Effect of different inoculation methods in modern and historic cultivars of soybean suitable for cultivation in Sweden

Sabina Juhlin Muñoz

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Swedish University of Agricultural Sciences, SLU
Department of Crop Production Ecology
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Supervisor:	**Fede Berckx, Swedish University of Agricultural Sciences, Department of Crop Production Ecology**
Assistant supervisor:	Alexander Menegat, Swedish University of Agricultural Sciences, Department of Crop Production Ecology
Examiner:	Martin Weih, Swedish University of Agricultural Sciences, Department of Crop Production Ecology

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Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Aquatic Science and Assessment

Abstract

The inclusion of soybean in Swedish crop rotations would enable an increase in legume production in Sweden. Previous attempts to introduce soybean as a crop in Sweden have failed, but in recent years, new attempts have been made under experimental conditions. Soybean cultivars suitable for cultivation in Sweden belong to maturity group (MG) 000, as well as historically Swedish-bred soybean cultivars, such as Bråvalla. However, for these cultivars to fix N, compatible rhizobia such as *Bradyrhizobium japonicum* are needed. These are not native to Swedish soils and have to be introduced through inoculation. Inoculating seeds can have several beneficial effects on soybean traits, but this varies with the interaction between soybean cultivar, rhizobial strain- or species and environmental factors. Inoculants can have different formulations which have different properties and may affect rhizobial survival and inoculation effect. The aim of this thesis was to evaluate the effects the inoculation methods no-, liquid- or peat inoculation had on phenotypic traits and N-fixation in soybean cultivars suitable for cultivation in Swedish climate in both field- and greenhouse conditions. The peat- and liquid inoculants containing *B. japonicum*, as well as uninoculated control, were tested in a field trial on Gotland, Sweden, on the soybean cultivars Abaca, Gallec, Sussex, and Todeka (all MG 000), and in a greenhouse experiment in Uppsala, Sweden, on the cultivars Abaca, Gallec, Sussex and Bråvalla (historic). Inoculation of the seeds resulted in higher N content and %Ndfa in both the field trial (significant increase) and the greenhouse experiment compared to the uninoculated plants. The peat – and liquid formulations did not differ in performance in the greenhouse experiment, but the peat inoculant did overall perform better than the liquid inoculant in the field trial. This may be an effect of the protective properties of peat supporting rhizobial survival in field conditions. Inoculation of the seeds did not have a significant effect on the traits: plant height, height of the lowest node, leaf biomass, or N content in the leaves at flowering (field trial). However, inoculation significantly affected: TKW, yield, root biomass, stem biomass, root-/ stem biomass ratio, nodule weight, nodule number, and protein yield compared to uninoculated plants. The responses to the inoculants varied between the environments and between cultivars. In comparisons between the greenhouse experiment and the field trial, the traits affected by rhizobial survival (N content, nodule number and nodule weight) were enhanced in the greenhouse experiment, while traits probably limited by light intensity (TKW and yield) were superior in the field trial. The results showed that the interaction between cultivar x inoculation method had a significant effect on: root biomass, stem biomass, root-/stem biomass ratio, nodule number, nodule weight (GH), TKW, yield (GH), N content in biomass and seeds and %Ndfa in biomass and seeds. This thesis demonstrated that peat inoculation was superior over liquid inoculation in Swedish field conditions and that the MG 000 cultivar Sussex in comibination with LegumeFix had the greatest potential for generating high yield and protein yield out of the tested combinations, hence being suggested to Swedish farmers wanting to try soybean cultivation. However, further research is needed to confirm these results.

Keywords: Soybean, *Glycine Max*, Sweden, *B. japonicum*, inoculation, phenotypic traits, nitrogen fixation, nodulation, liquid inoculum, peat inoculum.

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Abbreviations and glossary

BNF	Biological N-fixation – the process where atmospheric N_2 is turned into NH_3 by bacteria which can produce the enzyme nitrogenase.
CFU	Colony forming unit – an estimate of the number of viable cells able to form colonies.
FT	Field trial
GH	Greenhouse
GLMM	Generalized linear mixed-effects model
Leghemoglobin	A protein produced during symbiosis between a legume and N-fixing bacteria which buffers the O_2 concentration in nodules and gives the nodules a red color.
LMM	Linear mixed-effects model
MG	Maturity group – of soybean cultivars divided into MG 000 (early maturation) to MG X (late maturation).
^{15}N	A stable isotope of N with a concentration of 0.3663 atom% in the atmosphere.
Ndfa	N derived from atmospheric N_2 – can be decided by measuring the ^{15}N in a plant sample.
Nitrogenase	An enzyme catalysing the process where atmospheric N_2 is converted into NH_3.
Nodules	Organs where N-fixation occurs, developing on legume roots as a result of symbiosis with N-fixing bacteria.
Rhizobia	Bacteria able to fix N from the atmosphere in symbiosis with legumes.
RS ratio	Root biomass to stem biomass ratio
SNF	Symbiotic nitrogen fixation – a mutualistic relationship where N-fixing bacteria provide a plant with NH_3 and the plant provide the bacteria with fixed C.
TKW	Thousand kernel weight

1. Introduction

Legumes are plants able to fix dinitrogen (N_2) from the air, benefit biodiversity and diversify cropping systems, for example as a break crop in cropping systems dominated by cereals in the crop rotation (Fogelfors 2015; Blom 2022; Fang & Kong 2022). Sweden has roughly 2.5 million ha of arable land, out of which cereals were cultivated on about 962 000 ha and legumes on only 47 500 ha in 2022 (Karlsson 2023). The most cultivated grain legume crops in Sweden are peas (*Pisum sativum)* and faba bean (*Vicia faba)* (Karlsson 2023). According to a report from The Swedish Board of Agriculture (Blom 2022) there is potential to increase the production of legumes in Sweden, for example by replacing the importation of plant protein (mainly soya products for feed) with Swedish-grown legumes, which would require an increase of 140 000 ha of peas and faba bean cultivation. But the fact that there are two grain legume species dominating the legume cultivation in Sweden becomes a problem since both species propagate the same diseases, such as root rot of the peas (*Aphanomyces euteiches*) and root rot (*Phytophtora pisi*). Consequently, the crops are suggested to only appear once every seven to eight years in the crop rotation (Fogelfors 2015; Blom 2022; Jordbruksverket 2023). Therefore, it is of interest to diversify the species of legumes grown in Sweden, both to be able to grow them more often during a rotation and to increase the production of plant protein in the country. Soybean (*Glycine max* (L.) Merr.) is a crop cultivated for feed, food, green manure and biofuel that has the potential to be included in Swedish crop rotations (Gustafsson et al. 2013; Fogelberg 2021; Blom 2022). Additionally, soybean is not a host to the same problematic root diseases as peas and faba bean which would make it possible for farmers to cultivate grain legumes more often (Shang et al. 2000; Heyman et al. 2013; Pfender & Hagedorn 1982). However, to facilitate an increase of soybean production in Sweden, there is a need to further investigate agronomic practices for the crop in Swedish growing conditions (Gustafsson et al. 2013; Fogelberg 2021; Blom 2022).

1.1 Soybean

Soybeans are annual, self-pollinating plants with purple or white flowers depending on cultivar (Fogelfors 2015). They are legumes of the family Fabaceae, originating from East Asia with a domestication history dating back thousands of years and are

cultivated for food, animal feed, oil, fiber and biofuel (Day 2013; Britannica 2024). Soybean seeds have a high protein content of 35-40 % and also contain about 20 % fat, which in combination with a well-balanced amino acid profile makes the seeds good for human consumption, as meat- and dairy alternatives, as well as for animal feed (Day 2013).

In 2022 the five biggest producers of soybean in the world were Brazil, the USA, Argentina, India and China, together cultivating near 115 million ha of soybean (FAOSTAT 2023). In Europe the production of soybean is much smaller, the countries with the largest harvested areas in 2022 were Ukraine, Italy, Serbia, France and Romania, together cultivating soybean on about 2.4 million ha (FAOSTAT 2023). Even though soybean has been cultivated in many parts of the world, soybean cultivation is relatively new to Europe, especially in colder parts of Europe where cultivars adapted to high-latitude environmental conditions are needed (Zimmer et al. 2016).

Good growing conditions for soybean cultivated in Sweden are soils that gets warm and dry up early in the season, such as sandy soils (Fogelfors 2015; Fogelberg 2021) and soybean prefer soil pH 6 to 7 (Staton 2012). It is sown from the middle to the end of May, or when the soil temperature is above 10 °C and the plants are mature for harvest around the beginning of October. In Scandinavian conditions soybean commonly yield around 2 t/ha (Fogelberg & Recknagel 2017).

1.1.1 Soybean cultivars for Swedish climate

Soybean is originally a short-day plant, but plant breeding has resulted in a division of cultivars into 13 different maturity groups (MG). These range from 000 (very early) to X (late) depending on their sensitivity to photoperiods (Hartwig 1973). The cultivars in MG 000 reach maturity early and are adapted to grow at higher latitudes, i.e. have low photoperiod-sensitivity and are able to reach maturity in areas where the growing season coincides with long days (Criswell & Hume 1972). Additionally, the maturity is affected by temperature, where cultivars in MG 000 require a lower amount of growing degree days to reach maturation than the cultivars in higher MG's (Fogelberg & Recknagel 2017; Liu et al. 2017). In a study by Fogelberg (2021) it was concluded that soybean cultivars of MG 000 are the most suitable cultivars for securing yield in Swedish climate. It was also concluded that soybean can be cultivated up to a latitude of 59 °N (Stockholm area), which are the more southern parts of Sweden with a land area ranging between the latitudes 55 °N and 69 °N. Additionally, it was suggested to test the MG 000 cultivars in Swedish growing conditions and how cultivars perform in different regions prior to scaling up the production (Fogelberg 2021).

Except for MG 000 cultivars, there are soybean cultivars developed in Sweden suitable for Swedish soybean cultivation. In the 1940's the seed company Algot Holmberg & Sons started a breeding program in Fiskeby, Sweden, for developing soybean cultivars suitable for the Swedish climate. The plant breeder Sven Holmerg developed cultivars with abilities such as tolerance to low temperatures during the growing season, early maturation and adaptation to growing seasons with long days (Olsson 1997). Holmberg & Sons released several cultivars, such as Fiskeby I-V, Bråvalla and Träff. Fiskeby V was the highest-yielding cultivar developed at Fiskeby, but Bråvalla and Träff had extremely early maturation, maturing 8 respectively 12 days earlier than Fiskeby V. Even though Swedish cultivars of soybean were developed, the crop never got any spread in Swedish farming. This is thought to be because soybean still gave uncertain and low harvests in the Swedish climate compared to other crops. Hence, the breeding program was phased out at Fiskeby (Olsson 1997). In recent years there have been several attempts at bringing soybean back as a crop in Swedish farming and studies have shown that there can be successful cultivation of soybean in Sweden (Fogelberg 2021; Fogelberg & Mårtensson 2021). Current research projects, such as the IMPULSE project at the Swedish University of Agricultural Sciences, also aim to evaluate, for example, where in Sweden soybean can be cultivated and how the crop is affected by different cultivation strategies.

1.2 Rhizobia

1.2.1 Symbiotic nitrogen fixation: how and why?

Legumes are able to establish symbiotic relationships with soil inhabiting alpha- and beta- Pseudomonadota (previously Proteobacteria), collectively named rhizobia (Sprent et al. 2017; Kuzmanović et al. 2022). The symbiosis is a mutualistic relationship, where the legume provides the rhizobia with C derived from photosynthesis and, in return, receives plant-available N, a form of biological nitrogen fixation (BNF) referred to as symbiotic nitrogen fixation (SNF) (Wagner 2011). This symbiotic interaction occurs in nodules, which are plant organs created on the roots of the legume host upon initiation of the symbiosis (Figure 1). Inside the anaerobic environment of the nodules, the N fixing bacteria capture atmospheric N in the form of N_2 and reduce it to NH_3 (Hirsch 1992). The reaction (Equation 1) is possible through catalysation by the enzyme nitrogenase (Hoffman et al. 2014).

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16\ P_i \qquad (1)$$

Nitrogenase is degraded by O_2 and therefore an environment with low levels of free O_2 must be maintained for the N-fixation to function (Downie 2014).

Simultaneously, the reduction of N_2 is an energy-intensive process and requires a lot of O_2 for the production of ATP to fuel the reaction (Downie 2005). The solution for both problems is leghemoglobin, a form of hemoglobin found in the root nodules created only when the legume is in a symbiotic relationship with rhizobia. Leghemoglobin has a high affinity for O_2 and buffers the O_2 concentration in the nodules, but it can simultaneously provide enough O_2 for the bacteria to carry out oxidative phosphorylation to produce ATP (Downie 2014; Singh & Varma 2017). Leghemoglobin has proven to be crucial for SNF in legumes (Ott et al. 2005) and the red colour it gives the nodules is a sign of active nitrogen fixation. If the nodules are coloured green, dark or white it is a sign of ineffective nodulation and can indicate low SNF in the nodules (Unkovich et al. 2008).

Figure 1. Phenotype of a soybean root with root nodules. *Photo: Sabina Juhlin Muñoz.*

Soybean can derive N both as soil mineral N and through BNF. From germination and up to about 20 days after emergence, the soybean relies on N from the seed (Miladinović et al. 2011). If rhizobia able to initiate symbiosis with soybean are present in the soil, nodules start to appear on the roots roughly a week after germination. It takes the nodules about 10-14 days to supply the plant with sufficient amounts of N (Miladinović et al. 2011). The N-fixation increases rapidly when the soybean enters its reproductive stage (flowering) and culminates around the onset of pod filling, but this might vary between cultivars (Zapata et al. 1987; Pitumpe Arachchige et al. 2020). About 87 % of the N a soybean plant fixes is fixed during its reproductive stage (Zapata et al. 1987). N-fixation is an energy-intensive process and requires more energy allocation from the plant than uptake of soil

mineral N. Hence, if N is present, the plant will prioritize that source of N, resulting in reduced N-fixation (Abendroth et al. 2006; Tamagno et al. 2018).

1.2.2 Legume – rhizobia specificity

Rhizobia are a diverse group of bacteria, consisting of several different genera. There is a host-specificity between rhizobia and the host plants, meaning not all rhizobia have the ability to nodulate all species of legumes. Both the specificity of symbiotic partner and the effectiveness of the BNF vary between rhizobial species or - strains and legume hosts (Perret et al. 2000; Solomon et al. 2012; Zimmer et al. 2016). There may be narrow and broad host-specificity, both for legumes and rhizobia (Young & Johnston 1989). The rhizobia species able to effectively nodulate soybean are found in the genera *Rhizobium*, *Ensifer* (previously *Sinorhizobium)*, *Bradyrhizobium* and *Mesorhizobium* (Nakei et al. 2022). This paper will further investigate the soybean-nodulating species *Bradyrhizobium japonicum*.

1.2.3 Nodule formation and regulation

The process of nodule formation is initiated when legumes release compounds such as flavonoids into the soil, which attract free-living rhizobia to accumulate around the roots of the legume (Gage 2004). The rhizobia attach to the root hairs and produce nodulation (Nod) factors (lipochito-oligosaccharides). These Nod factors are polymers that can have many modifications, thereby being specific to the rhizobia producing them (Dénarié et al. 1996). When the Nod factors are recognized by the host legume, an infection process is initiated where the rhizobia are allowed to pass into the root hairs, and later the root, through an infection thread formed through an invagination of the root hair (Gage 2004; Ferguson 2013). The infection thread connects to a cluster of undifferentiated cells near the root cortex, called nodule primordium, allowing the bacteria to enter the cells (Gage 2004). The bacteria are enveloped in a plant membrane structure called symbiosome and differentiate into bacteroids which produce nitrogenase and are specialized at N-fixation in the mature nodules (Emerich & Krishnan 2014). Nodules deteriorate with age and are replaced by new nodules after a few weeks (Ferguson 2013).

Two different kinds of nodules exist: indeterminate and determinate. The two kinds of nodules differ in many properties, one of them being indeterminate nodules have a persistent meristem located in the apex, while determinate nodules have a meristem only active during early development. Consequently, the indeterminate nodules get an elongated shape, while the determinate nodules have a round shape (Hirsch 1992; Gage 2004). It is not the rhizobial strain, but the host plant, that determines which type of nodules are formed (Hirsch 1992). Indeterminate nodules

are formed on temperate legumes, while determinate nodules are often formed on legumes of tropical origin (Gage 2004). Hence, soybeans create determinate nodules in symbiosis with rhizobia (Hirsch 1992).

Nodules are concentrated on the soybean roots in the upper layers of the soil (around 20 cm depth), but can be found at over 1 m depth, varying with factors such as soil type and soybean cultivar (Grubinger et al. 1982). The number of nodules a soybean plant forms is affected and regulated by different environmental factors, such as drought (Sinclair et al. 1988), salinity (Singleton & Bohlool 1984), temperature (Lindemann & Ham 1979), pH (Ferguson et al. 2013), soil mineral N (Abendroth et al. 2006) and soil P and K (Jones et al. 1977). Environmental factors also affect the survival of rhizobia in the soil and the effectiveness of BNF. However, optimal environmental conditions for nodulation, rhizobial survival and effectiveness in N-fixation may vary depending on the origin of the *Bradyrhizobium* strain (Zhang et al. 2003; Asadi Rahmani et al. 2009).

