



Swedish University of
Agricultural Sciences

Interactions between *Rhizobium*, antagonistic bacteria and fungal pathogens in faba bean

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Master's thesis – 30 hec – Second cycle, A2E
Biotechnology – Master's Programme
Uppsala 2014

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Credits: 30 hec

Level: Second cycle, A2E

Course code: EX0565

Programme: Biotechnology – Master's Programme

Place of publication: Uppsala

Year of publication: 2014

Online publication: <http://stud.epsilon.slu.se>

Keywords: *Vicia faba*, beneficial plant bacteria, fungal pathogens, antagonism, interactions, microbial community

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Forest Mycology and Plant Pathology

Abstract

Plant associated micro-organisms such as nitrogen-fixing *Rhizobium*, and plant growth-promoting rhizobacteria (PGPR) *Pseudomonas* spp. and *Serratia* spp. are well recognised for their vital role in soil fertility and plant health. Most cultivated soils contain large populations of such micro-organisms. A diversity of interactions occur between rhizobia and PGPR with leguminous plants in natural conditions, however, their associations and functions in field crops such as faba bean is less studied. In Sweden, faba bean cultivation is increasing because of increasing demand for self-sufficiency of native protein feed. Two of the most serious diseases to limit the yield of faba beans are chocolate spot disease and Ascochyta blight.

This thesis contains several studies. One study was done on the microbial communities naturally associated with faba bean seeds of two different cultivars. In another study, the role of selected bacteria for health of faba bean was examined. Furthermore, the antagonistic ability of *Rhizobium* and PGPR towards faba bean pathogens, and the compatibility and root colonization competence of the two beneficial bacteria was investigated.

Cultivation-dependent and cultivation-independent approaches used to study the seed microbial community structure revealed high diversity, which seemed to be affected by the health status of the seeds. Enterobacteriaceae dominated the bacterial flora and *Serratia*, *Pseudomonas* and *Burkholderia* were among the numerous genera that inhabited the seeds. Higher bacterial growth in cv. Aurora seed exudates compared to cv. Fuego suggested differences in the chemical composition between the two varieties. Both rhizobial and PGPR isolates were shown to be antagonistic but PGPR proved to be stronger antagonists than rhizobia. *Serratia proteamaculans* S4 was strongest antagonist and produced both diffusible and volatiles antifungal metabolites.

In greenhouse, rhizobia and PGPR colonised roots of both cultivars after single or co-inoculation but the level of colonisation differed depending on the cultivar and the isolate combination. Colonisation by *Rhizobium leguminosarum* was stimulated by fluorescent *Pseudomonas* sp. Positive effects of seed bacterisation with *Rhizobium* in greenhouse were evident in terms of emergence and plant growth in both Aurora and Fuego. The results presented here highlight the importance of multifarious interactions between bacteria, plant and pathogens for sustainability in crop production of faba bean.

Abbreviations

PGPR	Plant growth-promoting rhizobacteria
AMF	Arbuscular mycorrhizal fungi
SDW	Sterile distilled water
PBS	Phosphate buffered saline solution
CTAB	Hexadecyltrimethylammonium bromide
PDA	Potato dextrose agar
PDB	Potato dextrose broth
KBA	King's B medium agar
YMA	Yeast mannitol agar
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
ITS	Internal transcribed region
BLAST	Basic local alignment search tool
NCBI	National Centre for Biotechnology Information
Cfu	Colony forming unit

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Background

In Sweden faba bean (*Vicia faba*, åkerböna in Swedish) is grown mostly in the southern and western parts of the country. Swedish faba bean production is increasing, primarily because self-sufficiency of protein forage crops is given high priority. Organic farming relies on a diverse crop rotation with legumes, due to their break crop effects in terms of reduced disease incidence and increased yield in the next crop mainly due to their nitrogen fixing ability. The nutritional requirements and ability to fix nitrogen (N) of faba beans are comparable to e.g. peas.

Severe attacks of fungal diseases in faba beans have been reported, particularly in Western Sweden. The most serious pathogens are *Botrytis fabae*, which causes chocolate spot disease (chokladfläcksjuka in Swedish), *Ascochyta fabae*, the cause of ascochyta blight (bönläcksjuka in Swedish) and *Peronospora viciae*, causing downy mildew (bönladmögel in Swedish) as they limit the yield of faba beans. Other pathogens such as *Fusarium* spp., *Pythium* sp. and *Rhizoctonia solani*, and bacteria are additional potential threats to faba bean cultivation but their contribution to yield losses is not known. With increased and intensified cultivation the problems with several diseases are expected to increase.

Beneficial micro-organisms inhabiting different soils and plants are important determinants of soil fertility and plant health. Nitrogen-fixing *Rhizobium* well known for symbiosis with leguminous crops is of crucial importance in organic farming. Besides being plant growth-promotion, rhizobia can also inhibit fungal pathogens. Beneficial bacteria belonging to e.g. *Pseudomonas* spp. and *Serratia* spp. are occupants of the same niche as rhizobia. In earlier studies at the Department of Forest Mycology and Plant Pathology, SLU, plant growth-promoting bacteria with yield increasing and antifungal potential have been isolated. They have been demonstrated to reduce plant pathogen disease severity, improve seed emergence and increase yield in field (Alström and Andersson 2011, 2012). However, the effects of these bacteria when inoculated alone or in different combinations on faba beans are not known.

In this thesis, the microbial communities associated with faba bean and the role of beneficial bacteria for plant growth and health of faba bean were investigated. The studies were carried out in the laboratory and in the greenhouse to determine the effects on faba bean plant growth and pathogen inhibition of selected bacterial isolates belonging to *Rhizobium* and other

rhizosphere bacteria, *Serratia*, *Pseudomonas* spp. The effects were determined using bioassays. Interactions, as a result of single /or co-inoculations on two different faba bean cultivars, were explored.

The goal of the studies was to identify criteria that are important for selection of bacteria for consistent efficiency in faba bean cultivation in field conditions. The goal was also to get an idea of what microorganisms coexist with field-grown faba bean. The study is limited two cultivars, a few bacterial isolates and was done under controlled conditions.

1 Literature Review

1.1 Faba bean

Faba bean is an important grain legume in Europe together with lupine, pea, soya bean, chickpea and lentil (Metayer, 2004). The nutritional value of faba bean is high, and is considered to be superior to peas or other grain legumes. (Crépon *et al.*, 2010). Faba bean is also grown for green manure and can significantly enhance yields of cereals or other crops when used as break crop (Wani *et al.*, 1994).

Faba bean is an excellent nitrogen fixer (Sahile *et al.*, 2008) and is rich in proteins, carbohydrates, B-group vitamins and minerals. The protein content of faba beans ranges from 20% to 41%, values depending on the cultivar (Chavan *et al.*, 1989). In total, faba bean seeds contain 51% to 68% of carbohydrates, the major proportion of which is constituted by starch (41–53%) (Cerning *et al.*, 1975). Faba beans are a good source of dietary minerals, such as phosphorus, potassium, calcium, sulphur and iron. Calcium content ranges from 120 to 260 mg/100 g dry mass also depending on the cultivar (Chavan *et al.*, 1989).

Faba bean is used as livestock feed and human food. It is eaten as a staple food in Egypt and other Mid-Eastern countries and its consumption may increase with the increasing population of Mid-Eastern people e.g. in the U.S. (Oplinger *et al.*, 1990).

In water deficit environment, major traits for faba bean to produce high yield are early flowering, pod and seed set to enable access to more soil water during post flowering before the onset of terminal drought (Siddique *et al.*, 2001). Faba beans can tolerate any type of soil but grow best on heavier-textured soils. (Duke, 1981)

1.2 Fungal Pathogens of faba bean

Faba bean is affected by fungal pathogens that may limit the crop yield. Different pathogenic fungi cause different fungal diseases in faba bean. In Sweden, the most common ones are chocolate spot disease (caused by *Botrytis fabae*), Ascochyta blight (caused by *Ascochyta fabae*) and downy mildew (caused by *Peronospora viciae*).



Figure 1. Three important fungal diseases of Faba bean.

<http://apps.rhs.org.uk/advicesearch/profile.aspx?pid=89> (chocolate spot)

<http://www.dpi.vic.gov.au/agriculture/pests-diseases-and-weeds/plant-diseases/grains-pulses-cereals/ag0151-ascochyta-blight-of-faba-bean> (ascochyta-blight)

<http://www.gettyimages.se/creative/mildew-stock-photos> (downy mildew)

Chocolate spot caused by *Botrytis fabae* Sard. and *B. cinerea* Pers.Fr. is the most important disease of faba bean worldwide. It can be residue-borne or seed-borne (Harrison, 1988). The first symptoms are discrete dark-brown spots surrounded by an orange brown ring on leaves, flowers and stems (Soddard *et al.*, 2010). The pathogen is spread with wind and rain splashes. Crop rotation with a minimum of 2 years break, thorough incorporation of crop residues to prevent the spread of spores to adjacent fields during the next spring, moderately dense crops, weed control, well-drained fields and well-balanced fertilization are some of the control measures.

Ascochyta blight, also known as leaf, stem, and pod spot, is another major disease of faba bean. This disease is caused by *Ascochyta fabae* (Jellis and Punithalingam, 1991) and can be seed- or residue borne. Lesions on leaves are round and those on stems elongated; both usually grey in colour and presenting distinctive rings of black pycnidia. The septate pycnidiospores are elongated and slightly curved, 3-6 μm x 10-26 μm (Kohpina *et al.*, 1999).

The disease is seed borne and can survive on seeds up to 3 years and on the plant residues the survival data varies between 4 and >12 months (Dixon G R, 1981). Control measures are practically the same as for *B. faba* but use of disease-free seeds is also recommended.

Downy mildew is caused by *P. viciae*, which is an oomycete and not a true fungus. It survives as oospores in soil and plant residues for several years (Dixon G R, 1981) and is spread by rain and wind within a field. Survival through the seeds cannot be ruled out (Smith I M, 1988). Humid and relatively cool weather favour the disease and yield losses can become significant (Gunnarsson A, 1987). Crop rotation with >3 years break between susceptible crops to allow destruction of oospores, deep plowing and removal of contaminated plant debris from field are recommended measures. *Phytophthora pisi*, a root pathogen of pea crop has recently been found to be a new root pathogen of faba bean. Both tolerant and susceptible cultivars to *P. pisi* have been reported in faba bean (Heyman, 2013).

Other fungi such as *Fusarium* sp., *Pythium* sp. and *R. solani* and some root pathogens can also be pathogenic, which implies that more faba bean cultivation means more problems with several of these (Baudoin, 2006; Infantino *et al.*, 2006; Salt, 1982). *Cercospora zonata* is another fungal plant pathogen which attacks faba bean. The pathogen mainly affects leaves, but may also affect stems and pods of faba bean. Its symptoms resemble with that of chocolate spot and ascochyta leaf spot (*Ascochyta faba*). Furthermore *Alternaria alternata* causes dark brown leaf spots. Its symptoms can also be mistaken for chocolate spot. (<http://www.dpi.vic.gov.au/agriculture/grain-crops/crop-production/growing-faba> bean).

