatments consisted of weedy (W) and weed-free (WF) conditions. Fallow plots, buckwheat and spring wheat reference crops were included in the experimental design.



3.1.2 Species, seed material and inoculation

Faba bean seeds were not inoculated, as the species is generally able to nodulate effectively with native rhizobia in temperate European soils, and inoculation is not commonly applied under Swedish field conditions (Fogelberg, 2023). Soybean and lupin seeds were inoculated with LiquiFix (Legume Technology Ltd., UK) using the crop-specific formulations. The inoculant was applied to the seeds as part of the sowing procedure, at most 24h before sowing. Colony Forming Unit concentrations of the inoculant were not quantified, as inoculation followed standard manufacturer recommendations rather than experimental adjustment of rhizobial cell density.

Plants were excavated at flowering (BBCH 61–67), and ten taproots per plot were collected and frozen at −20 °C in labelled bags immediately after sampling. All samples used in this thesis were retrieved from frozen storage.

3.2 Analysis of root samples

Root nodule samples consisted of all visible nodules collected from ten taproots per plot. Nodules were counted and weighed on a sample basis, and nodule fresh weight (g) was recorded using an OHAUS Explorer balance with a precision of 0.01 g.

For image-based assessment of nodule traits, samples were photographed using a Nikon camera mounted on a fixed stand at a height of 50 cm. A ruler was included in each image to enable scale calibration in ImageJ (v. 1.53t). Images were converted to 16-bit format, brightness was increased, and images were thresholded to generate high-contrast binary images. The scale was set using the ruler (1 cm), after which nodules were quantified using the Analyze Particles function. Output variables included total nodule area and nodule number. Where overlapping nodules were not correctly separated, the watershed function was applied prior to particle analysis. An example of the image processing results before and after segmentation is shown in Figure 2.

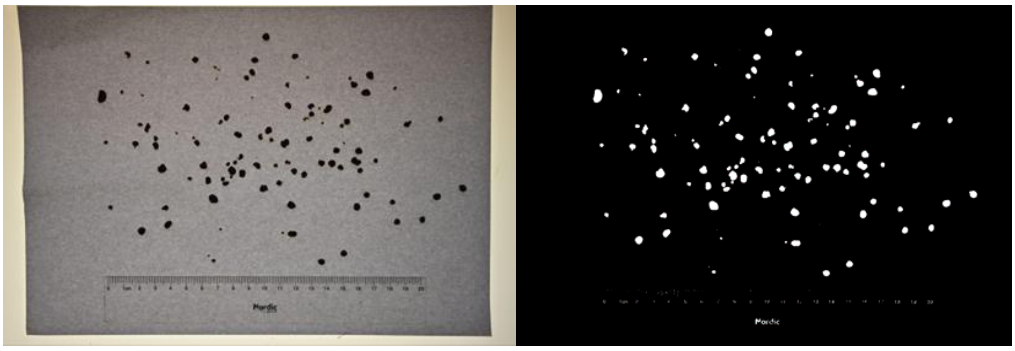


Figure 1. Soybean nodules before and after image processing.

3.3 %Ndfa and mass fraction N (%)

Subsamples of harvested grain and separated biomass, i.e. leaves and stems, from each plot were oven dried, milled, and send for analysis at the Stable Isotope Laboratory (SLU Umeå). The %Ndfa was estimated using the ^{15}N natural abundance method, which is widely applied for assessing SNF in field-grown legumes (Unkovich et al., 2008; Cox, 2022).

The Stable Isotope Laboratory (SLU Umeå) provided measurements of $\delta^{15}N$ and total nitrogen concentration for both legume and reference samples, which were used to calculate %Ndfa. A B value of −2.32‰ was used for faba bean, −1.83‰ for soybean and 6.15‰ for lupin. Mass fraction N (%) represents the total nitrogen concentration in plant tissue, expressed on a dry weight basis.

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of } N_2 \text{ fixing legume}}{\delta^{15}\text{N of reference plant} - B} \times \frac{100}{1}$$

Equation 1. Calculation of %Ndfa using the ^{15}N natural abundance method. B represents the $\delta^{15}N$ value of a fully fixing plant and was taken from the literature for each species (Unkovich et al., 2008). The Stable Isotope Laboratory (SLU Umeå) provided measurements of $\delta^{15}N$ and total nitrogen concentration for plant and reference samples, which were used to calculate %Ndfa. Mass fraction N (%) represents total nitrogen concentration in plant tissue.

3.4 Statistical analysis and visualisation

Data management was carried out using Microsoft Excel (2021) for organising nodule counts, fresh weights and image-derived variables. Some variables, including nodule weights and nodule areas, were stored as comma-separated lists within individual cells. These values were processed in Python (v. 3.x) to calculate summary statistics (number of observations, mean and standard deviation) for each plot sample.

Statistical analyses, including LLMs and analysis of covariance (ANCOVA), were conducted in JMP Pro 17. Models included fixed and random effects, and statistical inference focused on fixed effects relevant to the research questions. A significance level of $\alpha = 0.05$ was applied throughout. For each species (faba bean, soybean and lupin), separate LMMs were fitted for total nodule weight per sample (g) and %Ndfa. Site, weed treatment and their interaction were included as fixed effects, while block and replicate were included as random effects to account for the experimental design and hierarchical field structure. Results are presented as F-tests with associated p-values. Random effects were included to account for background variation among experimental units and were not interpreted biologically. The LMM was specified as:
Response = site + weed treatment + site * weed treatment + random effects (block/replicate).

To examine the relationship between total nodule weight per sample and nodule number, and to test whether this relationship differed among species and sites, ANCOVA was performed. Total nodule weight was used as the dependent variable, nodule number as a continuous covariate, and species and site as categorical factors. The ANCOVA model was specified as:
total nodule weight ~ nodule number * species * site. Descriptive statistics (mean, standard deviation, range and sample size) were calculated to summarise data distribution and are presented in Appendix 1.