1.2.4 Effectiveness of nitrogen fixation

One way to assess how effective the SNF has been in providing a legume with N is through the "^{15}N natural abundance method", where the percentage of plant N derived from atmospheric N_2 (%Ndfa) is estimated. N exists in two stable isotopes: ^{14}N, the more common isotope, and ^{15}N, the less common isotope expressed as the percentage of the total N present (atom% ^{15}N), as shown in Equation 2 (Unkovich et al. 2008).

$$\text{atom\% } ^{15}\text{N} = (^{15}\text{N}/ (^{15}\text{N} + ^{14}\text{N})) \times 100 \quad (2)$$

It is possible to estimate a sample's difference in atom% ^{15}N compared to that of the air, as the air has a near constant concentration of 0.3663 atom% ^{15}N (Mariotti 1983). The difference in atom% ^{15}N is expressed as δ^{15}N (‰), as defined by Equation 3. Consequently, a legume with N derived exclusively from N-fixation has a δ^{15}N close to 0 ‰, whereas a plant with N mostly derived from the soil will have a similar δ^{15}N as the soil nitrogen. A δ^{15}N value between the two indicates the legume has derived N both through N-fixation and from the soil (Unkovich et al. 2008).

$$\delta^{15}\text{N (‰)} = \frac{sample\ atom\%\ 15\text{N} - 0.3663}{0.3663} \times 1000 \quad (3)$$

The δ^{15}N can be used to estimate the amount of Ndfa a legume sample contains. Additionally, either the δ^{15}N of a reference plant (a legume without nodules or a plant belonging to another family than legumes grown simultaneously as the N_2 fixing legume), or the δ^{15}N of the soil where the N_2 fixing legume has been grown

is needed for the estimation (Equations 4 & 5) (Unkovich et al. 2008). A reference plant and soil N should have a similar $\delta^{15}N$ value, with regard to the above-mentioned reason that a plant with all its N derived from soil N will have a $\delta^{15}N$ similar to that of the soil N.

$$\%Ndfa = \frac{\delta 15N \text{ of soil N} - \delta 15N \text{ of sample}}{\delta 15N \text{ of soil N} - \delta 15N \text{ of N2}} \times 100 \quad (4)$$

$$\%Ndfa = \frac{\delta 15N \text{ of reference plant} - \delta 15N \text{ of sample}}{\delta 15N \text{ of reference plant} - \delta 15N \text{ of N2}} \times 100 \quad (5)$$

There are differences in $\delta^{15}N$ within a plant, also called isotopic fractionation, where the $\delta^{15}N$ in the shoots often is lower than the $\delta^{15}N$ of the whole plant. In field experiments, it can become problematic to acquire whole plants since roots are easily torn off at sampling, resulting in calculations of Ndfa often being based on the $\delta^{15}N$ content of shoots. To correct for the isotopic fractionation within a plant, the $\delta^{15}N$ of N_2 in Equation 5 is swapped for a B-value (Equation 6), which is a value preferably acquired from a legume deriving all its N through N-fixation (from symbiosis with the same rhizobial strain(s) as the sample plants) and collected at the same development stage as the sample plants (Unkovich et al. 1994, 2008).

$$\%Ndfa = \frac{\delta 15N \text{ of reference plant} - \delta 15N \text{ of sample}}{\delta 15N \text{ of reference plant} - B} \times 100 \quad (6)$$

The B-value is influenced by legume species, rhizobial strains (legume host associations), and the site where the plants are cultivated (Unkovich et al. 1994, 2008). Consequently, it is suggested to grow plants to estimate the B-value for each independent study where %Ndfa is assessed, but example values can be acquired from the literature.

1.3 Inoculants

1.3.1 Inoculation of seeds and its phenotypic effects on soybean

An inoculum is a medium containing microorganisms (in this study *B. japonicum*) and there is a broad range of different formulations of commercial inoculants that can be used for legume cultivation (Stephens & Rask 2000). There are several scenarios where an inoculant is needed to establish nodulation and effective BNF in legumes. Rhizobia occur naturally in many soils, but if a legume species

previously has not been grown in a soil, or if it was a long time ago, there will most likely be an absence of symbiotically compatible rhizobia and a need for inoculation (Giller 2001; Fogelfors 2015). There might also be scenarios where there are compatible rhizobia but the population is not sufficient to effectively nodulate the legume or where there are indigenous bacteria with less efficient SNF with the cultivated legume compared to the bacteria in the inoculant (Giller 2001). *B. japonicum* is not native to Swedish soils, thus, inoculation of soybean seeds is recommended in Sweden. However, it was concluded in a master thesis by Andersson (2014) that *B. japonicum* can survive in Swedish soils and that there is no need for re-inoculation of soybean with *B. japonicum* if soybean have been cultivated in a field up to two years before (Andersson 2014).

Research has proven inoculation of soybean with *Bradyrhizobium* inoculants before sowing increases yield compared to cultivating uninoculated plants and also affects other yield components such as thousand kernel weight (TKW), number of pods per plant, N uptake, seed yield and above-ground biomass (Solomon et al. 2012; Leggett et al. 2017). However, the effect an inoculant has varies depending on the interaction between the soybean cultivar and the rhizobia species or - strain in the inoculum. In several studies, the interaction has proven to affect phenotypic traits such as protein content, yield, protein yield, number of nodules per plant and nodule dry weight (Hume & Blair 1992; Luna & Planchon 1995; Solomon et al. 2012; Zimmer et al. 2016). In other words, the effect an inoculant has on the traits of one soybean cultivar may not be applicable to another cultivar.

1.3.2 Peat - and liquid inoculants

According to Brockwell & Bottomley (1995), one of the main objectives of inoculation is to make sure as much rhizobia as possible survive between the application of the inoculant and the formation of a legume rhizosphere that the rhizobia can inhibit. As previously mentioned, the success of SNF and the response of inoculation in soybean often depend on the selection of a suitable rhizobial strain or – species (Perret et al. 2000; Solomon et al. 2012; Zimmer et al. 2016), but a successful inoculum formulation is vital to promote the survival of the rhizobia, both during storage and in the soil (Stephens & Rask 2000). Inoculant formulations promoting higher rhizobial survival have proven to increase yield, nodule number and nodule mass in soybean (Hume & Blair 1992). Smith (1995) reviewed that there are several criteria a good carrier of bacteria should meet, including that it preferably should be sterile, have a high water holding capacity, be non-toxic to the microorganisms and the envrionement, be biodegradable and have a neutral pH. Additionally, it should be uniform both chemically and physically, sustain the survival and growth of the bacteria and later allow fast release of the microorganisms to the soil (Smith 1995). However, when it comes to the

development of commercial inoculants, it is often a difficult step to get a rhizobia strain effective in test conditions to function in a user-friendly product and be equally efficient under field conditions (Stephens & Rask 2000). There are several different formulations of commercial inoculants, where peat (the most common one) and liquid formulations are available in north-western Europe (Stephens & Rask 2000; Miladinović et al. 2011; Pannecoucque et al. 2018). Peat inoculants consist of sterilized - or unsterilized peat (choice of manufacturer) to which the rhizobia is added and are often applied directly to the seed (Stephens & Rask 2000). The viability of the bacteria is often higher in sterilized carriers compared to unsterilized carriers (Stephens & Rask 2000; Temprano et al. 2002). Peat has properties such as a high surface area and water holding capacity which are beneficial for both the survival and growth of bacteria (Tittabutr et al. 2007). However, there are downsides to peat, such as varying quality of the material (Bashan 1998) and the fact that the peat may come off the seeds as they go through the machinery at sowing (Deaker et al. 2004). The latter problem can be solved by adding an adhesive to the peat, but it is time-consuming for the farmers (Smith 1995). Liquid inoculants are based on water, oil or polymers and can be applied to the soil or directly to the seed (Stephens & Rask 2000; Xavier et al. 2004). Beneficial properties of liquid formulations are that they are easily combined with modern seeding equipment and that the inoculant sticks better to the seeds when they pass through machinery (Deaker et al. 2004; Tittabutr et al. 2007). Additionally, the liquid formulations enable a more even seed coverage compared to peat formulations (Smith 1995). However, the survival of the rhizobia in liquid formulations has in some cases been reported to be poor (Tittabutr et al. 2007) and there is no carrier protection for the bacteria (Bashan 1998). Therefore, there are benefits and downsides to both types of inoculum formulations, and the two formulations have been reported to impact soybean traits like nodulation and yield equally in several studies (Thao et al. 2002; Albareda et al. 2008; Schulz & Thelen 2008; Pannecoucque et al. 2018). Something to take into consideration is that commercial inoculants are often produced to function optimally in the US and that their efficacy therefore needs to be tested under other growing conditions (Pannecoucque et al. 2018). For example, Zimmer et al. (2016) reported that the commercial liquid inoculant Radicin No. 7 did not result in nodulation of soybean in cold field conditions in Germany, while the same inoculant managed to do so in pot trials.

In a meta-analysis by Thilakarathna and Raizada (2017), where the authors studied how different rhizobia inoculants affected traits in soybean under field conditions, the authors concluded that there are research gaps both regarding the interactions of soybean cultivar x rhizobia strain in the inoculants and in how to optimize inoculation methodologies. Additionally, the authors stressed the importance of

studying SNF under field conditions using techniques like ^{15}N isotope analysis instead of only assessing SNF from traits such as seed N content and nodule number (Thilakarathna & Raizada 2017). This thesis aims to investigate some of these research gaps.

1.4 Aims and hypotheses

The aim of the thesis was to evaluate the effects the inoculation methods no-, liquid- or peat inoculation had on phenotypic traits and N-fixation in soybean, specifically in soybean cultivars suitable for cultivation in Swedish climate (MG 000 and historic). Additionally, the effects were assessed in two different environments: field- and greenhouse conditions. The following questions and hypotheses were addressed:

(1) Does the inoculation method influence the effectiveness of N-fixation (%Ndfa) and total N content in soybean?

Hypothesis 1: Inoculation of the seeds with *B. japonicum* should increase the effectiveness of N-fixation and N content in soybean compared to plants where the seeds are not inoculated. Additionally, the inoculation method (peat – or liquid inoculation) is expected to influence the %Ndfa and N content in the soybean plants since one of the carriers may have superior properties for inoculation effectiveness and – success in the given environmental conditions.

(2) Does the inoculation method have varying effects on phenotypic traits, such as plant height, stem biomass, thousand kernel weight, number of nodules and yield?

Hypothesis 2: Soybean traits have responded positively to inoculation compared to uninoculated plants, for which reason the inoculated plants are expected to respond with increased plant height, stem biomass, thousand kernel weight, number of nodules and yield compared to uninoculated plants in the experiments. Additionally, the inoculation method (peat – or liquid inoculation) is expected to influence the responses in the phenotypic traits since one inoculation method may have superior properties for inoculation effectiveness and - success in the given environmental conditions.

(3) Does the inoculation method affect the effectiveness of N-fixation and phenotypic traits differently in the greenhouse compared to field conditions?

Hypothesis 3: Environmental conditions affecting rhizobial survival, nodulation and BNF (such as temperature and soil moisture content) should be more favorable in a greenhouse setting, where many environmental factors can be controlled, compared to field conditions. Therefore, it is expected that the effectiveness of N-fixation will be greater in greenhouse conditions compared to field conditions. I also expect to see an increase in the traits that can be compared in this study, such as higher TKW, yield and nodule number in plants from the greenhouse experiment compared to plants from the field trial.

(4) Lastly, do different cultivars respond to the abovementioned treatments in a similar way?

Hypothesis 4: The interaction of plant genotype x rhizobia strain will influence the responses in phenotypic traits, N content and %Ndfa in the assessed soybean cultivars.

Ultimately, these results will create a deeper knowledge of the suitability of modern MG 000 and historic cultivars bred for Scandinavian climates, for soybean to be an attractive crop for Swedish farmers.

2. Methodology

The experimental procedures consisted of two experimental set ups: a field trial conducted on Gotland during the summer and autumn of 2023, and a greenhouse experiment conducted throughout the autumn and winter of 2023. In total, the experiments featured five different cultivars of soybean: four cultivars in maturity group 000: Abaca, Gallec, Sussex and Todeka, and one historic cultivar: Bråvalla (Table 1).

Table 1. Soybean cultivars used in this thesis*. The cultivars were used in the field trial on Gotland summer 2023 and in the greenhouse trial at Ultuna during autumn/winter 2023. *Only used in the greenhouse experiment. [+] Only used in the Gotland field trial.*

Cultivar	Maturity group	Country of origin
Abaca	000	Austria
Bråvalla*	Historic	Sweden
Gallec	000	Switzerland
Sussex	000	Germany
Todeka [+]	000	Germany

In both the field trial and the greenhouse experiment, the seeds were treated with either peat inoculum, liquid inoculum, or no inoculum. For liquid inoculation and peat inoculation the products LiquiFix and LegumFix, respectively, provided by Legume Technology LTD, UK (Legume Technology LTD), were used (Table 2). According to the manufacturer LiquiFix contains a culture of *Bradyrhizobium* bacteria formulated with a polymer adhesive and nutrients, while LegumeFix contains a mixture of peat and rhizobia. The products exist for several different crops and contain different species of N-fixing bacteria, depending on the target crop for inoculation. The products developed for soybean contain the species *B. japonicum*. Independent of the inoculation method, the amount of bacteria per seed was the same (Table 2). However, due to variance in seed size (thousand kernel weight, TKW), as a consequence of differential soybean cultivar genotypes, there might be a difference in the amount of bacteria per seed.

Table 2. Inoculum products used in this thesis*. The inocula were used in the field trial on Gotland summer 2023 and in the greenhouse trial at Ultuna during autumn/winter 2023.*

Plant species	Type of inoculum	Product name	Rhizobia species	Concentration at manufacturer (g CFU/mL)
Soybean	Liquid	LiquiFix	*B. japonicum*	5 x 10^9
Soybean	Peat	LegumeFix	*B. japonicum*	5 x 10^9

2.1 Field trial

The field trial was conducted at Gotland Grönt Centrum (57°32'N, 18°25'E), Sweden, from May to October in 2023 (Figure 2). Four different cultivars of soybean were grown in the field trial: Abaca, Gallec, Sussex and Todeka, with three different methods for inoculation: liquid inoculation with *B. japonicum*, peat inoculation with *B. japonicum* or no inoculation. The soybean seeds were inoculated using liquid inoculum or peat inoculum at most 24 h before planting, or only a seed coating as provided by the seed supplier.

Figure 2. Soybean plants in the fieldtrial at Gotland summer 2023*. Foto credit: Fede Berckx.*

The field trial was divided into 48 plots, which measured 2 m x 6 m, consisting of four replicates of each inoculation treatment and cultivar. The plots were organized in a randomized complete block design, as illustrated in Figure 3. Additionally, buckwheat (*Fagopyrum esculentum*) was grown as reference plants to be used in the ^{15}N natural abundance method to calculate the %Ndfa in the plants later on. The experiment was sown on May 15th with a seeding rate of 65 plants/ m^2. The tillage method used in the field was inversion tillage in the autumn, followed by seedbed preparation in the spring with a cultivator. The crop previously grown in the field was spring barley and the trial was surrounded by a cultivation of faba bean (*Vicia faba*). The soil in the experimental field consisted of 50 % sand, 30 % silt, 16 % clay, 3.5 % mull and had pH 7.9. Soil samples were taken and the amount of nutrients and the nutrient classes were analyzed for each plot by Agri Lab AB and can be found in the appendices (Appendix 1, Appendix 2 & Appendix 3). No fertilizers or pesticides were used in the field trial, but the experiment was covered with a cloth to protect the seeds and young seedlings from birds. Additionally, the experiment was hoed twice before row closure for weed control.

Figure 3. Figure of the field trial setup on Gotland (57°32'N, 18°25'E) in 2023. *Four different soybean cultivars were grown; Abaca, Susssex, Todeka and Gallec, with three different inoculation methods; peat inoculation, liquid inoculation or no inoculation. The field trial was organized in four blocks in a fully randomized block design where four replicates of each combination of cultivar and inoculation method were included.*

2.2 Greenhouse experiment

In autumn 2023, a greenhouse experiment was conducted on the campus of the Swedish University of Agricultural Sciences (SLU) in Ultuna, Uppsala (59°49'N, 17°39'E). The greenhouse trial included four varieties of soybean (Abaca, Bråvalla, Gallec and Sussex), treated with two different inoculum formulations of rhizobia bacteria: peat or liquid, or no inoculum at all (see Tables 1 & 2).

2.2.1 Greenhouse setup

The soybean plants were grown in trays on a bench in the greenhouse, as shown in Figure 4. The bench held 12 trays with eight pots in each tray, and each tray held only one cultivar of soybean to mimic field condition density. Additionally, the trays held either inoculated plants or uninoculated plants to avoid contamination with *B. japonicum* to the uninoculated plants.

Figure 4. Set-up of the soybean plants grown in the greenhouse trial in Uppsala in autumn/winter of 2023.

Each of the 12 trays was given a number from 1 to 12. To randomize the greenhouse setup, ChatGPT was used to create a randomized list of the tray numbers. Thereafter, the list of the randomized tray numbers was paired with a list of inoculum treatments, organized in the order: no inoculum, inoculum, inoculum. ChatGPT then randomly paired each of the cultivars with one of the tray numbers

with no inoculum and with two tray numbers with inoculum. The pots in each tray were named A to H. Lastly, ChatGPT was asked to randomize the liquid and peat treatments between the two trays of each cultivar containing inoculated plants. Figure 5 displays the complete greenhouse setup.