1.3 Faba bean as a host of beneficial microorganisms

Plants have a complex network of interactions with microorganisms; some of which are beneficial while others are harmful. Beneficial microorganisms are those that can fix atmospheric nitrogen (N), decompose organic wastes and residues, detoxify pesticides, suppress plant pathogens, enhance nutrient acquisition, and produce bioactive compounds such as hormones and enzymes that stimulate plant growth (Sturz and Nowak, 2000 and Hardoim *et al.*, 2008). Beneficial microorganisms can play a key role in important ecosystem functions for plants and soil (Whipps, 1997; Raaijmakers *et al.*, 2009; Rosa *et al.*, 2011).

Current control strategies are not always effective to control e.g. soil-borne fungal diseases in different crops. Beneficial microorganisms have been shown to improve plant rooting

through, (i) the production of phytohormones which influence root development and its growth and ii) pathogen control (by producing antimicrobials, antibiotics), and (iii) the indirect effects due to the enhanced availability of nutrients and growth regulators. The application of beneficial microorganisms has been considered to reduce the use of agrochemicals.

1.3.1 Arbuscular mycorrhizal fungi

Most plants are able to naturally form symbiosis with mycorrhizal fungi. The arbuscular mycorrhizal fungi (AMF) are the most common type found in agricultural crops (Barea *et al.*, 1993). AMF improves the bio-availability of nutrients, in particular phosphorus. (Jakobsen, 1999; Vassilev *et al.*, 2001). They can also produce phytohormones. (Mohammadi *et al.*, 2008). Positive results after joint inoculation of AMF and bacteria, *Pseudomonas* sp. (Gamalero *et al.*, 2004), with *Bacillus circulans* (Singh and Kapoor, 1998), and *Burkholderia* sp. (Ruiz-Lozano and Bonfante, 1999) were found in terms of enhanced plant growth and nutrition.

Faba bean is shown to be mycotrophic (Babikova *et al.*, 2014) and different studies on interactions between faba bean and AMF have demonstrated increase in biomass production and photosynthetic rates by increasing the ratio of phosphorus and nitrogen accumulation (Ishac *et al.*, 1994; Jia *et al.*, 2004). Arbuscular mycorrhizal fungi can increase the spread of Plant Growth-Promoting Rhizosphere bacteria (PGPR) throughout the rhizosphere (Morrissey *et al.*, 2004; Toljander *et al.*, 2007). Bacteria harboured by AMF (Mycorrhiza-helper bacteria) have shown to stimulate mycorrhizal development (Bharadwaj *et al.*, 2007; Frey-Klett *et al.*, 2007; Dames and Ridsdale, 2012).

1.3.2 Nitrogen-fixing rhizobia

Rhizobium is well-known as the primary symbiotic fixer of nitrogen. Nitrogen is required by all living organisms for the synthesis of proteins, nucleic acids and other nitrogen-containing compounds. *Rhizobium* infects the roots of leguminous plants, and forms nodules where the nitrogen is fixed. The bacterial enzymes supply a constant source of reduced nitrogen to the host plant and the plant provides nutrients and energy for the activities of the bacterium. Specificity genes in the *Rhizobium* strains determine their ability to infect a specific legume species or even cultivar.

Faba bean supports rhizobia that fix nitrogen from the air. It produces a positive nitrogen balance, increasing the soil nutrition significantly. Faba bean crop can fix up to 350 kg N/ha totally, of which up to 160 kg N/ha is removed in harvested grains. Up to 270 kg fixed N/ha will contribute to soil N after harvest (Rochester *et al.*, 1998). Besides stimulation of nodule formation and nitrogen fixation, application of rhizobia can result in enhanced germination, larger root area and longer roots (Hassan and Abakeer, 2013; Zahir *et al.*, 2004). It has been reported that inoculation with indigenous *Rhizobium leguminosarum* bv. *viciae* significantly increased the growth and seed yield of faba bean (Khosravi *et al.*, 2001, 2004 and Carter *et al.*, 1994). Furthermore, inoculation with nitrogen-fixing *Rhizobium/Bradyrhizobium* in combination with *Azospirillum* or *Azotobacter* led to changes in total content of K, P, Ca, Mg, Fe, B, Mn, Zn and Cu. It also resulted in significant stimulatory effects on nodulation and plant growth of faba bean (Rodelas *et al.*, 1999) and soybean (Vessey, 2003). Shaban and El-Bramawy (2011) studied effects of combined treatments and found that the treatment with *R. leguminosarum* in combination with *T. harzianum*, significantly increased plant height, number of branches/ plant, number of pods/ plant, 100-seed weight and seed yield in faba bean in comparison with single inoculation.

Inoculation of rhizobia is usually not required for faba bean; particularly if the soil had previously been sown with this crop, because most cultivated soils contain large populations of indigenous rhizobia (Murinda and Saxena, 1985; Jensen, 1987 and Patriquin, 1986).

1.3.3 Plant Growth-Promoting Rhizobacteria

Soil is a natural environment for different microorganisms that are closely connected with the life of plants by stimulating or inhibiting their growth and development (Barabasz, 2004).

Most of the plant-associated bacteria are derived from the soil environment. Plant growth-promoting rhizobacteria (PGPR, Kloepper and Schroth, 1978; Bashan and Holguin, 1998) can stimulate plant growth, increase yield, reduce pathogen infection, as well as reduce biotic or abiotic plant stress (Welbaum *et al.*, 2004; van Loon and Bakker, 2005 and Lugtenberg and Kamilova, 2009). Use of PGPR is of interest for application in agriculture either as biofertilisers, as biopesticides and for phytoremediation (reviewed in Sturz *et al.*, 2000; Berg, 2009; Lugtenberg and Kamilova, 2009 ; Weyens *et al.*, 2009). Nevertheless, in some cases PGPR cannot give the desired effects in the field due to insufficient root colonization, which is as an important step required for exhibiting beneficial effects (Lugtenberg *et al.*, 2001). To improve the efficiency and reliability of inoculant strains it is important to understand, not

only mechanisms responsible for plant growth promotion, but all steps involved in plant colonization by PGPB.

Root exudates attract different types of soil microorganisms (Walker *et al.*, 2003). Because of this, PGPR have to be highly competitive to successfully colonize the root zone. In order to do this, they secrete siderophores and lytic enzymes and other metabolites that may reduce the growth of phytopathogens present in the rhizosphere. Siderophores have a high affinity for iron; these can inhibit the growth of pathogens by sequestering iron from the soil. Well known examples of bacterial compounds with antibiotic potential include 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, phenazine, pyoluteorin, pyrrolnitrin, thiotropocin, tropolone, cyclic lipopeptides, rhamnolipids, oligomycin A, kanosamine, zwittermicin A, and xanthobaccin (Trust, 1975; Kintaka *et al.*, 1984; Thomashow and Weller, 1988; Défago, 1993; Maurhofer *et al.*, 1994; Milner *et al.*, 1995; Milner *et al.*, 1996; Kim *et al.*, 1999; Nakayama *et al.*, 1999; Nielsen *et al.*, 2002; Raaijmakers *et al.*, 2002; Souza *et al.*, 2003). It has been shown that bacterial strains that secrete one or more of these metabolites are better suited to compete with the native microflora associated with plant hosts (Compant *et al.*, 2005; Haas and Défago, 2005; Raaijmakers *et al.*, 2008; Lugtenberg and Kamilova, 2009).

The application of beneficial microorganisms including rhizobia for increasing crop productivity is of great interest for sustainability in agriculture. Mixtures of these are suggested to be better adapted to meet environmental changes, protect against a broad range of plant pathogens, increase the genetic diversity of bio-control systems that persist longer in the rhizosphere, enhance the efficacy and reliability of their beneficial effects by allowing the combination of various mechanisms of actions without the need for genetic engineering (Duffy and Weller, 1995; Janisiewicz, 1988; Pierson and Weller, 1994).

1.4 Effect of beneficial microorganisms on faba bean pathogens

Faba bean is affected by fungal pathogens which reduces the yield of crop and quality of crop. Antagonistic bacteria which are present in the soil can suppress the development of different bean pathogens. *Pseudomonas fluorescens* has been recognized as an effective bio-control strain in numerous studies (Kang *et al.*, 2006). Several modes of action for antagonistic PGPR, *Pseudomonas* spp. have been reported, including production of different antimicrobial compounds and induction of plant defence mechanisms (Ramamoorthy *et al.*, 2002). Strains of fluorescent pseudomonads have demonstrated the ability to stimulate seed germination,

shoot and root development of different crops, including faba bean. Some strains seem to have the ability to destroy the fungal cell wall by secreting lytic enzymes and inhibit the growth of fungal pathogens by secreting hydrogen cyanide and antibiotics such as pycocyanin and phenazine.

El-Batanony *et al.* (2007) found that the cultural filtrates of *R. leguminosarum* showed synergistic potential with AMF in the bio-control of *Rhizoctonia solani*, *Fusarium solani*, and *Fusarium oxysporum* of faba bean. Similar experiences have been reported on beneficial effects of *P. fluorescens* in terms of increased shoot, root dry weights and number of pods, and induced disease resistance against root rot in faba bean (Abdelaziz *et al.* 1996; Samavat *et al.* 2011).

Some rhizobia enhance nodule formation and nitrogen fixation in leguminous plants. Interactions between strains of rhizobia and faba bean genotypes have been reported, whereby one strain may be very efficient with one genotype, but conceivably inefficient on another genotype (Mytton *et al.*, 1977). Other rhizosphere bacteria, PGPR, that do not form symbiosis with plants have potential to promote plant growth. Growth promotion by PGPR can be direct through mechanisms such as production of plant hormones and facilitating acquisition of nutrients, and/or indirect through antagonism and induced resistance towards plant pathogens. (Ramamoorthy *et al.*, 2002). These abilities in terms of synergistic or additive interactions is not fully explored in faba bean and thus has been given attention in this study with a long term aim to increase faba bean productivity in a sustainable manner.

Interactions between rhizobia, PGPR and plants are influenced by both abiotic and biotic factors. Examples of abiotic factors are soil, temperature and moisture, chemical properties of the soil and soil type. Biotic factors include interactions with plant pathogens, antagonists and total microbial communities sharing the same niche (Zhang *et al.*, 2010). As a result, the faba bean productivity may be influenced by microbial communities indigenous to both seeds and rhizosphere.

1.5 Objectives of the study

The objectives of this study were to identify and clarify what microorganisms are associated with faba bean, using both classical and molecular approaches. We also wanted to explore the

suppressive ability of *Rhizobium* spp. and antagonistic PGPR isolates towards faba bean pathogens. Furthermore, we wanted to investigate the compatibility of *Rhizobium* with PGPR and compare their colonization competence in faba bean. The investigations were guided by the hypothesis that the co-inoculation of *Rhizobium* with compatible PGPR will reduce the impact of pathogenic fungi in faba bean cultivations and reduce the levels of seed-borne inoculum. Various interactions treated in this thesis between *Rhizobium*, PGPR and fungal pathogens in faba bean are illustrated in Figure 2.