Pearson correlation coefficients (r) were calculated to assess the linear relationship between mass fraction N (%) and %Ndfa (%) within each species. Boxplots and scatterplots were used to visualise group differences and relationships among variables.

3.5 Limitations

Several methodological limitations apply to the dataset. Data on %Ndfa were not available for all species × site combinations (e.g. soybean at Uppsala), resulting in uneven sample sizes among species and analyses. Correlation and ANCOVA analyses were therefore conducted using only samples with complete data. The study is based on data from the 2021 growing season only, and no conclusions can be drawn regarding between-year variation. In addition, plot-level soil and climate data were not available and could not be included in the statistical models.

3.6 Organisation of the results

Results are first presented for the effects of site and weed treatment on total nodule weight and %Ndfa within each species. This is followed by analyses of relationships between nodulation traits and nitrogen fixation across species. Finally, overall patterns are summarised to highlight general trends in nodulation and SNF under the studied field conditions.

4. Result

4.1 Effects of site and weed treatment on nodulation and %Ndfa

4.1.1 Faba bean

In faba bean, total nodule weight was not significantly affected by site, weed treatment, or their interaction (Table 3). Numerical variation among sites was observed, with higher median values at Uppsala compared to Gotland and markedly lower values at Alnarp; however, these differences were not statistically supported. These patterns are illustrated in Figure 3.

Similarly, %Ndfa was not significantly influenced by site, weed treatment, or their interaction (Table 4). Median %Ndfa values were slightly higher at Uppsala compared to Alnarp, but without statistical significance. Weed-free and weedy treatments showed largely overlapping distributions for both variables, indicating no consistent treatment-related differences (Figure 3).

Table 3. Effects of site, weed treatment and their interaction on total nodule weight per sample (g) in faba bean analysed using a LMM.

Source	Nparm	DF	DFDen	F ratio	Prob > F
Site	1	1	0.804	28.4588	0.1606
Weed	1	1	2.059	2.5195	0.2499
Site*Weed	1	1	2.079	3.2910	0.2065

Table 4 Effects of site, weed treatment and their interaction on %Ndfa in faba bean analysed using a LMM.

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Weed	1	1	2.026	0.3063	0.6350
Site	2	2	5.732	0.8280	0.4832
Site*Weed	2	2	4.111	0.0661	0.9370

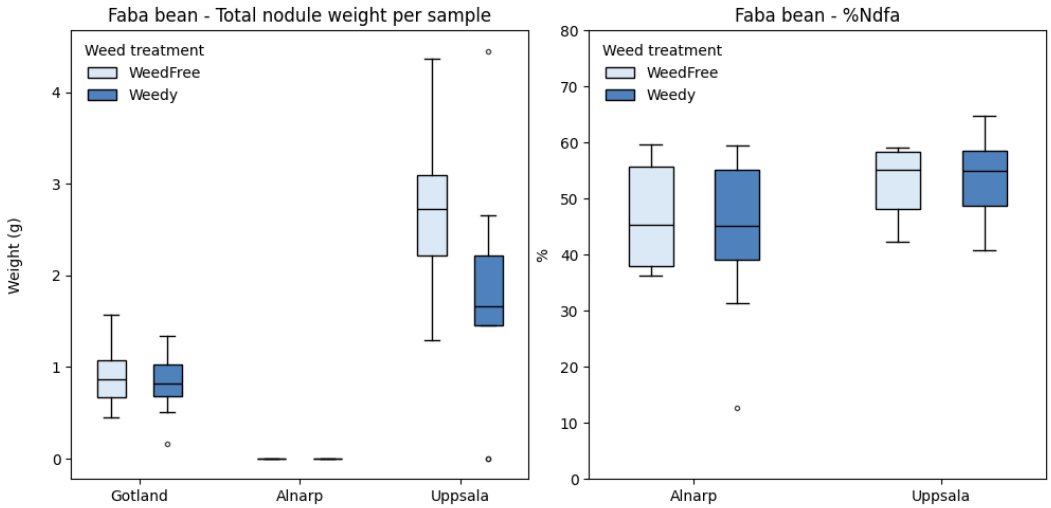


Figure 2. Total nodule weight and %Ndfa in faba bean across sites and weed treatments. Boxplots show total nodule weight per sample at Gotland and Uppsala, and %Ndfa at Alnarp and Uppsala. Circles indicate observations outside the interquartile range.

4.1.2 Soybean

In soybean, total nodule weight showed no significant response to site, weed treatment, or their interaction (Table 5). Numerical differences between sites were observed, with higher median values at Uppsala compared to Alnarp, although these differences were not statistically supported. These patterns are illustrated in Figure 4.

For %Ndfa, measurements were available only at Alnarp; therefore, site effects could not be evaluated. Weed treatment did not significantly influence %Ndfa at this site (Table 6). Weed-free and weedy treatments showed largely overlapping distributions, with no consistent treatment-related differences (Figure 4).

Table 5. Effects of site, weed treatment and their interaction on total nodule weight per sample (g) in soybean analysed using a LMM.

Source	**Nparm**	**DF**	**DFDen**	**F Ratio**	**Prob > F**
Site	1	1	6.229	2.2558	0.1820
Weed	1	1	8.352	2.4570	0.1540
Site *Weed	1	1	7.081	2.3989	0.1649

Table 6. Effect of weed treatment on %Ndfa in soybean at Alnarp analysed using a LMM.