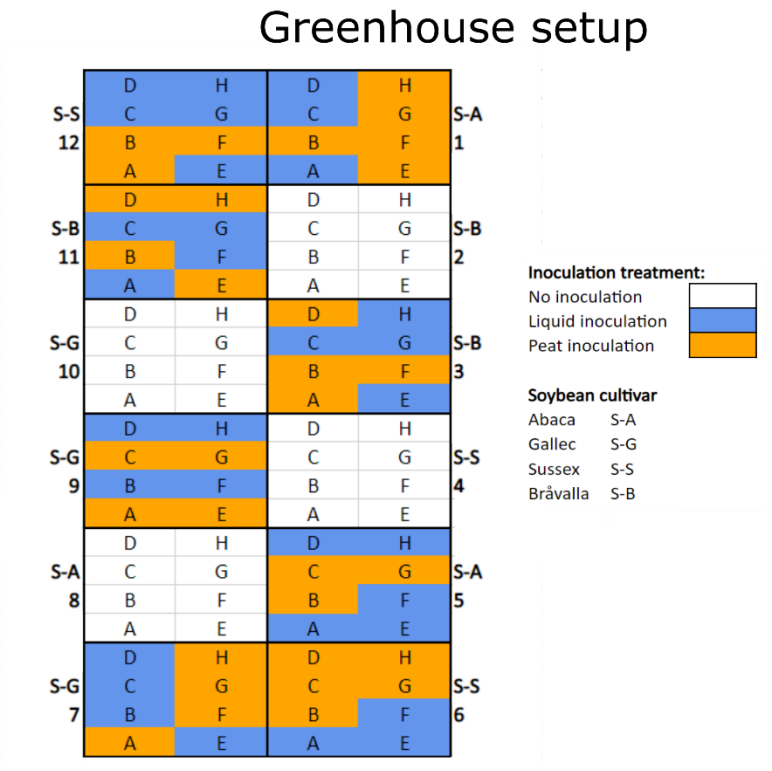

Figure 5. Figure of the greenhouse setup. *The experiment ran in Ultuna from September to December in 2023. The figure shows the setup of the soybean trial with the cultivars Abaca (S-A), Gallec (S-G), Sussex (S-S) and Bråvalla (S-B). The trays were marked 1 to 12 and the pots within each tray were marked A to H. The inoculation treatment of each seed is indicated by the colours white, blue or orange for the treatments no inoculum, liquid inoculum or peat inoculum respectively.*

The plants were rotated one step in a clockwise direction every 7 to 8 days, as there were gaps between the lights over the benches, resulting in unequal light dispersal to the trays. The temperature conditions in the greenhouse were set at 25 °C during the day and 20 °C at night. The lights were switched on for 14 h a day, between 6AM and 8PM. At sowing, the height of the lamps was adjusted to make the light intensity at pot level at least 400 to 600 µmol. The height of the lights was then adjusted to the height of the plants as they developed. The humidity in the greenhouse was set to 50 %. The plants were watered when the pots seemed dry. Sticky traps were put out on the trays and around the greenhouse due to problems with flies and thrips infesting the plants. Additionally, nematodes were added to the soil of each pot on two occasions to manage the fly larvae hatching in the soil.

2.2.2 Inoculation and planting

Pots were filled with 1100 g (+/- 10 g) of soil meant for germination of seeds, S-jord (Hasselfors Garden, Sweden). According to the manufacturer, S-jord has pH of 5.5 – 6.5 and consists of black peat, white peat, perlite, sand/ rock flour, mineral fertilizers and "Rotkraft" (concentrated, natural humic acids) (Hasselfors Garden n.d.). The added mineral fertilizers are summarized in a table in Appendix 4. The pots were placed on plastic trays, with eight pots on each tray. The trays were then put into the greenhouse and watered to moisten the soil.

Seeds of Abaca had been treated with a seed coating, over one year before the start of the experiment, explaining the blue/green colour of the seeds in Figure 6. The recommended shelf life is up to one year, and therefore it was assumed all the rhizobia would have died at the start of the experiment. The seeds were inoculated with either peat inoculum (LegumeFix), liquid inoculum (LiquiFix) or no inoculum. Inoculation and planting were performed on the same day. The peat inoculum was poured into a beaker and moistened with a few drops of water. The seeds were stirred in the peat until they were evenly covered. The seeds were picked out with a spoon. The liquid inoculum was mixed around in its transportation container and poured into a beaker. The seeds were stirred in the liquid and then picked out with a spoon. The seeds were airdried for at least 1 h before planting.

***Figure 6. Soybean seeds after inoculation**. The varieties from left to right are: Abaca, Gallec, Sussex and Bråvalla. The treatments from top to bottom are: liquid inoculum (blue), no inoculum (white) and peat inoculum (orange).*

Three seeds were planted in each pot on Thursday, September 28 of 2023, marking Day 1 of the experiment. All seeds with the same treatment were planted at the same time to avoid contamination between the treatments, starting with the uninoculated seeds. The seeds were planted in a triangle shape in the middle of the pots. After emergence (day 7) excess plants were removed from each pot, leaving one plant per pot. Once the plants got taller (day 35), wooden sticks were put in the pots for plant support.

2.3 Measurements of phenotypic traits

2.3.1 Sampling

Field trial- Flowering

About two and a half months after sowing, on July 17th (at flowering), ten plants from each of the plots were sampled. The plant height and the height of the lowest node were measured for each plant individually. Followingly, the shoots and leaves from each plant were separated and dried upon arrival in Uppsala. The roots from each plant were also collected and stored in a freezer at -20 °C upon arrival in Uppsala. Additionally, the crop density was measured per plot and soil samples were taken for analysis of soil type, pH and nutrient content and stored at -20 °C.

Field trial- Harvest

On October 12th, 150 days after sowing, ten plants from each of the 48 plots were collected from the field trial on Gotland. Before assessing the yield traits, the plants were dried.

Greenhouse experiment

The greenhouse experiment ended at day 83. On the day of harvest, the height of the plants (from soil level to the highest node) and the height of the lowest node (from soil level) were measured and rounded off to the nearest 0.5 cm. Followingly, the shoots of the plants were cut off at soil level and put in separate plastic bags, in which they were later dried in an oven at 50 °C for seven days. The roots were left in the pots overnight. The following day the roots were removed from the pots and any remaining soil was washed off with water. The roots were put in separate paper bags and dried in an oven at 50 °C for seven days.

2.3.2 Dry biomass of stem and leaves

Field trial - Flowering

Dry stems and leaves from each of the soybean plants sampled in the field trial at flowering were collected in separate paper bags. The samples were dried again in an oven at 30 °C the day before they were weighed and kept in the oven until right before weighing. The stems and leaves were weighed separately with an accuracy of two decimal places and then placed together in a paper bag.

Greenhouse experiment

Plastic bags containing the aboveground biomass for each plant sampled in the greenhouse were dried over night at 40 °C. Most of the plants lost their leaves at the end of the greenhouse experiment. Therefore, the leaves were removed from the plants with leaves still attached to them and the leaf biomass was not measured for the plants in the greenhouse experiment. Followingly, the stem biomass of each plant was weighed.

2.3.3 Nodule and root assessments

Field trial - Flowering

Soybean roots were collected from ten plants in each of the 48 plots in the field trial at flowering. One sample from plot 48 was lost during transportation to Uppsala. The roots were then kept in a freezer at -20 °C. Each root was taken out of the freezer and photographed with a camera of the model Nikon Z6 and camera objective 24-70/ 4S. If nodules were formed on the roots, those were removed, counted, and photographed. A small subsample of the root and one nodule was kept frozen at -20 °C, which could be used for future microbiome analysis. The nodules and roots were put in paper bags and then dried in an oven at 50 °C for at least two days. Lastly, the dry biomass of the roots and nodules was weighed separately with an accuracy of two decimal place. If the dry nodules weighed less than the accuracy of the scale (0.01 g), they were categorized as having a weight of 0.009 g. The nodule biomass was assessed as nodule biomass/ root biomass since many of the roots were torn-off at sampling, and, consequently, nodules attached to the torn off roots could not be accounted for. The root biomass was not assessed on its own for the same beforementioned reason.

Greenhouse experiment

The roots were dried overnight at 50 °C the day before measuring. Followingly, nodules and roots were assessed using a similar method as described for the samples in the field trial. The nodule biomass was assessed as nodule biomass/ root biomass to enable comparisons in nodule biomass with the field trial.

2.3.4 Yield traits

Field trial – harvest

The yield traits from the field trial were assessed as average values from ten plants in each of the 48 plots. For each plot, the total seed weight and TKW were measured. The total seed weight was then used to calculate the average yield per plant in each plot.

The grain yield for each replicate was supposed to be assessed for the whole harvested plots in the field trial, but due to an error in the process, the yield per replicate had to be calculated using the values of "yield per plant" measured for the plants sampled at harvest and the estimated plant density per plot.

Greenhouse experiment

In the greenhouse experiment, the yield traits were assessed for each plant. The yield traits: number of seeds, total seed weight (yield per plant) and TKW were measured.

2.4 Measurements for N assessment

2.4.1 Field trial

Nitrogen/chlorophyll measurements

At flowering, the chlorophyll content was measured for ten plants in each plot in the field trial using a chlorophyll meter of the model SPAD-502 (Konica Minolta Sensing Inc, Japan). The chlorophyll meter measures the absorbance of near-red and red wavelengths in the leaves and uses the absorbances to calculate a SPAD-value. The SPAD-values are proportional to the amount of chlorophyll in the leaves, which in turn is proportional to the N content in the leaves (Konica Minolta, Inc. 2009).

Sample preparation and analysis of ^{15}N content

The stems and leaves collected at flowering, and the biomass from the plants collected at harvest (stems, leaves and husks), were milled in a cutting mill (Retsch SM 200) equipped with a 0.5 mm sieve. Additionally, 100 beans from each plant collected at harvest were milled in a cyclone mill (Retsch TWISTER) equipped with a 0.5 mm sieve. Each milled sample was collected in a glass container, stirred around for homogenization, and about 1.5 mL was scooped into an Eppendorf tube.

The remaining parts of the milled samples were put in plastic cups with lids and stored. Between each sample, the mill was cleaned using a vacuum cleaner.

Once all the samples had been milled, they were sent for analysis of the ^{15}N isotopes in the plants at the SLU Stable Isotope Laboratory (SSIL), the Department of Forest Ecology and Management, at SLU Umeå, Sweden. At SSIL, an Elemental Analyzer - Isotope Ratio Mass Spectrometry (EA-IRMS) was performed on the samples. The instruments used for the EA-IRMS were an Isotope ratio mass spectrometer (DeltaV, Thermo Fischer Scientific) and an Elemental analyzer (Flash EA 2000, Thermo Fischer Scientific). The methodology was described by SSIL as follows: Dry mass was defined by oven drying at 70 °C for minimum 18 h. N of the dried samples was converted to N_2 by combustion. Mass spectrometric measurements on N_2 yielded the quantities listed in Table 3 marked with an asterisk. The results were corrected for drift and sample size effect (non-linearity). The information provided about each sample in the analysis report is in Table 3.

Table 3. Information provided about each sample in the analysis report from SSIL (SLU Umeå, Sweden) and their definition.

Quantities measured	**Definition**
ω_N *	Mass fraction of N (g N per g dry mass)
$\delta^{15}N$ *	15N/14N isotopic ratio expressed using the atmospheric nitrogen scale
F_N	Isotopic amount fraction $^{15}N/(^{14}N + ^{15}N)$; calculated from $\delta^{15}N$ using $R_{ref} = {}^{15}N/{}^{14}N = 1/272$

The information about the samples provided by the analysis report from SSIL was used to calculate the %Ndfa in the plants. In the calculations of %Ndfa in the plants sampled at flowering a lot of variety between the replicates was detected. Hence, the data was decided to be excluded from the report.

The calculations of %Ndfa in the samples from harvest (biomass and seeds) were made plot-wise, since the samples from each plot had been milled together. For each cultivar, a "reference plant" was calculated as the average $\delta^{15}N$ of the uninoculated plots in each block. The B-value of $\delta^{15}N$ -1.83 was taken from Appendix 3 in Unkovich et al. (2008). Followingly, the calculations of %Ndfa per plot were done according to Equation 6. In the cases where the calculations resulted in a negative %Ndfa, the values were corrected to 0.

Protein yield

The protein yield was calculated using the estimated yield per plot and N seed content. The conversion factor of 6.25 to calculate soy protein from N content was used (Krul 2019). The calculations were made according to Equation 7.

Protein yield = Yield x Seed N x 6.25 (7)

2.4.2 Greenhouse experiment

Nitrogen/chlorophyll measurements

Every seven to eight days, the chlorophyll content was measured in each of the soybean plants in the greenhouse experiment using a chlorophyll meter of the model SPAD-502 (Konica Minolta Sensing Inc, Japan). When taking the measurements, two leaves (the newest and fully developed leaves) were measured on each plant and an average SPAD-value of the two was used for later calculations and visualization of variations in SPAD-values over time.

Phenotypic comparisons

Photos were taken of the plants on days 54 (November 20) and 65 (December 1) of the greenhouse experiment with a camera of the model Nikon Z6 and camera objective 24-70/ 4S. For each variety, one representative plant of each treatment was chosen and photographed next to each other for comparisons of visual signs of N deficiency.

2.5 Data handling, statistics and data visualization

For data processing and visualization, the statistical software program R with R Studio was used (version 4.3.0) (R Core Team 2023). The raw data was first checked for normal distribution. Normally distributed data was fitted with a linear mixed-effects model (LMM): nodule biomass/root biomass (field trial), yield per plant (field trial), %Ndfa in biomass and seeds and protein yield. Non-normally distributed data were fitted with a generalized linear mixed-effects model (GLMM) using poisson-distribution for integer data: nodule number, number of pods, number of nodes, and gamma-distribution for numeric data; biomass, heights, TKW, and yield per plant (greenhouse). The models are provided in the lme4 R package (version 1.1.35.1) (Bates et al. 2015). The random effects included in the models depended on the dataset: “block” and “treatment” (inoculum and cultivar combination) for data collected at flowering in the field trial; “block” for data from plants collected at harvest of the field trial; “tray number” and “replicate” for greenhouse data. The Akaike’s Information Criterion (AIC) was used to evaluate

which link resulted in the best fitted GLMM (Bozdogan 1987). The normality of the data was evaluated with Q-Q plots and residual plots and once normality was confirmed, an analysis of variance (ANOVA) was performed on the models. The R package emmeans (version 1.10.0) (Lenth 2024) was used to create estimated marginal means (emmeans) from the models. Followingly, pairwise multiple comparisons (Sidak test) of the estimated marginal means were performed for data where significance was detected with the R package multcomp (version 1.4.25) (Bates et al. 2015), providing a compact letter display (cld) for the comparisons. The data were visualized using the R package tidyverse (version 2.0.0) (Wickham et al. 2019). Raw data were used for the visualization, with estimated marginal means from the GLMM´s and LMM's added to the figures.

3. Results

3.1 Phenotypic traits

3.1.1 Biomass of root and stem

Field trial

Inoculation of the seeds did not have a significant effect on the stem biomass at flowering in any of the cultivars in the field trial (Appendices 5 & 7). The root biomass was not assessed for the plants in the field trial due to torn-off roots at sampling, as mentioned previously.

Greenhouse experiment

In the greenhouse experiment, the responses to the inoculum treatments in stem – and root biomass varied between the cultivars, and the traits were significantly affected by cultivar x inoculum interactions (Appendix 8). Inoculation of the seeds solely affected the stem biomass in the cultivar Sussex, where both liquid- and peat inoculation resulted in a significantly lower stem biomass compared to uninoculated plants (Figure 7). The inoculation method had an effect on the root biomass in more cultivars, where significantly higher root biomass was found in uninoculated plants compared to plants treated with peat inoculum in the cultivars Abaca, Gallec and Sussex (Figure 7). Moreover, peat inoculation resulted in significantly lower biomass compared to liquid inoculation in Abaca. Notably, liquid inoculation of seeds resulted in a significantly lower root biomass relative to uninoculated plants solely in the cultivar Sussex. In Bråvalla, the inoculation treatments showed no effect on the root biomass.

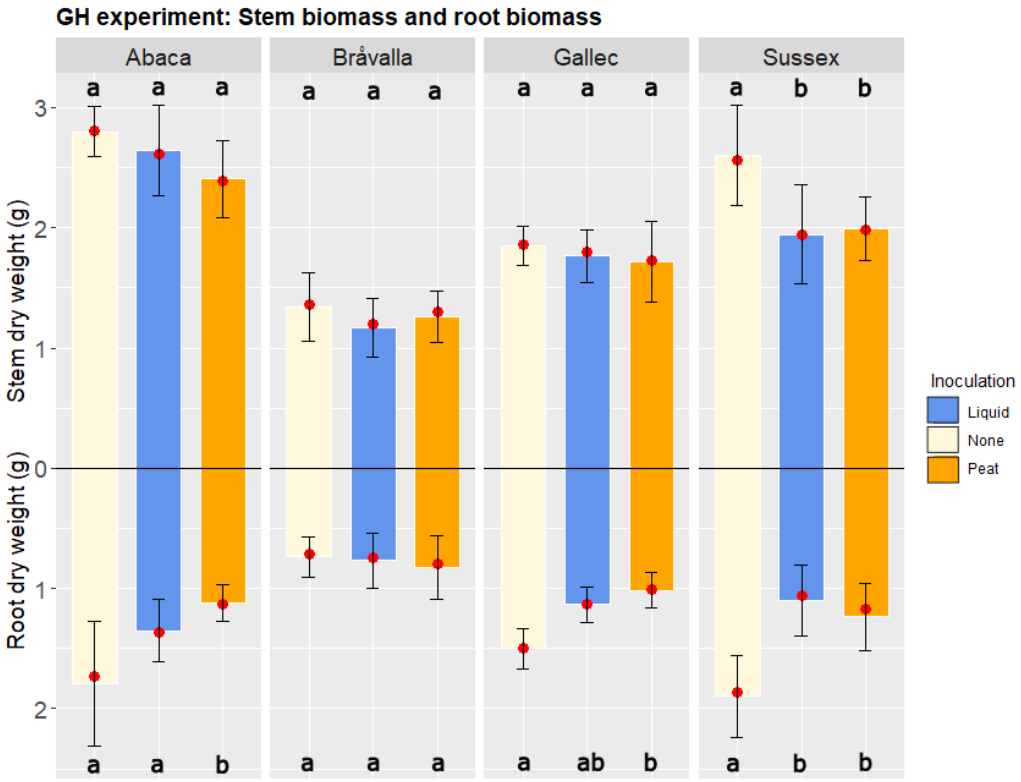

Figure 7. Effect of inoculation on dry weight of soybean cultivars. *Bar plots visualizing mean dry weight of roots (lower half of plot) and dry weight of stem (upper half of plot) derived from raw data for the four soybean cultivars Abaca, Bråvalla, Gallec and Sussex, treated with liquid-, peat- or no inoculum sampled from the greenhouse experiment in Uppsala, Sweden. Error bars show the standard deviation of the raw data. Red dots indicate means derived from data fitted with GLMMs. Significant differences between treatments within each cultivar ($p < 0.05$) are indicated by compact letter display.*

The root-to-stem biomass ratio (RS ratio) of the plants in the greenhouse experiment was significantly affected by inoculation in all cultivars except Bråvalla. A significantly lower RS ratio was seen when seeds were treated with peat inoculum compared to no inoculum in the cultivars Abaca and Gallec (Figure 8). Liquid inoculation resulted in a lower RS ratio in Sussex compared to no inoculation. There was no significant difference in RS ratio between liquid- and peat inoculation in any of the cultivars. The cultivar x inoculum interaction had a significant effect on the RS ratio (Appendix 8).

