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Bakteriesamhällen associerade med rötter till blålupin (*Lupinus angustifolius* L.): diversitet i förhållande till jordens ursprung och effekt på växtens tillväxt

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Abstract

Bacteria play several important ecological functions in soil and in relation to plants. Legumes, such as the recently introduced grain legume narrow-leafed lupin (*Lupinus angustifolius* L.), develop N_2-fixing root nodules in response to infection by soil bacteria generally called rhizobia but can also interact with other soil bacteria. To investigate the identities and diversities of root nodule bacterial communities, seedlings of two cultivars of narrow-leafed lupin (Galant and Bora) were inoculated with a range of soil samples. The samples were collected from fields at three locations where four different farming practices were applied. They were described as organic farming, as integrated conventional and organic farming, a crop demonstration site and a low-intensive agricultural field having a natural population of *Lupinus polyphyllus*, a wild relative of *L. angustifolius*. In the experiment, half the numbers of seedlings were in addition to soil also inoculated with a pure culture of *Bradyrhizobium* sp. to ensure root nodule formation. Seven weeks after inoculation, bacteria were extracted from the rhizosphere of young roots, the exterior of root nodules (nodulesphere) and the interior of surface-sterilized root nodules. The extracts were used for extraction of DNA and for isolation of bacteria into pure culture. Community diversity analysis was carried out using terminal restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA gene. Bacterial isolates were identified using PCR of the 16S rRNA gene and the 16S-23S-internal transcribed spacer region. All soil samples were found to lack compatible nodule-inducing *Bradyrhizobium* strains except the soil with *L. polyphyllus*, while all seedlings inoculated with the *Bradyrhizobium* isolate formed root nodules. The T-RFLP analysis showed that a certain restriction fragment, corresponding to *Bradyrhizobium*, dominated in the nodules, which is expected since no nodules were formed unless plants were inoculated with *Bradyrhizobium*. Additional restriction fragments represented other bacteria in the communities, which suggest that there exist diverse bacterial communities in and associated with the root nodules. Of the three root zones, the nodulesphere was found to have the most diverse bacterial communities and varied among soils. Some of the bacterial isolates that were identified were found to belong to species that are considered to be plant-growth promoting bacteria. The functional roles of the members of these bacterial communities need further investigation.

Popular Science

In modern agriculture, artificial fertilizer and products for disease and pest control are in high demand to increase the yield. However, excessive use of synthetic chemicals has resulted in environmental pollution and can threaten human health. Can we utilize the force of nature to solve these problems? The discovery of root nodules of legume plants inspired scientists to study plant growth promoting microorganisms. Legume plants, such as soybean, groundnut and clover, are main agricultural crops worldwide. Some bacteria (rhizobia) can form a symbiosis in the form of root nodules with legumes that makes it possible to take up atmospheric nitrogen (N_2), and make it available for the host plant to support plant growth. Later, other plant growth promoting bacteria have been discovered. However, those interactions between plant and microbes are complicated and the distribution of the microorganisms is related to the habitat types and environment conditions. Hence, it is important to understand the functional roles of these symbiotic interactions and utilize them to improve agricultural practice and human life. In this project, the bacterial communities present in different soil types and that are related to the root and root nodules of narrow-leafed lupin were studied.

Table of Contents

1 Introduction

Bacteria are the oldest organisms on earth with huge species diversity and abundant populations. Bacteria participate in many ecosystem functions, such as improving soil structure and affecting nutrient cycling as free-living bacteria or in symbiosis with plants. On the other hand bacteria may be pathogenic to plants or mammals. The appearance and abundance of bacterial communities in soil are variable due to the different environment conditions and habitat types present (Horner-Devine et al., 2003) where factors such as soil structure, concentrations of different nutrients and host plant exudates play important roles. The diverse bacterial communities in soil may affect plant growth and development by various microbe-microbe and plant-microbe interactions.

Several beneficial plant–microbe interactions are present in nature. The most well-known symbiotic interaction between bacteria and plants is that of legumes and rhizobia. This interaction results in a nitrogen-fixing symbiosis in the form of root nodules. This symbiotic interaction has been intensively investigated, especially between rhizobia and those legume species that are important in agriculture. In this mutualistic interaction, rhizobia elicit root nodule formation and synthesize nutrients that support plant growth and in return the plant supplies the rhizobia with compound for energy and building blocks in metabolism. Other soil bacteria such as plant growth promoting bacteria (PGPB), can collaborate with rhizobia to promote nodule formation and enhance disease resistance of plants by competing with pathogenic microbes or somehow strengthening plant defense. In crop production of legumes, rhizobia and other beneficial bacteria communities can serve as bio-fertilizers to support plant growth, increase the yield, and reduce the risk of environmental pollution caused by excessive use of artificial fertilizers and fungicides. Hence, it is of ecological and economical importance to study the diversity and effects of bacterial communities that associate with plants and support growth.

2 Research Background

2.1 Narrow-leafed lupin (*Lupinus angustifolius* L.)

Lupins (*Lupinus* sp.) belong to the *Leguminosae* family and include more than 280 species (Eastwood et al., 2008). Lupins have been used as grain crops and horticultural plants worldwide for a long time. As a model plant in my study, I selected narrow-leafed lupin (*Lupinus angustifolius* L.), which is a newly introduced crop legume in Sweden. Narrow-leafed lupin was firstly considered as a potential crop in the Scandinavian region (Finland) in 1938 (Kurlovich and Hovinen, 2007) due to its possibility to be cultivated in sandy soil, grow during a short season (but with long day light) and also because it prefers cool temperatures (Aniszewski, 1993). Sweden shares similar

Figure 1. Narrow-leafed lupin (*Lupinus angustifolius*). (Photo by Fang)

environment and climate conditions where the perennial lupin (*Lupinus polyphyllus*) commonly is grown as a horticultural plant. Lupin provides more options for crop cultivation and plant breeding. In Finnish soils lupins may need inoculation of rhizobia to support growth especially in the first field planted (Kurlovich and Hovinen, 2007). Occurrence of rhizobia in Swedish soils that are compatible with lupins is under investigation (P-O Lundquist, pers. comm.).

Narrow-leafed lupin is an annual plant with blue flowers and elongate-narrowed leaves (Figure 1), usually reaching 20-60 cm in height. The seeds can be harvested in the same year. The existence of rhizobia that form a nitrogen-fixing symbiotic interaction with lupins can promote plant growth and result in higher yield. Moreover this cultivation can improve the fertility of the soil. Further, as a new cultivar in Sweden, narrow-leafed lupin may lack pathogens that cause disease providing further advantages for crop rotation (Boström, 2004).

2.2 Root nodule based nitrogen-fixing symbiotic interaction

The establishment of root nodule based nitrogen-fixing symbiosis is a quite complex process, and the phenotype of the nodules is mostly under plant control (Masson-Boivin et al., 2009). Nodules can be round, determinate, elongate or of unregulated shape, and appear single or clustered.

With low nutrient (nitrogen) content in soil, plant roots will exude flavonoid and isoflavonoid compounds (i.e. genistein, naringenin and luteolin), which serve as chemotactic signals to attract rhizobia and induce the *nod* genes (Miklashevichs et al., 2001; Vessey et al., 2004). Expression of *nod* genes in bacteria will initiate the synthesis of the nodule-inducing nod factor (Miklashevichs et al., 2001), lipochito-oligosaccharides required for host specific recognition and induction of root nodule formation by the host plant (Vessey et al., 2004). The acceptance and activity of the nod factor in the host plant results in several responses such as, root hair deformation due to swelling and curling, development of infection threads (an intrusion originating from the cell wall) to create entrance for bacteria, cell divisions below the infected root hair and expression of nodule specific genes (ENOD genes) during nodule development (Vessey et al., 2004; Tang et al., 1992). However, not all legumes develop infection threads, some are penetrated by bacteria via gaps or epidermal cells. Infection threads were not observed in root hairs of lupin (Tang et al., 1992) and González-Sama et al. (2004) found that in white lupin bacteria enter through the space between the cell walls of the root base and the neighbouring epidermal cells. Narrow-leafed lupin may share a similar infection process as for white lupin. The bacteria are then released inside the root and live as bacteroids in the cytoplasm of cortical cells, colonize the infection zones by division of the invaded cells, and form nodule vascular tissue to connect to the xylem and phloem for nutrient transport (Tang et al., 1992).

The bacteria that have *nif* genes can make nitrogenase enzyme that convert atmospheric nitrogen to a useable form, ammonia. The process is:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$
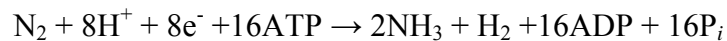
The nitrogenase is sensitive to high concentrations of oxygen (O_2) and rapidly becomes inactivated. Oxygen is necessary for bacteroids and plant mitochondria to make ATP, but nitrogenase will be inhibited by high concentrations of O_2 (Kimball, 2011). Therefore the O_2 concentration needs to be well regulated by both the plant and the bacteria to balance this conflict well. Bacteroids and plants together produce leghemoglobin to regulate the O_2 concentration (O'Brian et al., 1987; Santana et al., 1998). Leghemoglobin has a red color and a similar structure as myoglobin, the iron-containing oxygen-transport protein in muscles of animals. The fresh, mature and nitrogen-fixing nodules contain high amounts of leghemoglobin which make them red colored inside.