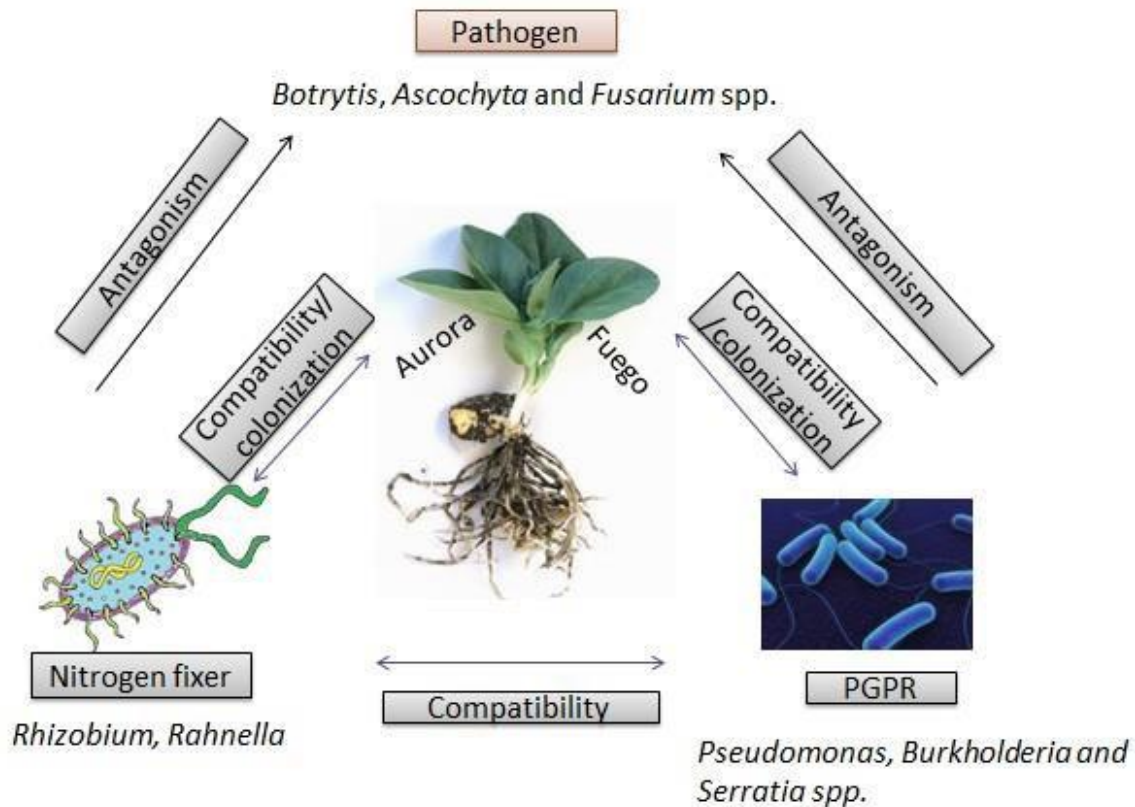


Figure 2. Different interactions between *Rhizobium*, PGPR and fungal pathogens in faba bean studied in this thesis.

Because of the different possible interactions between faba bean plant, *rhizobium* and PGPR, it was important to include more than one faba bean cultivar and different bacterial strains in our study. Two different cultivars of faba bean, Fuego and Aurora and bacteria from different groups, PGPR (e.g. *Burkholderia*, *Pseudomonas*, *Serratia*) and nitrogen-fixing bacteria (e.g. *Rhizobium*, *Rahnella*) were selected. Within each genus more than one strain was included in this study.

The microbial community was studied using both cultivation–dependent and cultivation–independent approaches. The antagonistic ability towards fungal pathogens was investigated by co-inoculation on different nutrient media. Furthermore, the compatibility of *Rhizobium* and other faba bean bacteria with antagonistic PGPR by co-inoculation was determined on nutrient agar. Bacterial compatibility was further studied by inoculating selected bacteria on faba bean seed exudates. Studies of the compatibility and colonization competence of PGPR and *Rhizobium* spp. was conducted in greenhouse by single and co-inoculation of bacteria and use of selective agar. Also, the bacterial effects on with faba bean plant growth and development were studied in greenhouse by sowing bacterized seeds.

2 Materials and Methods

2.1 Plant material

Seeds of two cultivars of *Vicia faba* Aurora and Fuego were obtained from Eurofins, Linköping for this study. The health status of the seed lots according to Eurofins are summarised in Table 1. Seemingly disease-infested and non-infested seeds from both cultivars were used for the comparative study on microbial load of seeds and for new isolations of bacteria and fungi with antagonistic potential.

Table 1. Germination potential and seed health of two faba bean cultivars used in the study.

Variety	Symptoms	Germination potential	Field Infestation
Aurora	Infested	86%	<i>Ascochyta</i> 12%, <i>Fusarium</i> 1%
Aurora	Non- infested	65%	not known
Fuego	Infested	not known	not known
Fuego	Non-infested	96%	not known

(Source: Eurofins)

2.2 Micro-organisms used in the study

Three plant pathogenic fungi, *Ascochyta pisi*, *Botrytis cinerea* (strain B05.10) and *Fusarium graminearum* (strain 1104-14) were used for the studies on direct and indirect antagonism. The fungi were obtained from our own collection at the Department of Forest Mycology and Plant Pathology, SLU, Sweden. *Botrytis cinerea* and *F. graminearum* have a broad host range which includes cereals and legumes (Lim and Cole, 1984; Skipp *et al.*, 1986). However,

pathogenicity of these fungi in faba bean was not investigated within the framework of this study.

Different rhizobia (ÅB4, ÅB17 and *R. leguminosarum*) and PGPR *Serratia proteamaculans* (S4), *Serratia plymuthica* (S412, S414), and *Pseudomonas fluorescens* (ARLS510 and FVC70) with antagonistic potential isolated in earlier studies at the Department of Forest Mycology and Plant Pathology, SLU, Sweden (Alström unpublished) were also obtained from our own collections. Some new isolates of bacteria obtained from faba bean seeds were also included in this study (see section 2.3.1). Their effects on faba bean or its pathogens are not known.

All fungal and bacterial isolates were checked for contaminants and multiplied on suitable nutrient substrates before conducting different experiments.

Two commercial *rhizobium* inocula for 1) pea, faba bean and vetch, 2) garden bean were obtained from Agroecology Lab, Hovmantorp, Sweden. These were included in the available form in greenhouse experiments.

2.3 Microbial community in faba bean

With an aim to facilitate the selection of antagonistic PGPR for successful compatible interaction with rhizobia in faba bean in field conditions, the type of micro-organisms that associate with the faba bean seeds were investigated. To get a more complete picture of the type of micro-organisms in seeds, both cultivation-independent and cultivation-dependent approaches were used.

Fifteen seeds of each of the two cultivars Aurora and Fuego, which were selected to be seemingly healthy or naturally infested with pathogenic micro-organisms, were processed before and after surface-sterilization. Seeds were first soaked overnight in distilled water. Surface-sterilisation was then performed by incubating them in 5% sodium hypochlorite solution mixed with 3 drops of Tween 20 for 5 minutes in laminar flow under gentle shaking followed by rinsing five times in sterile distilled water (SDW) After this, the seeds were submerged in 70% ethanol for 2 minutes also under gentle shaking and 5 times rinsing in SDW. The sterilised seeds were homogenized aseptically and lyophilised.

For cultivation-dependent analysis of microbial communities, 0.1 gm, of each freeze-dried sample from above was serially diluted in phosphate buffer saline solution (PBS, 1 tablet in 1000 ml of water yields, Medicago AB; 0.14 M NaCl, 0.0027 M KCl, 0.010 M phosphate buffer; pH 7.4) and spread aseptically on sterile malt agar (MA, per litre distilled water; 30gm malt extract and 15 gm agar, Difco), half strength potato dextrose agar (PDA; per litre distilled water, 19.5g potato dextrose agar and 7.5 gm agar, Difco) for fungi and diluted tryptic soybroth agar (TSBA; per litre distilled water; 10gm tryptone soya broth, 15 gm agar, Difco) for bacteria.

All inoculated plates were incubated at 20 °C and 48 hrs for seven days. After incubation the bacterial and fungal colonies were counted as they appeared.

2.3.1 Isolation and purification of seed-borne micro-organisms

Morphologically different colonies from the above plates were further isolated and purified for identification. TSBA was used to purify the bacterial colonies and PDA for fungal colonies. All cultures obtained were incubated and maintained in darkness at 20°C until tested. Yeast mannitol agar (YMA) with congo red, used for differentiating nitrogen-fixing *Rhizobium* from non-rhizobia bacteria. (Graham, 1969; Barbara *et al.*, 1983, per litre distilled water; 10gm mannitol, 0.4 gm yeast extract, 0.2 gm MgSO₄.7H₂O and 0.1 gm NaCl 15gm agar and one pinch of congo red) was used in this study for preliminary differentiation of rhizobia from non-rhizobia (Figure 3).



Figure 3 YMA with congo red distinguishing red *Rhizobium* from non-*rhizobium* colonies

All purified bacterial colonies were inoculated on YMA and incubated at 20°C in dark. The colonies showing red colour were considered as rhizobia but further identity was confirmed by DNA based molecular methods. Eight isolates Aic1, Fic4, Fic5, Fic6, Fic7, and Fic8, Anic1, Anic4 were then selected for further tests with respect to their antagonistic ability and compatibility with PGPR.

The fungi were also identified by molecular methods. The pure cultures of fungi were freeze-dried and homogenized aseptically before DNA extraction and further processing. For cultivation-independent analysis of microbial communities in seeds, the lyophilized seed samples from above were processed using DNA-based molecular method.

2.3.2 DNA amplification, purification, sequencing and sequence analysis from seed

Fungal DNA was extracted using cetyltrimethyl ammonium bromide (CTAB, 3%)-chloroform method (Gardes and Bruns, 1993). Fungal specific primer ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (forward, Sigma-Aldrich) (Gardes and Bruns, 1993) and the universal primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (reverse, Sigma-Aldrich) (White *et al.*, 1990) were used to amplify the fragment of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA.

For identification of bacteria, the universal primer used for 16S rRNA were 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (forward, Sigma-Aldrich) (Lane D J, 1991) and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (reverse, Sigma-Aldrich) (Sergio E M and William E H, 2009). Amplification was performed on a PCR machine using 47.6 µl reaction mixture which contained 10 µl MQ water, 5 µl buffer (RB), 5 µl dNTP, 1 µl forward primer, 1 µl reverse primer, 1.5 µl MgCl₂, 0.3 µl Taq-polymerase and 23.8 µl template DNA. Thermal cycle for ITS was programmed at 94°C for 5min as initial denaturation. 35 cycles were followed for denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. Final extension was performed at 72°C for 7min. Thermal cycle for 16S rRNA was programmed at 94°C for 3 min as initial denaturation. 35 cycles were followed for denaturation at 94°C for 45 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 30 seconds. Final extension was performed at 72°C for 7min.

The amplified PCR products were checked using agarose gel-electrophoresis, and then purified using AMPure PCR purification kit (Beckman Coulter, USA) according to the manufactures instructions. All samples were sent to Macrogen for Sanger sequencing.

The nucleotide sequences were edited using SeqMan DNA Star and the edited sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank sequence database. Identification was performed by comparing them with sequences of the ITS region and 16S rRNA region and using BLASTN algorithm (www.ncbi.nlm.nih.gov/BLAST/blast.cgi) (Altschul *et al.*, 1997). Sequences that showed 98-100% similarity and 98-100% query cover were considered to be the closest matches to the already available sequences. The names with highest species identity from the GenBank were thus assigned and their accession numbers were recorded.