Source	**Nparm**	**Df**	**DfDen**	**F ratio**	**Prob > F**
Weed	1	1	6.317	3.0521	0.1287

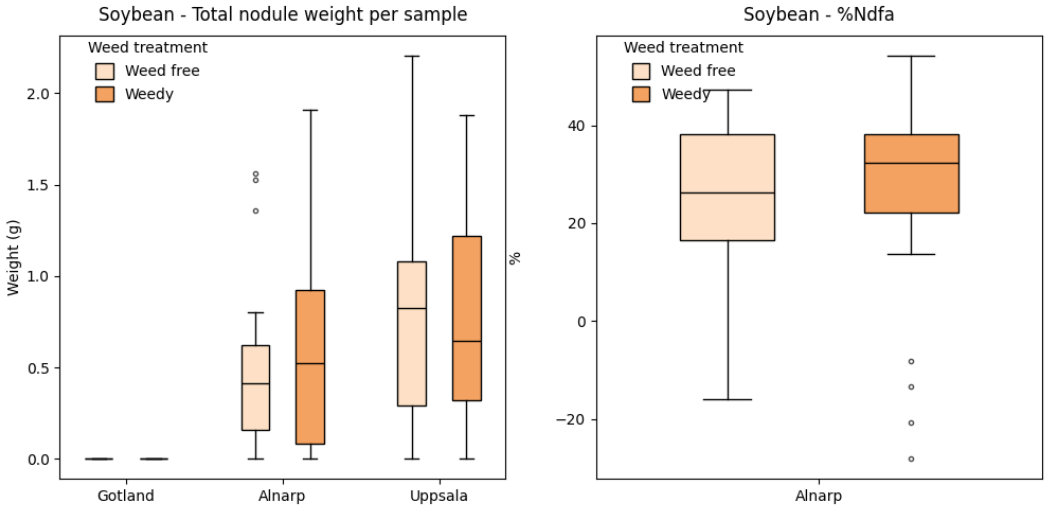


Figure 3. Total nodule weight and %Ndfa in soybean across sites and weed treatments. Boxplots show total nodule weight per sample at Gotland, Alnarp and Uppsala, and %Ndfa at Alnarp. Circles indicate observations outside the interquartile range.

4.1.3 Lupin

In lupin, total nodule weight showed a significant response to site (Table 7), with higher values observed at Uppsala compared with Gotland and Alnarp. Weed treatment and the site × weed interaction did not significantly influence total nodule weight. These patterns are illustrated in Figure 5.

For %Ndfa, measurements were available only at Uppsala; therefore, site effects could not be evaluated. Neither weed treatment nor site × weed interaction significantly affected %Ndfa (Table 8). Weed-free and weedy treatments showed largely overlapping distributions, with no consistent treatment-related differences (Figure 5).

Table 7. Effects of site, weed treatment and their interaction on total nodule weight per sample (g) in lupin analysed using a LMM.

Source	**Nparm**	**DF**	**DFDen**	**F ratio**	**Prob > F**
Site	2	2	1.292	76.7544	0.0454*
Weed	1	1	15.3	0.1275	0.7259
Site *Weed	2	2	7.252	0.0149	0.9853

Table 8. Effects of site, weed treatment and their interaction on %Ndfa in lupin analysed using a LMM.

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Weed	1	1	7.218	0.5685	0.4747
Site	1	1	7.829	0.3961	0.5470
Site * Weed	1	1	10.29	0.2867	0.6037

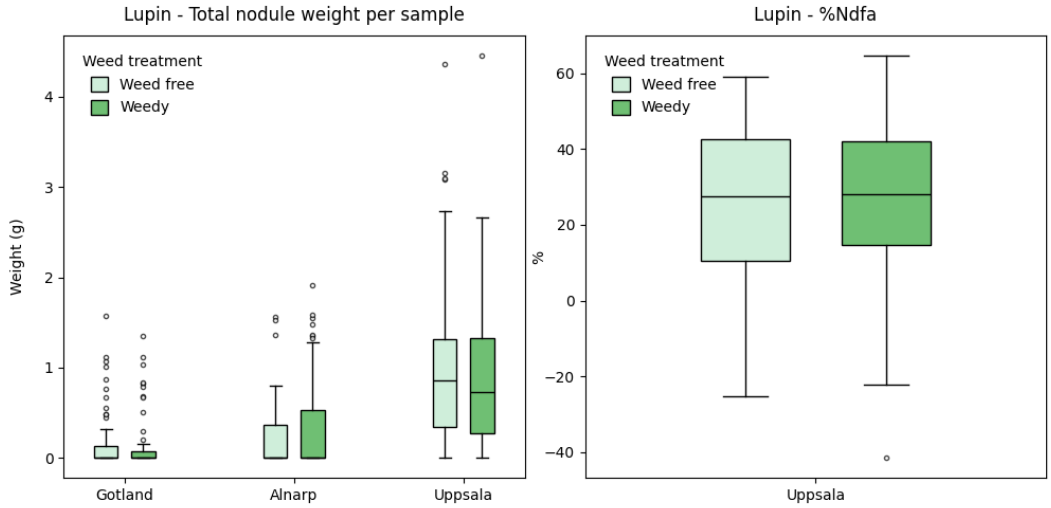


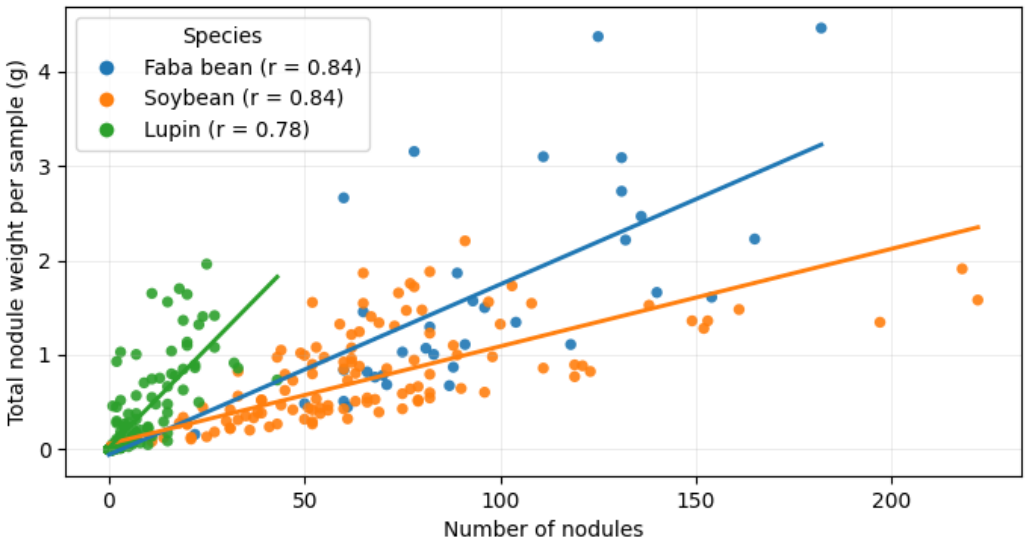
Figure 4. Total nodule weight and %Ndfa in lupin across sites and weed treatments. Boxplots show total nodule weight per sample at Alnarp, Gotland and Uppsala, and %Ndfa at Uppsala. Circles indicate observations outside the interquartile range.

