Swedish University of Agricultural Sciences
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Department of Plant Biology and Forest Genetics

Nodulation of the N_2-fixing legume narrow-leafed lupin (*Lupinus angustifolius* L.): soil inoculation methods, root nodule development and molecular identification of rhizobia

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Abstract

The following paper is a study on the interaction between *Lupinus angustifolius* L. and N_2-fixing nodulating bacteria in selected Swedish soils. Bacteria being able to induce N_2-fixing symbiotic root nodules on legume plants are generally called rhizobia. The main objectives were to investigate (1) the presence of rhizobia compatible with two cultivars of *L. angustifolius,* Bora and Galant in an agricultural soil in Skåne and a soil in Uppsala where the perennial lupin *L. polyphyllus* is growing, (2) the development of lupin root nodules, (3) taxonomic identity of the nodulating rhizobia using DNA sequencing and (4) effects of various pre-treatments of the inoculum as well as seed treatments on the nodule formation in *L. angustifolius*. Two experiments were conducted by growing plants in controlled light and temperature during May to August 2010, in the growth rooms at the Department of Plant Biology and Forest Genetics, SLU.

The Skåne agricultural soil and the Uppsala perennial lupin soil induced nodules in both cultivars. The number of nodules per plant using a seed dipping inoculation technique was similar for both soils. However, in the experiment where soil suspension was added as inoculum to the pot the number of nodules was lower for the perennial soil suggesting that mineral nitrogen or another soil factor inhibited nodule formation**.**

Using a method where seeds were inoculated by dipping into the soil suspension, the effect of drying soil in room temperature before use as inoculum and the effect of time after germination on nodule formation were investigated. Fresh soil induced a higher number of nodules in *L. angustifolius* than the dry soil indicating a decline of the infective population size or diversity in the dry soil. Seeds sterilized immediately prior to inoculation and seeds germinated for one day gave plants with higher nodule number compared to seeds germinated for two days.

Sequences of a part of the 16S-23S internal transcribed spacer showed that the nitrogen fixing rhizobium of both soils had highest identity to *Bradyrhizobium* sp in the *B. japonicum* group. However, a phylogenetic analysis showed good separation of the two clusters formed by the rhizobia of the two soils suggesting that the *Bradyrhizobium* sp in the Skåne agricultural soils is genetically different from that in the Uppsala perennial lupin soil.

Key words: *DNA sequence analysis, inoculation, lupin species, N_2 fixation, root nodule formation, Bradyrhizobium, symbiosis*

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1 Introduction

1.1 Legumes and biological N_2 fixation

Already the Romans knew that legumes could be used not only as a yield crop but also as a crop that could give benefits to subsequent crops (Graham and Vance, 2003). In modern agriculture, it is very well known that the input of N is essential for plant growth and crop yields. In eco-farming as well as conventional farming the N input through biological N_2 fixation is a very important aspect. Nitrogen fixation most often occurs between members of the plant family Leguminosae and a group of soil bacteria known as rhizobia (Taiz and Zeiger, 2006; Ngom *et al.,* 2004; Martyniuk and Oroń, 2008). Root nodules are specialized structures of the plant host that contain nitrogen-fixing bacteria (Taiz and Zeiger, 2006). However, successful biological N_2 fixation by a legume depends on a number of factors and particularly the presence of compatible bacteria in the soil.

N_2-fixing legumes are important agricultural plants and belong to the family Leguminosae. World-wide there are more than 17,000 species (annuals as well as perennial trees). Even more, some of the most important crops in the world e.g. soybean or beans, are legumes and they are as such major sources of protein in food and fodder (Graham and Vance, 2003). In many ecosystems availability of nitrogen (N) is the important limiting factor for plant growth (Taiz and Zeiger, 2006). Biological fixation of atmospheric N_2 by legumes in symbiotic association with rhizobium bacteria can provide a substantial amount of N into agricultural systems. Biological N_2 fixation is the only biological process that provides N into the soil sphere where it can act as crop fertilizer.

1.2 Narrow-leafed lupin

Narrow-leafed lupin (*Lupinus angustifolius* L.) also known as blue lupin belongs to the tribe Genisteae, subfamily Papilionoideae, family Leguminosae. The plant is an annual and grows about 60-150 cm in height. It usually has blue, in some cases also white flowers. In agriculture, it is used for forage as well as for silage and as grazing in early spring. Growing period duration is from 100 up to 180 days. The plant can be grown both as a winter annual in Mediterranean environments or as summer annual in temperate regions. It flowers between April and June

(Ecocrop, 2007). The plant contains high protein values and can be used as a pulse legume crop or as a green manure. It can also improve soil by increasing the P availability. Despite the large agricultural and ecological benefits that *L. angustifolius* could bring (Trujillo *et al.,* 2005) the plant has been insufficiently studied. Among the many possibilities that nitrogen fixing plants could offer are for example: biofertilizing, increasing availability of nutrients and disease control, but they remain relatively untouched (Cocking, 2003). In Sweden, the cultivation of lupin has not yet been fully established.

In Sweden, the number of cultivated legume species is small including pasture legumes like white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) and lucerne (*Medicago sativa*), pulse legumes like pea (*Pisum sativum*) and faba bean (*Vicia faba*). The recent introduction of the narrow-leafed lupin (*L. angustifolius*) in Sweden can be regarded as promising since it is not affected by so-called root-rot causing pathogenic fungi, which cause significant damage to pea. Therefore, *L. angustifolius* can be a potential candidate to be used in Swedish agriculture to meet the goal of achieving increased organic farming and sustainable agriculture. However, our knowledge about the diversity and distribution of rhizobia compatible with *L. angustifolius* in Swedish soils is very limited. A preliminary study has been conducted to investigate agricultural soils collected from six different locations in Sweden for the presence of rhizobia compatible with seven cultivars of *L. angustifolius* (Hossain *et al.,* personal communication). One soil in Skåne identified to contain rhizobia compatible with *L. angustifolius* was used in the present study.

Lupinus is a genus having about 170 species. In Sweden, Norway and Finland a garden lupin (*L. polyphyllus*) originating from North America has in recent years become established in marginal areas and road-sides (Fremstad, 2006). Since it is possible that rhizobia compatible with this species also could be compatible with *L. angustifolius* it is relevant to investigate this and to identity this bacterium. In the present study, nodules of *L. polyphyllus* and a soil from Uppsala, where the perennial lupin *L. polyphyllus* grows naturally, were together with the Skåne agriculture soil used to examine nodulation of *L. angustifolius* in a series of experiments.

Figure 1. Narrow-leafed lupin (*Lupinus angustifolius* L.)

1.3 Factors influencing biological N_2 fixation in lupins

The amount of N_2 fixed by bacteria in a legume plant is affected by several aspects of which availability of N_2-fixing bacteria is one (Date, 1970). Also, a high variability in the interaction between *Lupinus* lines and *Bradyrhizobium* strains has been shown (Robinson *et al.,* 2000). A high potential for selecting improved plant-microbe combinations was suggested. This gives a strong incentive to try to obtain efficient rhizobial strains and to inoculate lupins in agriculture.

Soil factors such as soil pH can also affect nodulation significantly. Experiments in Polish soils have shown that nodule formation was significantly lower in soils with a pH below 6.0 (Martyniuk and Oron, 2008). In contrast, specifically for *L. angustifolius* other researchers found that cultivation at a pH above 6.0 resulted in a significant reduction in nodulation and caused N limitation to the host plant (Tang and Robson, 1993; Mihajlović *et al.,* 2008). Further, soil content of P also influences root nodule formation with high soil P leading to an increase in the number of root nodules per plant in *L. angustifolius* (Jessop *et al.*, 1989).