2.3 Classification of symbiotic nodule-inducing nitrogen-fixing bacteria

The general term for soil bacteria inducing root nodules on legumes is "Rhizobium". These bacteria were initially separated into two genera, fast-growing *Rhizobium* and slow-growing *Bradyrhizobium* due to the variant physiology characteristics. Later, some non-rhizobial species were found that also can induce nodule formation in legumes. Overall, these nodulating bacteria (with more than 70 species) belong to 12 genera and were classified into two groups: α-proteobacteria (*Rhizobium, Bradyrhizobium, Devosia, Phyllobacterium, Mesorhizobium, Shinella, Sinorhizobium, Ochrobactrum, Azorhizobium, Methylobacterium*) and β-proteobacteria (*Cupriavidus and Burkholderia*) (Masson-Boivin et al., 2009). They are gram-negative bacteria with complex genome structure (5-8Mb) that contains circular chromosomes, small mega-plasmids and other plasmids (Martinez, 1990). The genetic variation between species or strains is due to differences in the size and number of plasmids, and in the DNA sequence of different genes (Young et al., 2006).

Bradyrhizobium spp. (*Lupinus*) are the major species that can induce nodule formation in lupin roots (Jordan, 1984), and they are slow-growing compared to other rhizobia. Other rhizobia species like *Orchrobactrum lupinii* (Trujillo et al., 2005), *Mesorhizobium loti* (González-Sama et al., 2004) and *Phylobacterium trifolii* (Valverde et al., 2005) can also infect *Lupinus* and induce nodule formation.

2.4 Bacterial communities in soil associated with legume growth

Besides the nodulating bacteria, there are diverse bacterial communities present in the rhizosphere associated with legumes. Some are free-living bacteria, some prefer to colonize roots or live in the rhizosphere to absorb root exudates and can be beneficial or pathogenic to the host plant.

The beneficial bacteria, usually known as plant growth promoting rhizobacteria (PGPR), can support plant growth in several ways. This group of bacteria includes the rhizobia but

also contain other bacteria such as *Pseudomonas* spp and *Paenibacillus* spp. They have a wide host range, some can fix nitrogen as free-living bacteria (Heulin et al., 1994), can increase the tolerance of plant for abiotic and biotic stress (Timmusk and Wagner, 1999), or produce antibiotics that can antagonize pathogens (Rosado and Seldin, 1993). Currently, PGPR have been mainly studied as potential biocontrol agents to prevent different plant diseases. Other effects of PGPR can be the production of phytohormones as indole-3-acetic acid (IAA) and cytokinins or oxidation of hydrogen (H_2) produced during N_2 fixation (Maimaiti et al., 2007).

Two pathogenic bacteria have been recorded to infect legumes but most pathogens for legumes are fungi according to Porta-Puglia and Aragona (1997) and database search results. The two species *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* can cause bacterial blight in beans and lupins by forming necrotic spots on leaves and cause wilting.

The richness and evenness of the bacterial communities in the rhizosphere can be influenced by soil type, pH, and nutrient content but the amount and composition of plant root exudates was shown to play the key role (Marschner et al., 2004). A study focused on cluster roots of white lupin (Weisskopf et al., 2005) showed that the structure and abundance of bacterial communities in root and rhizosphere soil were significantly associated with the developmental stages of cluster roots. Especially the mature cluster root had a strong impact on the selection of bacterial communities, and this result can be attributed to the metabolic differences during root development where high citrate and proton exudation were produced by the mature roots.

2.5 Agricultural practices affect bacterial diversity in soils

In modern crop production, different agricultural farming systems are utilized such as organic, conventional and integrated farming. Especially organic farming is getting more popular due to its hopefully more safe and environmental friendly produce due to the restricted use of chemical control for plant diseases, choice of disease resistant cultivars and use of organic fertilizers. However, organic farming can also cause lower yields and weed problems. If diverse microbial communities occur in soil, these can manage to enhance nutrient cycling, antagonize pathogenic microbes and bio-fertilize the crops. These farming systems that include different cultivars and field managements due to farmer's choice can result in varying performance of bacterial communities.

In organic farming, the high input of organic material in the field can benefit the microbial populations by providing suitable living conditions and enough nutrient resources (Stockdale et al., 2002). However, some studies showed that organic farming result in lower organic material content in soil compared to conventional farming (Girvan et al., 2003; Stark et al., 2004), and may be related to the lower yield and lower plant residues returned into the soil. In conventional farming, plant protection relies mostly on chemical pesticides. Fungicides and herbicides may be harmful to symbiotic microbes and reduce the diversity of host plant species in the field, and this can result in lower bacterial species

diversity than in organic farming systems (Bünemann et al., 2006). Orr et al. (2010) measured the activity of N_2-fixing bacterial communities during one year, and found that it is difficult to verify whether the bacterial communities are more diverse in organic or conventional farming. This result showed that the diversity of bacterial communities could be more influenced by factors such as sampling times and preceding crops than the farming system.

2.6 Methodology for species identification and phylogenetic analysis

The development of molecular technology has provided powerful tools to support bacterial taxonomy and understand the evolutionary history (Woese, 1987). It is based on PCR amplification of specific genes, such as the genes encoding 16S ribosomal RNA (for the 30S subunit, size ca 1500 bp) and 23S rRNA (for the 50S subunit, size ca 2900 bp) genes. The function of these rRNAs is to interact with specific proteins and assemble the 30S and 50S subunits forming the core structure of functional translation-competent ribosomes (Schluenzen et al., 2000). The ribosomes have important functions in the translation of the genetic information during the synthesis of protein in living cells and affects bacterial growth (Noller et al., 2005) and exist in all bacteria (Woese, 1987).

The 16S rRNA gene is the most commonly sequenced gene for phylogenetic analysis and often provides species-specific sequences. 16S rRNA sequences provide a rapid and high yielding fingerprint method to analyze phylogenetic variation and identify species and it was used to distinguish fast and slow growing rhizobia genera (Willems and Collins, 1993). Analysis of the 23S rRNA gene is mainly used to provide the information of genera establishment (Terefework et al., 1998).

Another commonly used sequence for phylogenetic studies is the 16S-23S rRNA internal transcribed spacer region (ITS) located between the structural rRNA coding gene sequences. It has been frequently used in recent years, due to its more variant characteristics in length and sequence (Gürtler and Stanisich, 1996).

2.7 Species diversity profiling: Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

In soil and root tissues, the bacterial communities are quite diverse and abundant. As a cost-effective and high throughput method, T-RFLP profiling was introduced to handle large amount of samples for diversity investigations. It was first described by Liu et al. (1997), developed for analysis of the 16S rRNA gene sequence, and later became widely utilized to study bacterial diversity in a wide range of environmental samples.

The T-RFLP method utilizes the PCR amplified products (commonly the 16S rRNA region for bacteria) labeled with a fluorescent dye (such as 6-FAM, HEX, ROX) at the 5′-end. The PCR fragments are digested with restriction enzymes. Because the position of the restriction enzyme sites in the amplified regions in general differ between different species,

the lengths of the terminal restriction fragments (TRFs) with the fluorescent dye will also vary between different members of the communities. Nevertheless, certain even not so closely related bacteria may share the restriction site position with the same restriction enzyme. This problem was solved by utilizing multiple enzymes for the comparison and maximizes the divergence of TRFs. Digested samples are analyzed by electrophoresis to separate TRFs and detected in an automatic sequencer and compared with size standard markers.

The T-RFLP files are read by a software such as Peak Scanner (Applied Biosystem). It can provide both graphic and quantitative results. A typical T-RFLP pattern is shown in Figure 2. Dye/sample peak, Size, and Area in point were the three columns that we mainly looked at. Different peaks can represent different genotypes of bacterial communities and are separated according to the fragment size, since the restriction site in the amplified region of different communities can differ. "Area in point" means the peak area of TRFs of the community in the sample, and represents the abundance of that community's population. Thus according to the T-RFLP result, it will be easy to analyze both richness and evenness of bacterial community diversities. Data handling need to be combined with statistical analysis, and this was well evaluated and illustrated in several papers (Li et al., 2007; Zhang et al., 2008).
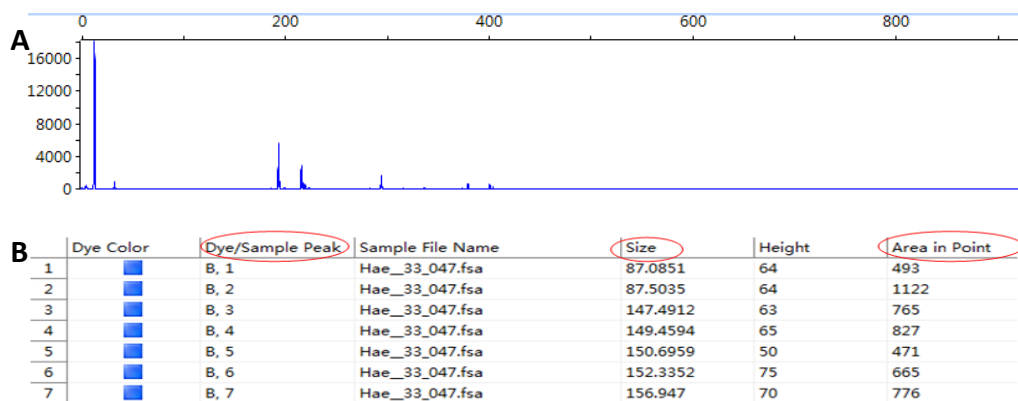
Figure 2. Example of T-RFLP results. A: the graphic result of detected TRFs, each peak with different size (X axis) represent a taxon where a higher peak means a more abundant population. B: data table of T-RFLP results where the three columns mainly used for analysis are highlighted.