4.2 Species-specific relationships between nodulation traits and nitrogen fixation

4.2.1 Structural patterns in nodulation: nodule number and biomass

Total nodule weight was strongly associated with nodule number and species. Species ($F = 95.27$, $p < 0.0001$) and nodule number ($F = 802.30$, $p < 0.0001$) had significant main effects, and a significant interaction between nodule number and species ($F = 64.38$, $p < 0.0001$) indicated species-specific differences in scaling between nodule number and total nodule biomass.

Across all species, total nodule weight increased with increasing nodule number ($r = 0.63$, $p < 0.0001$). When analysed separately, strong positive correlations were observed for faba bean ($r = 0.84$), soybean ($r = 0.84$), and lupin ($r = 0.78$). The slope of the relationship was steepest in faba bean, intermediate in lupin, and flattest in soybean (Figure 6).



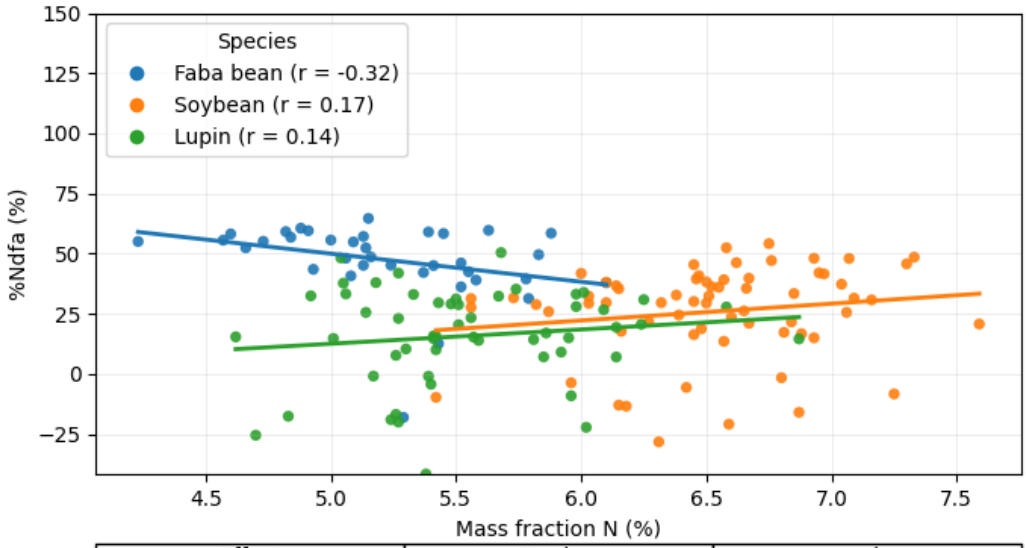
Effect	F-value	p-value
Species	95.271	0.0
Nodules	802.295	0.0
Nodules × Species	64.375	0.0
Residual	nan	nan

Figure 5. Nodule number and total nodule weight across species. Scatterplots show the relationship between nodule number and total nodule weight per sample (g) for faba bean, soybean and lupin. Lines represent fitted linear regressions, and Pearson correlation coefficients (r) are shown in the legend.

4.2.2 Plant nitrogen status and fixation efficiency

Species had a significant effect on %Ndfa ($F = 32.37$, $p < 0.0001$), whereas mass fraction N (%) did not ($F = 0.72$, $p = 0.398$). The interaction between species and mass fraction N was not significant ($F = 2.40$, $p = 0.095$), indicating no evidence of species-specific differences in slope.

Associations between plant nitrogen concentration and %Ndfa were weak (Figure 7). Faba bean showed a moderate negative correlation ($r = -0.32$), while soybean ($r = 0.17$) and lupin ($r = 0.14$) showed weak positive correlations. Overall, plant nitrogen concentration was not strongly associated with the proportion of nitrogen derived from atmospheric fixation.



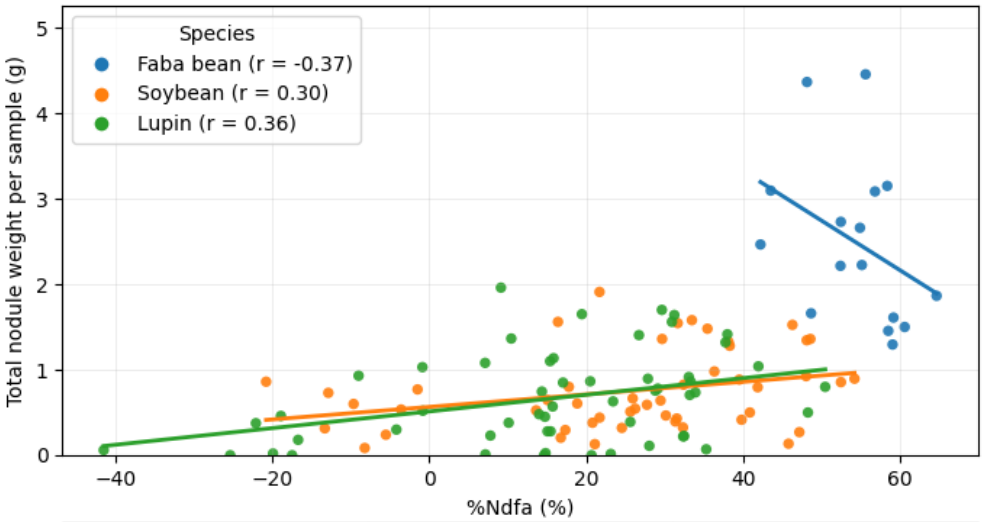
Effect	F-value	p-value
Species	32.371	0.0
Mass fraction N	0.72	0.398
Mass fraction N × Species	2.395	0.095
Residual	nan	nan