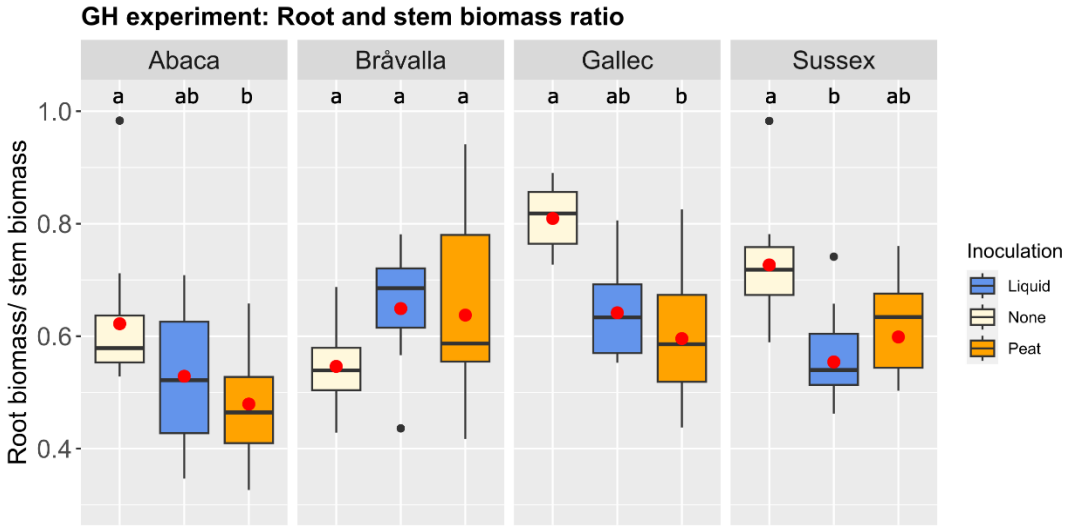

Figure 8. Effect of inoculation on root to shoot biomass. *Boxplots visualizing raw data of inoculation effect on root:stem ratio for soybean cultivars Abaca, Bråvalla, Gallec and Sussex sampled from the greenhouse experiment in Uppsala, Sweden. Black dots indicate outliers from raw data. The red dots indicate the estimated mean from the fitted GLMM. Significant differences between inoculum treatments within each cultivar ($p < 0.05$) are indicated by compact letter display.*

3.1.2 Nodule biomass/root biomass

Field trial

The inoculation method had a significant effect on the average nodule -/ root biomass ratio per plot in the field trial (Appendix 7). Peat inoculation resulted in a significantly higher nodule -/root biomass ratio compared to uninoculated plants in the cultivars Gallec, Sussex and Todeka (Figure 9). There were no significant differences in nodule -/root biomass ratio between plants inoculated with liquid- and peat inoculum. However, liquid inoculation did not increase the nodule -/root biomass ratio compared to uninoculated plants in any of the cultivars. No response in nodule-/root biomass ratio was seen from inoculation in the cultivar Abaca in the field trial.

Greenhouse experiment

The effects of the inoculation methods on the nodule -/ root biomass ratio in the greenhouse varied between cultivars (Appendix 8), but inoculation with both peat -and liquid formulations resulted in a significant increase in nodule -/root biomass ratio compared to uninoculated plants in all cultivars (Figure 9). In the cultivar Bråvalla, peat inoculation resulted in a significant increase in nodule -/root biomass ratio compared to plants inoculated with liquid inoculation.

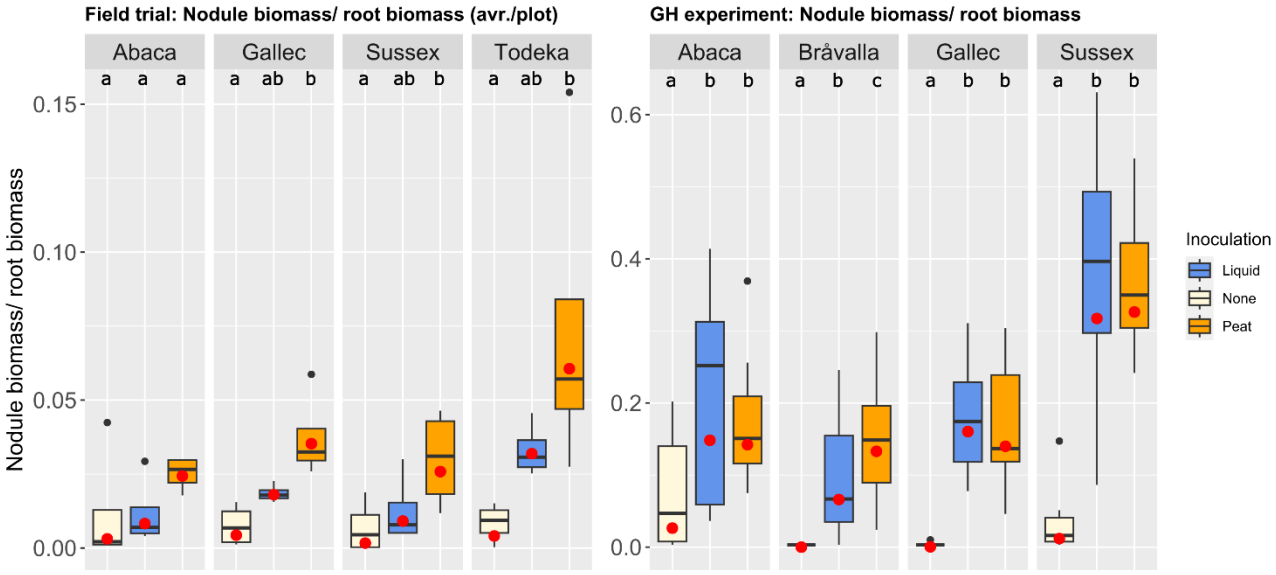

Figure 9. Effect of inoculation on nodule to root biomass. *Boxplots visualizing raw data of inoculation treatment effect on (left) average dry nodule biomass per plot/ average dry root biomass per plot for the soybean cultivars Abaca, Gallec, Sussex and Todeka sampled at flowering in the field trial at Gotland, and (right) nodule dry biomass/ root dry biomass for soybean cultivars Abaca, Bråvalla, Gallec and Sussex sampled from the greenhouse experiment in Uppsala. Black dots indicate outliers from raw data. The red dots indicate the estimated mean from the fitted LMM (left) and GLMM (right). Significant differences between inoculum treatments within each cultivar ($p < 0.05$) are indicated by compact letter display.*

3.1.3 Number of nodules

Field trial

The effect of inoculation on the number of nodules per plant varied between cultivars in the field trial, and the interaction of cultivar x inoculum treatment significantly influenced the number of nodules (Appendix 7). The number of nodules per plant was not affected by inoculation in the cultivar Abaca (Figure 10). In the cultivars Gallec and Sussex, the number of nodules significantly increased with peat inoculation compared to liquid- or no inoculation. Lastly, the cultivar Todeka responded to both inoculation methods, both liquid- and peat inoculation significantly increasing the number of nodules per plant compared to no inoculation (Figure 10).

Greenhouse experiment

Inoculation with both peat- and liquid-based inoculants resulted in a significant increase of number of nodules compared to no inoculation in all soybean cultivars in the greenhouse experiment (Figure 10). However, the effect of the inoculants varied between the cultivars, and cultivar x inoculum treatment interaction had a significant effect on the number of nodules per plant (Appendix 8). In the cultivars

Bråvalla and Sussex, there were significant differences in the number of nodules between peat- and liquid inoculation, where peat inoculation resulted in a significant increase in the number of nodules per plant. In the cultivars Abaca and Gallec, there were no significant differences in nodule number between liquid- and peat inoculum treatments.

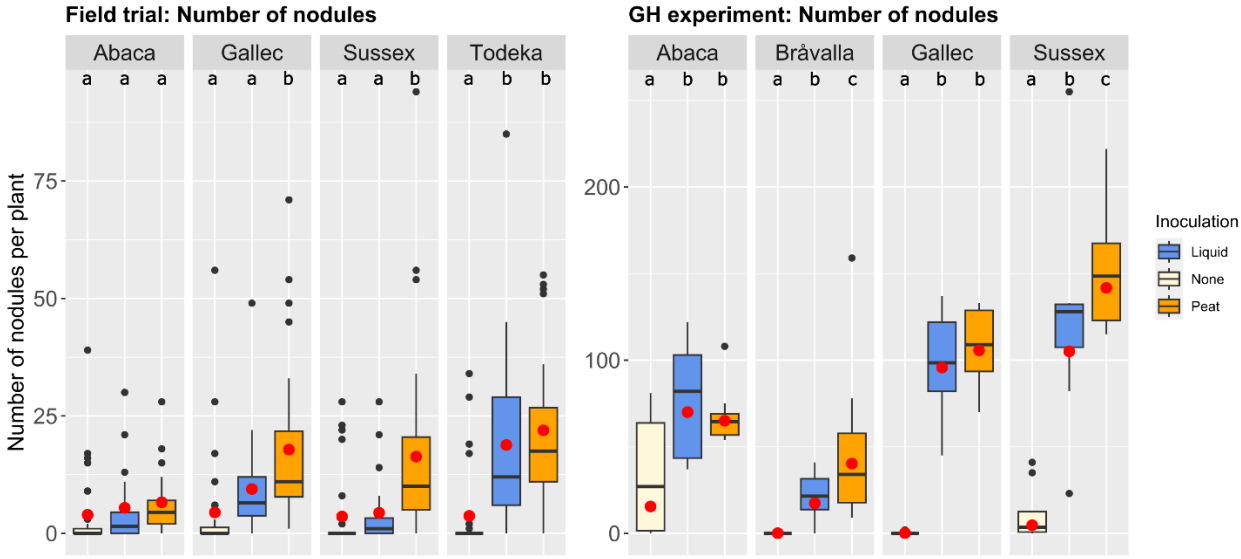

Figure 10. Effect of inoculation on nodule number. *Boxplots visualizing raw data of inoculation treatment effect on number of nodules per plant for (left) soybean cultivars Abaca, Gallec, Sussex and Todeka sampled at harvest in the field trial at Gotland and, (right) soybean cultivars Abaca, Bråvalla, Gallec and Sussex sampled from the greenhouse experiment in Uppsala. Black dots indicate outliers from raw data. The red dots indicate the estimated mean from the fitted GLMM's. Significant differences between inoculum treatments within each cultivar ($p < 0.05$) are indicated by compact letter display.*

3.1.4 Thousand kernel weight

Field trial

In the field trial, significant increases in the TKW were seen as a result of inoculation. However, there were varied responses to the inoculation methods between the cultivars, with a significant interaction effect of cultivar x inoculum treatment (Appendix 7). In the cultivars Abaca and Sussex, solely peat inoculation resulted in significantly higher TKW compared to uninoculated plants (Figure 11). The TKW of the cultivar Todeka was highly affected by inoculation and both liquid- and peat inoculation resulted in significantly higher TKW (increase of 41% respectively 36%) compared to no inoculation. In the cultivar Gallec, neither peat inoculum nor liquid inoculum showed any effect on TKW. There were no significant differences in TKW between plants treated with liquid- or peat inoculum in all four cultivars.

Greenhouse experiment

The inoculum treatment had a significant effect on TKW in the greenhouse experiment (Appendix 8). Liquid inoculum and peat inoculum had no significantly different effects on TKW (Figure 11). However, liquid inoculation resulted in a significantly higher TKW in Abaca compared to uninoculated plants, whereas peat inoculation did not. The cultivar x inoculum treatment interaction did not have a significant effect on TKW in the greenhouse experiment (Appendix 8).

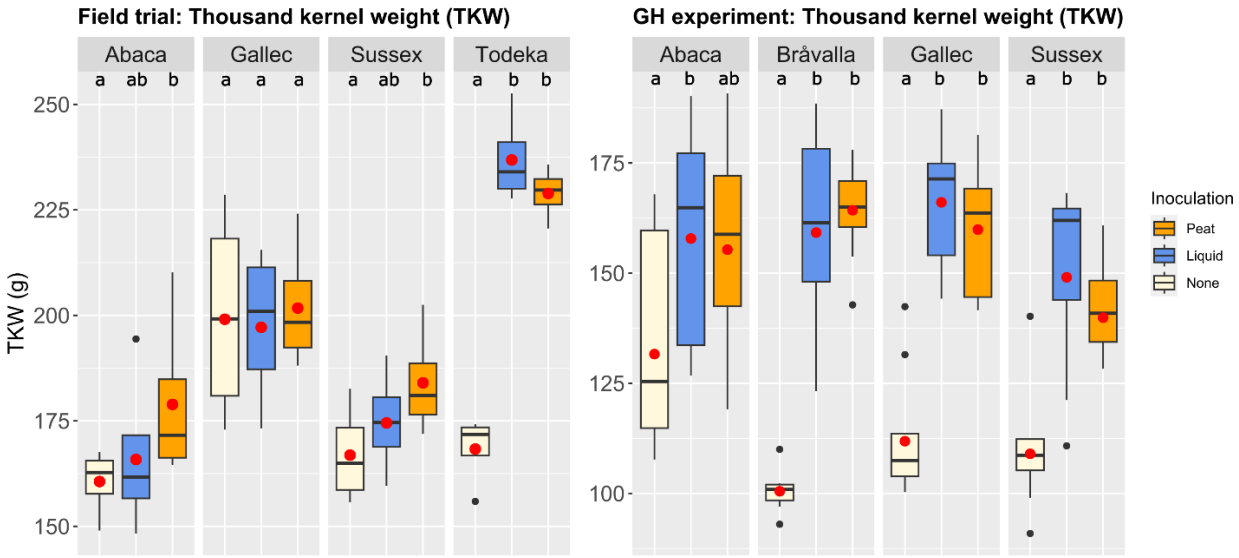

Figure 11. Effect of inoculation on TKW. *Boxplots visualizing raw data of inoculation effect on TKW for (left) soybean cultivars Abaca, Gallec, Sussex and Todeka sampled at harvest in the field trial at Gotland, and (right) soybean cultivars Abaca, Bråvalla, Gallec and Sussex sampled from the greenhouse experiment in Uppsala. Black dots indicate outliers from raw data. The red dots indicate the estimated means from the fitted GLMM's. Significant differences between inoculum treatments within each cultivar ($p < 0.05$) are indicated by compact letter display.*

3.1.5 Yield

Field trial

In the field trial, there were no differences in yield between the different treatments. In the ANOVA, there was a significant effect on yield per plant caused by inoculum treatment ($p < 0.05$) (Appendix 7). However, this did not show in the post-hoc (Sidak tests), which is why there is no compact letter display in Figure 12. In the field trial, there was no significant effect of the interaction of cultivar x inoculum treatment on yield per plant.

Greenhouse experiment

Comparisons of yield between the different treatments in the greenhouse revealed that inoculation resulted in significantly higher yield in all cultivars compared to

the uninoculated plants (Figure 12). Notably, peat inoculation resulted in a significantly higher yield compared to liquid inoculation in the cultivar Bråvalla. In the greenhouse experiment, there was a significant effect of cultivar x inoculum treatment on yield per plant (Appendix 8).

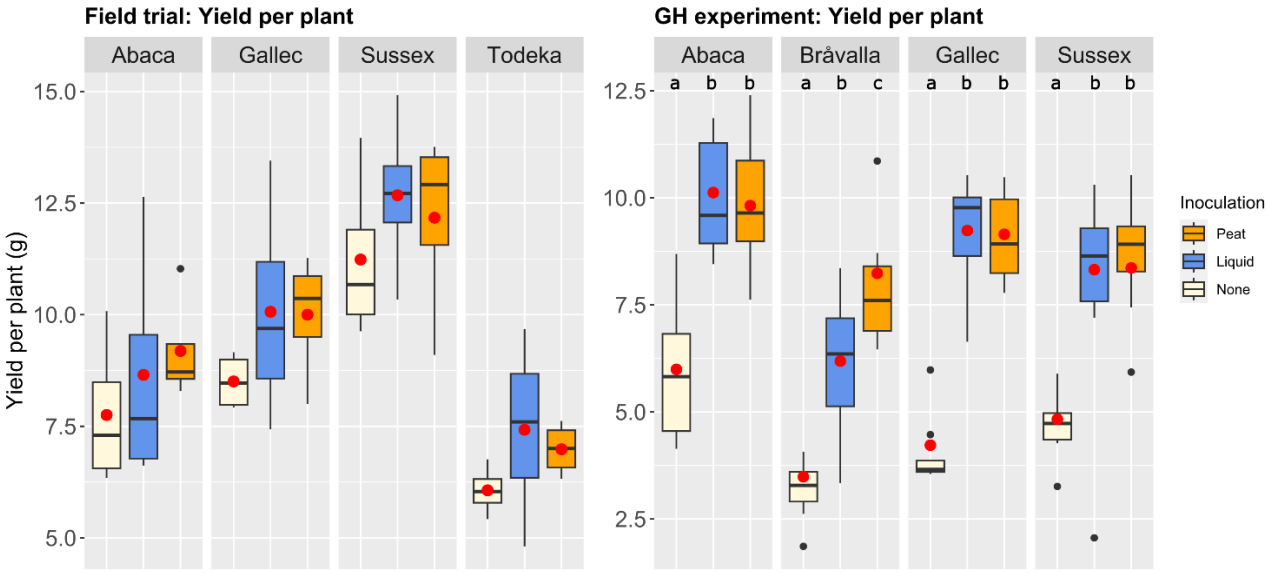

Figure 12. Effect of inoculation on yield per plant. *Boxplots visualizing raw data of inoculation effect on yield per plant for: (left) soybean cultivars Abaca, Gallec, Sussex and Todeka sampled at harvest in the field trial, and (right) soybean cultivars Abaca, Bråvalla, Gallec and Sussex sampled from the greenhouse experiment in Uppsala. Black dots indicate outliers from raw data. The red dots indicate the estimated means from the fitted LMM (field trial) and GLMM (greenhouse experiment). Significant differences between inoculum treatments within each cultivar ($p < 0.05$) are indicated by the compact letter display.*

3.1.6 Phenotypic traits not affected by inoculation method

In the field trial, there was no significant effect caused by inoculation on the traits: plant height, leaf biomass or the height of the lowest node (Appendix 5). In the greenhouse experiment, inoculation did not have an effect on plant height or the height of the lowest node (Appendix 6). Any differences in these phenotypic traits within or between cultivars were either caused by cultivar specific properties or not affected by the explanatory variables in this study (Appendices 7 & 8).