3 Aims

One aim was to investigate the community diversity in three root areas (nodules, nodulesphere and rhizosphere) and to study if they are affected by different soils. Another aim was to isolate bacteria from inside root nodules, identify them and re-inoculate on the plant to investigate any effects on root development. In addition, parameters such as cultivar variation, inoculation methodology and DNA extraction methods were evaluated.

4 Materials and Methods

4.1 Plant cultivars

Two commercially available cultivars of *L. angustifolius*, cv. Bora (Saatzucht Steinach GmbH & Co KG, Germany) and cv. Galant (DLF-Trifolium A/S, Denmark) purchased from Olssons Frö AB, Helsingborg, Sweden were used in the experiments. Galant is a branched cultivar giving higher yield potential and better competition with weeds but has slightly uneven seed maturation. Bora has medium to early seed maturation. It has good resistance to mycosis and viral diseases and can be grown on light to medium soils.

4.2 Soil samples

Table 1. Fields from which soil samples were collected for this study.

Soil name	Abbreviation	Location	Position	Description	Soil type
Organic 1	O1	Logården		Organic farm field (No.2)	Clay
Organic 2	O2	Logården	58°20′19′′N	Organic farm field (No.24)	Clay
Integrated 1	I1	Logården	12°38′8′′E	Integrated farm field (No.33)	Clay
Integrated 2	I2	Logården		Integrated farm field (No.54)	Clay
Ekhaga	E	SLU, Uppsala	59°49′55′′N, 17°48′21′′E	The demonstration site where peas were growing	Clay
Pustnäs	P	Uppsala	59°48′15′′N, 17°40′25′′E	A site where perennial lupin grows around the field	Sandy-clay

Four soil plots were selected from two different agricultural fields representing two different farming systems at the Logården farm in south-west Sweden. In each farming system, the two soil plot samples were chosen to have different structure and abundance of bacterial communities according to earlier research (Enwall et al., 2010, Wessén at al., 2011). The Logården soil samples were collected at the same time as in those two studies in spring 2007 and stored at -20°C. The Ekhaga soil sample was collected from five plots and mixed in September 2010 and stored at +4°C. The Pustnäs soil sample was collected in September 2010 and stored at +4°C. The experiment was carried out in December 2010.

4.3 Experimental design

Table 2. Design of experiment 1.

Soil types	Cultivation practice	Number of cultivars	*Bradyrhizobium* inoculation (-/+)	Inoculation type (dip in soil suspension)	Number of replicate pots	Total pots per treatment
	Organic 1	2	2	1	3	12
Logården	Organic 2	2	2	1	3	12
	Integrated 1	2	2	1	3	12
	Integrated 2	2	2	1	3	12
Ekhaga		2	2	1	3	12
Pustnäs		2	2	1	3	12
No inoculation		2	2	1	3	12

This greenhouse experiment was carried out using two cultivars, six soils, and with or without inoculation with *Bradyrhizobium* sp (Br) all in three replicates for each treatment (Table 2). To half of the pots a *Bradyrhizobium* sp (Br) isolate (isolate #14 from perennial lupin, provided by Dr Mohammad Zabed Hossain, SLU) was added to ensure nodule formation if Br did not exist in all soil samples, and this made it possible to study bacterial communities from soils associated with root nodules.

To investigate the effects of the type of inoculum added, three different inoculation methods (dipping, adding and pellet) were compared in a small experiment with two organic soils and the Galant cultivar (Table 3). The soil samples could contain nutrients that suppress nodule formation and inoculation methods may influence the availability of bacteria to attach to plant roots. Dipping would let the microorganisms (MOs) attach to the roots with less nutrients added to the seedlings root. Adding an inoculum suspension could provide more abundant MOs but also more nutrients. Pelleting was tested to remove the nutrients and increase the number of MOs as much as possible.

Table 3. Design of experiment 2.

Soil types		Cultivars	*Bradyrhizobium* inoculation (-/+)	Adding soil suspension or adding bacterial pellet	Replicates	Total pots
Logården	Organic 1	1	2	2	3	12
	Organic 2	1	2	2	3	12
	No inoculation	1	2	1	3	6

4.4 Seed treatment and planting

4.4.1 Seed sterilization
Healthy and intact seeds were chosen, and incubated with 5% sodium hypochlorite (NaClO) solution for 15 min and rinsed with autoclaved MilliQ water at least 3 times. The seeds were put in a new petri dish with autoclaved MilliQ water and left to germinate in a tissue culture room without light for two days.

4.4.2 Pots and substrates
The seedlings were grown in a Leonard jar-type of pot with an upper pot containing the substrate and connected by a cloth to a lower pot containing MilliQ water. Before planting the pots were autoclaved and autoclaved sand-vermiculite added as the substrate. In addition, the pot was put in an open-ended elongated plastic bag to avoid the spread of bacteria and plant shoot contact between different samples.

4.4.3 Inoculation methods and planting
The Br isolate was cultured in 20 ml YMA liquid medium (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.1 g, yeast extract 0.4 g per L, pH 6.8, adjusted with MilliQ water and autoclaved (Vincent, 1970)) for 3 days. The liquid medium was centrifuged to collect the pellet which was resuspended in autoclaved MilliQ water and centrifuged again to remove residual culture medium. The washing with MilliQ water was repeated two more times, and then the pellet was adjusted to a 20 ml suspension.

The working procedure was to first plant five seedlings in each pot, to work with one soil at a time and first prepare the pots without Br inoculation. Soil suspensions were prepared by adding MilliQ water as 1 g per 10 ml. To the soil suspensions including Br inoculation, 1 ml Br suspension was added to the soil suspension but otherwise shared the same procedure as without Br inoculation. Here is the procedure for inoculation:

1. Dipping: seedlings of different cultivars were put in separate tubes and 1 ml soil suspension per seedling used was added to each tube. The replicates with the same treatment were inoculated together and kept on slow shaking for 30 min. The suspension was discarded and the seedlings put into the pots by sterilized forceps.
2. Adding: seedlings were directly planted in pots. Each pot received 1 ml soil suspension per seed.
3. Pellet: soil suspensions (1 ml per seed used in the experiment) were filtered and centrifuged at 1000 rpm for 20 min. The supernatant was removed and the pellet resuspended with MilliQ water. The pellet was diluted to the original volume. Each pot received 1 ml soil suspension per seed.

No inoculation (control) pots just received autoclaved MilliQ water instead of soil suspension, and pellet, but underwent the same procedure as above. After one week, two weak seedlings were removed leaving three plants in each pot.

4.5 Plant harvest

After 7 weeks plants were harvested. Control samples and samples without Br inoculation were harvested first. Sterilized tools were used and gloves changed between different soil samples. All the samples were photographed. Plant shoots were cut and dried at 65 °C for 24 h, and weighed to measure dry biomass. Plant roots were gently moved out from the substrate and slightly shaken. The presence of nodules in each sample was recorded, the nodules then collected and put into tubes with autoclaved MilliQ water. The tubes were vortexed to suspend the outside bacteria communities around the nodules into the water to obtain the nodulesphere suspension. The nodulesphere is defined as the thin region that surrounds the root nodule which is the interaction area of soil, plant and microorganisms that associate with root nodules. The nodule samples were transferred to new tubes and weighed. The nodules bacteria were surface-sterilized with 5% $NaClO_3$ solution for 10 min, then rinsed with autoclaved MilliQ water for more than 5 times and then stored at -20°C. Root samples were root tips that were not close to the nodules and were picked into tubes with MilliQ water and vortexed to make a rhizosphere suspension. These nodules, nodulesphere and rhizosphere samples were kept at -20°C for later use.

4.6 Isolation of root-nodule bacteria

4.6.1 Sterilization of nodules and extraction of a bacterial suspension
For each sample, 4-5 nodules were picked, 100 µl homogenization solution added and the nodules crushed with a sterilized metal stick before transfer of the supernatant into a filter tube. This step was repeated and all residues transferred into the filter tube and centrifuged at 1,000 rpm. The bacterial suspension was collected in the bottom of the tube.

4.6.2 Culture and purification of bacterial isolates

An aliquot (10 µl) of the bacterial suspension of each sample was pipetted into a new 2 ml Eppendorf tube, and diluted with 40 µl autoclaved MilliQ water. Vortexing was used to suspend the bacterial cells. Plates having the medium YMA Congo-red (same components as YMA liquid plus 15 g Agar and 1 ml Congo red (0.25 g/L) (Vincent, 1970)) and TSA-10 medium (Tryptic soy broth 10 g and Bacto agar (Difco), adjusted to 1 L with MilliQ water) were used for isolation. The bacterial suspensions (20 µl diluted bacteria) were evenly spread with a sterilized triangle bar. YMA CongoRed medium was used for *Bradyrhizobium* culturing and selection. TSA-10 medium was used to culture general bacterial communities.