Figure 6. Mass fraction N (%) and %Ndfa across species. Scatterplots show the relationship between faba bean, soybean and lupin. Lines represent ordinary least squares regressions fitted separately for each species. Pearson correlation coefficients (r) are shown in the legend. Results from an ANCOVA testing for species-specific differences in the relationship are indicated.

4.2.3 Nodule biomass as a predictor of nitrogen fixation

Species ($F = 72.94$, $p < 0.0001$) and %Ndfa ($F = 7.39$, $p = 0.008$) had significant effects on total nodule weight. In addition, the interaction between %Ndfa and species was significant ($F = 4.25$, $p = 0.017$), indicating that the relationship between nitrogen fixation and nodule biomass differed among species.

Across species, correlations varied in direction (Figure 8). In faba bean, total nodule weight was negatively associated with %Ndfa ($r = -0.37$), whereas positive associations were observed in soybean ($r = 0.30$) and lupin ($r = 0.36$). These patterns demonstrate contrasting species-specific associations between %Ndfa and total nodule weight.



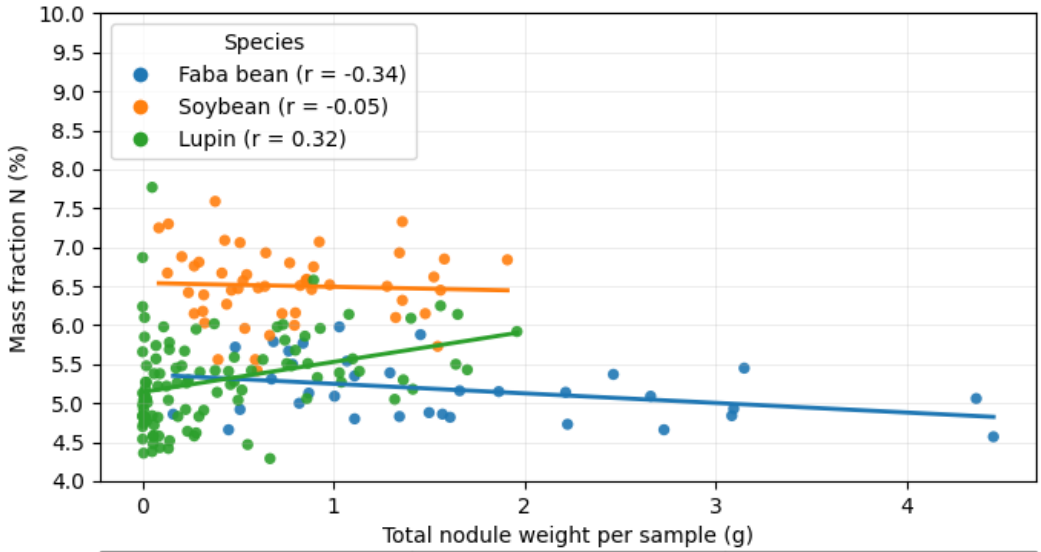
Effect	F-value	p-value
Species	72.941	0.0
%Ndfa	7.394	0.008
%Ndfa × Species	4.249	0.017
Residual	nan	nan

Figure 7. %Ndfa and total nodule weight across species. Points represent individual plot samples for faba bean, soybean, and lupin. Solid lines indicate species-specific linear regressions. Pearson correlation coefficients (r) are shown for each species. Results from an ANCOVA testing the effects of species, %Ndfa, and their interaction on total nodule weight are presented below the plot.

4.2.4 Nitrogen concentration and nodulation investment

Species had a significant effect on mass fraction N ($F = 109.92$, $p < 0.0001$), whereas total nodule weight alone did not ($F = 0.78$, $p = 0.379$). However, the interaction between species and total nodule weight was significant ($F = 7.69$, $p = 0.001$), indicating species-specific differences in the relationship.

Species-specific correlations showed contrasting patterns (Figure 9). Total nodule weight was negatively associated with mass fraction N in faba bean ($r = -0.34$) and soybean ($r = -0.05$), whereas a positive association was observed in lupin ($r = 0.32$).



Effect	F-value	p-value
Species	109.922	0.0
Total nodule weight	0.778	0.379
Total nodule weight × Species	7.685	0.001
Residual	nan	nan

Figure 8. Mass fraction N (%) and total nodule weight across species. Scatterplots show the relationship between faba bean, soybean and lupin. Lines represent ordinary least squares regressions fitted separately for each species, and Pearson correlation coefficients (r) are reported in the legend. Results from an ANCOVA testing for species-specific differences in the relationship are shown below the figure.

4.3 Summary of results

Across the three legume species, weed treatment had no significant effect on total nodule weight or %Ndfa, and no consistent weed-related responses were detected. Site effects differed among species. In faba bean and soybean, neither total nodule weight nor %Ndfa was significantly influenced by site, whereas lupin exhibited a significant site effect on total nodule weight, with higher values at Uppsala compared to Gotland and Alnarp.

Across species, total nodule weight increased with increasing nodule number, although the strength of this association differed among species, being strongest in faba bean and soybean and weakest in lupin. Associations between plant nitrogen status and SNF were generally weak. While mass fraction N (%) was not significantly related to %Ndfa, species-specific differences emerged in the relationship between %Ndfa and total nodule weight, which was negative in faba bean but positive in soybean and lupin. A similar pattern was observed between total nodule weight and mass fraction N (%), with negative associations in faba bean and soybean and a positive association in lupin.