1.4 Improving nodule formation and N_2 fixation through inoculation

Inoculum can be added to seed or soil in order to ensure sufficient infection and high efficiency of bacteria. In many established areas rhizobia are already present in soil. On the other hand, the bacteria may also be less effective. When new soil is taken into use for legume cultivation, highly compatible bacteria should be introduced to secure a high crop yield. To increase the practice of inoculating legumes, highly effective inoculants should be used. Nevertheless, also educational motivation of the farmers plays an important role. In natural soils, there are several strains present with different compatibilities with their host (Simms and Taylor, 2002) and the inoculant strain needs to be competitive enough against these bacteria. In case a legume is introduced to new

agricultural soil and compatible rhizobia are absent it may be crucial to inoculate with compatible rhizobia. Nevertheless, the great variation found in the plant-microbe interactions suggests that it should be fruitful to give specific recommendations of inocula depending on host plant genotype and soil condition.

1.5 Identification of rhizobia

Molecular methods are very informative to investigate the identity and diversity of rhizobia in N_2-fixing root nodules. The 16S ribosomal rRNA gene and the 16S-23S intergenic spacer have been used extensively to classify bacteria (e.g. Boyer *et al.,* 2001). PCR primers to conserved sites in these genes have been developed that can be applied for taxonomic and ecological studies.

1.6 Aims of the study

Since *L. angustifolius* is newly introduced to Sweden basic knowledge about cultivation and inoculation in relation to N_2 fixation is lacking. In an ongoing project on N_2 fixation and lupins in Sweden, research objectives regarding N_2-fixing bacteria compatible with lupins in Swedish agricultural soils are to investigate the geographic distribution of lupin-compatible bacteria, their effectiveness at inducing root nodules and promoting plant growth and their genetic relatedness. To obtain initial information the present study specifically addressed the following questions:

- Do two different soils, an agricultural soil from Skåne and soil where the perennial lupin *L. polyphyllus* grows, induce different number of nodules on *L. angustifolius*?
- Do two cultivars of *L. angustifolius* (Bora and Galant) differ in nodule production and plant growth when inoculated with these two soils?
- Do seed treatment (sterilization, germination stage) and soil inoculum treatment (fresh soil and dry soil) affect root nodule production and plant growth?
- What is the identity of nodule-forming bacteria compatible with *L. angustifolius* in these two soils and do they differ genetically?

2 Materials and methods

2.1 Experiment 1

2.1.1 Soils and plants used

In this experiment two soils were used as inocula to examine nodulation in *L. angustifolius*: one soil was collected from an agricultural field of Skåne and the other one was from Valsätra, Uppsala where perennial *L. polyphyllus* is grown (Fig. 2). In addition, inoculum prepared from crushing of nodules collected from *L. polyphyllus* grown in the latter soil was also used. Inoculum collected from nodules of *L. polyphyllus* was used as positive control and we were also interested to see whether rhizobium nodulating this plant species is also potentially compatible with *L. angustifolius*.

Two commercially available cultivars of *L. angustifolius*, cv. Bora (SAATZUCHT STEINACH GmbH & Co KG, Germany) and cv. Galant (DLF-TRIFOLIUM A/S, Denmark) were used in these experiments. Galant is a branched cultivar giving higher yield potential and competition against weeds but a slightly more uneven seed maturation. Bora is a variety with medium to early seed maturation. It has good resistance to mycosis and viral diseases and can be grown on light to medium soils. Seeds of these two cultivars were obtained from Ullalena Boström of the Department of Crop Production Ecology, SLU, Uppsala.

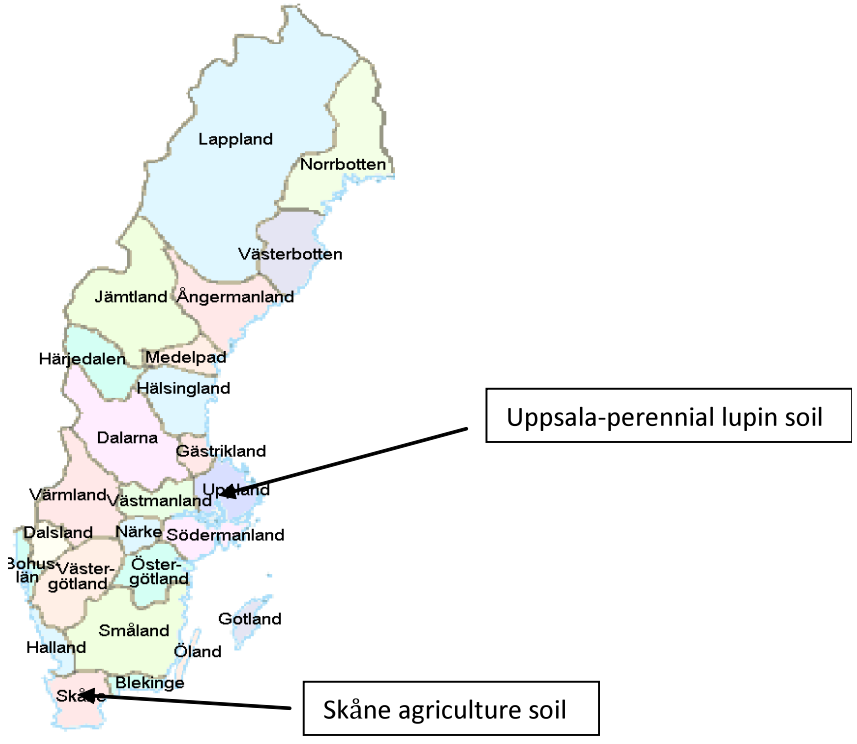

Figure 2. The map of Sweden showing soil sampling sites.

2.1.2 Seed sterilization

Seeds were sterilized by soaking in 5% sodium hypochlorite for 10 min with gentle shaking. Then, the sodium hypochlorite was removed and seeds were rinsed with autoclaved MQ water thrice. Seeds were then kept overnight at 28^{o}C.

2.1.3 Preparation of inoculum

Ten g of each soil type was mixed with 150 ml autoclaved MQ water on a shaker for 30 min. Five ml was taken from this suspension with the help of an electric pipette and applied to seedlings in each pot. After application of inocula, 50 ml water was added to the plants.

2.1.4 Growing plants

A mixture of sand and vermiculite (75:25, v/v) was used to grow plants. After mixing properly the mixture was filled into a metal box and autoclaved. The mixture was then divided into ½ L pots for growing. To cover the bottom holes of the pot a sterilized piece of cloth (4cm x 4cm) was used. Each pot was covered with a polythene bag of 3 L in a volume that helped to ensure aseptic conditions. Three replicates of each treatment were covered with a single polythene bag. The total number of pots was 96 (3 types of inoculum X 2 genotypes X 4 harvesting times) including controls with each 3 replicates.

On May 11, 2010, three seeds each of the two cultivars were sown per pot. After emergence of the seedlings, plants were thinned to one seedling per pot. During the growing period, the plants were watered when necessary. The experiment was conducted in a growth room under controlled temperature and day length: temperature during the day (from 07.00 to 23.00) was 24^{o}C and 18^{o}C during the night period (from 23.00 to 07.00).

2.1.5 Harvesting of plants

Plants were harvested on 18 June, 28 June, 9 July, and 19 July 2010. After harvest, the height and weight of plants were measured. Shoots were cut and put into paper bags marked carefully and dried in an oven at 65^{o}C for 24 h to weigh dry mass. Plant roots were washed gently so that nodules were not lost and then stored at -20^{o}C. Root systems were taken out of the freezer and nodules were separated, counted and measured for weight per plant.

2.2 Experiment 2

2.2.1 Soils and plants used

The same two soils that were used in Experiment 1 (as already described in Experiment 1) were used in Experiment 2. Fresh soil, one day-air dried soil and sterilized soil autoclaved twice at 24 hr interval were used to examine the effects of soil treatments on nodulation of lupin plants. The importance of seed sterilization and seedling germination stage was also investigated in Experiment 2. After sterilization (Figure 3a- procedure is already described in Experiment 1), some seeds of each cultivar were kept for one day (Figure 3b) and some for two days (Figure 3c) of germination in a temperature controlled room (28^{o}C). On the day of sowing, some seeds of both cultivars were sterilized to be sown immediately. Seeds without sterilization were also sown in this experiment.