YMA CongoRed medium plates were incubated at 28°C and TSA-10 at 20°C in darkness. The bacterial growth time and colony characteristics on the plates were checked after 8 days. Different bacteria colonies were selected as isolates according to the size, form, elevation, margins, color, texture, and opacity (Smibert and Krieg, 1994). *Bradyhizobium* colonies usually take 5 days to appear and with specific morphologigal characteristics such as typical round and pulvinate shape, slightly pink and opaque color (Figure 3).

Purification of isolates from the bacterial mass was made by using an autoclaved toothpick to slightly touch the selected colony, and with the streak plate technique to spread the bacteria in a new plate. For the *Bradyrhizobium* isolates the YMA Congo-red medium was used, but for the other isolates the TSA-10 medium was used. After two days of incubation, the presence of purified bacterial isolates was checked, and re-purified if more than one type of bacterial colonies appeared. A single isolated colony was then picked from the purified isolate plate, and cultured in 4-5 ml YMA liquid medium in a 15 ml tube for 2-3 days at 28 °C. After the bacterial isolates successfully grew in the liquid

Figure 3. *Bradyrhizobium* growing on YMA Congo red medium.
(Photo by Fang)

medium, a 100 µl aliquot of each isolate was transferred into a new 2 ml Eppendorf tube that contained 100 µl 20% glycerol. Those isolate samples were stored at -70°C for long term use. The remaining liquid culture isolate was centrifuged to collect the bacterial pellet, the supernatant poured out carefully and the pellet washed with autoclaved MilliQ water, by centrifugation to discard the medium. The pellet was re-suspended with autoclaved MilliQ water, transferred into a 2 ml Eppendorf tube and kept at -20°C for later identification.

4.7 Identification of bacterial isolates based on DNA sequences

4.7.1 DNA extraction of bacterial isolates

The isolate suspensions were centrifuged at maximal speed for 10 min to collect the pellet, 100 µl lysis solution (0.2 M NaOH, 0.1 % SDS) added before incubation at 100°C for 10 min followed by cooloing on ice. To the lysed samples 900 µl autoclaved MilliQ water was

added, made into a suspension and centrifuged at max speed for 10 min. The supernatant was carefully transferred into a new 1.5 ml Eppendorf tube and stored at -20°C.

4.7.2 PCR amplification of bacterial isolates

The bacteria that were isolated from nodules and cultivated on TSA-10 medium were analyzed using the 16S rDNA sequence amplified using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGT TACGACTT-3') (Weisburg *et al.*, 1991) resulting in a product length of around 1500 bp. Candidate Bradyrhizobia samples were analysed using the ITS sequence amplified by using the primers 1492F (5'-AAG TCGTAACAAGGTAGCC-3') and 482R (5'-GCTTTTCACCTTTCCCTC AG-3') (Willems *et al.*, 2001) resulting in a product of around 1000 bp. The PCR reactions for amplification of 16S and ITS were run in a total volume of 25 µl as follows.

Master mix	µl per 25 µl reaction	PCR-Program	
ddH$_2$O	20.2	94°C	2 min
Buffer (10x)	2.5	94°C	30 s
Mg^{2+}	0.75	54°C	30 s
dNTP (5 mM)	0.5	72 °C	90 s (16S)/60 s (ITS)
F primer (20 µM)	0.25	72°C	7 min
R primer (20 µM)	0.25	4°C	
Platinum Taq DNA polymerase (Invitrogen) (5U/µl)	0.1		
Template DNA	0.5		

(54°C / 72°C cycle steps) 40 cycles

The PCR amplifications were run in a S1000^{TM} Thermal Cycler (Bio-Rad) instrument. The PCR products were analyzed by electrophoresis in agarose gels (1% agarose and ≤0.1% Ethidium bromide, 1xTBE buffer).

Each PCR product (10 µl) was used for sequencing (Macrogen Inc., Netherlands). The 27Forward direction was sequenced for the 16S samples and the 1492Forward for the ITS samples. Sequencing results was aligned and edited by using CLC sequence viewer (CLC bio A/S). The sequence quality was checked and kept the most accurate fragment.

The phylogenetic tree for different isolates was constructed by using CLC sequence viewer. The edited sequences of the 16S rRNA gene of the samples were analyzed using the Ribosomal Database (http://rdp.cme.msu.edu/seqmatch), and the ITS sequence were analyzed using blastn (Altschul et al., 1990) search in GeneBank to identify the genus, species or strains.

4.8 Investigation of bacterial communities using T-RFLP

Firstly, the cost-efficiencies of DNA extraction methods were evaluated only with the nodule samples for the T-RFLP analysis. To compare the diversity of bacteria communities, two extraction methods were tested, lysis and kit.

4.8.1 General method by use of lysis solution

For nodule samples, the bacterial suspensions of root nodules that were extracted before in 4.6.1 were utilized. Each sample received 100 µl wash solution before vortexing, and centrifugation at 13 000 rpm for 10 min. The supernatant was removed, and lysis solution added. The following steps were the same as for the DNA extraction of the isolates. DNA concentrations were measured by using a NanoDrop spectrophotomer at 260 nm.

4.8.2 DNA extraction using a kit

The nodules (4-5 intact nodules, not the extracted bacterial suspension), nodulesphere and rhizosphere samples were extracted by using FastDNA Spin Kit for soil (MP Biomedicals). Extracted DNA samples were labeled and stored at -20 ˚C.

4.8.3 PCR amplification of T-RFLP samples

The nodules, nodulesphere and rhizosphere samples for T-RFLP also amplified the 16S region but were labeled with a fluorescent dye and of shorter length (fD1(27F)-FAM: 5'-AGAGTTTGATCCTGGCTCAG-3' (modification 5'-FAM) (Weisburg et al.,1991) and 926R: 5'-CCGTCAATTCCTTTRAGTTT-3' (Muyzer et al., 1995). The generated fragment had a length around 900 bp. The PCR conditions for the T-RFLP samples was the same as for the bacterial isolate samples, but the PCR reaction had a total volume of 50 µl and the extension time was decreased to 1 min. The primers and PCR products of T-RFLP samples were covered with aluminum foil since the fluorescein is light sensitive. The PCR results were analyzed using agarose gel electrophoresis.

4.8.4 Digestion of PCR products for T-RFLP

For each PCR product of the T-RFLP samples, three enzymes were used: HaeIII, MspI and HhaI (New England Biolab) and digestions were prepared as below. All three digestions of the PCR products were incubated for 2h at 37°C and kept in darkness all the time. The digestion was terminated by increasing the temperature to 80°C for 20 min.

Different digestions (total volume of each sample is 50 µl)

HaeIII	Fluorescently labelled PCR product	12 µl
	10 x reaction buffer 2	5 µl
	HaeIII (10 units/µl)	1 µl
	ddH$_2$O	32 µl
MspI	Fluorescently labelled PCR product	12 µl
	10 x reaction buffer 2	5 µl
	MspI (20 units/µl)	0.5 µl
	ddH$_2$O	32.5 µl
HhaI	Fluorescently labelled PCR product	12 µl
	10 x reaction buffer 4	5 µl
	BSA (NEB 100x = 10 mg/ml)	0.5 µl
	HhaI (20 units/µl)	0.5 µl
	ddH$_2$O	32 µl

The digestion results were checked in an agarose gel (10 µl digest and 2 µl loading dye).

A 10 µl aliquot of each digest was sent for fragment analysis using an ABI 3730XL capillary sequencer (Applied Biosystem). The fluorescently labelled TRFs were separated and detected, and the sizes were determined by comparison with a 1000 bp size standard labelled with X-Rhodamine (MapMarker). The T-RFLP results were viewed by using the Peak Scanner Software (Applied Biosystem). The data were exported as an Excel file.

4.9 Re-inoculation of isolated bacteria

According to the sequencing result of each soil sample, the interesting root nodule bacterial isolates were selected and re-inoculated on two days old seedlings (three replications for each isolate). Root development was examined by counting the lateral root numbers after two weeks.

4.10 Statistical analyses

All the plant production results were statistically analyzed using GLM ANOVA analysis (SAS 9.2) to compare the differences of plant growth performance. T-RFLP data was analyzed using Detrended Correspondence Analysis (DCA) in PC-ORD (version 5.0, MjM Software Design).

5 Results

5.1 Shoot biomass and nodule formation comparisons
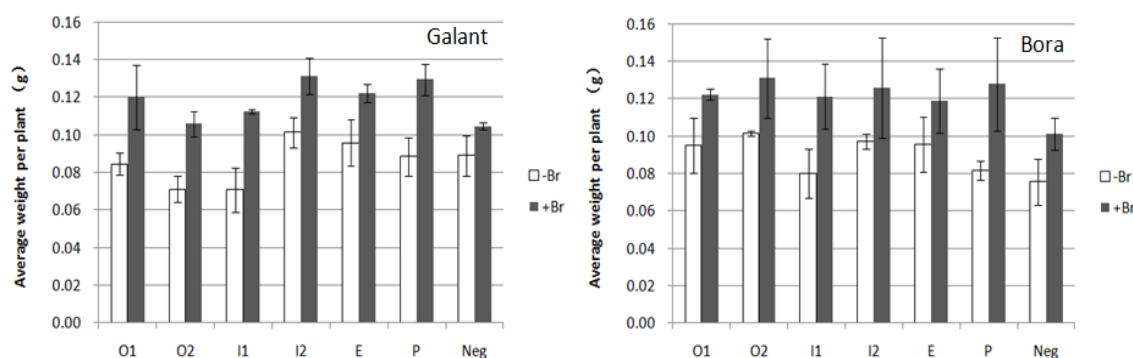
Figure 4. Shoot dry weight of two cultivars (Galant and Bora) of *Lupinus angustifolius* inoculated with 6 different soils and either without or with inoculation with Br. Averages ± SE, n=3 pots.