5. Discussion

In this section, the results are discussed in relation to the following research questions. The discussion is structured to address these questions in sequence.

1. How are nodule number and total nodule biomass related to the amount of nitrogen fixed?
2. To what extent does weed management influence nodulation and SNF?
3. How do field sites affect nodulation and nitrogen fixation capacity?
4. Do species differ in their responses to weed management and site conditions?

5.1 Relationship between nodule development and nitrogen fixation

The main finding was that nodule biomass was not consistently associated with SNF under Swedish field conditions. Although total nodule weight increased with nodule number in all species, higher nodule biomass was not consistently linked to higher %Ndfa, and Hypothesis 1 was therefore not supported.

Similar discrepancies between nodulation traits and fixation outcomes have been reported under field conditions, where structural characteristics explain only part of the variability in SNF (Zimmer et al., 2016; Salvagiotti et al., 2008). This suggests that nodule size or number alone may provide limited insight into functional fixation under heterogeneous field environments.

The relationship between nodulation and atmospheric nitrogen dependence is influenced by plant nitrogen status and environmental context (Jensen et al., 2010; Peoples et al., 2009). The absence of a consistent structural–functional association in the present study may therefore reflect regulated fixation rather than a strictly proportional response to nodulation intensity (Ciampitti & Salvagiotti, 2018; Ferguson et al., 2019). Nitrogen fixation responds to host nitrogen demand and carbon availability, and nodules can remain present even when nitrogenase activity is reduced (Udvardi & Poole, 2013; Sickerman et al., 2019). From this perspective, the negative association observed in faba bean ($r = -0.37$) could be consistent with situations in which greater access to soil nitrogen reduces proportional atmospheric dependence (Thies et al., 1991; Salvagiotti et al., 2008). However, without direct measurements of soil nitrogen uptake or absolute fixation rates, this interpretation remains tentative.

Interpretation is further shaped by the response variable. Because %Ndfa reflects relative nitrogen source partitioning rather than absolute fixation (Unkovich et al., 2008), shifts in soil nitrogen uptake may alter proportional atmospheric

dependence even if total fixation changes little. Under heterogeneous field conditions, the ^{15}N natural abundance method may also dampen detectable associations between nodulation and fixation (Unkovich et al., 2008). Unequal species × site representation in the dataset adds further uncertainty when comparing slopes across species.

Species-specific patterns reinforce the complexity of the structural–functional relationship. Soybean showed weak associations between nodule biomass and %Ndfa, which is consistent with documented autoregulation of nodulation and fixation (Ferguson et al., 2019). Differences in nodule type may also contribute to the observed variability. Determinate nodules, such as those of soybean, develop and senesce synchronously, whereas indeterminate nodules in faba bean and lupin maintain developmental gradients with zones of differing activity (Oldroyd et al., 2011; Udvardi & Poole, 2013). Structural measurements at a single growth stage may therefore integrate nodules with variable physiological activity, potentially obscuring earlier or transient differences in fixation.

Taken together, the findings suggest that structural nodulation traits were not consistently aligned with proportional atmospheric nitrogen dependence in this study. Rather than indicating an absence of functional relevance, the results may reflect the interaction between regulation, nitrogen availability and methodological constraints documented in previous field-based assessments (Salvagiotti et al., 2008; Unkovich et al., 2008).

5.2 Effect of weed competition on nodulation and nitrogen fixation

Weed management was not statistically associated with variation in total nodule weight or %Ndfa in any of the three species, and Hypothesis 2 was therefore not supported. This differs from experiments conducted under high weed density or strong shading, where reductions in nodulation and nitrogen fixation have been reported (Khalifa et al., 2020; Piekarczyk, 2023). In such systems, competition may reduce light interception and carbon assimilation, thereby limiting the assimilate supply required to sustain nitrogen fixation (Minchin & Witty, 1997). The absence of statistically supported differences in the present study may therefore reflect lower competitive intensity relative to those conditions.

Because weed biomass and shading were not quantified, the magnitude of competitive pressure cannot be directly compared with previous studies. Nitrogen fixation represents a substantial carbon investment for the host plant (Salvagiotti et al., 2008), yet moderate reductions in assimilate supply may not necessarily lead to proportional declines in fixation if plant nitrogen demand remains high

(Ferguson et al., 2019). Legumes regulate fixation in response to internal nitrogen status and external nitrogen availability (Ciampitti & Salvagiotti, 2018; Ferguson et al., 2019), which may buffer both structural nodulation and proportional atmospheric nitrogen dependence under moderate stress.

At the same time, the absence of statistically significant effects does not necessarily indicate the absence of biological responses. Under field conditions, spatial heterogeneity in soil nitrogen availability and microclimate can reduce statistical sensitivity (Zimmer et al., 2016; Unkovich et al., 2008). Because %Ndfa reflects relative nitrogen source partitioning rather than absolute nitrogen accumulation (Unkovich et al., 2008), shifts in total plant growth or soil nitrogen uptake may occur without detectable changes in proportional atmospheric dependence (Salvagiotti et al., 2008). It is therefore possible that weed competition influenced aboveground biomass or carbon assimilation without proportionally altering nitrogen source partitioning, although such relationships were not directly analysed here.

Sampling at BBCH 61–67 represents a single developmental stage. Early competitive effects on nodule initiation or carbon allocation may therefore not be reflected in nodule biomass measured at flowering. Differences in nodule developmental patterns between determinate and indeterminate types (Oldroyd et al., 2011) may also influence how rapidly structural or functional responses become detectable.

Environmental background conditions may have further moderate competitive effects. Early-season soil temperatures typical of Nordic systems can constrain rhizobial infection and nitrogen fixation (Schmidt et al., 2015; Fogelberg & Mårtensson, 2021). If fixation was already operating under temperature-related constraints, additional carbon competition may have had limited marginal influence on proportional nitrogen dependence.