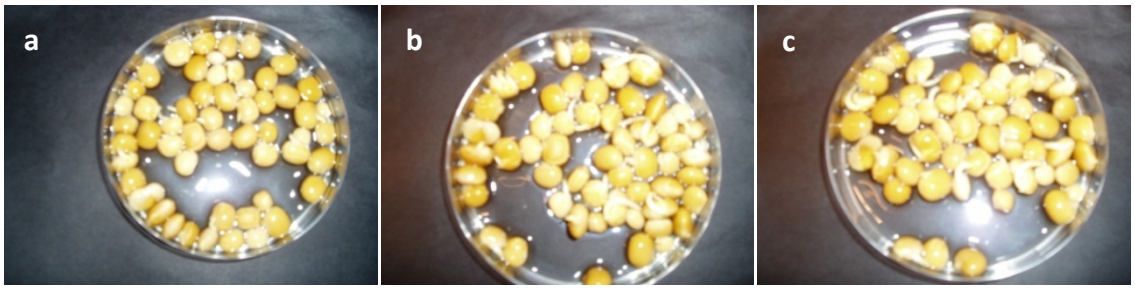

Figure 3. Seeds immediately after sterilization (a), 1 day of germination (b) and 2 days of germination (c)

Seeds of *L. angustifolius* cv. Bora obtained from Ullalena Boström, Department of Crop Production Ecology, SLU, Uppsala and *L. polyphyllus* bought from local shop were used in this experiment.

2.2.2 Preparation of inocula

Ten g soil was mixed with 150 ml autoclaved MQ water, incubated on a shaker for 30 min and filtered through a 25 µl size filter cloth. The suspension was then kept overnight. Seeds were dipped into inocula for 30 min and sown in the autoclaved sand-vermiculite mixture (75:25, v/v) on June 11, 2010.

2.2.3 Growing plants

Eight inoculated seeds per pot were sown. Each pot was protected with a plastic bag of 3L in volume. Plants were watered with MQ water when necessary till harvesting. No replicate pots for each treatment were used.

Figure 4. Plants were grown in pots. The plants were first covered with 3L plastic bags. After approximately 1 week, the plastic bags were pulled down like the picture shows.

2.2.4 Harvest of plants

Harvest was done on August 3, 2010, 53 days after inoculation. After harvest plant height was noted and the shoot was cut and separated from the roots. Shoots were dried in an oven at 65^{o}C for 24 hours. The root material was stored at -20^{o}C.

2.3 Analysis of nodule forming rhizobial bacteria

2.3.1 Picking of nodules

Nodules were washed carefully with a gentle flow of water to remove sand and vermiculite. Excess water was removed with tissue paper. Nodules were separated from the root system with the help of forceps and scalpel. Nodule number was counted and biomass was measured. Average nodule size per plant was also determined by dividing the mass of all nodules by total number of nodules. Nodules were then stored in an 1.5 ml eppendorf tube at -20^{o}C for further use.

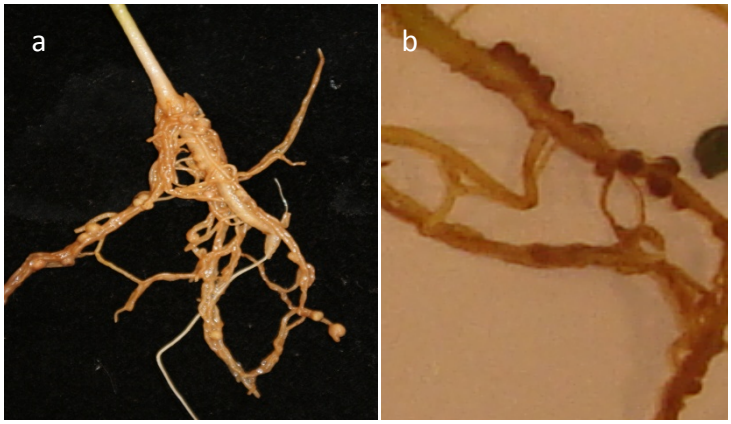

Figure 5. Root systems with symbiotic root nodules of *Lupinus angustifolius* (a) and *L. polyphyllus* (b).

2.3.2 Surface sterilization of nodule

Surface sterilization of root nodules started with soaking the nodules in 5% sodium hypochlorite for 10 min. Nodules were then rinsed for five times with autoclaved MQ water. Nodules were then soaked in 70% alcohol for 1 min and rinsed five times with autoclaved MQ water.

2.3.3 Extraction of DNA from nodule bacteria

A nodule was put into a tube and 100 µl of PBS was added. The nodule was crushed with a sterile metal rod. The suspension was taken up with a pipette and passed through a filter of 25 µm pore size in a construction with a 1 ml syringe, tip and filter (Figure 6). The filtrate was then collected in an 1.5 ml tube. To get more homogenate suspension, an additional 100 µl of PBS was added and passed through the filter.

The filtrate was centrifuged at 13,000 rpm for 15 minutes. The supernatant was then discarded, and the remaining pellet contained bacteria. The pellet was washed with washing solution, and centrifuged again for 15 min at 13,000 rpm. The supernatant was discarded. Then, lysis solution (0.2M NaOH and 0.1% SDS solution) was added to the pellet. The solution was boiled in a heating block at 100^{o}C for 10 minutes and immediately cooled on ice. The supernatant was transferred to a new tube for later use in PCR. The extracted DNA extracted thus was then stored at -20^{o}C till PCRs started.

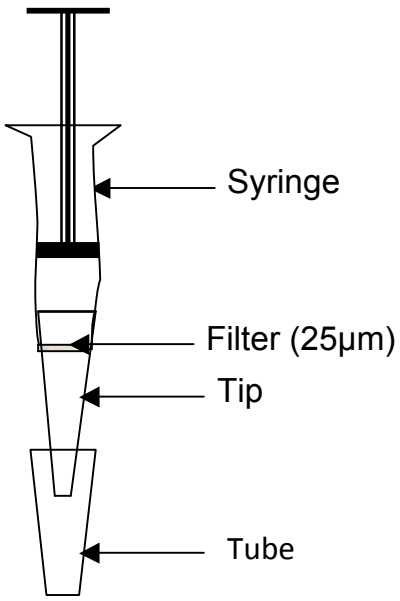

Figure 6. Filtering of crushed nodule homogenates to obtain bacterial suspensions

2.3.4 PCR of the 16S-23S internal transcribed spacer

Part of the ITS gene was amplified in this experiment to study nodule forming rhizobium bacteria. This gene was amplified by using primers f16-23-ITS1492 (5′-AAGTCGTAACAAGGTAGCC-3′) and r16-23-ITS-482 (5′-GCTTTTCACCTTTCCCTCAC-3′) (Willems *et al.*, 2001). A PCR mixture of 25 µl in volume contained 2.5 µl 10X PCR Buffer (Invitrogen), 1 µl 50 mM $MgCl_2$ (Invitrogen), 0.5 µl 10mM dNTP (Fermentas), 0.5 µl each of forward and reverse primers, 0.1 µl of *Taq* DNA polymerase (Platinum® *Taq* DNA polymerase, Invitrogen), 1 µl template DNA and 18.9 µl autoclaved MQ water.

A total of 35 cycles of reactions were done with the initial denaturation temperature of 94^{o}C for 2 min, denaturation at 94^{o}C for 30 seconds, annealing at 54^{o}C for 30 seconds, extension at 68^{o}C for 90 seconds, and final extension at 68^{o}C for 4 min and final storage at 12^{o}C.

After finishing PCR, the band size of the PCR product was confirmed by agarose gel (1% agar in 0.5X TBE solution) electrophoresis at 80 V for 35 min. Prior to electrophoresis, Gel-Red (0.5 µl 30 ml^{-1}), which fluoresces when subjected to UV light, was added to the gel to visualize the amplified DNA fragments. 1.5 Kb marker was used to compare the PCR product with. Then a digital image of the DNA band was taken.