The result of shoot dry weight per plant for each sample (Figure 4) showed that only + and - Br inoculation (P<0.0001) were significantly associated with biomass production between different samples (Table 4). It can be seen that the shoot biomass production of

both Bora and Galant cultivars were improved for all treatments with the Br inoculation compared to non-inoculated samples. The negative controls for both cultivars were similar at about 0.1 g per plant with Br inoculation.

The two cultivars also showed significant association with the difference of biomass production (P<0.05) (Table 4). For the Galant cultivar, two samples (O2 and I1 without Br inoculation) had lower values than the negative control. Bora had more similar shoot biomass productions for the six different samples, especially with Br inoculation.

Table 4. The GLM ANOVA analysis of different sources that affect shoot biomass.

Source	F Value	Standard error	Pr > F
Br	37.81	0.0033	<.0001¤
Cultivar	4.63	0.0033	0.0359 #
Soil	1.85	0.0061	0.1069
Soil*Cul	0.88	0.0087	0.5143
Cul*Br	1.05	0.0046	0.3100
Soil*Br	0.61	0.0087	0.7219
Soil*Cul*Br	1.10	0.0123	0.3749

*Interaction between different sources
¤P<0.0001, significantly affect the variance
#P<0.05, affect the variance

The shoot biomass results between organic and integrated soils were different, but the difference also varied among the cultivars. O1 and I2 had higher biomass production of Galant cultivar +/- Br inoculation than O2 and I1 Bora samples where O1 and I1 had lower shoot biomass production, and no big difference for I and O samples when inoculated with Br.

Previous soil analysis (Enwall et al., 2010, Wessén at al., 2011) showed that the selected Logården soil samples O1 and I1 had more diverse bacterial communities than O2 and I2. Hence, it is difficult to verify whether the diversity of bacterial communities of organic agricultural practices will enhance plant growth. The growth vigor of each plant is different, and the long term storage of material in the freezer may cause losses of some bacteria. Further, the differences among the soil samples in bacterial communities with possibly different functions could all influence the plant growth.

All samples with Br inoculation showed nodule formation. Without Br inoculation only samples of Pustnäs soil had nodule formation, showing that this soil contained nodulating bacteria. Narrow-leafed lupin root nodules are round in shape, commonly occurs as single or clustered nodules and fresh nodules are red-colored inside (Figure 5).
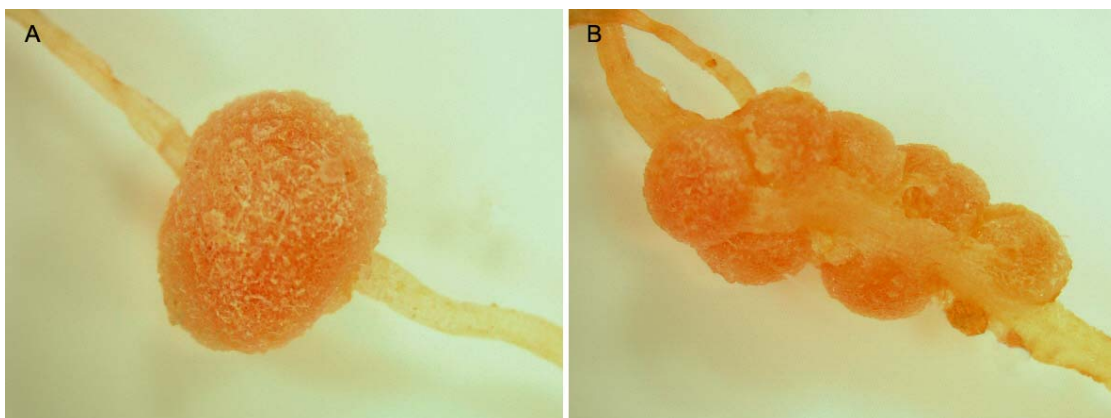

Figure 5. Root nodules of narrow-leafed lupin either as a single root nodule (A) or as a cluster of root nodules (B). (Photos by Fang)
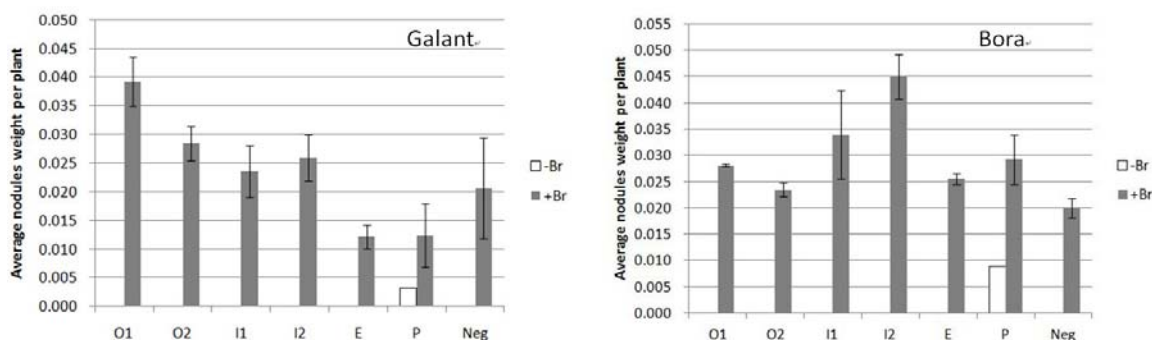
Figure 6. Root nodule formation by two cultivars of *L. angustifolius* (Galant and Bora) inoculated with six different soil suspensions or with *Bradyrhizobium*. Note that no nodules were formed in response to only soil inoculation without Br inoculation except for the Pustnäs soil, in which both Galant and Bora cultivars had one replicate pot each with plants having nodules.

According to Figure 6, the average weight of root nodules varied more between different soils than the plant weight. The Bora cultivar produced more root nodules than Galant in most of the soil besides O1, O2, and the negative control with Br inoculation. Compared with the shoot biomasses, except O1, O2, I1 (Galant and Bora, +Br), other samples showed that Galant produced less (I2, E and P) or similar (Neg) levels of root-nodules but still had similar shoot biomass or even much higher than Bora. The reason why Bora and Galant had different levels of nodules between different samples may be due to that the preference of nodule inducing and growth promoting bacteria communities of different cultivars can differ. Also in E and P soils, both Galant and Bora samples showed less nodule formation (especially Galant, less nodules than negative control), but had higher biomass production. Without Br inoculation both Galant and Bora produced less nodules in Pustnäs soil than plus Br inoculation.

The statistical analysis of root nodule production (Table 5) indicated that the soil types were significantly associated with different amount of nodule production. Besides, the existences of Br and the interaction between soils, Br also played a role on root nodule production (P<0.0001). Interestingly, the negative controls of both cultivars showed very similar results. Different cultivars, and the interaction of different factors also influenced the nodule production (P<0.05).

Table 5. The GLM ANOVA analysis of different sources that affect nodule production.

Source	F Value	Standard error	Pr > F
Br	453.61	0.0008	<.0001¤
Cultivar	15.26	0.0008	0.0003 #
Soil	6.03	0.0015	<.0001¤
Soil*Cul	3.47	0.0022	0.0057 #
Cul*Br	13.48	0.0012	0.0006 #
Soil*Br	6.62	0.0022	<.0001¤
Soil*Cul*Br	3.19	0.0031	0.0093 #

*Interaction between different sources
¤P<0.0001, significantly affect the variance

From the result of experiment 2, different inoculation methods gave somewhat different results of shoot biomass and nodule production (Figure 11 and 12) with significant association (P<0.05, Table 7 and 8). Addition of a soil suspension resulted in the highest shoot biomass production. Only O1 samples showed no difference among the three methods with Br inoculation. Dipping of soil suspensions resulted in less biomass production than for the other two methods that may be due to loss of bacterial communities and nutrients from soil compared to the other two methods.
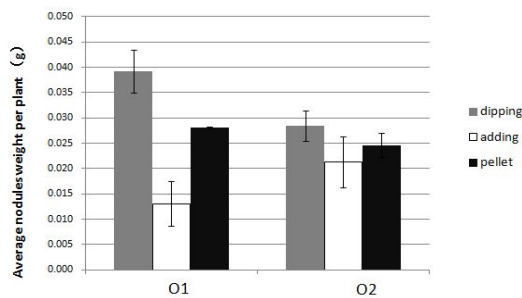

Figure 11. Comparison of nodule formation with three different inoculation methods (dipping, adding suspension and adding pellet) tested for O1 and O2 soils together with inoculation with Br.
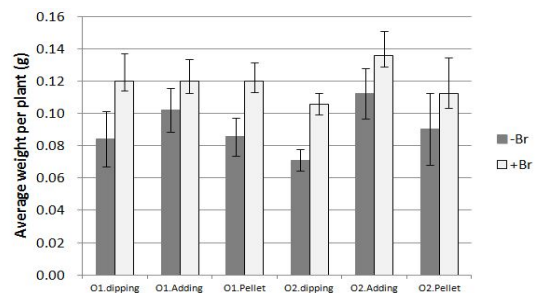

Figure 12. Comparisons of Lupin's shoot dry biomass with three inoculation methods (dipping, adding suspension and adding pellet) tested by using O1 and O2 soils.