Together, the findings did not statistically support differences in nodulation or proportional atmospheric nitrogen dependence under the studied field conditions. However, the lack of quantified weed intensity and multi-stage measurements limits detection of subtle or transient effects on nitrogen fixation.

5.3 How do the field sites (Uppsala, Alnarp, Gotland) affect nodulation and nitrogen fixation capacity?

Site was statistically associated with variation in total nodule biomass in lupin, whereas faba bean and soybean did not show statistically supported differences among locations. This pattern provides partial support for Hypothesis 3, as site-related variation was observed for nodulation in lupin but not in the other species, and site effects were not consistently reflected in %Ndfa. Environmental context was therefore related to structural nodulation in a species-dependent manner.

Soil temperature, moisture, pH and mineral nitrogen availability are known regulators of early symbiotic establishment and nodule development (Zahran, 1999; Schmidt et al., 2015). Because nodulation requires substantial carbon investment, variation in site-specific growth conditions may influence carbon allocation to the symbiosis (Salvagiotti et al., 2008; Ferguson et al., 2019). The stronger structural response observed in lupin may be consistent with reports describing its sensitivity to soil chemical constraints and nutrient imbalances (Birchall et al., 1995; Huyghe, 1998; Ding et al., 2019). However, detailed plot-level soil measurements were not available, and the relative contribution of individual edaphic factors cannot be determined.

Despite structural differences in lupin, site was not consistently associated with differences in %Ndfa. This pattern aligns with the observation in Section 5.1 that structural nodulation does not necessarily correspond to proportional atmospheric nitrogen dependence. Nitrogen fixation is regulated by whole-plant nitrogen status and carbon supply (Ferguson et al., 2019), and fixation efficiency per unit nodule mass depends on internal physiological processes rather than nodule size alone (Minchin & Witty, 1997; Udvardi & Poole, 2013). It is therefore possible that environmental variation influenced early symbiotic establishment without proportionally altering nitrogen source partitioning at the time of sampling.

Because nodulation and %Ndfa were assessed at a single developmental stage, potential temporal differences in fixation dynamics among sites were not captured. In addition, incomplete species × site coverage limits direct comparison of functional responses across all locations.

Together, the findings indicate that site variation was more clearly reflected in structural nodulation than in proportional atmospheric nitrogen dependence under the examined field conditions. However, the absence of detailed soil and microclimatic data constrains mechanistic interpretation of the observed site-specific patterns.

5.4 Species-specific regulatory responses

Species differed in their responses to site conditions, whereas no species showed a statistically supported response to weed management. Hypothesis 4 was therefore supported with respect to site effects but not weed competition.
Beyond differences in site responsiveness, species also differed in how nodule biomass is related to %Ndfa. The significant species × %Ndfa interaction indicates that the association between structural investment and proportional atmospheric nitrogen dependence varied among species rather than following a uniform pattern. This reinforces the broader observation in Sections 5.1–5.3 that nodulation intensity alone did not consistently explain functional fixation outcomes.

In faba bean, total nodule biomass was negatively associated with %Ndfa. Because %Ndfa reflects relative nitrogen source partitioning rather than absolute fixation (Unkovich et al., 2008), proportional atmospheric contribution may decline if soil nitrogen uptake increases (Salvagiotti et al., 2008). The concurrent negative association between nodule biomass and mass fraction N (%) is consistent with growth-related nitrogen dilution (Salvagiotti et al., 2008; Peoples et al., 2009). This pattern may reflect greater soil nitrogen uptake in more vigorous plants, altered nitrogen allocation, or regulatory adjustment of fixation relative to plant nitrogen status (Ferguson et al., 2019), rather than reduced symbiotic capacity per se. However, absolute fixation and soil nitrogen uptake were not directly quantified, and alternative interpretations cannot be excluded.

Soybean exhibited a weak positive association between nodule biomass and %Ndfa. Such limited structural–functional coupling is consistent with documented autoregulatory control of nodulation and fixation (Ferguson et al., 2019). Determinate nodules and systemic nitrogen feedback mechanisms may buffer structural variation, resulting in relatively stable proportional atmospheric dependence (Oldroyd et al., 2011; Ciampitti & Salvagiotti, 2018). Alternatively, the weak association may reflect regulatory adjustment of fixation relative to soil nitrogen uptake, resulting in limited variation in proportional atmospheric dependence (Ferguson et al., 2019; Ciampitti & Salvagiotti, 2018).

Lupin displayed a moderate positive association between nodule biomass and %Ndfa, suggesting a closer alignment between structural investment and proportional fixation under the studied conditions. Lupin has been reported to achieve substantial atmospheric reliance under favourable environmental conditions (Lucas et al., 2015; Huyghe, 1998), although nodulation in the present study was sensitive to site variation (Ding et al., 2019).

The observed pattern may therefore reflect interaction between species-specific physiological regulation and environmental responsiveness rather than structural investment alone.

Across Sections 5.1–5.4, structural nodulation traits were variably related to proportional atmospheric nitrogen dependence, and this relationship appeared to depend on species identity and environmental context rather than nodulation intensity alone. Nodule biomass was not consistently associated with fixation performance under the studied field conditions. Because the dataset covers only one growing season (2021), it cannot be determined whether these species-specific relationships would remain similar under contrasting climatic conditions and soil nitrogen dynamics.

6. Conclusions

This study indicates that species identity and site-related environmental variation exerted a stronger influence on nodulation and SNF than weed management under the studied Nordic field conditions. As no statistically supported weed effects were detected, interspecific differences and geographic context accounted for most of the observed variation, with structural site effects most evident in lupin.