3.2 Nitrogen assessment in the field trial

3.2.1 SPAD measurements

The inoculation treatments did not have an effect on the SPAD-values when the plants in the field trial were measured at flowering (Appendix 5).

3.2.2 Nitrogen content in biomass and seeds

Biomass N

The inoculum treatments had a significant effect on the N content in the biomass of the plants sampled in the field trial at harvest, but the effect of the inoculation methods varied between the cultivars (Appendix 7). In the cultivars Abaca and Sussex, peat inoculation resulted in significantly higher N content, whereas liquid inoculation did not (Figure 13). In the cultivar Gallec, inoculation had no effect on N content. On the other hand, in Todeka, both liquid- and peat inoculation resulted in significantly higher N content. There were no significant differences in N content between plants treated with liquid- or peat inoculation in any of the cultivars.

Seed N

Significant differences in seed N were found between the treatments in each cultivar of the plants sampled in the field trial at harvest. However, the interaction between cultivar and inoculum treatment had a significant effect on seed N (Appendix 7), meaning there were different responses to the inoculants between the cultivars. In the cultivars Abaca and Sussex, peat inoculation resulted in significantly higher N content in the seeds compared to both uninoculated plants and plants inoculated with liquid inoculum (Figure 13). In Gallec, neither liquid- nor peat inoculation affected the N content in the seeds. In Todeka, both liquid- and peat inoculation resulted in significantly higher N content in the seeds and there were no significant differences between plants treated with liquid inoculum or peat inoculum.

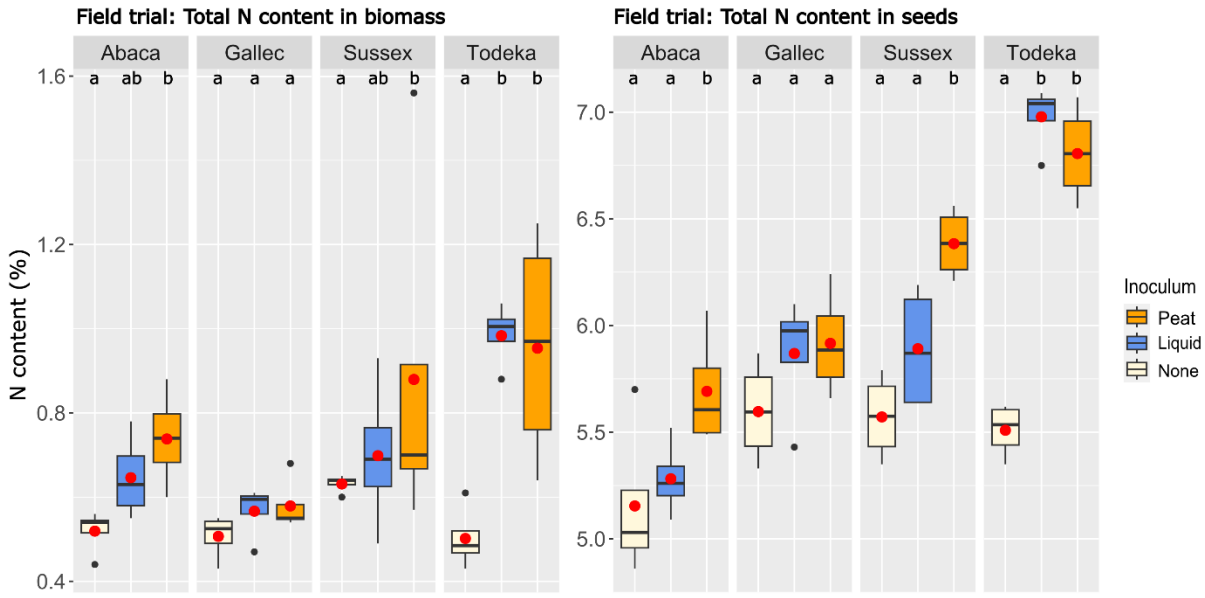

Figure 13. Effect of inoculation on N content in biomass and seeds. *Boxplots visualizing raw data of inoculation effect on nitrogen content per g dry mass (%) in biomass (left) and seeds (right) for*

the soybean cultivars Abaca, Bråvalla, Gallec and Sussex sampled at harvest in the field trial. Black dots indicate outliers from raw data. The red dots indicate the estimated means from the fitted GLMM's. Significant differences, calculated on the estimated means, between inoculation treatments within each cultivar ($p < 0.05$) are indicated by the compact letter display.

3.2.3 Percentage nitrogen derived from atmosphere in biomass and seeds

Biomass

Significant differences in %Ndfa in the biomass were found between the treatments in each cultivar. Peat inoculation resulted in a significantly higher amount of Ndfa in the biomass compared to uninoculated plants in all four soybean cultivars (Figure 14). Liquid inoculation resulted in a significantly higher amount of %Ndfa compared to uninoculated plants in the cultivar Todeka. In the cultivars Abaca and Sussex, inoculation with peat resulted in significantly higher %Ndfa in the biomass of the plants compared to the liquid inoculum. In the cultivars Gallec and Todeka, there were no significant differences in %Ndfa in the biomass between plans treated with liquid- and peat inoculum. The %Ndfa in the biomass of plants sampled at harvest was significantly affected by the interaction of cultivar x inoculum treatment (Appendix 7).

Seeds

The different inoculation methods resulted in significant differences of %Ndfa in the seeds within each soybean cultivar. Peat inoculation resulted in a significantly higher amount of Ndfa in the seeds compared to uninoculated plants in all four soybean cultivars (Figure 14). Liquid inoculation resulted in a significantly higher amount of Ndfa in the seeds compared to uninoculated plants in all soybean cultivars except for Abaca. Significant differences in %Ndfa in the seeds were seen between liquid- and peat inoculation in the cultivars Abaca and Sussex, where peat inoculation resulted in a significantly higher amount of %Ndfa in the seeds compared to liquid inoculation. The Ndfa in the seeds was significantly affected by the interaction of cultivar x inoculum treatment (Appendix 7).

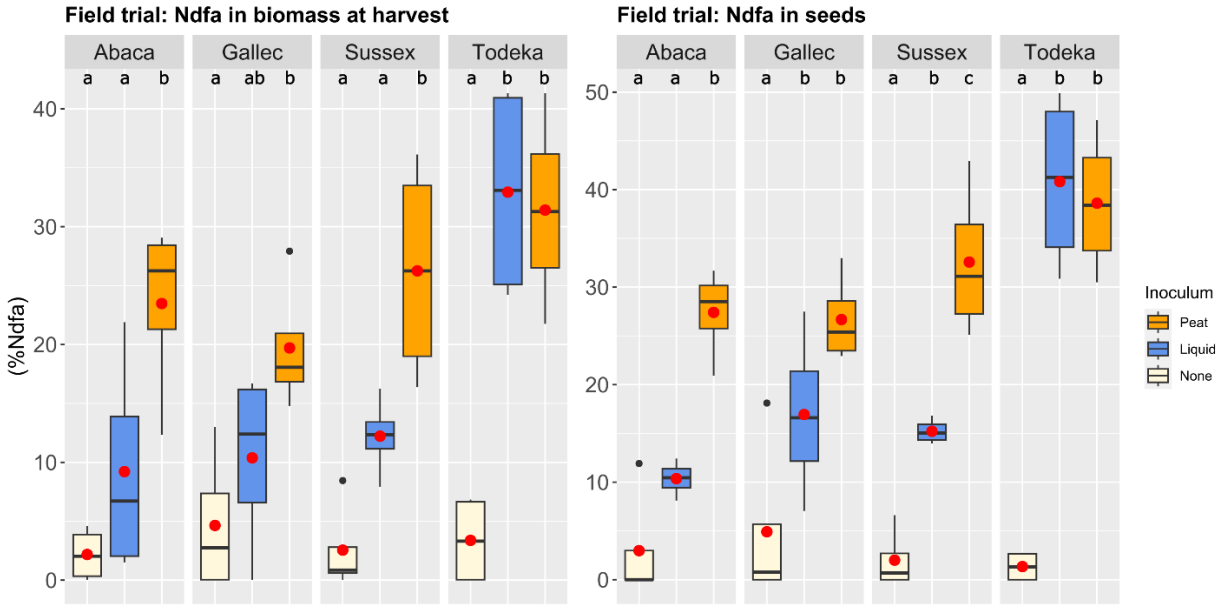

Figure 14. Effect of inoculation on %Ndfa in biomass and seeds*. Boxplots visualizing raw data of inoculation treatment effect on Ndfa in (left) biomass and (right) seeds for the soybean cultivars Abaca, Gallec, Sussex and Todeka sampled at harvest in the field trial. Black dots indicate outliers from raw data. Red dots indicate the estimated means from the fitted LMMs. Significant differences, calculated on the estimated mean, between inoculation treatments within each cultivar ($p < 0.05$) are indicated by the compact letter display.*

3.2.4 Protein yield

The protein yield was assessed both per plant and in kg/ha. The protein yield per plant was significantly affected by the inoculation method (Appendix 7). Significant differences between the inoculation methods were seen in the cultivar Todeka, where liquid inoculation resulted in a significantly higher protein yield per plant compared to uninoculated plants (Figure 14). Peat inoculation did not result in a protein yield per plant significantly different from neither liquid inoculation nor uninoculated plants in the cultivar Todeka. Inoculation did not result in significant differences in protein yield per plant compared to uninoculated plants.

The effect of inoculation changed when the plant density was included in the protein yield. Inoculation still had a significant effect on protein yield (Appendix 7). In the cultivar Sussex, the protein yield was significantly increased by peat inoculation (Figure 14). Inoculation with liquid inoculum did not result in a protein yield significantly different from peat inoculation or uninoculated plants. The protein yield in kg/ha was not affected by inoculation with peat – or liquid inoculum in the cultivars Abaca, Gallex and Todeka.

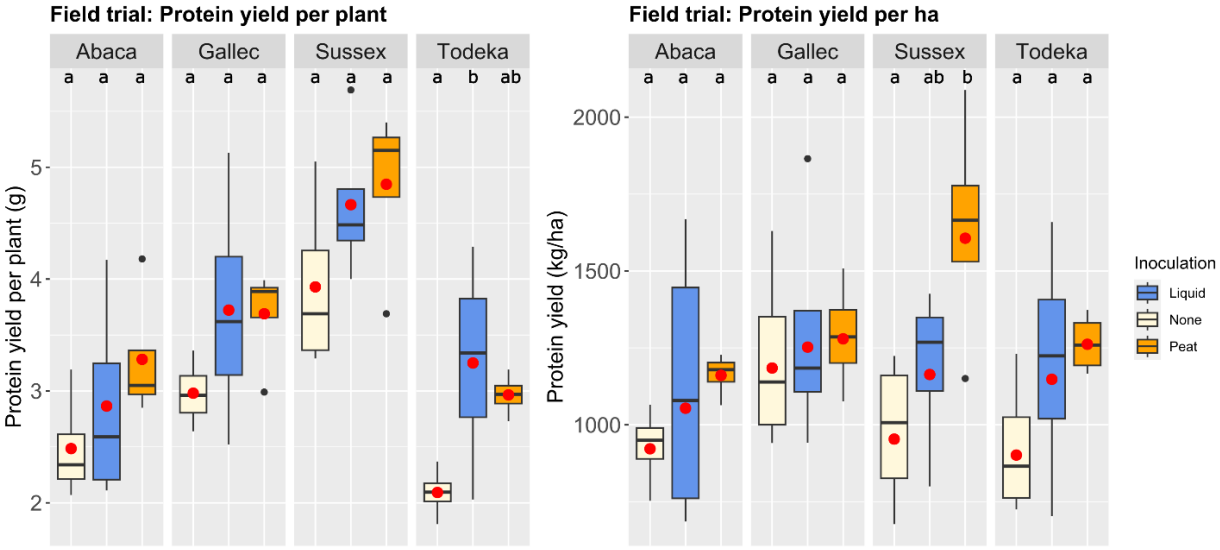

Figure 15. Effect of inoculation on protein yield. *Boxplots visualizing raw data of inoculation treatment effect on (left) protein yield per plant biomass and (right) protein yield in kg/ha for the soybean cultivars Abaca, Gallec, Sussex and Todeka sampled at harvest in the field trial. Black dots indicate outliers from raw data. Red dots indicate the estimated means from the fitted LMM's. Significant differences, calculated on the estimated mean, between inoculation treatments within each cultivar ($p < 0.05$) are indicated by compact letter display.*

3.3 Nitrogen assessment in the greenhouse experiment

3.3.1 Greenhouse experiment – weekly measurements

The weekly SPAD measurements used for N assessment in the greenhouse experiment showed differences in N content in the leaves between inoculated and uninoculated plants of each cultivar. The SPAD-values did not vary between treatments up to the measurements on day 36 in all cultivars (Figure 16). On day 43, the SPAD-values had started to decrease in the uninoculated plants of Abaca, Bråvalla and Gallec. The decrease in SPAD-value was later in Sussex, evident on day 50. From days 43 to 50, the SPAD-values continued to decrease in all four cultivars until the end measurements. The inoculated plants showed an increase in SPAD-value from the first measurements on day 15 to the measurements on day 57. After day 57 the SPAD-values decreased in the inoculated plants of Abaca, Bråvalla and Gallex, whereas the SPAD-value stagnated in Sussex until the end measurements. There were no evident differences in SPAD-values and -dynamics between plants inoculated with peat – or liquid inoculum.

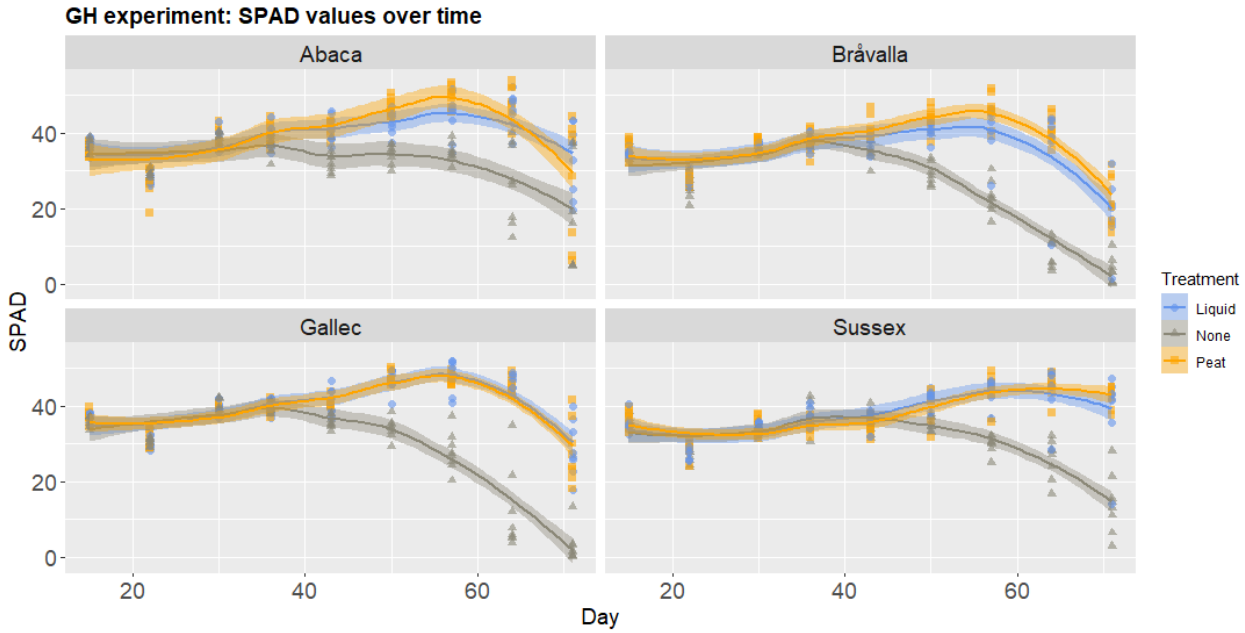

Figure 16. Effect of inoculation on SPAD-values over time. *Time series of SPAD (y-axis) measured over time (Day, x-axis) measured on four different soybean varieties (Abaca, Bråvalla, Gallec and Sussex) every seven to eight days in a greenhouse experiment conducted in Uppsala in autumn/winter of 2023. The cultivars were treated with liquid inoculum (blue line, round shape), peat inoculum (orange line, square shape) or were uninoculated (brown line, triangle shape). The measurements started on day 15 and finished on day 71 of the experiment. Where SPAD measured 0 there were no leaves left on the plants to measure. For the lines local polynomial regression fitting (loess) was used. The coloured areas around the lines are 95% confidence intervals.*

3.3.2 Visual comparisons of N deficiency

Chlorotic leaves were visible on Bråvalla at day 54 in the plants without inoculum treatment (Figure 17). The uninoculated plants of Abaca, Gallec and Sussex had leaves of a lighter green colour compared to the inoculated plants on day 54 of the experiment. No difference in overall colour was seen between the plants treated with peat inoculum and liquid inoculum. On day 65 of the experiment, the uninoculated plants of all cultivars had visible chlorotic leaves. In Bråvalla and Abaca, a few chlorotic leaves were visible on both the liquid- and peat inoculated plants. In comparisons within all cultivars, there were no visual differences between plants treated with liquid- or peat inoculum on day 65.