For root nodule production, only the different soils and the interaction with inoculation methods showed a significant effect (P<0.05). Dipping produced the highest nodule than adding and pellet. The reasons could be that the nutrients in the soil suspension or other soil compounds can affect the plant-microbe interaction, and compared to the bacterial population size, affect the autoregulation of nodulation (AON) (Li et al., 2009). However for the study of root nodule formation it is still better to choose dipping as inoculation method, since this experiment was more focused on the plant growth associated bacteria and reduced the affection of nodules formation inhibitor.

Table 7. The GLM ANOVA analysis of different sources that affect shoot biomass for inoculation method test.

Source	F Value	Standard Error	Pr > F
Inoculation	3.68	0.0059	0.0417 #
Soil	0.01	0.0048	0.9041
Br	16.73	0.0048	0.0005 #
Soil*Br	0.04	0.0068	0.8514
Soil*Ino	1.26	0.0084	0.3041
Br*Ino	0.38	0.0084	0.6886
Soil*Br*Ino	0.15	0.0118	0.8621

*Interaction between different sources
#P<0.05, affect the variance

Table 8. The GLM ANOVA analysis of different sources that affect nodules production in inoculation method test.

Source	F Value	Standard error	Pr > F
Inoculation	3.33	0.002594	0.0778
Soil	8.37	0.002118	0.0160 #
Soil*Ino	14.8	0.003668	0.0010#

*Interaction between different sources
#P<0.05, affect the variance

5.2 T-RFLP analysis

Galant cultivar samples were selected for T-RFLP analysis. The T-RFLP results were edited by deleting TRFs < 50bp and >900 bp in size and peak areas contributing with <1% to exclude background and undigested PCR fragments (Zhang et al., 2008). Edited TRF results were analyzed by using PC-ORD (version 5.0, MjM Software Design) to obtain quantitative information among bacterial communities (Li et al., 2007).
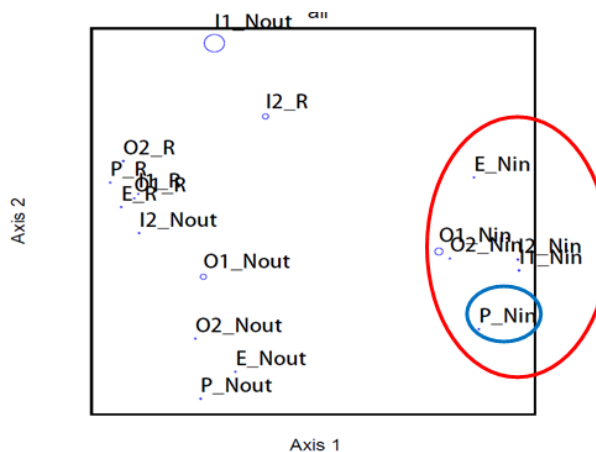

Figure 13. Detrended Correspondence Analysis of bacterial communities at three root zones of different soil samples. (Nin:inside nodule community; Nout: nodulesphere, outside nodule community; R: rhizosphere, outside root tip community)

5.2.1 Detrended Correspondence Analysis (DCA) of T-RFLP data

Comparison of differences among bacterial communities in these three root zones was done by drawing a DCA graph (Figure 13). It showed that inside root nodules, bacteria communities were different and separated from nodulesphere and rhizosphere samples.

The inside nodule community composition also showed variation between different soils. The root nodules that were inoculated with Pustnäs soil (natural population of *L. polyphyllus*) was different from the Ekhaga soil, corresponding to a domesticated field without natural lupin growth. The four soils selected from the Logården farm also showed that organic farming soils resulted in different bacterial communities in root nodules compared with integrated soils. Interestingly, within the same farming system, the two replicate soils for each farming system were similar although they came from different places in the farm which had different structure and abundance of bacterial communities and also different soil conditions (Enwall et al., 2010, Wessén at al., 2011). The results showed that the bacterial communities within the nodules were different due to the difference in farming system.

5.2.2 Community diversity of root nodule samples

Table 10. The summary of combined root nodule TRFs obtained using three enzymes.

Samples	Richness	Evenness	Diversity	Samples	Richness	Evenness	Diversity
O1_Nin_kit	49	0.827	3.220	O1_Nin_lysis	42	0.846	3.163
O2_Nin_kit	32	0.745	2.581	O2_Nin_lysis	28	0.771	2.569
I1_Nin_kit	20	0.780	2.338	I1_ Nin_lysis	30	0.792	2.693
I2_Nin_kit	26	0.685	2.232	I2_Nin_lysis	37	0.777	2.807
E_Nin_kit	41	0.851	3.159	E_ Nin_lysis	29	0.779	2.623
P_Nin_kit	37	0.756	2.731	P_ Nin_lysis	26	0.700	2.281

Explanation: Nin= Nodule internal space, kit = DNA extracted by using a kit, lysis =DNA extracted by using lysis buffer.
Richness = number of different TRFs of each sample, Evenness = Diversity / ln (Richness),
Diversity = - sum (Pi*ln(Pi)) = Shannon`s diversity index

The diversity of TRFs inside root nodules between different soil inoculums (each soil had two samples that used two different DNA extraction methods) is summarized in Table 10. The highest diversity index value and TRF number was found in O1_Nin_kit samples and O1_Nin_lysis, which represents a more diverse bacterial community in nodules from the O1 soil. The higher richness values in the O1 samples also showed bigger population sizes of bacteria communities than other samples. The lowest diversity index was found in the I2_Nin_kit sample. The sample from soil of organic cultivation (O1 and O2) had more diverse bacterial communities than the samples from plants inoculated with soils from Integrated cultivation (I1 and I2). The I1_Nin_kit and the I2_Nin_kit had the lowest diversity of the bacterial communities. A comparison of the two different DNA extraction methods showed that the O1, O2, E and P samples when extracted with the kit had higher diversity and richness value than the samples extracted with the lysis method. The opposite situation was found for the the I1 and I2 samples, where more diverse and abundant bacterial communities were found when the lysis solution was used to extract DNA from the samples compared with the kit method. In conclusion, the comparison of two different extraction methods for these six soils showed that samples from four soils that were extracted by a kit gave more diverse bacterial communities. The kit method seem to more efficiently extract bacterial DNA especially for some rare bacteria species and yield more purified DNA template for PCR reaction.

5.2.3 Community diversity of nodulesphere samples

Table 11. Summary of combined TRFs of 3 enzyme digests of nodulesphere samples.

Samples	Richness	Evenness	Diversity
O1_Nout	71	0.929	3.962
O2_Nout	72	0.936	4.002
I1_Nout	58	0.909	3.692
I2_Nout	48	0.841	3.256
E_Nout	65	0.913	3.811
P_Nout	73	0.951	4.080

Nout = Nodulespere,nodule outside space

Among the nodulesphere samples the highest diversity, richness and evenness values of TRFs were found for nodules of plants inoculated with the Pustnäs soil (Table 11). For both organic and integrated samples the O1_Nout and O2_Nout had more diverse bacterial communities than I1_Nout and I2_Nout in the nodulesphere zone with both higher richness and diversity value.

5.2.4 Community diversity of rhizosphere samples

In rhizosphere samples (Table 12), both I1_R and I2_R showed higher diversity values than the other samples which were totally different compared with the nodule and nodulesphere results. The lowest bacterial community diversity was found in the P_R sample with both lowest richness and diversity index value.

Table 12. Summary of combined TRFs of 3 enzymes of rhizosphere samples.

Samples	Richness	Evenness	Diversity
O1_R	47	0.849	3.267
O2_R	49	0.816	3.176
I1_R	65	0.838	3.500
I2_R	60	0.903	3.695
E_R	60	0.768	3.145
P_R	44	0.739	2.795

R = Rhizospere, root tip outside space

5.2.5 Comparison of bacterial community diversity at different root zones

A comparison of diversity index values in three root zones (Figure 14) showed more diverse communities in the nodulesphere samples, except for the I2 rhizosphere samples. The diversities in the nodule zone are less than the other two root zones in O2, I1 and I2 samples, and similar as rhizosphere in O1, E and P samples. The figure also showed that organic samples have higher diversity value in nodules and nodulesphere parts than integrated samples, but in rhizosphere samples the integrated samples showed more diverse communities.
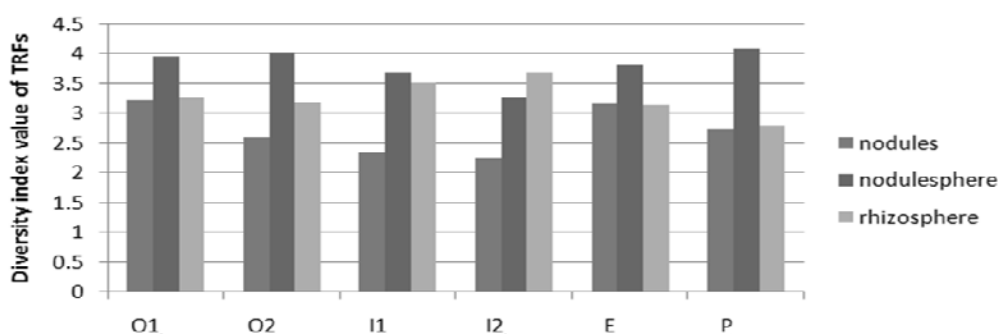

Figure 14. Diversity index values of bacterial communities at different roots zones of six different soil samples extracted using kit.