2.4 *In vitro* interactions between the bacterial and fungal pathogens

In total, 15 different bacterial isolates were selected for this study. Of these, 10 new isolates originated from the faba beans above and five isolates; ARLS510, S4, S412, S414 and FVC70 were obtained from our own collection at the Department of Forest Mycology and Plant Pathology. These isolates have in earlier studies been shown to be antagonistic to several plant pathogens such as *Rhizoctonia solani*, *Phytophthora infestans*, *Verticillium longisporum*, *Fusarium culmorum*. They also have plant-growth promoting potential in agricultural crops (Bharadwaj *et al.*, 2012; Neupane *et al.*, 2013; Alström unpublished). However, their effects on faba bean and its pathogens and their compatibility with rhizobia are not known. The purity of all isolates was confirmed before they were multiplied on TSBA. Both direct and indirect effects of these bacteria were investigated on the fungal pathogens. For the purpose, two day old fresh cultures of bacterial and 5-10 days old fungal cultures were used.

For the direct interaction assay, a uniform size of an agar plug (5mm diameter) with active mycelium of each pathogen of was placed in the centre of a PDA petri plate (8.2 cm diameter) and the bacteria were inoculated twice on opposite side of the centre. Control plates contained fungus only. All the plates were sealed by parafilm and incubated in dark at $21\pm 1^{\circ}\text{C}$.

For the indirect interaction assay, only *Botrytis* and *Ascochyta* were included. The procedure for fungal and bacterial inoculations was same as for the direct assay except that the bacteria

were streaked on TSBA and the fungi on PDA. The plates with bacteria were inverted on the plates with the fungi and the joints were sealed carefully with parafilm to enable evaluate the involvement of possible volatile compounds in antagonism. Controls were prepared in an identical way but with fungi on PDA and un-inoculated TSBA. All the treatments in the direct and indirect assay were arranged in three replicates per treatment. The direct antagonistic effect was graded as 0=no inhibition, 1=weak, 2=moderate and 3=strong inhibition based on the fungal growth in treatment combination plates when compared to the growth in the control plates. The fungal colony growth was measured as diameter in the indirect antagonism assay.

2.5 Assessment of compatibility between different bacteria on agar

The antagonistic PGPR (ARLS 510, S412, S414, S4, FVC70) and selected bacteria originating from faba bean (ÅB4, ÅB17, Aic1, Anic1, Anic4, Fic4, Fic5, Fic6, Fic7, Fic8) were subjected to *in vitro* compatibility test using two approaches 1) streak method and 2) spot method.

All fresh bacterial cultures of faba bean bacteria were suspended in PBS (approximately 1×10^7 colony forming units/ml) in an identical manner and both tests were performed on TSBA. For the streak method, the procedure was as described by Anandaraj *et al.* (2010). For the spot method, *Rhizobium* and other faba bean bacterial isolates (100 µl each) were spread on the TSBA surface. The PGPR was then inoculated in spots 2hrs after spreading of *Rhizobium* and faba bean bacterial isolates. The test was done in three replicates. The co-inoculated plates were incubated at room temperature ($21 \pm 10^\circ\text{C}$) for 3 days. Absence of an inhibition zone growth of around the spotted colony was considered as a compatible interaction (Anandaraj *et al.*, 2010).

2.6 Assessment of compatibility of the bacteria with faba bean *in vitro*

Thirty seeds each of variety Aurora and Fuego were soaked for 20hr in 150 ml sterile PBS solution at 4°C . The seed exudates were filtered separately through sterile filters of pore size $0.22 \mu\text{m}$. Five bacterial isolates; S4, ARLS510, ÅB4, *R. leguminosarum* and Commercial 2 were selected for this experiment. According to the manufacturer, Commercial2 is based on *Rhizobium* bacteria. To facilitate correct comparison of competence of the bacteria, attempts were made to isolate the rhizobia from inoculum using standard nutrient substrates and/or YMA. Only one bacterial colony could be isolated from eight different attempts that included

inoculation on yeast extract mannitol broth/ agar commonly used for isolating rhizobia. Surprisingly, its identity was shown to be fluorescent *Pseudomonas* and not *Rhizobium* spp. However, the isolate was purified and included in the study.

Seed exudates were transferred aseptically in 24-well plates (1ml/well). Bacteria were suspended separately in PBS solution and then inoculated aseptically in each well (10µl/well). Plates were incubated for 24 hr while shaking at 120 rpm. Control wells contained only exudates. Four replications were prepared for each treatment. After incubation, colony forming units (cfu/ml) were estimated in all wells as a parameter for comparative competence. For cfu estimation, 1ml of each suspension was serially diluted in PBS and spread aseptically on TSBA. Plates were incubated in dark at 20°C, colonies were counted.

2.7 Bacterial interactions with faba bean in greenhouse

2.7.1 Effect of bacteria on faba beans in greenhouse

Faba bean bacteria originating from the seeds (Anis12 and ÅB17), the isolate *R. leguminosarum* and the two commercial inocula (Commercial 1 and 2) were chosen in this study. All bacteria were multiplied on TSBA. Twenty µl of each bacterium was suspended in 100ml PBS solution. Faba bean seeds of the both varieties were submerged separately in bacterial suspensions for two hours before sowing them in pots (20 cm length x 8cm width, 2 seeds /pot) filled with perlite. Seeds for control pots were submerged in PBS solution only before sowing.

All treatments were arranged in six replicates and pots were placed in a greenhouse with 12hr photoperiod, temperature at 20°C and relative humidity of 80%. Plants were watered and fertilized regularly and monitored twice a week with regard to shoot length, shoot dry weight, root infection in a uniform manner. Final observations were made at the end of six weeks when plants were harvested. Shoots were weighed after drying them overnight at 80°C.

2.7.2 Assessment of bacterial colonization of faba bean roots in greenhouse

Colonization competence was determined for PGPR in comparison to rhizobia in a separate greenhouse experiment. Twenty µl of each of two PGPR (ARLS510, S4) and two rhizobia (ÅB4 and *R. leguminosarum*) were suspended in 100ml PBS. The seeds were surface-decontaminated by repeated rinsing in running tap water for half an hour to minimize the

interfering effect of native microflora on the inoculated bacteria. The seeds of the two varieties were then submerged for one and half hour either in single bacterial suspensions or different mixtures of rhizobia and PGPB suspensions. Control seeds were treated with PBS solution only.

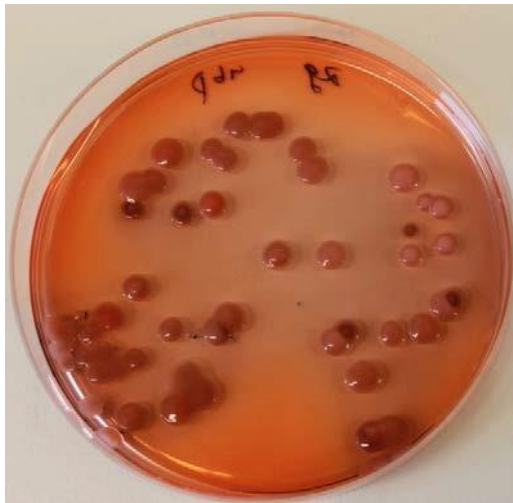


Figure 4(a) YMA with congo red distinguishes red colonies of *Rhizobium* from others

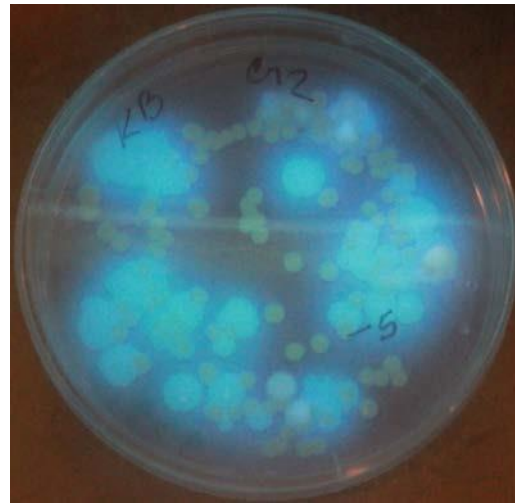


Figure 4(b) KBA distinguishes fluorescent *Pseudomonas* from non-fluorescent colonies

The treated seeds were sown in plastic bags (32 x 5) cm (length/breadth respectively in cm, one seed per bag) filled with vermiculite. The experiment was placed in the greenhouse with same growth conditions as above. Fifteen days after emergence the plants were harvested to collect roots for assessing colonization competence of the bacterial inocula. Roots from three plants were sampled, homogenized in PBS solution. The root suspensions thus obtained were serially diluted and the appropriate dilutions were spread on TSBA, YMA and King's Medium B (KBA, King *et al.*, 1954). Yeast mannitol agar and KBA were used to differentiate the rhizobia from non-rhizobial colonies and fluorescent from the non-fluorescent bacterial colonies respectively (Figures 4a & 4b). All plates were incubated for 48 hr in dark at 20°C. At the end of the incubation period, the colonies appearing on different media were recorded. Red colonies appearing on YMA were considered belonging to rhizobia and fluorescent colonies appearing on KBA were considered belonging to fluorescent *Pseudomonas*. The roots were dried at 80°C and all the data on number of bacteria (cfu) was transformed to cfu /gram dry weight of roots.

2.8 Statistical analysis

Statistical analysis of the data was performed using JMP Statistical Software, version 10.0.0. One way ANOVA was used to test for significant differences between different treatments. Comparisons of means within treatments were made using Tukey-Kramer HSD.

3 Results and Discussion

3.1 Microorganisms associated with the faba bean seeds

The aim of this study was to determine the number and type of micro-organisms that are closely associated with faba bean seeds. The results are summarized in tables 2 and 3. In general, the bacterial counts were higher before than after surface-sterilization of seeds. Naturally infested seeds harboured higher population of bacteria than the apparently healthy seeds indicating different health status of seeds. A clear cultivar difference was also evident irrespective of the health status of the seeds. Significantly low or no bacteria were detected in the surface sterilized seed tissue. This is indicative of either absence or below detection limit of bacteria inside the seeds (Table 2).

The surface-sterilization procedure followed in this study seems to eliminate all the bacteria from the seed surfaces except in infested seeds of Aurora where the bacterial load was reduced from 38×10^3 cfu/ml to 1.3×10^3 cfu/ml but not completely eliminated. Nakagawara (1998) found that even micro molar concentrations of sodium hypochlorite are enough to reduce bacterial populations significantly, which was also obtained in this our study. Treatment with 70% ethanol was included as an extra step for surface sterilization in our study, but it did not seem to increase the efficiency of the sterilization procedure indicating need of including more effective treatment either e.g. hydrogen peroxide or silver nitrate solution.

Composition of fungi associated with faba bean seeds was also estimated as number of fungal colonies that appeared on PDA used for the purpose. However, their numbers were too small to draw any safe conclusions.

Table 2. Bacteria colonizing (cfu/g seed dried tissue) apparently healthy and naturally infested faba bean seeds before and after surface-sterilization.

Cultivar	Healthy seeds, x10 ³ cfu/g		Infested seeds, x10 ³ cfu/g	
	Untreated	Surface sterilised	Untreated	Surface sterilised
Aurora	8.3	0	38.0	1.3
Fuego	1.5	0	6.8	0

Morphologically different colonies of fungi and bacteria from the above plates were isolated and purified for identifying the microorganisms associated with seeds according to the cultivation-dependent approach. In total, 74 isolates from the cultivation-dependent approach were subjected to identification. For the cultivation-independent approach, total DNA from 8 different seed samples were analysed for both bacterial and fungal communities.