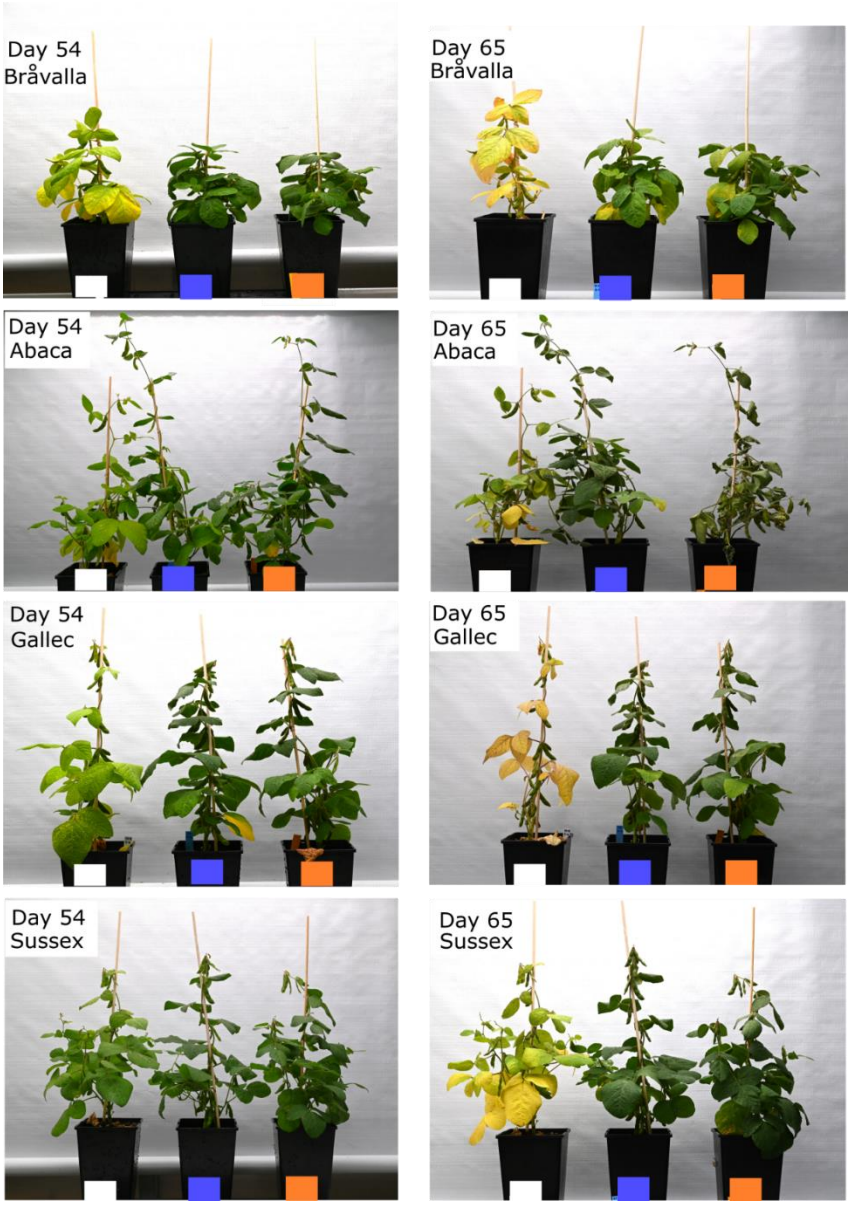

Figure 17. Effect of inoculation on visual symptoms of N deficency. *Photos taken of soybean plants from the greenhouse experiment at Ultuna during autumn/winter 2023. One representative plant of each treatment and cultivar were chosen and photographed at two different occasions: day 54 and day 65 of the experiment. White = no inoculum; Blue = liquid inoculum; Orange = Peat inoculum.*

4. Discussion

There is an interest in increasing the legume production in Sweden, for which soybean would be a crop of interest. One main benefit of cultivating legumes is their ability to fix their own N through symbiosis with rhizobia. To cultivate soybean in Sweden capable of SNF, there is a need to inoculate the seeds since rhizobia compatible with soybean are not native to Swedish soils (Giller 2001; Fogelfors 2015). Since soybean have been cultivated in Sweden to a very small extent, there is little experience on which inoculum products and soybean cultivars work well in Swedish cropping conditions. In this study, inoculation of soybean cultivars suitable for cultivation in Sweden had a significant effect on several traits, such as yield, TKW, nodule number, nodule weight and root weight, compared to uninoculated plants. However, the inoculants proved to have different symbiotic performance, where inoculation with peat as a carrier more frequently resulted in an improvement in the traits and a more successful BNF compared to uninoculated plants than having liquid as a carrier did. The superior effects of peat were more evident in the assessments from the field trial than in the greenhouse experiment. Additionally, the soybean cultivars did not respond similarly to the treatments, and the interaction effects of cultivar x inoculum were seen to significantly influence several traits, such as TKW, nodule number and root biomass, as well as the %Ndfa and N content in the plants (Appendices 7 & 8). Hence, there were combinations of soybean cultivars and inoculum products performing superior to other combinations. One combination that appeared superiorly in protein yield and yield, as well as showed stability in effective symbiosis in both field – and greenhouse conditions, was the cultivar Sussex treated with the peat inoculant.

4.1 Effect of inoculation method on plant N and phenotypic traits

One major purpose of inoculating legumes is to provide them with N-fixing bacteria for the plants to acquire N without having to add N fertilizer (Bashan 1998). Therefore, the difference in N content between uninoculated plants and inoculated plants in both the field trial (Figure 13) and greenhouse experiment (Figure 16) in this study was expected since the uninoculated plants were not provided with any

N-fertilizer during the experiment. In the greenhouse experiment, the higher N content in the inoculated plants compared to the uninoculated plants (Figure 16) was a sign that the N-fixation in the inoculated plants was effective enough to compensate for the lack of N in the soil, and even increase the N content in the leaves, while the plants solely dependent on soil N (Appendix 4) decreased in N content. Consequently, even though the %Ndfa was not measured for the plants in the greenhouse experiment, the %Ndfa in the inoculated plants should be higher than the %Ndfa in the uninoculated plants. Additionally, as seen in Figure 9 and Figure 10, the nodule weight and nodule number were significantly increased in the inoculated plants compared to the uninoculated plants in the greenhouse experiment, which can also be an indication of successful SNF in the inoculated plants (Thilakarathna & Raizada 2017). This supports the first hypothesis, where inoculated plants were expected to have an increase in N content and effectiveness of N-fixation compared to uninoculated plants, which was seen in both the field trial and the greenhouse experiment. However, in the field trial, the effect of the inoculation methods on %Ndfa in both biomass and seeds, and on N content in the seeds, varied between the cultivars (Figures 13 & 14). Peat inoculation proved to be the inoculation method with the most consistency in significantly increasing %Ndfa and N content in the soybean cultivars (Figures 13 & 14). Liquid inoculation did not show the same consistency, only significantly increasing the N content compared to uninoculated plants, both in the biomass and in the seeds, in the cultivar Todeka. In the first hypothesis, one of the inoculation methods was expected to have beneficial properties for success of the inoculation in a given environment, which is evident for peat in the field trial and therefore supports the hypothesis. On the contrary, there was no evident difference in N content in the leaves between plants inoculated with peat– or liquid inoculum in the greenhouse experiment (Figures 16 & 17), which does not support the first hypothesis. The varying performance of liquid inoculants can be confirmed by other studies, where they have been reported to perform both inferior (Zimmer et al. 2016) and equal (Pannecoucque et al. 2018) to peat inoculants in cold climates. However, comparisons between liquid- and peat-based inoculants in this study and other studies should be made with caution since the inoculants have varying compositions of both bacteria and carriers depending on the commercial product. Consequently, it may not only be the carrier in the inoculant that decides the effect of the inoculation, but also the rhizobial species or -strain. Yet, in this study, comparisons can be made between the same treatments in two different environments (treatments including Abaca, Gallec and Sussex), where it is evident that the liquid inoculant was able to effectively nodulate and establish SNF with all soybean cultivars in the greenhouse trial, and consequently, the liquid carrier may have lacked properties to make it equally effective in the field trial. On the other hand, the peat inoculant might have properties that supported rhizobial survival and nodulation success

more than the liquid inoculat did in field conditions, resulting in superior responses in the plants treated with the peat inoculant. For example, having peat as a carrier provides protection for the rhizobia and could also hold moisture better than a liquid (Tittabutr et al. 2007), which could be important traits for a carrier to have in a field with fluctuating environmental conditions. However, these properties might not give an advancement over the liquid formulation in favorable environmental conditions, thus resulting in a more equal performance between the two formulations in the greenhouse experiment.

Except for N-fixation and N content in the plants, several of the assessed phenotypic traits were affected by inoculation. The traits where inoculation affected at least one cultivar in either the field trial or the greenhouse experiment were stem biomass (GH), root biomass (GH), RS ratio (GH), nodule weight, nodule number, TKW and yield. The traits where inoculation did not have a significant effect were plant height, height of the lowest node, leaf biomass (FT) and SPAD-values at flowering (Figures 5 & 6). In several of the traits where inoculation did have a significant effect, the inoculation method influenced the response, which is consistent with the second hypothesis. As previously mentioned, liquid inoculation proved to be more efficient in the greenhouse experiment, which was also evident in the phenotypic traits. One sign of the liquid inoculation affecting the traits more in the greenhouse environment compared to the field trial could be seen in the nodule weight and nodule number (Figures 9 & 10). In the field trial, liquid inoculation did not result in a significant increase in nodule weight in any of the cultivars and the nodule number in any of the cultivars except for Todeka, while it significantly increased nodule weight and nodule number in all cultivars in the greenhouse experiment. Peat inoculation resulted in a significant increase in both nodule weight and nodule number compared to uninoculated plants in both the field trial and the greenhouse experiment, in all cultivars except for Abaca. A similar trend was seen for TKW, where liquid inoculation was not significantly different from uninoculated plants for all cultivars except Todeka in the field trial, while it significantly increased the TKW for all cultivars in the greenhouse experiment (Figure 11). Peat inoculation did on the other hand result in a significant increase in TKW in all cultivars in the field trial except for Gallec, for which inoculation did not have an effect. Therefore, peat inoculation proves to be more consistent with improving nodulation and TKW compared to liquid inoculation.

One notable result from the greenhouse was how inoculation affected the root biomass. In the greenhouse experiment, the root biomass was found to be significantly higher in uninoculated plants compared to inoculated plants (except for Bråvalla) (Figure 7), which contradicts the second hypothesis where an increase in the traits was expected from inoculation. In addition to a lower root biomass, the

SPAD-values indicated a lower N-content (Figure 16), there were visible signs of N-deficiency (Figure 17) and significantly lower nodule number and weight (Figures 9 & 10) in uninoculated plants compared to the inoculated plants. The higher root biomass in the uninoculated plants could therefore be a response to N-deficiency. As reviewed by Hermans et al. (2006), N-deficiency changes the allocation of carbon in the plants, leading to more carbon being transported to the roots and an increase in root growth. This also increases the root-to-shoot ratio in the plants (Hermans et al. 2006), which is consistent with the results in this study where the RS ratio was increased in the uninoculated plants compared to the inoculated plants in the greenhouse experiment (Figure 8). However, it should be pointed out that the allocation of biomass to the roots can be affected by other limiting factors than N-deficiency, such as poor water availability or deficiencies of other nutrients (Hermans et al. 2006; Poorter et al. 2012). The only cultivar where significant differences in root biomass between liquid – and peat inoculation showed was Abaca, where peat inoculation resulted in a significantly lower root biomass compared to liquid inoculation (Figure 7). This could be an indication of more efficient N-fixation in plants treated with peat inoculum compared to liquid inoculum. However, the SPAD-values between liquid- and peat inoculated replicates of Abaca (Figure 16), as well as the ocular assessment of the plants (Figure 17), did not show any differences between the two treatments. This can not be explained by nodule number or nodule biomass either, since no significant differences between liquid inoculation and peat inoculation of Abaca were seen in these traits (Figures 9 & 10).

Even though inoculation affects several phenotypic traits, the traits affecting the economic value of the crop are probably of most interest to the farmer. In this study, both the yield and the protein yield were assessed. The yield in the field trial was significantly affected by inoculation according to the ANOVA (Appendix 7) and the estimated means for the inoculated plants were slightly higher than for the uninoculated plants, but no significant differences between the treatments appeared in the post-hoc analysis (Figure 12). In the greenhouse experiment, the yield was significantly increased by both inoculation methods in all cultivars, and, additionally, the yield was significantly increased by peat inoculation compared to liquid inoculation in the cultivar Bråvalla (Figure 12). This shows that the inoculants have the capability of increasing the yield compared to uninoculated plants, supporting the second hypothesis. Nevertheless, the yield could not be assessed per plot in the field trial, which would have given better predictions of how the inoculants affected the yield in the plots as a whole, rather than randomly chosen individual plants in each plot. The protein yield was not affected by inoculation in the same extent as %Ndfa and N content were; it was only significantly increased by liquid inoculation in Todeka for g/plant and by peat

inoculation in Sussex for kg/ha (Figure 15). The reason for this is probably because the yield in the field trial was not as affected by inoculation as the N-fixation was. However, depending on the cultivar, inoculation did have an effect on the protein yield, showing the importance of the best performing soybean cultivar x inoculum treatment combination to get the highest possible protein yield. When assessing the protein yield in kg/ha, the estimated plant density for each plot was used to calculate the seed yield, which should not be considered the same as assessing the protein yield of the whole plots. This may also have caused the protein yield to appear high compared to similar experiments (Zimmer et al. 2016; Pannecoucque et al. 2018). Hence, the effect the inoculation treatments had on protein yield could be lower on a field scale compared to the findings in this study.

4.2 Effects of environment and interactions between Cultivar x Inoculum treatment

The effects of inoculation were influenced by the environment, which has also been reported in other studies (Zhang et al. 2003; Zimmer et al. 2016). As previously mentioned, the two inoculants performed more equally in the greenhouse experiment compared to the field trial where inoculation with peat resulted in superior performance in N content, %Ndfa, nodule number and nodule weight. Since these are traits directly linked to the symbiosis between *B. japonicum* and the soybean cultivars, the environmental conditions affecting nodulation, rhizobial survival and BNF were probably more beneficial in the greenhouse experiment compared to the field trial, which is consistent with the third hypothesis. However, the fact that the plants had fewer nodules and a lower nodule weight in the field trial compared to the greenhouse experiment could be an effect of there being more plant available N in the soil in the field trial plots (Appendix 3) compared to the restricting pots in the greenhouse experiment (Appendix 4). This might have resulted in the plants in the field trial using the N sources in the soil above N-fixation since N-fixation consumes more energy for the plants (Abendroth et al. 2006; Tamagno et al. 2018). The larger amount of available N in the soil in the field trial could also be a reason for the inoculation having less effect on traits such as yield and TKW in the field trial compared to the greenhouse experiment (Figures 11 & 12). However, it was evident that both %Ndfa and N content were significantly affected by inoculation in the field trial (Figures 13 & 14), thus the TKW and yield in the field trial might not have been affected by the N increase from inoculation. Therefore, another possible reason for the inoculation having less effect in the field trial is that there might have been other factors restricting the yield and TKW in the field trial than N, for example, water or other nutrients than N. An improvement for future experiments would be to include a treatment with N

fertilizer to detect if there are other factors than N limiting plant growth. If that were the case, there would be no beneficial effect of the inoculants (Thilakarathna & Raizada 2017).

When comparing the cultivars included in both the field trial and the greenhouse experiment, the baselines for both TKW and yield were almost exclusively lower in the greenhouse experiment compared to the field trial, which is not supported by the third hypothesis where an increase was expected in these traits in the greenhouse experiment compared to the field trial. Even though the environmental conditions in the greenhouse supported rhizobial survival and nodulation, the plants in the greenhouse could have been restricted by the lower light intensity from the greenhouse lighting compared to the sun in the field, which results in less efficient photosynthesis (Wimalasekera 2019). The light intensity also affects total biomass and seed yield negatively in soybean (Jumrani & Bhatia 2020) which would explain why the TKW and yield were higher in the field trial compared to the greenhouse experiment.

As previously discussed, the effect of the inoculation methods varied between cultivars, resulting in an interaction between soybean cultivar x inoculation method. The interaction had a significant effect on root biomass, stem biomass, RS ratio, nodule number, nodule weight (GH), TKW, yield (GH), N content in biomass and seeds and %Ndfa in biomass and seeds (Appendix 7 & 8). The fact that the interaction of plant genotype x rhizobia strain influences the responses in N content and %Ndfa, as well as in the phenotypic traits, does support the fourth hypothesis. However, there were several traits where the interaction did not have an effect: protein yield, stem biomass, nodule weight (FT), yield (FT), plant height, height of lowest node, leaf biomass and SPAD at flowering, which does not support the fourth hypothesis. Some of the results in this study are inconsistent with the findings of Zimmer et al. (2016), where the authors concluded that the interaction between soybean cultivar x *Bradyrhizobium* strain did not have a significant effect on %Ndfa, nodulation, grain yield and TKW, for which interactions were found in this study. Additionally, the authors found an interaction effect on protein yield, which was not seen in this study (Appendix 7). However, the authors did find that the interaction had a significant effect on the N (protein) content in the seeds (Zimmer et al. 2016), which complies with the results in this study. But, as mentioned previously, comparisons between studies can become difficult, since other soybean cultivars and inoculants were used in the study by Zimmer et al. (2016), as well as other experimental sites.