A total of 6 treatments ensuring each inoculum type and each cultivar type were selected to collect 3 nodules for each treatment totaling 18 nodules for DNA extraction and PCR followed by sequencing.

2.3.5 DNA sequencing

Ten µl of PCR product of each sample was sent to Macrogen, Korea for single extension sequencing using the forward primer (f16-23-ITS1492). After getting the sequencing results, sequences were checked for the sequencing quality. Most reliable parts of the sequences containing 862 to 865 nucleotides were searched against GenBank using Blast, aligned to each other and to representative rhizobia sequences and used for creating a phylogenetic tree. CLC sequence analysis software (www.clc.com) was used for alignment and building a phylogenetic tree.

2.4 Statistical tools

In Experiment 1, ANOVA statistical method was performed in order to interpret the empirical data. Two-way ANOVA was performed to compare the effects of main sources of variation such as inoculum types, genotypes and the interactions between these two factors on the nodule development as well as the plant growth parameters. Then one-way ANOVA was done to compare the effects of various factors within each of these main factors. For instance, the comparison between Bora and Galant genotypes was made by following one-way ANOVA, where each of the genotypes included various treatments (e.g. different soils, crushed nodule inoculum and times of measurement).

For Experiment 2, Paired t-tests were done to examine the significance of differences between various treatments. For instance, fresh soil and dry soil treatments were considered as pairs for each seed treatment of unsterilized, sterilized, 1 day germinated, and 2 day germinated seeds.

3 Results

3.1 Experiment 1

3.1.1 Nodule number

Nodules were formed in both soils and both cultivars (Figure 7). Soil type significantly influenced the number of nodules per plant (P = 0.0001), although cultivar types did not show any significant difference. When comparing all three inoculum types, Skåne agriculture soil produced more nodules in both cultivars than the perennial lupin soil and perennial crushed nodule inocula. The period after inoculation significantly influenced the number of nodules produced (P = 0.0084). Generally, nodule production increased over time after the inoculation.

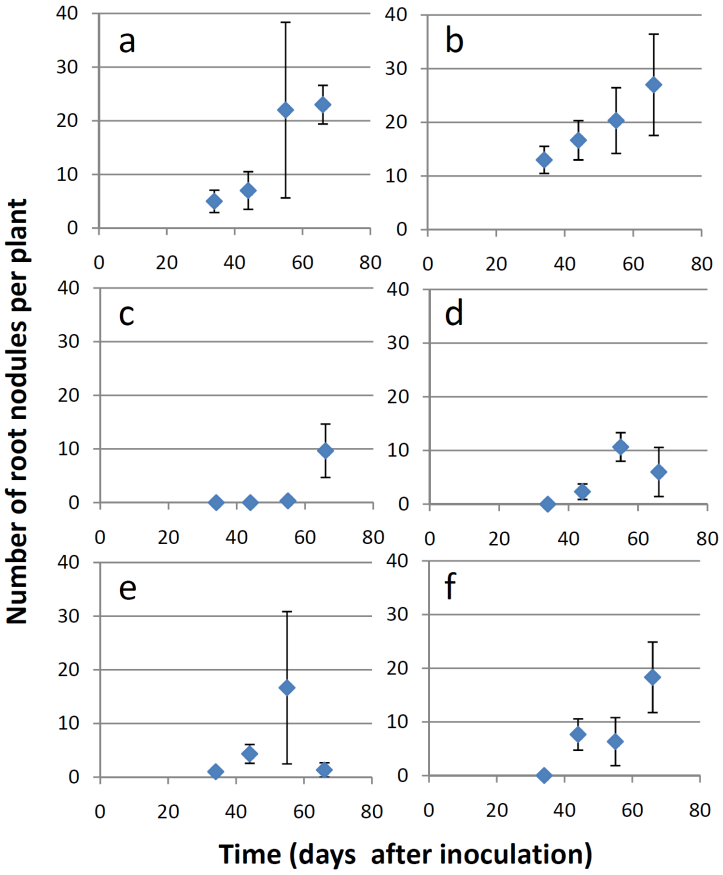

Figure 7. Effect of time on the number of root nodules produced per plant in *Lupinus angustifolius* cv. Bora (a, c and e) and Galant (b, d and f) after inoculation with Skåne agriculture soil (a and b), perennial lupin soil (c and d) and perennial crushed nodule (e and f).

3.1.2 Nodule mass

Soil type showed significant effect on the production of biomass of root nodules (P = 0.0079), however, cultivars did not differ significantly in this respect (Figure 8). Skåne agriculture soil produced higher biomass of nodule per plant in both cultivars (Bora and Galant) compared to the other two inoculum types (perennial lupin soil and crushed nodule of perennial lupin). Time after inoculation significantly influenced the nodule biomass (P = 0.0007), and it generally increased from the time after inoculation.

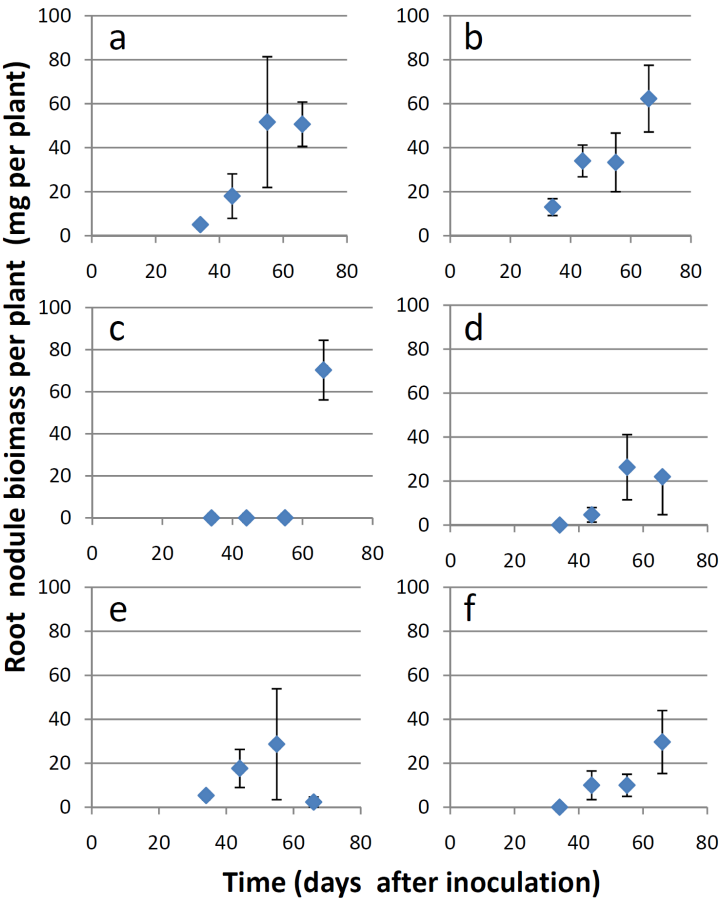

Figure 8. Effect of time on root nodule biomass (mg) per plant in *Lupinus angustifolius* cv. Bora (a, c and e) and Galant (b, d and f) after inoculation with Skåne agricultural soil (a and b), perennial lupin soil (c and d) and crushed nodule of perennial lupin (e and f).

3.1.3 Size of the nodule

It is interesting to note that nodule size (mg mass per nodule per plant) did not differ that much between the two cultivars (Bora and Galant) when inoculated with the Skåne agricultural soil (Figure 9). There was a significant interaction between inoculum type and the period after inoculation; although there is a general tendency of an increase in nodule number from the first day of inoculation to final harvest across cultivars, there is a size decrease over time in case of Bora inoculated with crushed nodule of perennial lupin (Figure 9).