Numerous studies report higher microbial diversity by cultivation-independent approach than by cultivation-dependent approach. (Richardet *et al.*, 2003; Ovreås *et al.*, 1998 and Hammes *et al.*, 2011). Our results on bacterial community structure in seeds by both approaches should be interpreted with care because most sequences particularly those obtained by the cultivation-independent approach were not good enough to blast in the NCBI database. Based on the sequences that were possible to be identified (98–100% similarity and 98-100% query coverage), nineteen genera belonging to *Bacillaceae*, *Burkholderiaceae*, *Microbacteriaceae*, *Xanthomonadaceae*, *Enterobacteriaceae*, *Flavobacteriaceae*, *Staphylococcaceae*, *Pseudomonadaceae* families were observed in Aurora compared to four genera from two families in Fuego. Further cloning and sequencing would be required to give a more complete picture of the bacterial community in faba bean in relation to cultivar differences. (Table 3)

Both *Ascochyta* and *Botrytis* were detected in the faba bean seeds. *Ascochyta* was detected only in Aurora, both in healthy untreated and surface-sterilized seeds and also infested untreated seeds while *Botrytis* was found in only infested surface-sterilized Fuego seeds. *Ascochyta* found in healthy surface-sterilized seeds indicate its seed-borne and endophytic nature (Noura *et al.*, 2012). Its absence in Fuego suggests that this cultivar may be tolerant to *Ascochyta*. Occurrence of faba bean resistance to *A. fabae* has been investigated by many researchers (e.g. Hanounik and Robertson, 1989; Sillero *et al.*, 2001) and it has been observed only in some cultivars.

Table 3: Bacterial genera identified in faba bean seeds using cultivation-dependent and cultivation-independent approaches

Cultivar	Untreated	Surface-sterilised
Aurora	<i>Bacillus sp</i>	
	<i>Burkholderia sp</i>	<i>Burkholderia sp</i>
	<i>Cenocepacia sp</i>	
	<i>Cepacia sp</i>	
	<i>Curtobacterium sp</i>	<i>Curtobacterium sp</i>
	<i>Dyella sp</i>	
	<i>Enterobacter sp*</i>	
	<i>Erwinia sp*</i>	
	<i>Flavobacterium sp</i>	
	<i>Kluyvera sp</i>	
	<i>Leclercia sp</i>	
	<i>Luteibacter sp</i>	
	<i>Pantoea sp*</i>	<i>Pantoea sp</i>
	<i>Rahnella sp*</i>	
	<i>Serratia sp</i>	
	<i>Staphylococcus sp</i>	
	<i>Tatumella sp</i>	
<i>Xanthomonas sp</i>		
		<i>Pseudomonas sp</i>
		<i>Stenotrophomonas sp</i>
Fuego	<i>Burkholderia sp</i>	
	<i>Klebsiella sp</i>	
	<i>Pantoea sp</i>	
	<i>Serratia sp</i>	

*: detected by both approaches

No asterisk: detected only by cultivation-dependent approach

Serratia and *Pseudomonas* were among several bacterial associates of faba beans found according to the cultivation-dependent approach (Table 3). Members of both of these were detected as naturally occurring components of bacterial flora of faba bean seeds. It is plausible that *Rhizobium*, inoculated or native, has to compete with members of Enterobacteriaceae as they seem to dominate the faba bean seed micro-flora. PGPR members of these two genera were thus included in our work to study compatibility and colonization efficiency in relation to *Rhizobium*.

3.2 Effect of faba bean seed exudates on bacterial growth

The bacteria included in this study were S4 (*Serratia proteamaculans*), ÅB4 (*Rhizobium* spp.), ARLS510 (fluorescent *Pseudomonas*), and an isolate of *Rhizobium leguminosarum*. Presence of fluorescent *Pseudomonas* in Commercial 2 was an unexpected finding. Fluorescent pseudomonads are a group of bacteria that have been reported from numerous soil and plant environments (Stutz *et al.*, 1986; Cuppels and Kelman, 1973). Their role in plant growth stimulation and plant disease protection has been well documented (Shweta *et al.*, 2008; Bagnasco *et al.*, 1998; Bakker *et al.*, 1987; Jayaswal *et al.*, 1992., Sullivan *et al.*, 1992).

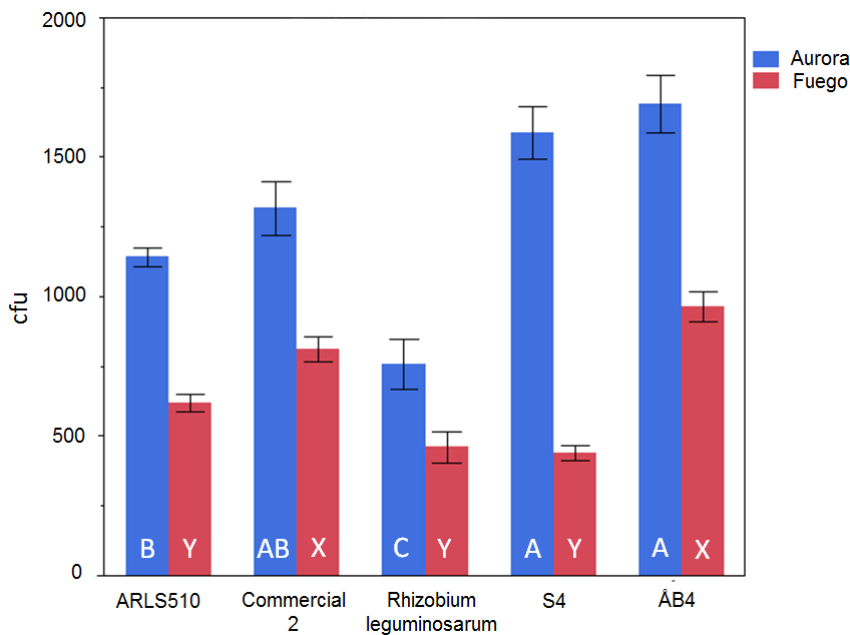


Figure 5. Effect of faba bean seed exudates on growth of rhizobia (ÅB4, *R. leguminosarum* and Commercial 2 and antagonistic PGPR (ARLS510 and S4). Each error bars is constructed using 1 standard error from the mean. The difference between the cultivars were significant, $p=0.0001$. Different letters indicate significant differences within a cultivar at $p < 0.05$, $n=4$. (Comparison of all pairs using Tukey-Kramer HSD).

The results from the bacterial growth in faba bean seed exudates demonstrated that all the isolates were able to grow on both seed exudates but to different extent depending on the isolate (Figure 5). The bacterial growth was higher in Aurora than in Fuego exudates ($p < 0.0001$). Differences in chemical composition of seed exudates may affect the size of the bacterial population in the two cultivars. Chemical analysis of the seed exudates of Aurora and Fuego is needed to confirm the basis of cultivar differences in this study.

3.3 *In-vitro* antagonistic potential of rhizobia and PGPR against fungal pathogens

Antagonistic activity was measured as the reduction in growth of fungal mycelium during the interaction with different bacterial isolates. This method is based on the hypothesis that either diffusible (cause of direct inhibition) and/or volatile compounds (cause of indirect inhibition) produced by the bacterium is responsible for inhibition of the fungal growth. In total 10 bacteria originated from faba beans (Aic1, Anic1, Anic4, Fic4, Fic5, Fic6, Fic7 and Fic8, ÅB4 and ÅB17) and five bacteria ARLS510, FVC70, S4, S412, S414 isolated from different plant in previous studies (Neupane *et al.*, 2012 ; Bharadwaj *et al.*, 2012; Alström unpublished). They were all tested for their additional antagonistic ability towards *Ascochyta*, *Botrytis* and *Fusarium* spp., all pathogenic to faba beans.

The results summarized in Figure 6 show that the three PGPR *Serratia* isolates; S4, S412, S414 exhibited strong direct inhibition of *Ascochyta* and *Botrytis* and moderate inhibition of *Fusarium* spp. The isolate, ÅB4 (*Rhizobium*) strongly inhibited growth of *Ascochyta*, moderately of *Botrytis* and only slightly of *Fusarium*. The remaining isolates exhibited moderate to weak or no inhibition of the three pathogens depending on the isolate and pathogen combination. (Figure 6)

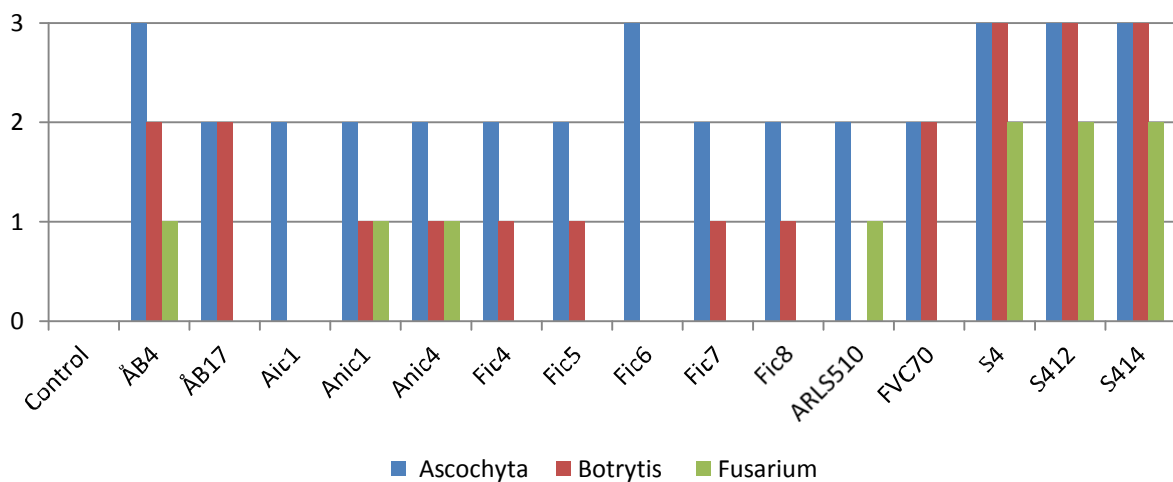


Figure 6. *In vitro* direct inhibition of growth of *Ascochyta*, *Botrytis* and *Fusarium* by two rhizobia (ÅB4 and ÅB17) in comparison to 13 different potentially antagonistic bacteria. N=3. All replicates showed the same score. Y axis: 0 = no, 1= weak, 2= moderate and 3= strong inhibition of the pathogen on PDA.



Figure 7a. Direct strong inhibition of *Botrytis* by S412. Inhibition was apparent long before the bacterium floated on the agar surface.



Figure 7b. Direct strong inhibition of *Ascochyta* by S414. Inhibition was apparent long before the bacterium floated on the agar surface.

Numerous reports are available on antagonism by different PGPR strains such as *Pseudomonas maltophilia* antagonistic towards *F. oxysporum*, *P. fluorescens* towards *Alternaria cajani*, *Curvularia lunata*, *Fusarium* sp., *Bipolaris* sp. and *Helminthosporium* sp. (Srivasthasa *et al.*, 2008) while *Bacillus* spp and *Pseudomonas* spp towards *Aspergillus* sp *Penicillium* sp and *Fusarium* sp (Nourozian *et al.*, 2006). Furthermore, *Paenibacillus polymyxa* was effective in suppressing germ tube growth of *B. cinerea* in a strawberry fruit pulp suspension culture through production of antibiotic compounds and enzyme production (Pichard *et al.*; 1995; Helbig, 2001).