There were examples in the results where combinations of cultivar x inoculum treatment were less successful in affecting a trait compared to the other

combinations, such as for the cultivar Gallec, for which the N content in both the seeds and the biomass was not affected by any of the inoculation methods in the field trial (Figure 13) and the nodule number and nodule weight of Abaca (Figures 9 & 10), which were not affected by inoculation in the field trial. However, the results from the greenhouse experiment show that both Abaca and Gallec could be effectively nodulated and fix N with the same inoculants. Therefore, the unsuccessful combinations are probably a consequence of other limitations in the field trial or that there was contamination of the inoculant to untreated plots. However, the %Ndfa in both the seeds and the biomass of Gallec was significantly increased by inoculation compared to uninoculated plants. Consequently, an increased N content in uninoculated plants due to contamination of inoculum to untreated plots of Gallec can be excluded, which could indicate that Gallec has been able to meet its N intake from the soil or that other factors might have restricted the effect of inoculation on N content in Gallec.

In the selection of the most efficient inoculum method, the final judgment should be made based on how the inoculant performs under field conditions, since it is ultimately in the field that the inoculant will be used on a commercial scale. Additionally, not just the environment but also the combinations of soybean cultivar x inoculum can result in differences in performance. This shows the importance of testing combinations of soybean cultivars and inoculants at different sites. For example, in Germany, Zimmer et al. (2016) could give different suggestions on combinations of inoculants and soybean cultivars depending on the intended end product of the beans (based on protein content) at different production sites. The authors also concluded the importance of effectiveness in commercial inoculants for the success of soybean cultivation in new areas. There have been successful attempts at selecting *Bradyrhizobium* strains and soybean cultivars to get the most efficient N-fixation for the environment in a specific area (Alves et al. 2003; Zhang et al. 2003). Zhang et al. (2003) tested *Bradyrhizobium* strains originating from cooler areas, which generated higher N-fixation in cool conditions compared to strains in commercial inoculants. For future research, an inoculant containing a *Bradyrhizobium* strain adapted for N-fixation in areas with short (cold) growing seasons would be of interest to test with cultivars of MG 000 in Sweden. Studies where inoculants of soybean have been tested in other cool climate areas also suggest more research on which soybean cultivar – and inoculant /*Bradyrhizobium* combinations are superior for effective symbiosis under cool soil conditions (Lynch & Smith 1993; Zimmer et al. 2016).

4.3 Which soybean cultivar was superior?

In this study, the difference in traits caused by only cultivar (soybean genotype) effect was not further investigated. The results could be used to interpret what cultivars, for example, are higher yielding in the experiments, such as Sussex in average being higher yielding in both grain and protein than Todeka in the field trial, independent of inoculum treatment (Figures 12 & 15). In this study, the MG 000 cultivar Sussex showed an even performance between both the field trial and greenhouse experiment, and additionally had the highest yield and protein yield without inoculation. Sussex has proved to be a high-yielding soybean variety with high adaptability to different tillage systems (Wijata et al. 2023). One downside with the cultivar Sussex is that it appeared to take longer to mature compared to the other tested cultivars, which is less suitable for the short growing season in Sweden. In the greenhouse experiment, the SPAD-measurements showed how the N content in the leaves stagnated in Sussex until the last measurements, compared to the other cultivars where the N content culminated on day 57 (Figure 16). This could be a sign of late maturation in Sussex, though leaf senescence is initiated at seed filling in soybean, resulting in the N being translocated to the seeds (Singh 2010). However, Sussex, in combination with the peat-based inoculant LegumeFix, was the highest-yielding combination in protein and also yielded high in grain. Additionally, the cultivar showed a good response in nodulation from peat inoculation, which, if eventual limiting factors had been provided, might had resulted in even better effects on yield and protein yield. However, to be sure of which soybean cultivars and inoculum products are more suitable for use in Swedish conditions, further studies are needed. For example, it would be necessary to test the combinations over several years, since the combinations may fluctuate in performance over time depending on the current environmental conditions during a year. It would also be of interest to test the combinations at several test sites to determine if factors such as soil type and soil pH would affect the performance of the soybean cultivar and inoculant.

4.4 Sources of error and areas of improvement

Nodule assessment

When assessing the nodules from both the field trial and the greenhouse experiment, all nodules on the roots were included in the data without minding the colour of the nodules. As mentioned previously, the colour of nodules may vary depending on whether there is active N-fixation in the nodules or not (Unkovich et al. 2008). Therefore, the results of nodule number and nodule weight in this study might have been overestimated since inactive nodules or nodules with poor SNF

could have been included in the data. Thus, an improvement to the methodology would be to only include nodules with colour that indicates active N-fixation.

Inoculum contamination

Nodules were found on the roots of uninoculated plants in all cultivars in the field trial and in all cultivars except Bråvalla in the greenhouse experiment (Figures 9 & 10). In the field trial this might have occurred due to contamination between the plots. The plots in the field trial lay close to each other, and the experiment was completely randomized, which consequently led to the placement of plots with inoculum treatment next to plots with uninoculated plants (Figure 3). The plants were sampled from the middle of the plots, but the rhizobia can spread in the soil, which might have resulted in contamination of the plants in the middle of the plots as well. This did result in some amounts of Ndfa in the uninoculated plants (Figure 14) and can therefore have affected the results in this study, especially regarding the effect of liquid inoculation since the difference between plants inoculated with liquid inoculum or no inoculum might have been bigger. In the greenhouse experiment, the nodules on the uninoculated plants were primarily found in Abaca (total of eight plants: 268 nodules or 1.2 g dry biomass), but also in Sussex (total of eight plants: 89 nodules or 0.53 g dry biomass). As mentioned previously, Abaca had a previous seed coating, which, if rhizobia still viable, might have led to the development of root nodules in Abaca. However, since there was also nodule formation on uninoculated plants of Gallec and Sussex, it is more likely to have occurred due to cross-contamination at sowing even though precautions were taken to avoid it, or the presence of soybean-compatible rhizobia in the soil used. Even so, nodule number and nodule weight still increased significantly with both liquid – and peat inoculation compared to uninoculated plants in all cultivars in the greenhouse experiment (Figures 9 & 10) and the N content was higher in the leaves of the inoculated plants compared to uninoculated plants (Figure 16). Therefore, the lack of contamination might have resulted in even bigger differences between untreated and inoculated plants in the greenhouse experiment, but the differences between inoculated and uninoculated plants were still evident.

Calculation for %Ndfa

When the calculations of %Ndfa were done according to Equation 6 the reference plants used were calculated as the average $\delta^{15}N$ of the uninoculated plots in each block. As previously discussed, there were nodules on uninoculated plants in the field trial, and when the plants were sampled at harvest, the roots were left in the soil. Consequently, we do not know if the reference plants were nodulated, which might have affected the estimations of %Ndfa in the field trial (Figure 14). Additionally, the B-value used for calculating the %Ndfa was taken from literature and was not grown for each cultivar x inoculation method combination in a medium

where the plants were solely dependent on N-fixation for provision of N, as suggested by Unkovich et al. (2008). Lastly, when the samples were milled before sending them for ^{15}N analysis, there was a risk of cross-contamination between the samples, which might have affected the results. Unkovich et al. (2008) suggest that a "blank" material can be used between samples, containing a plant material with low N and ^{15}N content, to reduce the risk of cross-contamination, which could be an improvement of the methodology in this thesis.

Greenhouse experiment

The plants in the greenhouse experiment started to lose their leaves as they were maturing and the leaves got mixed on the bench in the greenhouse, making it impossible to determine which leaves belonged to which plants and to assess the leaf biomass. To avoid this from happening and enable the assessment of leaf biomass, plastic bags (with holes for air) could have been put over each plant to collect the leaves as they fell off. Another flaw in the greenhouse experiment was the infestation of thrips and flies on the plants. Damage from the thrips was seen on the leaves, which might have influenced the SPAD-measurements as well as the photosynthetic capacity of the leaves. Whether the flies did any damage to the plants or not is unknown. Lastly, there is potential to test the effect of the inoculants compared to uninoculated plants that completely lack N in the greenhouse experiment, which cannot be done in a field trial where soil mineral N will be present to some extent. However, in this trial, soil containing N fertilizer was used as the potting medium in the greenhouse (Appendix 4), which both might have influenced nodulation in the inoculated plants (Abendroth et al. 2006; Tamagno et al. 2018) and could not represent a treatment completely lacking N for comparison. Therefore, a different potting medium could have been used, such as sand, to avoid any additional N in the soil.

5. Conclusions

The possibility of soybean cultivation in Sweden would enable Swedish farmers to grow grain legumes more frequently and, additionally, cultivate a crop with high protein quantity and quality properties for a wide range of uses. In this study, both soybean cultivars with potential for cultivation in Sweden, as well as different formulations of *B. japonicum* inoculants, were tested to evaluate their performance in field- and greenhouse conditions. Inoculation of soybean resulted in significantly higher %Ndfa and N content in both biomass and seeds in field conditions and in higher N content (SPAD-values) in greenhouse conditions compared to uninoculated control. The peat inoculant was more consistent in significantly increasing %Ndfa and N content in the seeds than liquid inoculation in the field trial, while the inoculants performed more equally in the greenhouse experiment. The same trend was seen in how nodule number and nodule weight were affected by inoculation in the two environments. An explanation for this could be that peat had beneficial properties for rhizobial survival in the field trial, but the more favorable environmental conditions in the greenhouse resulted in equal performance between the formulations. Inoculation of soybean was seen to significantly affect the phenotypic traits: TKW, yield, root biomass, stem biomass, RS ratio, nodule weight, nodule number and protein yield compared to the uninoculated control. However, these responses varied depending on the environment and soybean cultivar. Where there were significant differences between the treatments in a cultivar, peat inoculation almost always affected the trait in a superior way. Inoculation did not have a significant effect on the traits: plant height, height of the lowest node, leaf biomass and SPAD at flowering. In comparisons between the greenhouse experiment and field trial, the traits affected by rhizobial survival (N content, nodule number and nodule weight) were enhanced in the greenhouse experiment, while traits probably limited by light intensity (TKW and yield) were superior in the field trial. Lastly, the interaction effect between cultivar x inoculation method was significant for root biomass, stem biomass, RS ratio, nodule number, nodule weight (GH), TKW, yield (GH), N content and %Ndfa. The MG 000 cultivar Sussex in combination with the commercial peat inoculant LegumeFix resulted in the highest protein yield and second highest yield out of all combinations of cultivar x inoculation method, and also resulted in significantly increased nodule number, %Ndfa and seed N content compared to the liquid inoculant. The combination of Sussex and LegumeFix could be a possible suggestion for Swedish farmers wanting to try soybean cultivation. However, the combination needs to be assessed over a longer period of time and at more locations to be able to determine the evenness of the performance.

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Popular science summary

Legumes are special plants for many reasons, but two of their superior traits are that they can fix nitrogen (N) from the air in symbiosis with N-fixing bacteria (rhizobia) and contain high amounts of protein. There is an interest in increasing grain legume production in Sweden and one solution to do so would require a wider diversity of grain legume species cultivated in Sweden, for example soybean. Soybean is originally a tropical plant, but plant breeding has resulted in soybean cultivars able to grow in Sweden. However, the rhizobia compatible with soybean do not occur naturally in Swedish soils and therefore, to grow soybean able to fix N in Sweden, one must use an inoculant which is a product containing rhizobia. The purpose of this thesis was to evaluate how N-fixation and several traits in soybean cultivars suitable for cultivation in Sweden were affected by either not treating the plants with any rhizobia or by treating them with inoculants based on either peat or a liquid. Additionally, the effects of the treatments were looked at in two different environments: one field trial on the island of Gotland and one greenhouse experiment in Uppsala. The results showed that the N-fixation was more effective in the plants treated with an inoculant compared to the uninoculated plants in both the field trial and the greenhouse experiment, which also resulted in a higher N content in the inoculated plants. N is an important part of protein, and higher amounts of N in the seeds will therefore result in a higher protein content, which is wanted by the farmers. Except for N-fixation, the inoculants had an effect on several soybean traits such as yield, stem weight, root weight, the ratio between root - and stem weight, nodule weight, nodule number and protein yield. There were also several traits where no effect of the inoculants was observed. These traits were plant height, the height of the lowest node and leaf weight. The effect the inoculants had differed between the greenhouse experiment and the field trial, as well as between the different soybean cultivars. The peat-based inoculant is thought to work better in the field environment compared to the liquid inoculant since it can better protect the rhizobia in the more challenging field environment. In the greenhouse experiment, the environment was not as testing, and therefore the liquid-based inoculant worked just as well as the peat-based one in the greenhouse. The peat-based inoculant performed best overall and is suggested over the liquid inoculant based on the results of this study. One of the soybean cultivars tested, Sussex, performed well in combination with the peat-based inoculant and would be of interest for Swedish farmers to cultivate and further investigate.

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Appendix 1

Table with the results from the soil analysis showing the amount of nutrients in the soil and the corresponding soil classes (Kl) (where possible) in the soil for each of the plots in the field trial at Gotland in 2023. Separate tables for N content in the plots are found in Appendix 2 and 3.

Plot number	P-AL	Kl	K-AL	Kl	Mg-AL	K/Mg-AL	Ca-AL	Al-AL	Fe-AL	K-HCl	Kl	P-HCl	Kl	Cu-HCl
	mg/100g		mg/100g		mg/100g		mg/100g	mg/100g	mg/100g	mg/100g		mg/100g		mg/Kg
1	4,3	III	8,3	III	42,6	0,2	3485	12	10	136,0	3	40,2	2	13,0
2	3,6	II	9,7	III	48,4	0,2	4130	13	13	158,9	3	41,6	3	13,9
3	3,6	II	9,0	III	45,8	0,2	3926	13	12	157,0	3	41,0	3	14,6
4	3,5	II	8,5	III	45,9	0,2	3976	12	11	152,5	3	40,0	2	14,1
5	3,4	II	9,3	III	41,2	0,2	3706	12	10	158,2	3	40,9	2	14,5
6	3,8	II	9,0	III	43,1	0,2	3719	12	10	149,7	3	41,2	3	14,3
7	3,7	II	8,7	III	45,1	0,2	3985	12	10	144,8	3	39,5	2	14,3
8	4,1	III	9,1	III	43,1	0,2	3869	12	10	149,2	3	41,4	3	14,3
9	4,3	III	9,5	III	43,7	0,2	3865	12	10	159,7	3	42,1	3	14,6
10	3,5	II	8,5	III	48,7	0,2	4094	13	12	165,7	3	42,5	3	14,9
11	3,7	II	8,8	III	49,8	0,2	4180	13	12	173,5	3	42,0	3	15,3
12	3,3	II	8,8	III	49,8	0,2	4368	12	12	174,8	3	42,0	3	15,4
13	3,6	II	8,7	III	51,2	0,2	4465	12	13	163,7	3	43,2	3	15,0
14	3,9	II	8,7	III	50,1	0,2	4457	12	12	171,0	3	44,2	3	15,9
15	4,4	III	8,8	III	51,9	0,2	4557	13	14	164,0	3	44,6	3	15,3
16	5,2	III	8,3	III	48,8	0,2	4545	13	12	160,0	3	45,7	3	15,6
17	6,8	III	8,9	III	48,0	0,2	4493	13	12	159,1	3	48,4	3	15,5
18	7,0	III	8,1	III	45,0	0,2	4158	12	11	159,8	3	49,5	3	15,5
19	8,1	IVA	8,5	III	48,1	0,2	4565	13	12	160,9	3	52,7	3	15,8
20	7,8	III	7,8	II	46,7	0,2	4235	13	12	156,4	3	49,8	3	16,1
21	9,0	IVA	8,8	III	45,9	0,2	4292	13	11	157,6	3	50,9	3	16,3
22	8,0	III	8,1	III	45,3	0,2	4011	13	11	152,0	3	49,2	3	15,8
23	6,5	III	8,1	III	46,3	0,2	3929	13	11	146,7	3	45,8	3	15,1
24	5,9	III	9,0	III	47,4	0,2	3923	12	12	153,6	3	46,4	3	15,6
25	3,3	II	8,9	III	50,5	0,2	3888	14	12	165,7	3	40,7	2	14,0
26	3,4	II	9,8	III	51,3	0,2	4005	13	12	165,8	3	41,4	3	13,9
27	3,1	II	9,0	III	50,7	0,2	3829	13	12	169,8	3	39,9	2	14,4
28	3,0	II	8,6	III	52,2	0,2	4013	14	13	170,2	3	40,7	2	14,5
29	3,3	II	9,8	III	51,7	0,2	4061	14	12	173,9	3	41,7	3	14,9
30	3,6	II	9,9	III	51,3	0,2	3934	14	12	172,0	3	41,4	3	15,0
31	3,5	II	8,7	III	51,8	0,2	4037	14	12	164,8	3	41,8	3	14,6
32	4,0	II	10,8	III	52,1	0,2	4078	13	12	167,0	3	43,0	3	15,0
33	3,8	II	8,7	III	49,6	0,2	4068	13	11	161,6	3	41,8	3	14,9
34	4,3	III	10,2	III	49,6	0,2	4175	14	12	176,0	3	45,0	3	15,5
35	4,8	III	11,1	III	48,3	0,2	4212	14	12	177,1	3	44,3	3	15,6

36	4,6	III	10,2	III	47,5	0,2	4276	13	12	180,0	3	44,9	3	15,6
37	4,5	III	9,3	III	47,3	0,2	4388	14	12	178,2	3	44,0	3	15,9
38	5,0	III	9,1	III	45,4	0,2	4198	14	12	170,8	3	43,9	3	16,0
39	5,3	III	9,7	III	45,7	0,2	4242	14	12	169,0	3	45,2	3	15,8
40	6,2	III	10,3	III	43,7	0,2	4113	14	11	174,5	3	47,2	3	15,9
41	6,7	III	10,1	III	43,4	0,2	4241	14	11	175,2	3	48,5	3	16,1
42	7,0	III	9,2	III	44,4	0,2	4341	14	12	174,5	3	49,7	3	16,6
43	6,6	III	10,6	III	44,2	0,2	4308	14	12	186,2	3	49,5	3	16,4
44	8,0	III	10,1	III	46,0	0,2	4568	14	12	180,6	3	53,4	3	16,4
45	7,2	III	10,7	III	45,7	0,2	4230	14	11	179,2	3	50,3	3	16,3
46	7,6	III	10,4	III	44,5	0,2	4004	14	11	172,8	3	50,6	3	16,4
47	6,0	III	9,9	III	45,2	0,2	4007	14	11	169,7	3	47,5	3	15,9
48	6,5	III	11,1	III	45,8	0,2	3901	14	11	168,4	3	47,9	3	15,6

Appendix 2

Table with the results from the soil analysis showing the total N content (N-tot), dry matter (DM and the amount of NO3-N and NH4-N in the dry matter in the soil for each of the plots in the field trial at Gotland in 2023.