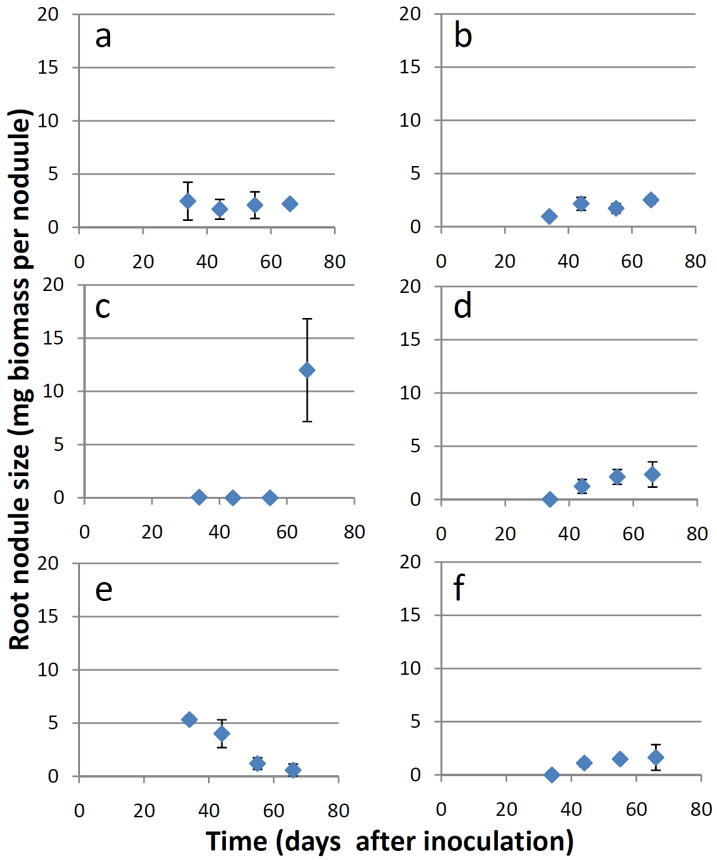

Figure 9. Effect of time on the average size of root nodules (mg biomass per nodule per plant) in *Lupinus angustifolius* cv. Bora (a, c and e) and Galant (b, d and f) after inoculation with Skåne agriculture soil (a and b), perennial lupin soil (c and d) and crushed nodule of perennial lupin (e and f).

3.1.4 Plant biomass

There was a significant effect of time on the plant shoot biomass ($P < 0.0001$), although no significant effects of soil type or cultivar type appeared (Figure 10). A significant ($P = 0.017$) interaction appeared between soil and cultivar type on the plant shoot biomass production; generally plant biomass increased from first sampling date to fourth and last sampling date in plants inoculated with Skåne agricultural soil and perennial nodule soil. Control plants (not treated with inocula) showed highest biomass production 55 days after inoculation and then growth ceased.

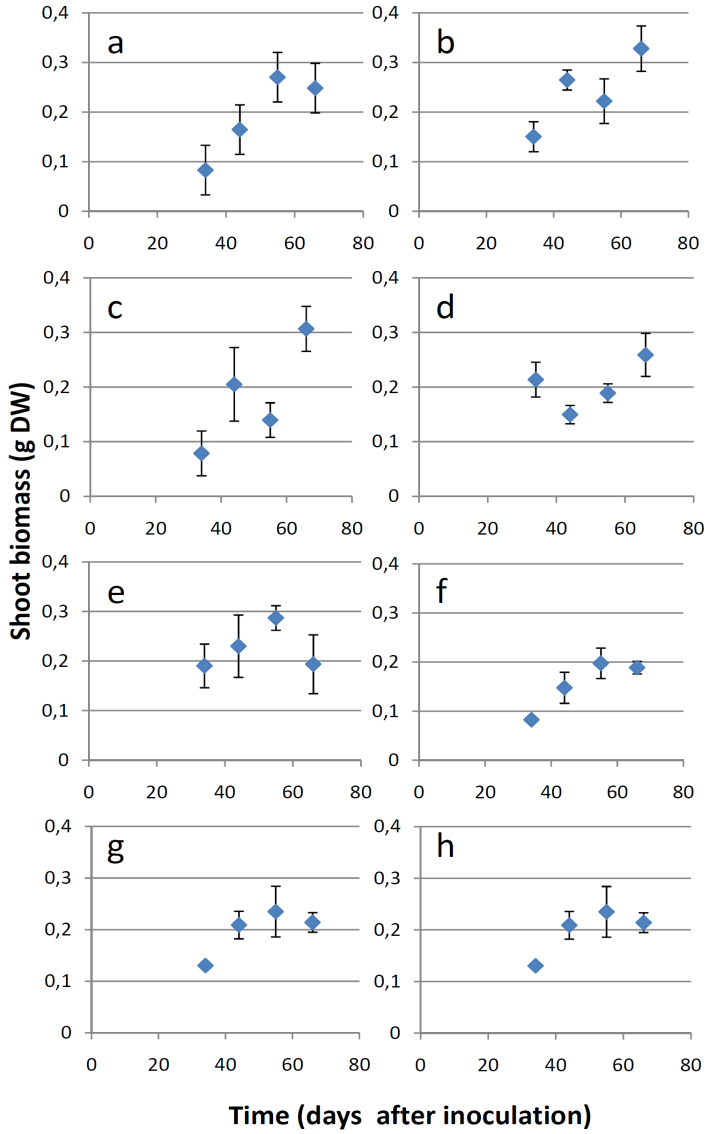

Figure 10. Effect of time on dry biomass of plant shoot in *Lupinus angustifolius* cv. Bora (a, c, e and g) and Galant (b, d, f and h) after inoculation with Skåne agricultural soil (a and b), perennial lupin soil (c and d), crushed nodule of perennial lupin (e and f) and control (no inocula; g and h).

3.1.5 Plant height

As shown in Figure 11, Bora plants showed significantly higher plant height than Galant (P = 0.0002), although no significant difference appeared between inoculum types in this response. There was a significant time effect on plant height; plant height significantly increased from 34 days after inoculation to 44, 55, and 66 days after inoculation.

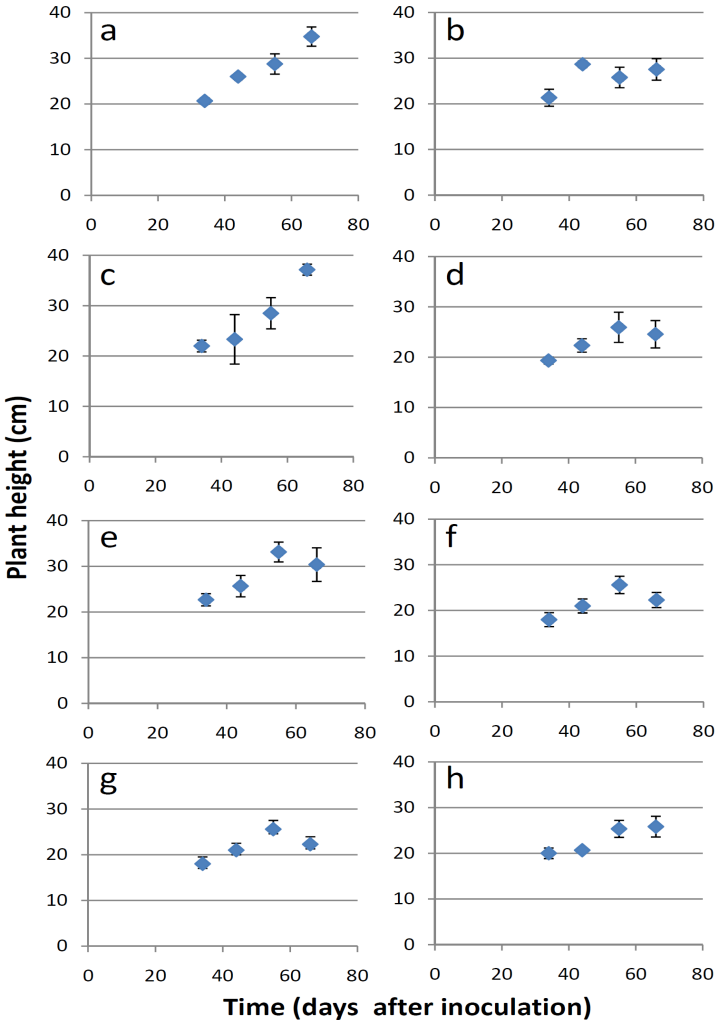

Figure 11. Effect of time on plant height (cm) in *Lupinus angustifolius* cv. Bora (a, c, e and g) and Galant (b, d, f and h) after inoculation with Skåne agricultural soil (a and b), perennial lupin soil (c and d), crushed nodule of perennial lupin (e and f) and control (no inocula; g and h).