5.2.6 The relationship between TRF diversity and plant growth performance

Regression analysis to examine the correlation between plant growth performance (shoot biomass and nodule production) (as x) and TRF diversity index (as y) of six different inoculated soil samples (cv. Galant, plus Br inoculation) in three different root zones.
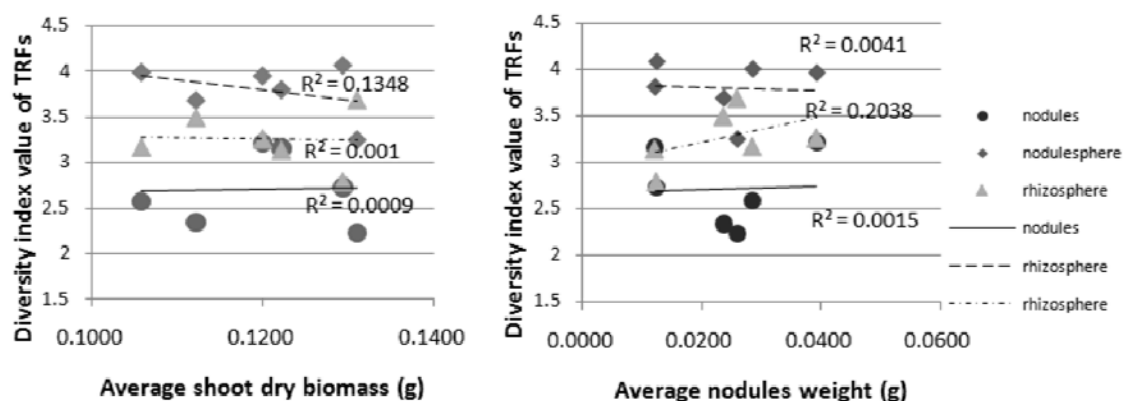

Figure 15. The polynomial trend line of shoot biomass or nodule production (x axis) vs. TRF diversity index (y axis) of three different root zones.

The bacterial community diversities in all three roots zones did not correlate to shoot and nodule production. Accordingly it is not possible to predict the plant growth performance simply by looking at the community diversity based on this study.

5.3 Sequencing and phylogenetic analysis of isolated bacteria

5.3.1 16S rRNA sequences

In total 29 non-*Bradyrhizobium* bacterial taxa were isolated from Galant root nodules (+Br) of 6 different soils. The sequences for these 29 bacterial isolates were aligned using a fragment of ~730 bp of high quality sequence. By utilizing the sequences that matched in the Ribosomal Database, the sequences of the isolates were clustered into different genera.
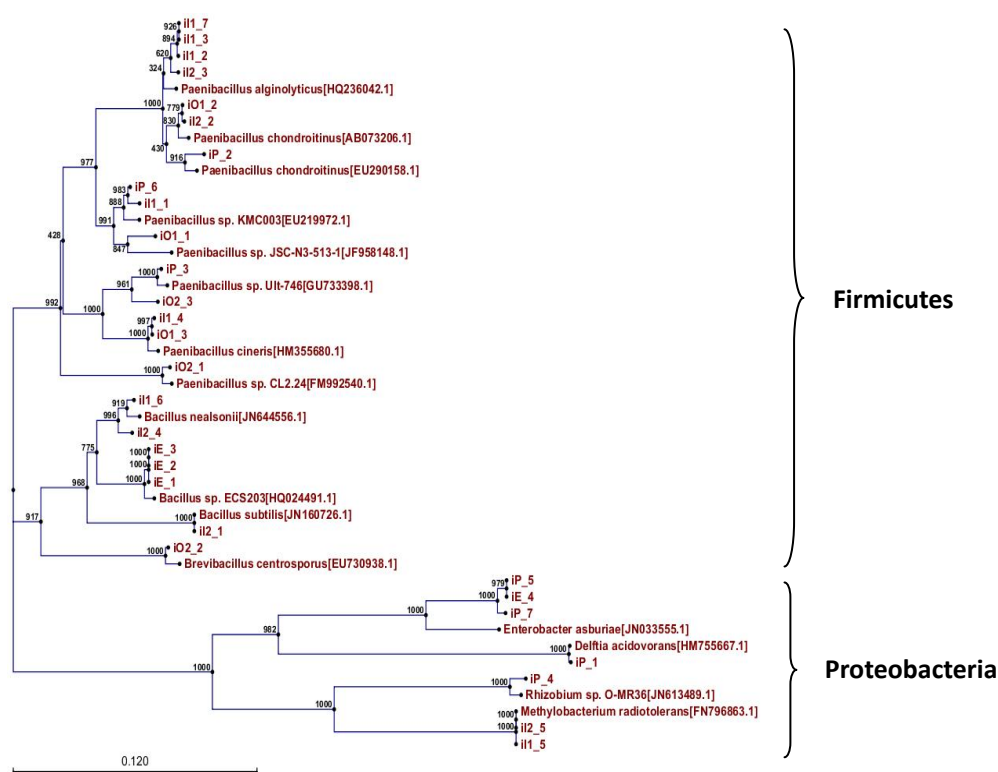

Figure 16. Neighbor-Joining tree constructed based on 16S rRNA sequences of 29 bacteria isolated from root nodules of *L. angustifolius* cv. Galant. The root nodules were obtained from plants inoculated with 6 soils. Bootstrap values after 1000 bootstrap replicates and branch length representing the genetic distance are shown. The sequences of the isolates were analyzed together with sequences of similar bacteria as found through Seqmatch (RDP). The phylogenetic analysis was carried out in CLC sequence viewer (CLC bio A/S).

However species names were not available for certain isolates. All sequences were used to make the phylogenetic tree. Bootstrap analysis of the neighbor-joining (Saitou and Nei, 1987) tree (Figure 16) showed genetic variation and evolutionary relationship of different species.

According to the sequence match results, the bacteria identified from root nodules of the six soil samples mainly belonged to genera often found to be plant-growth-promoting bacteria (*Bacillus, Paenibacillus, Brevibacillus, Enterobacter*; Vessey et al. 2003) and

rhizobia (*Methylobacterium* and *Rhizobium*; Masson-Bolvin et al., 2009), except *Delftia* which is common in soil and water but not known if it interacts with plants. In the *Paenibacillus* sp. group (Fig. 16), the isolates appeared to cluster into at least three different groups and may represent different species or strains of *Paenibacillus* sp, as supported by the bootstrap values. For other genera, as *Bacillus* it was confirmed that iI1_6 and iI2_4 in the same clade belong to the same species (*Bacillus nealsonii*), and the two isolates iI1_5 and iI2_5 were confirmed as the same species, *Methylobacterium radiotolerans*. The isolates that belonged to *Enterobacter* showed high similarity to each other.

A list of the genera or species of bacteria identified for each sample based on combining the sequence match result and the phylogenetic analysis is presented in Table 13. The identified isolates were quite different in the samples inoculated with different soils. All samples contained *Paenibacillus* communities except the Ekhaga sample.

Table 13. Identified bacteria genera or species present in 6 soils of Galant nodules based on 16SrDNA sequences and identified in the Ribosomal Database.

Soil	Genus (species)	Soil	Genus (species)
O1	*Paenibacillus* (3)	**O2**	*Paenibacillus* (2)
			Brevibacillus (1)
I1	*Paenibacillus* (5)	**I2**	*Paenibacillus* (2)
	Methylobacterium radiotolerans (1)		*Methylobacterium radiotolerans* (1)
	Bacillus sp.		*Bacillus subtilis* (1)
	Bacillus nealsonii (1)		*Bacillus nealsonii* (1)
P	*Paenibacillus* (3)	**E**	*Bacillus* (3)
	Rhizobium radiobacter (1)		*Enterobacter* (1)
	Enterobacter (2)		
	Delftia acidovorans (1)		

The number of isolates that belong to the same genus or species for each sample are shown in brackets.

5.3.2 ITS sequences
PCR products of the ITS from slow-growing bacteria on YM-medium were sequenced. Sequences were edited to remove low-quality regions (50 bp in the 5' end and 920-1020 bp in the 3´end) resulting in a sequence length of ~870 bp. The edited sequences were used to search GeneBank using blastn to collect the top ten matched sequences. The candidate *Bradyrhizobium* isolate from Pustnäs soil was found to be 98~99% identical to various *Bradyrhizobium* sp. and *Bradyrhizobium japonicum* isolates with 100% coverage. Hence, it can be confirmed that *Bradyrhizobium* present in the Pustnäs soils belong to the *Bradyrhizobium japonicum* species group.