All the 15 bacterial isolates were tested for indirect antagonism but only against *Ascochyta* and *Botrytis* spp. These bacterial isolates showed different degrees of growth inhibition (Figures 8a & 9a). S4 showed strongest inhibition of both *Ascochyta* and *Botrytis* than other *Serratia* isolates, S412 and S414. The bacterial isolates belonging to *Pseudomonas* (ARLS510, FVC70), *Rhizobium* (ÅB17) showed strong, moderate and weak or no inhibition for *Botrytis* and *Ascochyta* depending on the bacterium and pathogen combination. The faba beans isolates showed no indirect inhibition of *Ascochyta* or *Botrytis*. (Figures 8a & 9a).

In our study some bacterial isolates showed antifungal potential both through the mechanism of diffusible antagonistic substances and volatile metabolites depending on the bacterium and the pathogen combination. The diffusible substances include antibiotics (pyrrolnitrin) and siderophores (enterobactin and aerobactin) and volatiles include hydrogen cyanide and acetoin (Rakh *et al.*, 2011; Neupane *et al.*, 2013). What bacterial substances caused the inhibition of fungi was not investigated within the scope of this study.

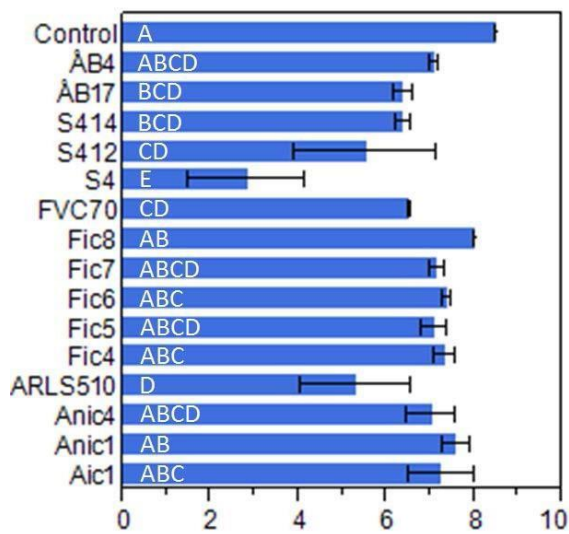


Figure 8a. *In vitro* indirect inhibition of growth of *Ascochyta* pathogenic to faba beans by two rhizobia (ÅB4 and ÅB17) in comparison to 13 other potentially antagonistic bacteria. (n=3). Each error bars is constructed using 1 standard error from the mean. Different letters statistically significant at P<0.05. (Comparison of all pairs using Tukey-Kramer HSD).

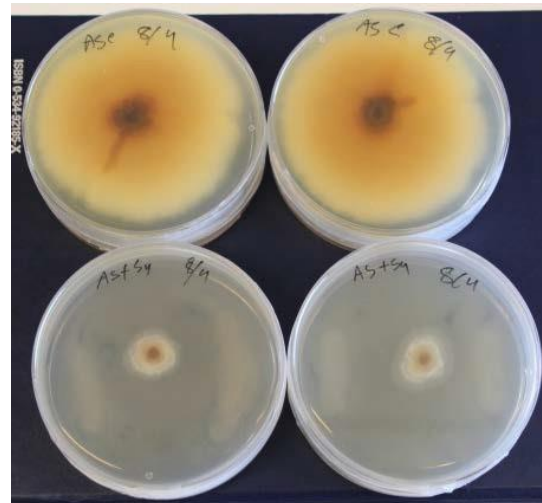


Figure 8b. Strong indirect inhibition of *Ascochyta* by S4. Control plates above row. The pathogen was inoculated on PDA and the bacterium on TSBA.

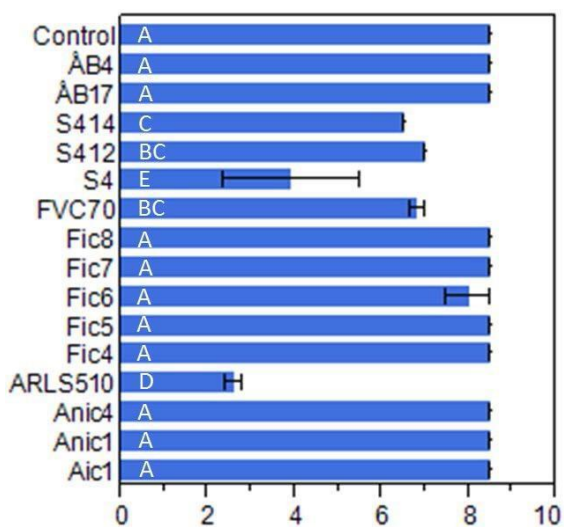


Figure 9a. *In vitro* indirect inhibition of growth of *Botrytis* by two rhizobia (ÅB4 and ÅB17) in comparison to 13 other potentially antagonistic bacteria. (n=3). Each error bars is constructed using 1 standard error from the mean. Different letters statistically significant at P<0.05. (Comparison of all pairs using Tukey-Kramer HSD).

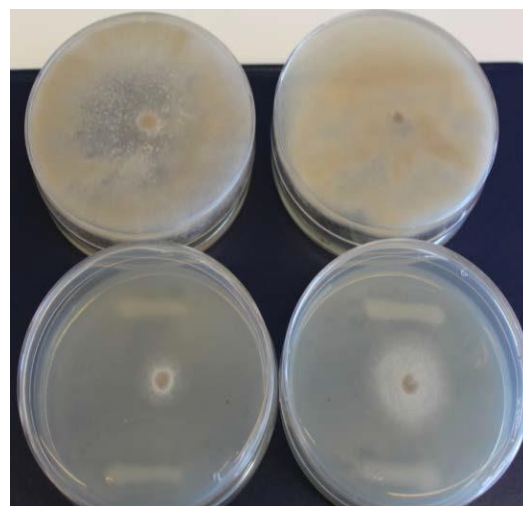


Figure 9b. Strong indirect inhibition of *Botrytis* by S4. The pathogen was inoculated on PDA and the bacterium on TSBA.

Studies by different researchers have demonstrated the ability of rhizobia to strongly inhibit *Fusarium solani*, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Macrophomina phaseolina* as well as *Rhizoctonia solani* and *Pythium* spp. pathogenic to various leguminous crops. (Shaban and El-Bramawy, 2011; Jensen *et al.*, 2002; Bardin *et al.*, 2004). In our study *Rhizobium* showed moderate to weak growth inhibition indicating the need for finding either more effective *Rhizobium* isolates or finding PGPR with strong biocontrol potential that are able to stimulate the efficiency of *Rhizobium*.

Attempt was made to explore the possible direct effect of the antagonistic bacteria on the morphology of the hyphal growth by light microscopy (Lecia DM 5500B). The mycelium was stained with 0.1% phenosafranin (Sigma Aldrich) and 3% KOH. The microscopic examination revealed wide-scale deformation of the mycelium in presence of bacterium indicating a stress response in the pathogenic fungus possibly by the bacterial metabolites (figure 10a). If the stress response was of same kind by the bacterial volatiles was not investigated. However, further studies are required to understand the stress mechanism and its impact on the pathogenicity of the fungus.



Figure 10a. Thickening of septa, swellings and deformation of *Ascochyta* mycelium in presence of S412. 40x



Figure 10b. Hyphae of *Ascochyta* (Control) 40x

3.4 Compatibility between different bacteria *in vitro*

For this study, the PGPR isolates ARLS510, S412, S414, S4, and FVC70 were selected and their compatibility with two isolates preliminary identified as *Rhizobium* was investigated *in*

in vitro using two methods, the spot assay and the perpendicular streak assay. Results were compared with compatibility between PGPR and ten additional bacterial isolates originating from faba bean seeds. Anandaraj and Leema (2010) found that *Rhizobium sp.*, *Bacillus megaterium* and *Pseudomonas fluorescens* were compatible with each other in cross streak plate assay. In our study, *Pseudomonas* spp. ARLS510 were found compatible with *rhizobium* ÅB4 and ÅB17 in agar spot plate assay (Table 4).

Results from the streak assay were not shown to be reliable and also difficult to interpret. S412 inoculants showed compatible with ÅB17 and Anic1 in agar spot plate assay. Both PGPR isolates, S414 and S4 were interpreted as incompatible with most of the bacterial inoculants as they induced a clear inhibition zone around their colony. The PGPR isolate FVC70 was found to be compatible with ÅB17 and the faba bean seed isolates; Aic1, Anic1, Anic4, Fic5 and Fic6. The compatibility in the case of remaining isolates was difficult to interpret in the test system used in this study and hence no clear conclusions can be drawn.

Table 4: Compatibility of rhizobia and faba bean seed bacteria with antagonistic PGPR bacteria using agar spot plate assay.

Faba bean bacteria	Identity	ARLS 510	S412	S414	S4	FVC70
ÅB4	<i>Rhizobium</i>	+	n.c	n.c	n.c	n.c
ÅB17	<i>Rhizobium</i>	+	+	-		+
Aic1	<i>Burkholderia</i>	n.c	n.c	n.c	-	+
Anic1	<i>Rahnella</i>	n.c	+	-	-	+
Anic 4	<i>Rahnella</i>	n.c	-	-	-	+
Fic4	<i>Burkholderia</i>	n.c	-	-	n.c	n.c
Fic5	<i>Burkholderia</i>	n.c	n.c	n.c	-	+
Fic6	<i>Burkholderia</i>	n.c	n.c	n.c	-	+
Fic7	<i>Burkholderia</i>	n.c	n.c	n.c	-	n.c
Fic8	<i>Burkholderia</i>	n.c	n.c	n.c	n.c	n.c

+ means compatible , - means incompatible and n.c means not clear

3.5 Bacterial interactions with faba bean in greenhouse

3.5.1 Effects on emergence and plant growth

Four potentially nitrogen-fixing bacterial isolates (Anis12, ÅB4, ÅB17 and *R. leguminosarum*) and two commercial *Rhizobium* inocula (Commercial 1 and 2) were inoculated on Aurora and Fuego seeds to study their effects on emergence and plant growth in a greenhouse experiment. The Aurora seeds used had natural infestation of *Ascochyta* (12% of the seeds infected), and *Fusarium* (1% of the seed infected), while the level of infestation in Fuego was not known.