3.2 Experiment 2

Experiment 2 was started with *L. angustifolius* cv. Bora and *L. polyphyllus* and two different soils: Skåne agricultural soil and perennial lupin soil from Uppsala. Since *L. polyphyllus* did not survive in the majority of treatments, data of this species is not presented in this study.

3.2.1 Effect of soil treatment and seed treatment on the number of nodule

Autoclaved soil used as inoculum source did not induce any nodules irrespective of seed treatments (Table 1). In case of fresh and dry soil as inoculum, there is a trend that fresh soil induced more nodules per plant than dry soil.

Out of 8 cases where seeds were inoculated with fresh and dry soils across two soil types in seven cases the number of nodules was higher in fresh soil than in dry soil. The average number of nodules per plant (7.94) was significantly (P = 0.009, Paired t-test, where fresh soil and dry soil treatments were considered as pairs for each seed treatment of unsterilized, sterilized, 1 day germinated, and 2 day germinated seeds) higher in fresh soils than in the dry soil (3.04).

Table 1. Effect of soil and seed treatments on the number of nodules per plant in *L. angustifolius* cv. Bora.

Seed treatment	Soil treatment[a]	Skåne agriculture soil	Uppsala perennial lupin soil
Unsterilized	Fresh	3.4 (5)[b]	11.6 (5)
	Dry	0.8 (6)	0.0 (7)
	Autoclaved	0.0 (5)	0.0 (5)
Sterilized	Fresh	8.4 (5)	8.0 (6)
	Dry	4.5 (6)	3.6 (5)
	Autoclaved	0.0 (5)	0.0 (5)
1 Day of germination	Fresh	8.3 (4)	14.2 (6)
	Dry	4.2 (6)	2.4 (5)
	Autoclaved	0.0 (6)	0.0 (4)
2 Days of germination	Fresh	5.0 (5)	4.6 (5)
	Dry	4.2 (5)	4.6 (5)
	Autoclaved	0.0 (6)	0.0 (3)

[a] Plants were cultivated in a sand-vermiculite mix and inoculated with Skåne agricultural soil and Uppsala perennial lupin soil.
[b]Values in brackets are total number of plants for each treatment.

It is interesting to note also that plants from sterilized seeds produced more nodules than plants from unsterilized seeds. The differences were detected also in the case of seeds germinated for 1

or 2 days. The plants grown by 1 day germinated seeds in combination with fresh soil treatment produced more nodules than 2 days germinated seeds with fresh soil treatment.

Some differences appeared in the ability to induce nodules between Skåne agricultural soil and perennial lupin soil. Especially, there was a large difference for unsterilized seed treatment and fresh soil treatment, where Skåne agricultural soil induced 3.4 nodules per plant, while it was 11.6 nodules per plant in perennial lupin soil. Some difference was detected also when seeds were treated with fresh soil after 1 day of germination. Absence of nodules in plants grown from unsterilized seeds and treated with autoclaved soil indicated that the seeds used in this study did not contain any nodule forming bacteria compatible with Bora.

3.2.2 Effect of soil and seed treatment on nodule biomass

The results showed that plants treated with a dry soil suspension produced a surprisingly high nodule biomass (Table 2), which contrasted with the number of nodules per plant in dry soil (Table 1). The results also showed that Bora produced a higher nodule biomass in Uppsala perennial lupin soil, when it was treated with fresh soil treatments, compared to Skåne agriculture soil. An exception in nodule biomass can be identified in 2 days of germination seed treatment, where Bora plants from Skåne agricultural soil gave higher nodule biomass than Bora plants, grown in Uppsala perennial lupin soil (Table 2).

The results also revealed that out of eight cases where seeds were inoculated with fresh and dry soils, five soils showed higher biomass production in fresh soil compared with dry soil treatments, two dry soils showed increased nodule biomass compared with fresh soils, and one soil showed equal biomass production between the two soil treatments. Average nodule biomass production per plant (46.0 mg) was significantly (P = 0.045, Paired t-test, where fresh soil and dry soil treatments were considered as pairs for each seed treatment of unsterilized, sterilized, 1 day germinated, and 2 day germinated seeds) higher in fresh soils than in the dry soil (22.5 mg).

Table 2. Effect of soil and seed treatment[a] on the nodule biomass (mg) per plant in *L. angustifolius* cv. Bora.

Seed treatment	Soil treatment	Skåne agricultural soil	Uppsala perennial lupin soil
Unsterilized	Fresh	20 (5)[b]	71 (5)
	Dry	7 (6)	0 (7)
	Autoclaved	0 (5)	0 (5)
Sterilized	Fresh	35 (5)	71 (6)
	Dry	28 (6)	7 (5)
	Autoclaved	0 (5)	0 (5)
1 Day of germination	Fresh	27 (4)	109 (6)
	Dry	27 (6)	48 (5)
	Autoclaved	0 (6)	0 (4)
2 Days of germination	Fresh	25 (5)	10 (5)
	Dry	33 (5)	30 (5)
	Autoclaved	0 (6)	0 (3)

[a]Experiment as presented in Table 1.
[b]Values in brackets are total number of plants for each treatment.

3.2.3 Effect of soil and seed treatment on nodule size

In most treatments, the nodule size of plants treated with dry soil were larger than for plants treated with fresh soil (Table 3).

Table 3. Effect of soil and seed treatment on the average root nodule size[a] in *L. angustifolius* cv. Bora.

Seed treatment	Soil treatment	Skåne agricultural soil	Uppsala perennial lupin soil
Unsterilized	Fresh	6 (5)[b]	6 (5)
	Dry	8 (5)	0 (7)
	Autoclaved	0 (5)	0 (5)
Sterilized	Fresh	4 (5)	8 (6)
	Dry	6 (6)	2 (5)
	Autoclaved	0 (5)	0 (5)
1 Day of germination	Fresh	3 (4)	8 (6)
	Dry	6 (6)	20 (5)
	Autoclaved	0 (6)	0 (4)
2 Days of germination	Fresh	5 (5)	2 (5)
	Dry	8 (5)	6 (5)
	Autoclaved	0 (6)	0 (3)

[a]Total nodule mass in mg per total number of nodule per plant calculated based on experimental data presented in Tables 1 and 2.
[b]Values in brackets are total number of plants for each treatment.

3.2.4 Effect of soil and seed treatment on the shoot dry biomass

Seed and soil treatments did not largely affect the production of shoot biomass, i.e. absence of nodules such as in the treatment with autoclaved inoculum did not result in lower biomass. There was no significant difference ($P = 0.294$) between fresh and dry soil treatments on the production of biomass per plant (Paired t-test, where fresh and dry soil treatments were considered as pairs for each seed treatment of unsterilized, sterilized, 1 day germinated, and 2 day germinated seeds) (Table 4).

Table 4. Effect of soil and seed treatment on the per plant dry shoot biomass (mg)[a] in *L. angustifolius* cv. Bora.