5.4 Effects of selected isolates on root development

In order to study effects of the isolated bacteria on plant root development (Fig. 17), six different isolates and one *Bradyrhizobium* isolate obtained from Galant root nodules were selected to represent different genera based on the DNA sequence identification. These 6 isolates belonged to different bacterial genera or species.
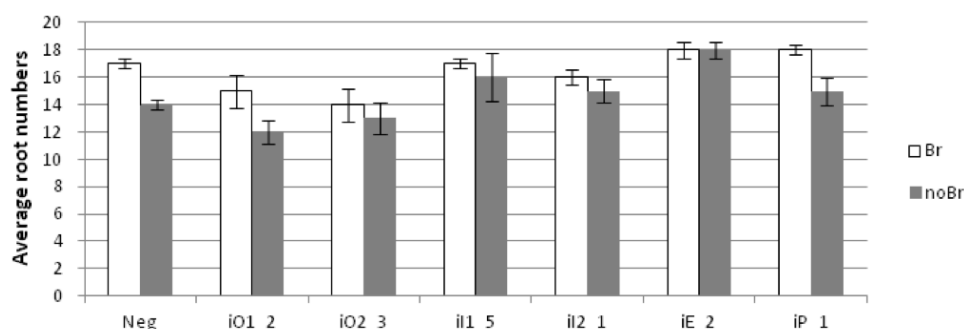

Figure 17. The effect of 6 selected isolates on Galant root growth development. Isolates were inoculated individually or together with a *Bradyrhizobium* isolate. The number or primary lateral roots were counted after two weeks. iO1_2: *Paenibacillus* sp.; iO2_3 *Brevibacillus* sp., iI1_5: *Methylobacterium radiotolerans*; iI2_1: *Bacillus subtilis*; iE 2: *Bacillus* sp.; iP 1: *Delftia*

According to the statistical analysis (Table 14), the isolates showed significant association with the variation in root numbers (P<0.0001). The number of roots per pant for almost all inoculations were increased by Br inoculation (P<0.05). The isolate iE_2 had significantly higher root numbers without Br inoculation compared to the uninoculated control, and no difference between samples with or without Br inoculation. The root numbers of the other three isolates (iI1_5, iI2_1 and iP_1) were not affected by Br inoculation.

Table 14. The GLM ANOVA analysis of the effect of 6 different isolates with Br inoculation on first lateral roots.

Source	F Value	Standard error	Pr > F
Br	15.43	0.2850	0.0005#
Isolates	7.4	0.2850	<.0001¤
Br*Isolates	1.46	0.8062	0.2195

*interaction between different sources
¤P<0.0001, significantly affect the variance
#P<0.05, affect the variance

6 Discussion

6.1 Plant growth performance and nodule formation in different soils

It is well known that N_2 fixing symbiosis between legumes and rhizobia can support plant growth and this was also found here where both cultivars had higher shoot biomass production when inoculated with the Br isolate. Only one soil (Pustnäs) was found to result in nodule formation in narrow-lupin roots and the soil contained a strain similar to the *Bradyrhizobium japonicum* group based on ITS sequencing. The plants inoculated with the Pustnäs soil had less biomass production without additional inoculation with Br so in this experiment the Pustnäs soil inoculum appeared to have a lower abundance of the natural *Bradyrhizobium* community in the soil than what the plant needed for maximum nodule formation under the current situation. All except for one soil used in this study did thus not contain rhizobia that was compatible with narrow-leafed lupin and could induce root nodules.   Hence, it would be advantageous to apply *Bradyrhizobium* for lupin cultivars during crop cultivation, especially for fields not previously cultivated with lupin. The nodule formation result (nodule weight per plant) did not show the same trend as biomass production. Low nodule production can still result in high biomass production (Ekhaga and Pustnäs). This may refer to the presence of different PGPR or other beneficial bacteria that can somehow improve the nitrogen-fixing efficiency.

6.2 Two different cultivars

The performances of the two cultivars varied both for shoot biomass production and nodule formation when inoculated with the same soil. This may due to dissimilar compositions of bacterial communities in the different soils, and or that the preference of bacteria communities varies between the two cultivars. However, Bora seems to produce more standard high biomass production when inoculated with Br in different soils. Further, to complete the information for the two cultivars, T-RFLP and isolate identification of the Bora cultivar is needed.

6.3 Effects of inoculation method

Inoculation methods were evaluated since the soil samples could contain nutrients that could affect nodule formation in positive as well as negative ways. Also, the method of adding inoculum may influence the availability of bacteria, rhizobia as well as other bacteria, to attach to plant roots. The method of dipping seedlings would let the microorganisms attach to the roots with less nutrients added to the seedlings root. Adding an inoculum suspension could provide more abundant microorganisms but also more nutrients. In addition, making a pellet of soil suspensions was tested since it would be a way to remove nutrients and at the same time increase the number of microorganisms as much as possible.

Using the dipping produced higher nodule weight per plant compared to the method of adding soil suspension or as soil suspension pellet. The reasons could be that the nutrients

in the soil suspension or other soil compound affected the plant-microbe interaction. It is also known that the plant controls nodule development through a process called autoregulation of nodulation (Li et al., 2009), which results in that the number and total weight of nodules are not proportional to the bacterial population size in the inoculum. In this experiment it is possible that the possibly increased number of rhizobia present in the pelleted soil suspension could not be reflected in increased number of root nodules due to the control by autoregulation. For the study of root nodule formation it seemed best to choose dipping as inoculation method, since this experiment was more focused on the plant growth associated bacteria and reduced the effect of nodule formation inhibitor.

6.4 Diversity analysis based on T-RFLP

The T-RFLP analysis highlighted that community diversity vary in different soils and root zones. Bacterial communities present inside the root nodules were different and separated from nodulesphere and rhizosphere areas, and the nodulesphere zone showed more diverse bacterial communities for most of the samples. The reason for different distribution of communities at different root zones could be that the bacterial communities that live in the soil samples have different preference of niches (living condition, root or nodules exudation etc.) and the colonization ability (free-living, symbiosis with plant, or pathogenic bacteria). The nodulesphere may be a more suitable area for bacterial growth. Similar communities were found inside nodules inoculated with soils with the same farming system (O1 and O2, I1 and I2). Although the structure and abundance of bacterial communities are different even in the same field, the specific communities associated with plant growth (nodule formation) may be related to the cultivation practice.

After comparing plant biomass and nodule production with diversity index, almost no correlation between diversity of bacteria communities vs. plant growth and nodule formation ability were found in this study. T-RFLP profiling is quite efficient and applicable to study the diversity of bacterial communities, but the TRFs diversity index still cannot represent the real diversity of bacteria communities (Li et al., 2007). Some TRFs that belong to the bacteria communities may be deleted as background or disturbance due to the quite small fragment size and peak area. Since the general diversity was so broad further testing could focus on some specific bacteria communities, such as N_2-fixing free-living bacteria or IAA producing bacteria by using specific primers. Next generation sequencing (NGS) techniques would also provide an option to both quantify and identify bacterial communities and be more powerful than T-RFLP. But T-RFLP is cheaper and can directly use the mixed digested DNA samples for analysis without losing communities during for example clone selection, but can only give a general overview of the microbial diversity. Use of a phylogenetic assignment tool based on searching the matched TRF sizes in the database of each restriction digest can extend the usability of the T-RFLP profile for species identification (Kent et al., 2003).

6.5 Evaluation of DNA extraction methods

For the two DNA extraction methods tested, the kit seems to be more efficient than the lysis solution for most samples. However, in this test the two methods used different nodules although within the same sample, so the bacteria communities might be different. To improve the evaluation, the samples should have the same origin and be evenly split. Also other DNA extraction methods could be tested to find out the most suitable method for further tests.

6.6 Identification of isolates

The isolation of bacteria from the root nodules and the identification based on 16S rDNA sequencing showed that the bacterial communities inside root-nodules were dominated by Bradyrhizobia but still were allowing other bacterial communities to live inside the nodules, especially bacteria that have been suggested to be growth-promoting bacteria. However, the isolates obtained may not represent all members of the bacterial communities in the nodules due to the isolation bias. *Paenibacillus* sp. were common in different nodule isolates, which may be due to that it was present in all samples, but also be due to that it grew fast and rapidly colonized the medium, so space and nutrient usage restrict growth of other bacteria. The methods for isolating bacteria are still limited and not all the bacteria can be cultured (Ritz 2007). When comparing the enormous number and variety of bacteria that exist in the environment, the fraction of bacteria that is cultivable is very small. However, cultivation is still useful for the identification of unknown bacteria of interest and for the purification of bacterial communities for other purposes. Application of further different media for selection of isolates would be advantageous. The selected bacterial isolates from root nodules (Galant) showed significant association with root numbers produced by seedlings, and had variable root growth performances. Two isolates, *Paenibacillus* and *Brevibacillus*, had even less root production than the negative control samples. But more information should be collected regarding nodule formation, shoot biomass production and root length to allow a systematic analysis of the effects.

6.7 Final conclusions

The exploration of symbiosis between plants and bacteria for agricultural production can improve plant growth and is also environment friendly. However, the interaction between rhizobia and legume plants is complex in nature. Because the intra-root-nodule area can also host additional bacteria, it is likely that the interaction between the plant and these communities and in different environmental conditions may affect the plant growth performance. Diversity of bacterial communities may have some effect on plant growth and root nodule formation, but the presence of bacterial communities preferred by plants and rhizobia are more important. Breeding of plant cultivars is also an important factor to improve this symbiosis interaction.

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