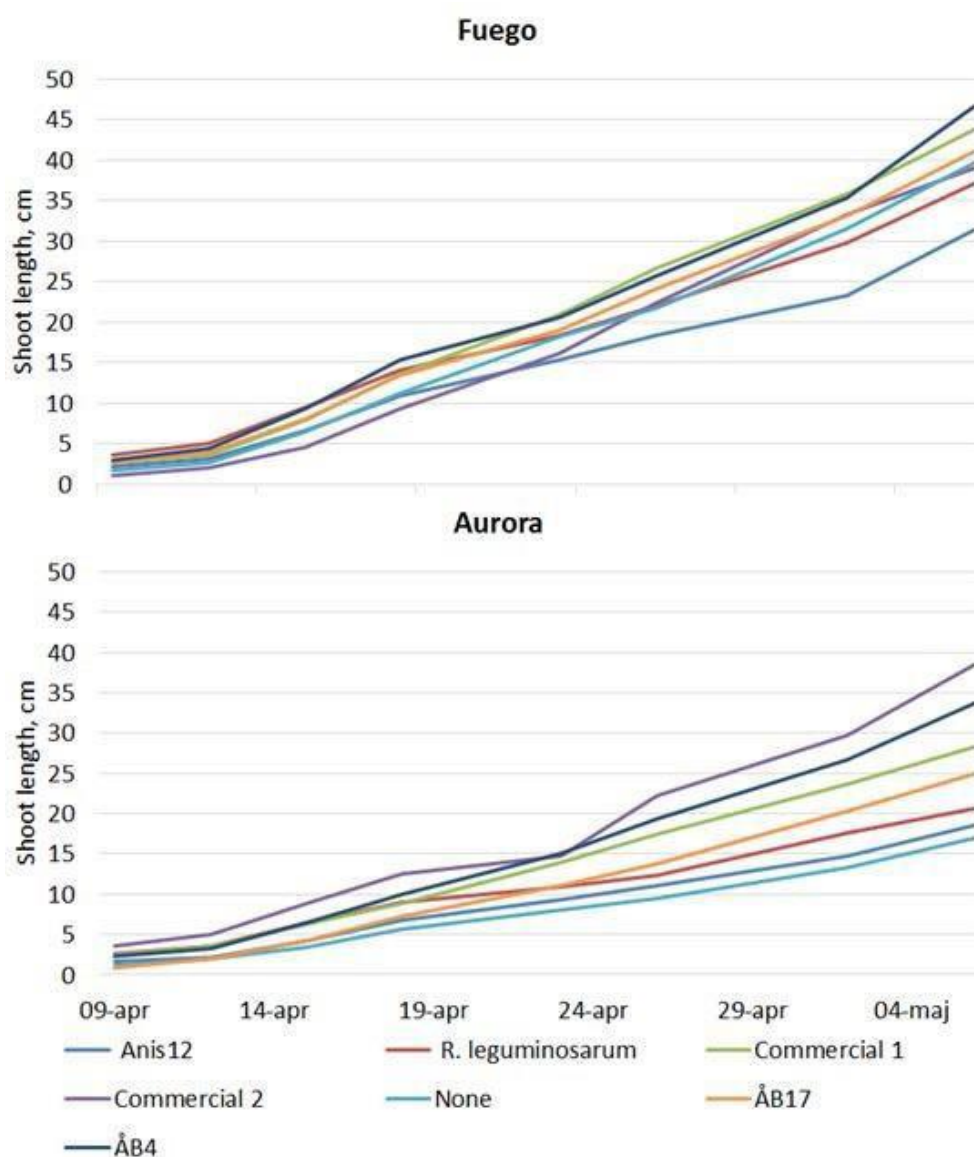


Figure 11a. Shoot growth of plants of Aurora and Fuego respectively after treatment with three faba bean bacteria and two commercial *rhizobium* inocula. N=6 (2 seeds/pot)

The three bacterial isolates were confirmed for their ability to form red colonies in YMA medium supplemented with congo red. Presence of *Rhizobium* was not checked in the two commercial inocula at the time of setting up the experiment.



Figure 11b. Effect of bacterial treatments on emergence and growth of plants of Aurora. N=6 (2 seeds/pot). Bottom row – control.

Effect of treatments on germination as observed two weeks after sowing was clear in Aurora treated with ÅB4 and commercial 2 (80%) compared to in control (60%). Aurora seeds used in the experiment were naturally infested with *Ascochyta* and the results indicate emergence improving potential of these bacteria. PGPR with emergence stimulation effect have been reported in canola (Kloepper *et al.*, 1988). However, this observation needs to be confirmed on high enough number of seeds to draw safe conclusions. Seed emergence in Fuego was, in general, high (100%) in control pots and no negative or positive effect by any of the bacteria was observed in this cultivar.

Our results on bacterial effects on faba bean growth are shown in Figure 11a. Statistical analyses of the effects of different treatments on shoot growth rate, determined as the slope of the regression line by plotting shoot height against the day of measurement, demonstrated that Commercial 2 significantly stimulated the growth in both cultivars in Aurora and Fuego and ÅB4 stimulated the growth of Aurora (Figure 12)

Nodulation occurred in most treatments but no differentiation was made between effective and non-effective nodules and their function was not studied within the framework of this thesis. Hence no data on nodule number or weight is included in this thesis.

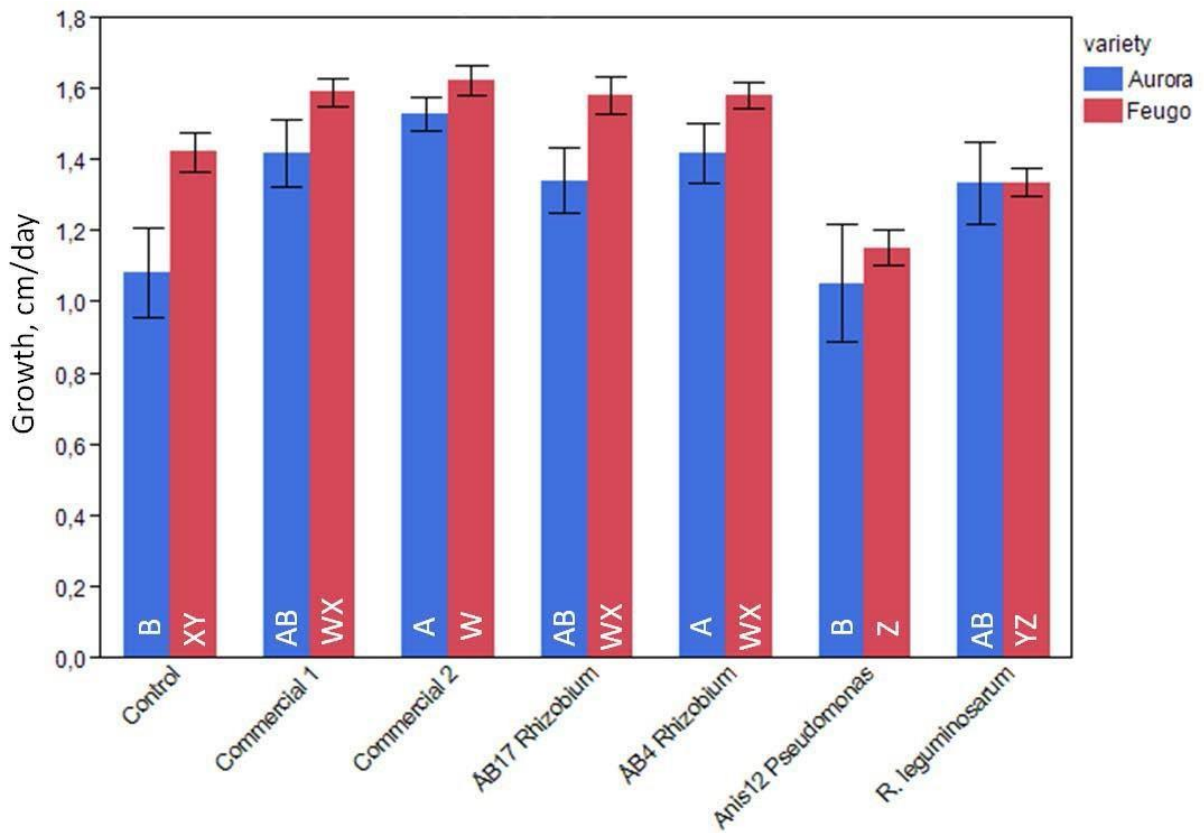


Figure 12. Growth rate of faba bean plants after treatment with three faba bean bacteria and two commercial rhizobium inocula. N=6 (2 seeds/pot). Each error bars is constructed using 1 standard error from the mean. Mean values indicated with different letters are statistically significant at $P < 0.05$ (Comparison of all pairs using Tukey-Kramer HSD).

Improved emergence and early plant growth of faba bean by AB4 may be due to its ability to inhibit fungal pathogens (Figure 6), produce phytohormones, exopolysaccharides, siderophores or induced systemic resistance but further studies are required in support of these hypotheses.

3.5.2 Effects on root infection in faba bean

In the above experiment, root infection was assessed as classes of infection in Aurora and Feugo plants. The results showed that the bacterial treatments reduced root infection compared to that in the control plants in both cultivars (figure 13a, 13b).

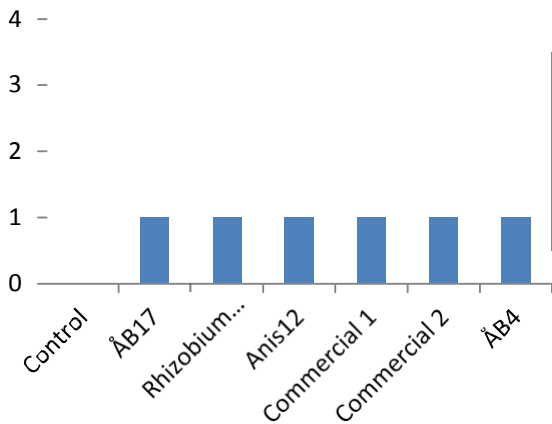


Figure 13a. Effect of four potentially nitrogen-fixing bacterial isolates and two commercial inocula on root infection in **Aurora** grown in perlite in greenhouse. Inoculations were made on seeds before sowing. Columns indicate the mean infection score N=6 (2 seeds/pot).

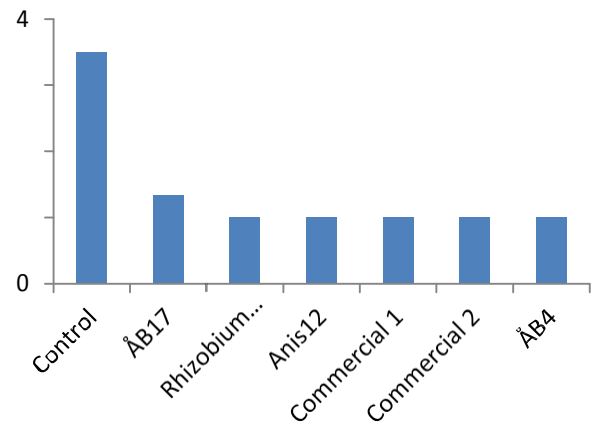


Figure 13b. Effect of four potentially nitrogen-fixing bacterial isolates and two commercial inocula on root infection in **Fuego** grown in perlite in greenhouse. Inoculations were made on seeds before sowing. Columns indicate the mean infection score N=6 (2 seeds/pot).



Figure 14a. Faba bean roots (control)



Figure 14b. Faba bean roots after treatment with *Rhizobium leguminosarum*

A study by Shaban and El-Bramawy (2011) reported that treatment of broad bean seeds with *R. leguminosarum* resulted in significant reduction in damping-off caused by different fungal pathogens such as *Fusarium oxysporum*, *Fusarium solani*, *Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii* as compared to in the untreated control plants. Huang and Erickson (2007) confirmed that, besides the disease control, seed treatment with *R.*

leguminosarum also improved plant growth. Samavat *et al.* (2011) found that the combined treatments of common bean seeds with rhizobia cultural filtrates and *P. fluorescens* isolates reduced root rot and damping-off severity. Furthermore, Kumar *et al.* (2001) showed that seed treatment with *P. fluorescens* isolates alone and together with a *Rhizobium* reduced the number of infected pea plants grown in *Fusarium oxysporum* infected soils. Thus, treatment of faba bean with effective strains of *R. leguminosarum* alone or in combination with other beneficial microorganisms may be preferred over the fungicides, because of their multiple potentials to fix nitrogen, control disease, improve of soil fertility, increase crop productivity besides reducing the negative environmental impact associated with chemical use (Jensen *et al.*, 2002; Huang and Erickson, 2007).