Seed treatment	Soil treatment	Skåne agricultural soil	Uppsala perennial lupin soil
Unsterilized	Fresh	190 (5)	209 (5)
	Dry	178 (6)	144 (7)
	Autoclaved	204 (5)	98 (5)
Sterilized	Fresh	238 (5)	198 (6)
	Dry	182 (6)	173 (5)
	Autoclaved	112 (5)	120 (5)
1 Day of germination	Fresh	222 (4)	133 (6)
	Dry	163 (6)	143 (5)
	Autoclaved	182 (6)	70 (4)
2 Days of germination	Fresh	159 (5)	110 (5)
	Dry	212 (5)	181 (5)
	Autoclaved	131 (6)	85 (3)

[a]Based on pooled sample per treatment divided by the number of plants in bracket and same experiment as in previous Tables.

3.2.5 Effect of soil and seed treatment on plant height

There was no significant difference in plant height among all soil treatments and seed treatments. Plants treated with fresh soil or dry soil did not differ significantly ($P = 0.295$) in height (Paired t-test, where fresh soil and dry soil treatments were considered as pairs for each seed treatment of unsterilized, sterilized, 1 day germinated, and 2 day germinated seeds) (Table 5).

Table 5. Effect of soil and seed treatment on the shoot height[a] in *L. angustifolius* cv. Bora.

Seed treatment	Soil treatment	Skåne agricultural soil	Uppsala perennial lupin soil
Unsterilized	Fresh	35 (5)[a]	30 (5)
	Dry	35 (6)	29 (7)
	Autoclaved	33 (5)	28 (5)
Sterilized	Fresh	36 (5)	31 (6)
	Dry	31 (6)	32 (5)
	Autoclaved	35 (5)	31 (5)
1 Day of germination	Fresh	29 (4)	35 (6)
	Dry	30 (6)	30 (5)
	Autoclaved	28 (6)	25 (4)
2 Days germination	Fresh	28 (5)	25 (5)
	Dry	40 (5)	31 (5)
	Autoclaved	26 (6)	27 (3)

[a]Based on pooled sample per treatment divided by the number of plants in bracket and same experiment as in previous Tables.

3.3 Identification and phylogenetic analysis

From plants inoculated with Skåne soil high-quality sequences were obtained from one nodule each for cv Bora and cv Galant and from two nodules for perennial lupin. From plants inoculated with Uppsala perennial lupin soil, high-quality sequences were obtained from two nodules for cv Bora plants. All four sequences from the Skåne soil were identical to each other and the two Uppsala perennial soil sequences were also identical to each other. The Skåne and Uppsala sequences differed in sequence at 15 positions and the Uppsala sequence had one 2-nucleotide deletion and one 1-nucleotide deletion compared to the Skåne sequence in the 862/865 nucleotide long sequence that was analyzed.

Using BLAST to search GenBank (blast.ncbi.nlm.nih.gov) all sequences were found to be 90 to 100% identical to various *Bradyrhizobium* isolates. Phylogenetic analysis in which the obtained sequences were analyzed together with published sequences of *Bradyrhizobium* spp isolates and

related organisms showed that they grouped together and specifically in the *B. japonicum* - *B. liaoningense* group (Figure 12).

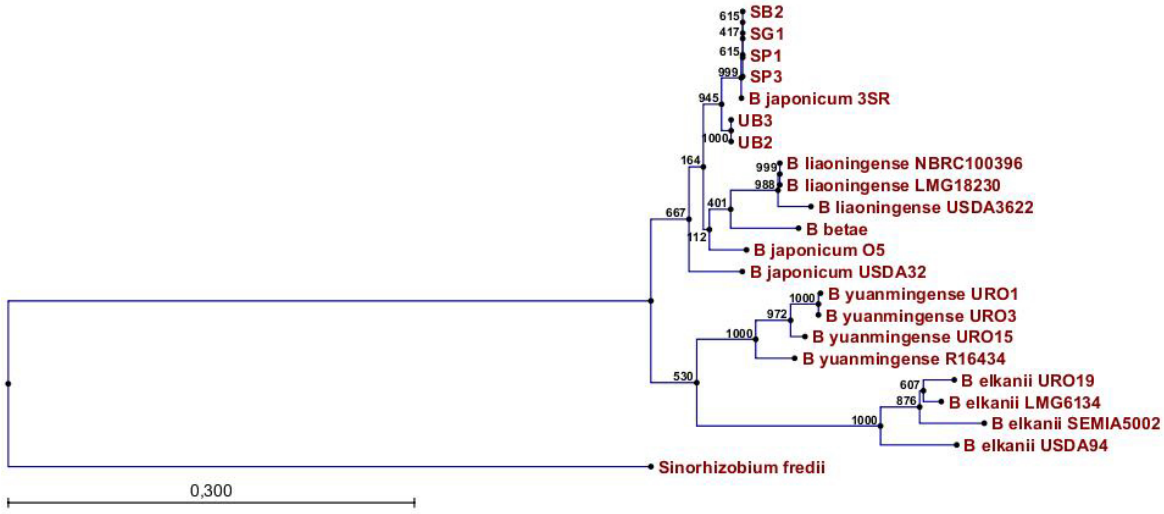

Figure 12. Neighbour-joining phylogenetic tree based on 16S-23S internal transcribed spacer sequences (ITS) showing the position of isolates in relation to representative strains of *Bradyrhizobium* sp. The sequences of this study were obtained from different nodules collected from either *L. angustifolius* cv. Bora (B) or cv. Galant (G) or from *L. polyphyllus* (P) that were cultivated in pots and inoculated with agricultural soil from Skåne (S) or perennial lupin soil from Uppsala (U). The sequences were 862 to 865 nucleotides long and in the 5´ end of the ITS. Bootstrap values for 1000 replications are indicated. The scale bar indicates 0.3 substitutions per nucleotide position.

We could also verify that the *Bradyrhizobium* strain found in the initial nodules picked from the field-grown plants and the strain later found in the nodules obtained in the experiment was the same strain based on the ITS sequence (data not shown). In addition, isolates obtained from the perennial nodules and grown in culture had the same ITS sequences as the original source.

4 Discussion

4.1 Compatible bacteria in the soils

Presence of rhizobia in soil and an efficient interaction with the plant host is essential for legumes. Here we compared two soils that were previously known to contain rhizobia compatible with lupins and investigated several aspects of inoculation and root nodule formation by *L. angustifolius*.

Two different methods to add the soil inocula to lupin plants were used in the two experiments. In Experiment 1 the plants were inoculated with a soil suspension (5 ml of suspension per pot) and thus carried all components of the soil including mineral nutrients. In Experiment 2 the plants were inoculated by dipping seeds in a similar soil suspension as in Experiment 1. The number of bacteria added was in this case those that attached to the seeds plus some carried over by the small volume of soil suspension attached to the seeds after dipping. The effect of any mineral nutrients (N, P etc) that are known to affect nodule formation and plant growth was minimized in Experiment 2 because of the choice of inoculation, while in Experiment 1 it is possible that the soil suspension contained mineral nutrients.

The Skåne agricultural soil and the Uppsala perennial lupin soil induced similar number of nodules per plant using the seed dipping inoculation technique. However, in the experiment where the soil suspension inoculum was added to the pot the number of nodules was lower for the perennial soil suggesting that mineral nitrogen or other soil factor inhibited the nodule formation. Further, nitrogen (NO_3^- and NH_4^+) and phosphorus in soil also influence nodule formation in legume plants; nitrogen inhibits nodule formation while phosphorus stimulates nodule formation. We do not have any information on nutrient contents in these two soils, but it is possible that it could have affected nodule formation. For two treatments in Experiment 2 the number of nodule-inducing bacteria in the Uppsala perennial soil was higher which seem reasonable since the soil was collected close to the roots of a growing perennial lupin and thus an active host plant. In rest of the cases in Uppsala perennial lupin soil the number of nodule-inducing bacteria was not so high. This makes it difficult to generalize about different seeds treatments other than that they give different results.