Across species, structural nodulation traits were not consistent predictors of functional nitrogen fixation. Increases in total nodule biomass were not uniformly associated with higher %Ndfa, suggesting that dependence on atmospheric nitrogen is regulated rather than strictly proportional to nodulation intensity. Fixation patterns instead reflected species-specific regulatory strategies interacting with nitrogen availability and environmental conditions. Faba bean exhibited relatively stable proportional fixation despite variation in nodulation, lupin showed environmentally responsive nodulation without consistent shifts in %Ndfa, and soybean displayed weak structural–functional coupling consistent with documented autoregulatory control. These contrasting responses suggest that species physiology influences how environmental factors translate into functional dependence on atmospheric nitrogen**.**

Overall, the results indicate that nodule biomass alone did not explain variation in nitrogen fixation under the studied field conditions, and that species identity and environmental context played a larger role. Although the study was conducted within a single growing season, the findings underscore that reliable evaluation of biological nitrogen fixation require**s** a functional assessment beyond structural nodulation traits**.** Strengthening functional assessment under realistic field conditions can enable more precise legume selection and site-adapted management strategies in Swedish agriculture, thereby supporting increased legume integration and contributing to a substantial reduction in reliance on external nitrogen fertilisers.

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Popular science summary

Nitrogen is essential for crop production, but modern agriculture often depends on mineral fertilisers that require a lot of energy to produce and can contribute to environmental problems. Grain legumes such as faba bean, soybean and lupin can obtain nitrogen directly from the air. They do this through a natural partnership with soil bacteria that live in small nodules on their roots. Together, the plant and the bacteria convert atmospheric nitrogen into a form the plant can use; a process known as biological nitrogen fixation.

In this study, nodulation and nitrogen fixation were examined in faba bean, soybean and narrow-leaf lupin grown under Swedish field conditions. Field experiments were carried out in 2021 at three locations in Sweden. The crops were grown under organic management, with plots kept either weed-free or weedy. Root nodules were measured, and the amount of nitrogen obtained from the air by the plants was estimated.

Differences in nodulation and nitrogen fixation were observed between the three legume species. In faba bean, nodulation and nitrogen fixation did not differ between sites or weed treatments. Soybean showed a similar pattern for nodulation. Nitrogen fixation in soybean was measured at one site only, which meant that differences between locations could not be evaluated. Lupin differed from the other species by showing differences in nodulation between sites. Higher total nodule weight was observed at one location, while very low nodulation was recorded at the other sites. Weed management did not result in detectable differences in nodulation or nitrogen fixation in any of the species studied.

Relationships between nodulation traits and nitrogen fixation were also examined. In all species, higher nodule numbers were associated with higher total nodule weight, while associations with nitrogen fixation varied between species and sites.

Overall, the text summarises how nodulation and nitrogen fixation were measured and how these traits varied among legume species grown under Swedish field conditions.

Appendix 1

Faba bean

Summary statistics for total nodule weight per sample (g) in faba bean across sites and weed treatments. Values are presented as mean, standard deviation (StdDev), minimum (Min), maximum (Max), and number of observations (N) for each site × weed treatment combination.

Site	Weed	Mean	StdDev	Min	Max	N
Gotland	WeedFree	0,885	0,361775	0,43	1,59	9
Gotland	Weedy	0,818333	0,347176	0,155	1,38	9
Uppsala	WeedFree	2,653889	0,922675	1,14	4,25	9
Uppsala	Weedy	2,01375	1,25851	0	4,425	8

Summary statistics for %Ndfa in faba bean across sites and weed treatments. Values are presented as mean, standard deviation (StdDev), minimum (Min), maximum (Max), and number of observations (N) for each site × weed treatment combination.

Site	Weed	Mean	StdDev	Min	Max	N
Alnarp	WeedFree	40,36043	23,52806	-17,9688	59,73054	9
Alnarp	Weedy	43,31278	14,73726	12,65625	59,58084	9
Uppsala	WeedFree	52,78303	6,637603	42,23013	59,18592	9
Uppsala	Weedy	53,49946	7,599718	40,80664	64,68647	9

Soybean

Summary statistics for total nodule weight per sample (g) in soybean across sites and weed treatments. Values are presented as mean, standard deviation (StdDev), minimum (Min), maximum (Max), and number of observations (N) for each site × weed treatment combination.

Site	Weed	Mean	StdDev	Min	Max	N
Alnarp	Weed Free	0,431111	0,309452	0,03	1,545	27
Alnarp	Weedy	0,688	0,528334	0,02	2,245	28
Uppsala	Weed Free	0,919333	0,531113	0,125	2,215	30
Uppsala	Weedy	0,891833	0,513389	0,11	1,885	30

Summary statistics for %Ndfa in soybean at Alnarp under weed-free and weedy conditions. Values are presented as mean, standard deviation (StdDev), minimum (Min), maximum (Max), and number of observations (N) for each weed treatment.

Site	Weed	Mean	StdDev	Min	Max	N
Alnarp	WeedFree	23,34354	18,41686	-15,8683	47,1875	31
Alnarp	Weedy	28,26691	19,56159	-28,1437	54,21875	34

Lupin

Summary statistics for total nodule weight per sample (g) in lupin across sites and weed treatments. Values are presented as mean, standard deviation (StdDev), minimum (Min), maximum (Max), and number of observations (N) for each site × weed treatment combination.

Site	Weed	Mean	StdDev	Min	Max	N
Alnarp	WeedFree	0,0075	0,015321	0	0,05	10
Alnarp	Weedy	0,009167	0,012007	0	0,03	6
Gotland	WeedFree	0,13775	0,154815	0	0,57	24
Gotland	Weedy	0,123778	0,22346	0	1	27
Uppsala	WeedFree	0,676852	0,506134	0	1,645	27
Uppsala	Weedy	0,668148	0,567563	0	1,95	27

Summary statistics for %Ndfa in lupin at Uppsala under weed-free and weedy conditions. Values are presented as mean, standard deviation (StdDev), minimum (Min), maximum (Max), and number of observations (N) for each weed treatment.

Site	Weed	Mean	StdDev	Min	Max	N
Uppsala	WeedFree	16,535	18,72657	-25,3855	48,27995	27
Uppsala	Weedy	15,22322	21,13076	-41,5184	50,47962	27

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