Plot number	**N-tot** g/kg	**DM** %	**NO3-N** mg/(100g TS)	**NH4-N** mg/(100g TS)
1	2,1	87,5	2,31	0,19
2	2,2	86,9	2,19	0,16
3	2,2	86,0	2,68	0,40
4	2,1	86,5	2,03	0,17
5	2,3	86,0	2,73	0,16
6	2,2	86,5	1,85	0,18
7	2,2	86,7	1,81	0,17
8	2,2	87,3	2,74	0,19
9	2,3	86,6	1,75	0,12
10	2,2	86,2	1,62	0,14
11	2,2	87,6	1,58	0,15
12	2,2	85,7	1,50	0,13
13	2,1	87,5	1,29	0,17
14	2,2	86,9	1,68	0,22
15	2,2	86,6	1,19	0,16
16	2,1	86,8	1,67	0,12
17	2,1	86,2	1,55	0,12
18	2,3	85,0	1,84	0,21
19	2,3	86,7	1,62	0,17
20	2,3	85,0	2,12	0,14
21	2,3	86,5	2,26	0,15
22	2,3	86,9	1,67	0,25
23	2,3	87,6	1,19	0,17
24	2,3	85,7	2,08	0,19
25	2,4	86,6	1,77	0,19
26	2,4	86,1	1,56	0,21
27	2,3	85,5	1,95	0,27
28	2,3	85,9	2,10	0,32
29	2,5	86,8	1,36	0,21
30	2,3	86,1	1,84	0,22
31	2,3	88,0	1,22	0,22
32	2,5	86,9	1,87	0,26
33	2,4	86,4	2,50	0,20
34	2,4	88,8	2,13	0,24
35	2,4	86,3	2,58	0,25
36	2,3	86,9	1,90	0,23
37	2,3	86,7	1,21	0,29

38	2,3	86,7	1,35	0,15
39	2,4	86,1	0,84	0,14
40	2,3	85,8	3,68	0,19
41	2,3	87,4	1,40	0,17
42	2,3	87,2	0,89	0,16
43	2,4	86,0	0,71	0,17
44	2,4	85,7	1,68	0,19
45	2,4	88,6	1,13	0,14
46	2,3	86,5	2,10	0,23
47	2,4	85,6	3,21	0,30
48	2,4	86,9	2,70	0,20

Appendix 3

Table with the results from the soil analysis showing the amount of NO_3, NH_4 and mineral N in kg/ha in the soil layers 0-30 cm, 30-60 cm and 0-60 cm for each of the plots in the field trial at Gotland in 2023.

Plot number	NO3-N 0-30cm	NH4-N 0-30cm	N-MIN 0-30cm	NO3-N 30-60cm	NH4-N 30-60cm	N-MIN 30-60cm	NO3-N 0-60cm	NH4-N 0-60cm	N-MIN 0-60cm
	kg/ha	kg/ha	kg/ha	kg/ha	kg/ha	kg/ha	kg/ha	kg/ha	kg/ha
1	92,3	7,6	99,8	103,8	8,5	112,3	196,0	16,1	212,1
2	87,6	6,6	94,2	98,6	7,4	106,0	186,2	14,0	200,2
3	107,4	16,0	123,3	120,8	18,0	138,7	228,1	33,9	262,1
4	81,1	6,8	87,9	91,2	7,7	98,9	172,3	14,5	186,8
5	109,3	6,4	115,7	123,0	7,2	130,2	232,3	13,6	245,9
6	74,1	7,1	81,2	83,4	8,0	91,4	157,5	15,1	172,6
7	72,6	7,0	79,5	81,6	7,8	89,5	154,2	14,8	169,0
8	109,7	7,8	117,4	123,4	8,7	132,1	233,0	16,5	249,5
9	70,1	4,9	75,0	78,9	5,5	84,4	149,0	10,4	159,4
10	64,9	5,7	70,5	73,0	6,4	79,3	137,9	12,0	149,9
11	63,2	5,9	69,2	71,1	6,7	77,8	134,4	12,6	147,0
12	59,8	5,3	65,2	67,3	6,0	73,3	127,1	11,4	138,5
13	51,5	7,0	58,5	58,0	7,8	65,8	109,5	14,8	124,3
14	67,3	8,9	76,1	75,7	10,0	85,6	142,9	18,8	161,8
15	47,6	6,4	53,9	53,5	7,2	60,7	101,1	13,5	114,6
16	66,9	5,0	71,9	75,3	5,6	80,9	142,2	10,6	152,8
17	61,8	4,9	66,7	69,6	5,5	75,0	131,4	10,3	141,7
18	73,7	8,5	82,1	82,9	9,5	92,4	156,5	18,0	174,5
19	64,8	6,9	71,6	72,9	7,7	80,6	137,7	14,6	152,3
20	84,9	5,8	90,7	95,5	6,5	102,0	180,5	12,3	192,7
21	90,2	6,1	96,4	101,5	6,9	108,4	191,7	13,1	204,7
22	66,8	10,0	76,9	75,2	11,3	86,5	142,0	21,3	163,3
23	47,4	6,9	54,3	53,3	7,7	61,1	100,8	14,6	115,4
24	83,4	7,6	90,9	93,8	8,5	102,3	177,2	16,1	193,2
25	70,6	7,7	78,3	79,4	8,7	88,1	150,1	16,4	166,5
26	62,5	8,3	70,9	70,3	9,4	79,7	132,8	17,7	150,6
27	77,9	10,9	88,8	87,7	12,3	100,0	165,6	23,2	188,8
28	83,9	12,7	96,6	94,3	14,3	108,6	178,2	27,0	205,2
29	54,3	8,4	62,7	61,1	9,4	70,6	115,5	17,8	133,3
30	73,7	8,9	82,6	82,9	10,0	92,9	156,6	18,9	175,5
31	48,8	8,7	57,6	54,9	9,8	64,8	103,8	18,6	122,4
32	74,7	10,3	85,0	84,1	11,6	95,6	158,8	21,9	180,7
33	100,1	7,8	107,9	112,6	8,8	121,4	212,6	16,6	229,3
34	85,3	9,7	95,0	96,0	10,9	106,9	181,4	20,5	201,9
35	103,3	9,8	113,2	116,2	11,1	127,3	219,6	20,9	240,5
36	76,1	9,4	85,4	85,6	10,5	96,1	161,6	19,9	181,5

37	48,2	11,7	59,9	54,3	13,1	67,4	102,5	24,8	127,3
38	53,9	6,0	60,0	60,7	6,8	67,4	114,6	12,8	127,4
39	33,6	5,6	39,2	37,8	6,3	44,1	71,5	11,9	83,4
40	147,3	7,7	155,0	165,7	8,6	174,3	313,1	16,3	329,3
41	56,0	7,0	62,9	63,0	7,8	70,8	118,9	14,8	133,7
42	35,7	6,3	42,0	40,2	7,1	47,3	75,9	13,4	89,4
43	28,3	7,0	35,3	31,8	7,9	39,7	60,1	14,9	75,0
44	67,2	7,4	74,6	75,6	8,4	83,9	142,8	15,8	158,5
45	45,2	5,7	50,9	50,8	6,4	57,3	96,0	12,2	108,2
46	83,8	9,3	93,1	94,3	10,5	104,8	178,1	19,8	197,9
47	128,5	12,1	140,6	144,6	13,7	158,2	273,1	25,8	298,9
48	108,0	7,8	115,8	121,5	8,8	130,3	229,4	16,6	246,0

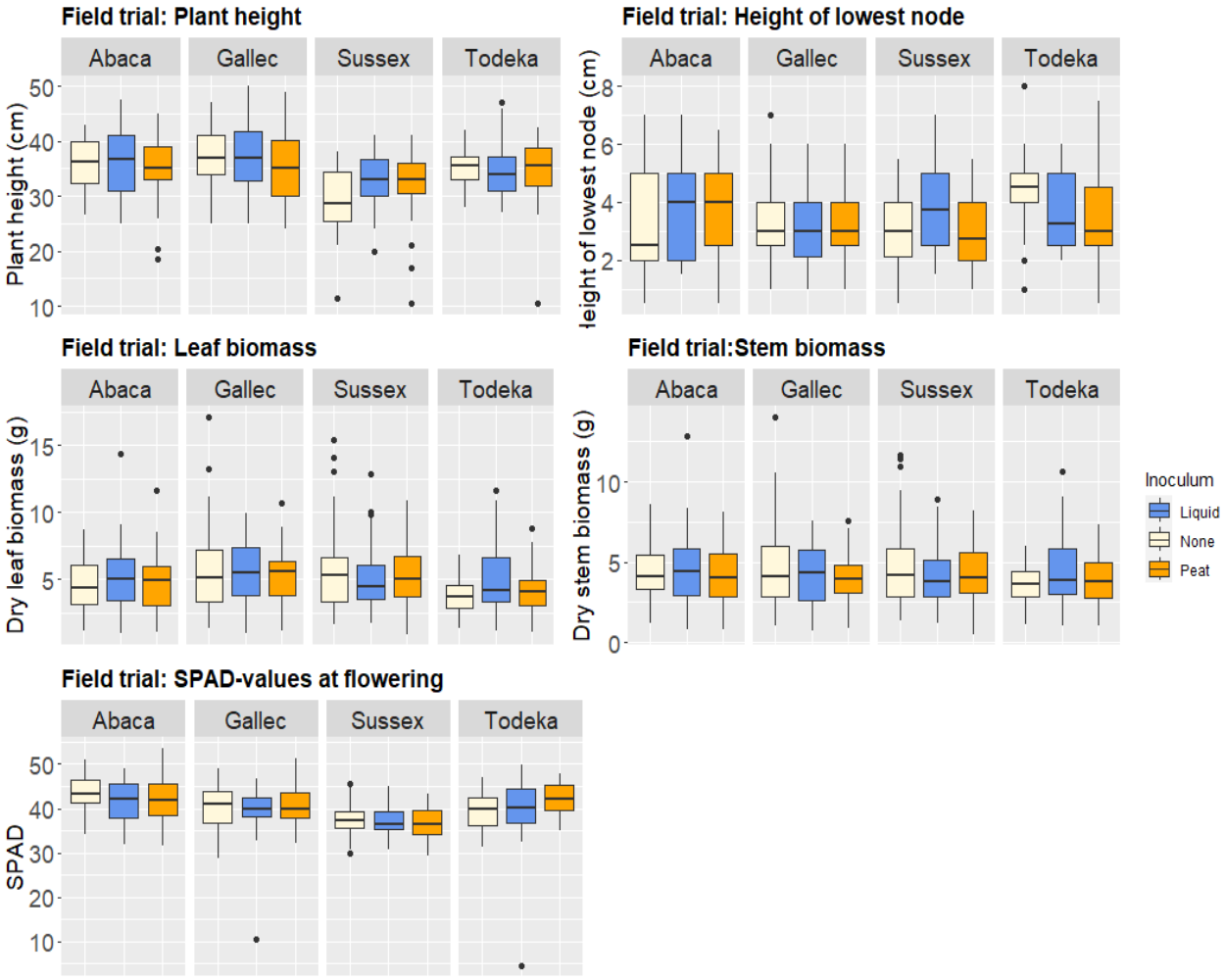
Appendix 4

Added nutrients from the manufacturer of the soil (s-jord) used for the greenhouse experiment in Ultuna 2023.

Nutrient	**Added amount in g/m^3**
Easily soluble nitrogen (N)	125
Boron (B)	0,3
Phosphorus (P)	65
Cupper (Cu)	1,1
Potassium (K)	140
Iron (Fe)	1,0
Magnesium (Mg)	220
Manganese (Mn)	1,5
Calcium (Ca)	1800
Molybdenum (Mo)	0,5
Sulfur (S)	70
Zink (Zn)	0,4

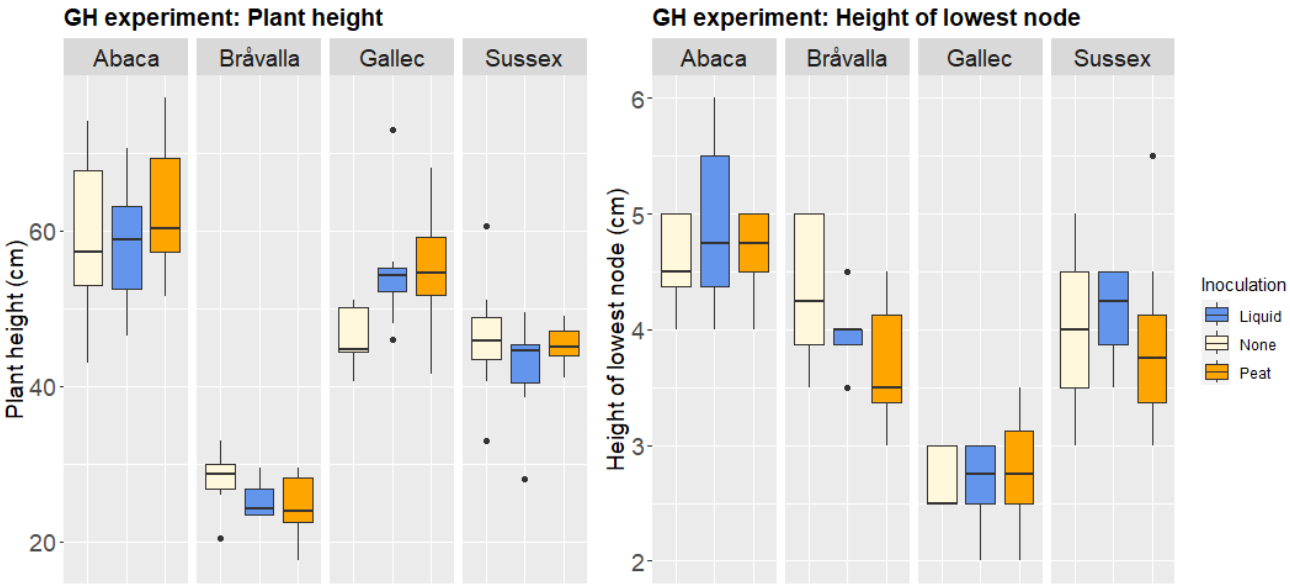
Appendix 5

Figures showing the measured traits in the plants sampled in the field trial at Gotland 2023 where inoculum did not have a significant effect on the response variable (plant height, height of lowest node, leaf biomass, stem biomass and SPAD-values at flowering) in the soybean cultivars Abaca, Gallec, Sussex and Todeka.



Appendix 6

Figures showing the measured traits in the plants sampled in the greenhouse experiment in Uppsala 2023 where inoculum did not have a significant effect on the response variable (plant height and height of lowest node) in the soybean cultivars Abaca, Bråvalla, Gallec and Sussex.



Appendix 7

Results from the analysis of variance for the measured traits in the plants sampled in the field trial on Gotland. The significance threshold level was set to $p < 0.05$. Significant p-values are indicated by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. P-values with no significance are indicated by "-".

Response variable	**Explanatory variable**	**DF**	**p- value**
Stem biomass	Cultivar (C)	3	-
	Inoculum (I)	2	-
	C x I	6	-
Nodule -/root biomass ratio	Cultivar	3	-
	Inoculum	2	***
	C x I	6	-
Number of nodules	Cultivar	3	***
	Inoculum	2	***
	C x I	6	***
TKW	Cultivar	3	***
	Inoculum	2	***
	C x I	6	***
Yield/plant	Cultivar	3	***
	Inoculum	2	*
	C x I	6	-
Plant height	Cultivar	3	***
	Inoculum	2	-
	C x I	6	-
Height lowest node	Cultivar	3	-
	Inoculum	2	-
	C x I	6	-
Leaf biomass	Cultivar	3	*
	Inoculum	2	-
	C x I	6	-

SPAD-values at flowering	Cultivar	3	***
	Inoculum	2	-
	C x I	6	-
N content in biomass	Cultivar	3	***
	Inoculum	2	***
	C x I	6	**
N content in seeds	Cultivar	3	***
	Inoculum	2	***
	C x I	6	***
%Ndfa in biomass	Cultivar	3	***
	Inoculum	2	***
	C x I	6	**
%Ndfa in seeds	Cultivar	3	***
	Inoculum	2	***
	C x I	6	***
Protein yield/plant	Cultivar	3	***
	Inoculum	2	***
	C x I	6	-
Protein yield (kg/ha)	Cultivar	3	-
	Inoculum	2	**
	C x I	6	-

Appendix 8

Results from the analysis of variance for the measured traits in the plants from the greenhouse experiment in Uppsala. The significance threshold level was set to $p < 0.05$. Significant p-values are indicated by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. P-values with no significance are indicated by "-".

Response variable	**Explanatory variable**	**DF**	**p- value**
Stem biomass	Cultivar (C)	3	*******
	Inoculum (I)	2	******
	C x I	6	*
Root biomass	Cultivar	3	***
	Inoculum	2	***
	C x I	6	***
Root-/stem biomass ratio	Cultivar	3	**
	Inoculum	2	*
	C x I	6	*
Nodule -/root biomass ratio	Cultivar	3	***
	Inoculum	2	***
	C x I	6	***
Number of nodules	Cultivar	3	**
	Inoculum	2	***
	C x I	6	***
TKW	Cultivar	3	*
	Inoculum	2	***
	C x I	6	-
Yield/plant	Cultivar	3	***
	Inoculum	2	***
	C x I	6	***
Plant height	Cultivar	3	***
	Inoculum	2	-
	C x I	6	-

Height lowest node	Cultivar	3	***
	Inoculum	2	-
	C x I	6	-

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