Comparing the two methods it seems that the inoculation by dipping in Experiment 2 resulted in a lower number of root nodules compared to the corresponding time-point in Experiment 1, which may be reasonable due to the lower volume and duration of inoculum exposed to the seedlings. Experiment 2 with inoculation by dipping the seeds may give a reasonable reflection of the actual population sizes of the soil inocula since the number of bacteria attached to the seedling would therefore be limited by the seedling size. In general, population size of the nodule forming rhizobia is one important factor determining the number of nodules formed. To get better estimates in the future of the number of the nodule-forming rhizobia present in soils additional methods such as the most probable number (MPN) dilution technique should be used.

The two cultivars Bora and Galant originate from two different plant breeding programs, produced by different seed companies and therefore have different genetic backgrounds. In the two early time-points of Experiment 1 there was a tendency towards higher root nodule number and root nodule biomass per plant for Galant when inoculation was with the Skåne agricultural soil. Whether there is a difference in compatibility between the root nodule-inducing bacteria and these two cultivars needs to be investigated. However, in view of the many molecular interactions leading to root nodule development and the knowledge of host specificity groups among rhizobia compatible with different legume species it is not unlikely that there are differences among lupin cultivars with regard to interactions with rhizobia in Swedish soils.

Host specificity of the nodule-forming bacteria is also an important factor in the ability of these bacteria to induce nodule formation. In this study it was found that the two soils, Skåne agricultural soil and perennial lupin soil of Uppsala, appear to contain two different compatible rhizobium strains. This is not surprising because of the geographic distance and the presence of a perennial host plant species in one soil. The rhizobium in the Uppsala soil is originating from a population associated with wild *L. polyphyllus*. In contrast, the Skåne agricultural soil was inoculated with commercial rhizobia a few years ago (Ullalena Boström, personal communication). These two rhizobial strains may have different compatibility with the two lupin species and among the two cultivars possibly seen in the early nodule formation. However, this needs to be studied further using pure isolates of these strains.

4.2 Effects of soil and seed treatments on nodule formation

Experiment 2 showed that soil treatments and seed treatments had clear effects on nodule formation. Fresh soil induced more nodules than dry soil which can be explained in the way that fresh soil supports better conditions for microbial growth. If soils are dried both the size of the population and the species richness of microbes are likely to decline because under extreme conditions (dry condition of soil here) few organisms can survive or at least remain active. The results of this study, therefore, recommend using fresh soil as inocula for studies on nodule formation in lupin plants.

A clear effect of germination stage of the seeds on the number of root nodules was observed. Seeds sterilized immediately prior to inoculation and seeds germinated for one day gave plants with higher nodule number compared to seeds germinated for two days. Since seeds germinated for two days produced longer root than seeds germinated for one day it was expected that after inoculation these plants would have higher contact with inocula resulting in a higher number of nodules. However, data of Experiment 2 showed that there was a lower number of nodules produced in plants, which had been germinated for two days. The reason might be that the plant has a control of or a certain duration time of susceptibility to infection by rhizobium that is of higher importance than the mere chance of contact to infecting agents. The results of the present study also suggest that one-day germination is quite enough prior to sowing seeds in the field or in experiments.

With regard to the effect of fresh and dry soil inoculum it was interesting to see that although the number of nodules per plant was lower for the dry inoculum the average root nodule size was bigger for dry compared to fresh inoculum. It could mean that the plant in conditions where the population size of infective rhizobia is smaller, as indicated by the lower number of nodules, can regulate the growth of those nodules that do form. This would lead to that the plant-bacteria symbiosis could fix sufficient amounts of N_2 although with fewer but larger nodules.

According to shoot biomass production the absence of nodules such as in the treatment with autoclaved inoculum did not result in lower biomass. During the short period that this experiment lasted the plant may have relied mostly on nitrogen obtained from the protein-rich seeds and not suffered greatly from nitrogen deficiency.

The results of the phylogenetic analysis showed that based on the ITS sequences the root nodule-inducing bacteria were different depending on soil origin and seem to be two different strains. The results also showed that both strains of bacteria could form nodules with both *L. angustifolius* and with *L. polyphyllus* as verified by sequencing PCR products amplified from nodules of both species.

Overall, the results of this study suggest that the two selected soils of Sweden studied here contain rhizobia that are compatible with *L. angustifolius* cv. Bora and Galant. Rhizobium compatible with perennial lupin can also be used as an inoculant for *L. angustifolius*. Both seed and soil treatment measures should be taken under consideration when growing legumes in the field and when evaluating nodulation capacity of soils under laboratory conditions.

5 Future perspectives

The study had also some limitations that could have affected the results that we got. The main purpose of this section is of course to provide recommendations for further research:

- In this study only two soils and two plant genotypes were studied. To generalize the findings of the diversity of rhizobia and their interactions with *Lupinus angustifolius*, it requires a broader study with more representative soil samples from Sweden and more cultivars.
- There could be some differences in growing plants in the field and in pots in a growth room. This is especially important for practical use of those plants. Therefore, a field level study is necessary as well to answer the questions addressed in this study.
- The nitrogen-fixing nodulating bacteria obtained from perennial lupin soil should be tested for their potential in nodulating other cultivars of *L. angustifolius*.
- Since the nodulating bacteria of Skåne agricultural soil and perennial lupin soil show differences in DNA sequences to some extent and may be different strains, it would be worthwhile to study them further with regards to e.g. their physiological N_2 fixation efficiency in symbiosis with *L. angustifolius*.

6 References

Boyer SL *et al.* (2001) Is the 16S-23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. Molecular Biology and Evolution 18: 1057-1069.

Cocking EC (2003) Endophytic colonization of plant roots by nitrogen-fixing bacteria. Plant and Soil 252: 169-175.

Date RA (1970) Microbiological problems in the inoculation and nodulation of legumes. Plant and Soil 32: 703-725.

Ecocrop (2007) Blue lupin. http://ecocrop.fao.org/ecocrop/srv/en/cropView?id=2519

Fremstad E (2006) NOBANIS-Invasive Alien Species Fact Sheet– *Lupinus polyphyllus*. Online Database North European and Baltic Network on Invasive Alien Species. www.nobanis.org

Graham PH and Vance CP (2003) Legumes: Importance and constraints to greater use. Plant Physiology 131: 872-877.

Jessop RS *et al.* (1989) Phosphorus fertilizer response in narrow-leafed lupin (*Lupinus angustifolius* L.) and chickpea (*Cicer arietinum*) compared to wheat (*Triticum aestivum*) in early growth. Fertilizer Research 19: 1-6.

Martyniuk S and Oron J (2008) Populations of rhizobia in some Polish soils not planted with legumes. Ekologija 54: 165-168.

Mihajlović V *et al.* (2008) Performance of Blue Lupin (*Lupinus angustifolius* L.) cultivars on a pseudogley soil in Serbia. Proceedings of the 12th International Lupin Conference, 14-18 Sept.2008; Palta JA, and Berger JB (eds).

Ngom A *et al.* (2004) A novel symbiotic nitrogen-fixing member of the *Ochrobactrum* clade isolated from root nodules of *Acacia mangium*. The Journal of General and Applied Microbiology 50: 17-27.

Robinson KO *et al.* (2000) Variability in plant-microbe interaction between *Lupinus* lines and *Bradyrhizobium* strains. Plant Science 159: 257-264.

Simms EL and Taylor DL (2002) Partner choice in nitrogen-fixation mutualisms of legumes and Rhizobia. Journal of Integrative Plant Biology 42: 369-380.

Taiz L and Zeiger E (2006) Plant Physiology. Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.

Tang C and Robson AD (1993) pH above 6.0 reduces nodulation in *Lupinus* species. Plant and Soil 152: 269-276.

Trujillo ME *et al.* (2005) Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp.nov. Applied and Environmental Microbiology 71: 1318-1327.

Willems A *et al.* (2001) Comparison of sequence analysis of 16S–23S rDNA spacer regions, AFLP analysis and DNA-DNA hybridizations in *Bradyrhizobium*. International Journal of Systematic and Evolutionary Microbiology 51: 623-632.

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