PGPR are known to enhance root development either directly by producing phytohormones, or indirectly by inhibiting plant pathogens through the production of different compounds and/or induced disease response. Defago *et al.* (1990) found that *P. fluorescens* suppressed black root rot disease caused by *Thielaviopsis basicola* in tobacco. In our study, three *Rhizobium* isolates, resulted in reduced root infection (figure 13a, 13b). The possible explanation behind root disease protection may be the production of pathogen inhibitory compounds/ enzymes. Another explanation is the possibility of rhizobia to induce systemic resistance mediated by their exo-polysaccharides (Abdelaziz *et al.*, 1996). Further study based on assays using selected substrates will be needed to explore this potential in bacteria in support of this hypothesis.

3.6 Comparative colonization of faba bean roots by *Rhizobium* and PGPR

Root colonization by introduced bacteria is an important step in the interaction of beneficial bacteria with the host plant. True root colonists can be considered those bacteria that colonize roots in competitive conditions.

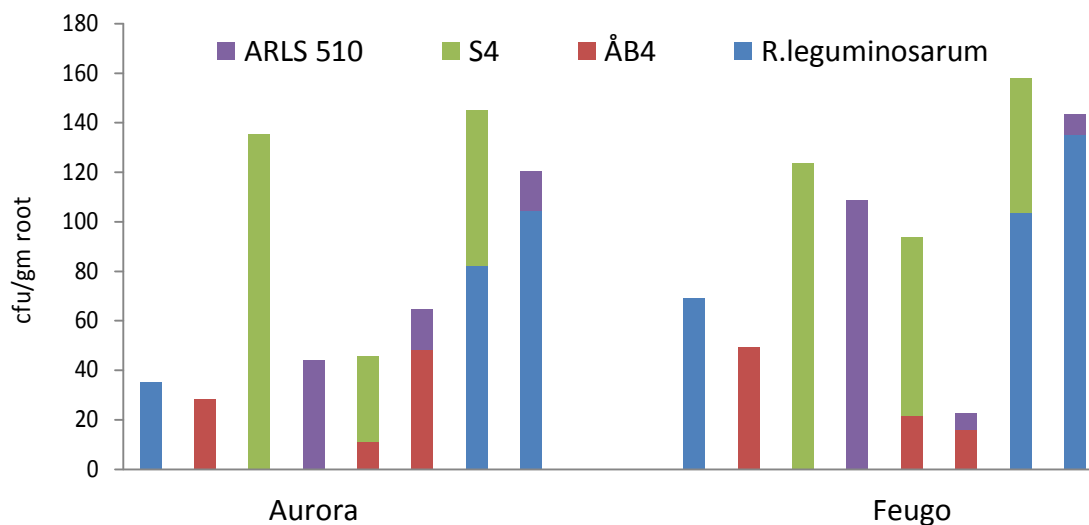


Figure 15. Colonization of roots (cfu/g root) of Aurora and Fuego respectively by two rhizobia and two antagonistic PGPR inoculated singly or in combination. n=3

Root colonization is a competitive process that is affected by characteristics of both the root bacteria and the host. The aim of the colonization competence experiment in faba bean was to confirm and quantify the competitive ability of rhizobia in presence of selected PGPR that was observed in *in vitro* compatibility assay described above. Two rhizobia (*R. leguminosarum* and ÅB4), and two PGPR isolates (ARLS510 and S4) were further investigated with respect to the two cultivars of faba beans (Aurora and Fuego) in a greenhouse experiment. The isolates were selected on the basis of their antifungal potential and observations on *in vitro* compatibility assay.

The approach of using specific culture media in this study allowed quantification of root colonization by the introduced bacteria. The results summarized and shown in Figure 15 and Table 5 demonstrated that all the four bacteria, when inoculated alone or in combination, successfully colonized roots of faba bean irrespective of the cultivar although the PGPR isolates originated from oilseed rape (Alström unpublished) and *Equisetum* sp. (Neupane 2013). S4 seems to be the best root coloniser of both cultivars while ARLS510 and *R. leguminosarum* seem to prefer Fuego than Aurora. The PGPR isolate, ARLS510 was shown to significantly stimulate the root colonization of *R. leguminosarum* in Aurora. Similar pattern was evident for *R. leguminosarum* in presence of S4 but this stimulation was not statistically significant. The pattern of colonization by these bacteria when co-inoculated was similar in Fuego. Co-inoculation of *R. leguminosarum* was stimulated in presence of both ARLS510 and S4 in Fuego but it was stronger in presence of ARLS510 than in presence of S4.

Table 5. Colonization of roots (cfu/g root) of Aurora and Fuego respectively by two rhizobia and two antagonistic PGPR inoculated singly or in combination. n=3. Mean values indicated with different letters are statistically significant at P<0.05 (Comparison of all pairs using Tukey-Kramer HSD).

Cultivar	Treatment	cfu	p-value	Mean comparison
Aurora	<i>R. leguminosarum</i>	35	0,1847	
Fuego	<i>R. leguminosarum</i>	69		
Aurora	ÅB4	28	0,1662	
Fuego	ÅB4	49		
Aurora	S4	135	0,526	
Fuego	S4	124		
Aurora	ARLS 510	44	0,0386	
Fuego	ARLS 510	109		
Aurora	<i>R.leguminosarum</i>	35	0,0186	A
	<i>R.leguminosarum</i> + ARLS 510	104		B
	<i>R.leguminosarum</i> + S4	82		AB
Fuego	<i>R.leguminosarum</i>	69	0,0933	-
	<i>R.leguminosarum</i> + ARLS 510	135		-
	<i>R.leguminosarum</i> + S4	104		-
Aurora	ÅB4	28	0,1054	-
	ÅB4 + ARLS 510	48		-
	ÅB4 + S4	11		-
Fuego	ÅB4	49	0,0041	A
	ÅB4 + ARLS 510	16		B
	ÅB4 + S4	21		B
Aurora	S4	135	0,0004	A
	S4 + <i>R.leguminosarum</i>	63		B
	S4 + ÅB4	34		B
Fuego	S4	123	0,0194	A
	S4 + <i>R.leguminosarum</i>	54		B
	S4 + ÅB4	72		AB
Aurora	ARLS 510	44	0,1015	-
	ARLS 510 + <i>R.leguminosarum</i>	16		-
	ARLS 510 + ÅB4	16		-
Fuego	ARLS 510	109	<0,0001	A
	ARLS 510 + <i>R.leguminosarum</i>	8		B
	ARLS 510 + ÅB4	7		B

Chabot *et al.* (1996) found that rhizobia were superior colonizers compared with other PGPR bacteria of maize and lettuce root inoculated with bioluminescent *R. leguminosarum* bv *phaseoli*. In our study, colonisation by *Rhizobium* was stimulated in presence of PGPR. (Figure 15). Wiehe *et al.* (1994) also perceived that *P. fluorescens* and *R. leguminosarum* b.v. *trifolii* colonized the surface of pea and lateral root emergence sites of lupine. This may be explained by a greater root exudation rate at these sites or they may be better adapted to the rhizosphere conditions. In our study, individual inoculation of *Serratia* S4 in both cultivars colonized the faba bean roots stronger than other isolates (figure 15).

According to Rougier M (1981), the root cap of maize produces an important quantity of mucilage, which contains polysaccharides. In contrast, other parts of the root exudate especially an extensive variety of soluble materials, particularly sugars and amino acids (Curl *et al.*, 1986). So the differences in root colonization between PGPR strains could be explained partly by their ability to utilize these compounds as a carbon nutrient. Strains that have the ability to preferentially use certain compounds present in root exudates have a competitive advantage over other bacteria. Bennett and Lynch (1981) studied co-inoculation of two microorganisms in cereal rhizosphere and suggested that the reason behind the superior colonization in mixed treatment could be that the metabolites of PGPR strains may contain factors that stimulated colonization by the other. Inoculated bacteria must be able to compete for acquisition of nutrients in their new habitat. Production of antagonistic compounds by PGPR, such as antibiotics, could also be partly involved in this nutritional competition and thus in the establishment of high enough bacterial populations in the rhizosphere (Weger *et al.*, 1995). Characterization of compounds released by ARLS510 that are specifically used by the *R. leguminosarum* in the root colonization process will give further information.

Conclusions

- The structure and population of the bacterial community in faba bean was diverse and seems to be affected by the health status of the seeds
- Enterobacteriaceae seems to dominate the bacterial flora of faba bean seeds. *Serratia*, *Pseudomonas* and *Burkholderia* were among several genera that were found in /on faba bean seeds. The genus *Erwinia* was also detected whose members have broad host range and are commonly known to cause stem and root rots. Its importance for faba bean yield is not known.

- Fungi were found to be present in faba bean seeds but their population was difficult to determine by the approaches used in this study. Both *Ascochyta* and *Botrytis* were present confirming their seed-borne nature.
- PGPR bacterial isolates known for antagonism against several other pathogens also exhibited both direct and indirect inhibition of fungal pathogens of vital importance for health of faba bean plants. They were superior to rhizobia with respect to antifungal activity thus highlighting the importance of developing co-inoculants based on multifunctional PGPR with bio-control potential.
- Both rhizobia and PGPR are able to grow in faba bean seed exudates but to different extent depending on the isolate and cultivar. This may be due to differences in their chemical composition
- Some PGPR were compatible with *Rhizobium* and enhanced their colonisation in faba bean roots.
- Selected isolates of rhizobia and antagonistic PGPR have potential to be exploited for improving germination, plant growth and plant health in faba bean
- Quality control of the commercial inocula seems to be important

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<http://apps.rhs.org.uk/advice/search/profile.aspx?pid=89>, chocolate spot.

<http://www.dpi.vic.gov.au/agriculture/pests-diseases-and-weeds/plant-diseases/grains-pulses-cereals/ag0151-ascochyta-blight-of-faba-bean>, ascochyta-blight.

<http://www.gettyimages.se/creative/mildew-stock-photos>, downy mildew.

Acknowledgements

First I acknowledge the support from the organizations CL Behms Foundation for legume cultivation and Ekhagastiftelsen for partial financing that made this work possible.

My special appreciation goes to my supervisor Associate Prof. Sadhna Alström for giving me the opportunity to work as a master student. I have always been impressed by your intelligence, how you make well time planning for project and lead the research. My respect goes on your patience in teaching, how you teach in understandable way. You were helpful in every sections of my thesis work. I learnt many things from you during my work. Your concern in everything including my illness and bad time gave me inner strength to go on.

My special appreciation also goes to my another supervisor Agr. Dr. Björn Andersson for giving me your support, advice, encouragement in my work. I am very grateful for your help in statistics. Thanks for all valuable discussions that I have been involved in, especially for reading and revising manuscripts and giving me good suggestions to make this better.

Thanks to my examiner Associate Prof. Malin Elfstand to reminds me friendly of making scientific writing understandable, and gave me a lot of useful suggestions to organize the structure of my thesis.

Thanks to my opponent Sofia Alvarez Nordström for her good questions which really inspired my thinking on this topic.

Thanks to Konstantia Gkarmirifor for her help in microscopic observation in my work. Also thanks to Rena Gadjievafor demonstrating me in molecular lab specially for DNA extraction and PCR. I would like to thank Katarina Ihrmark and Maria Jonsson for all their suggestions in lab.

Special thanks to my best friend Sultan Mahmud who gave me inspiration and strength when I was depressed in some situations. Your company inspired me to hold the life in a good way.

I would like to express my warm gratitude to Ashik who was always with me in difficult moment. Ashik, you have the magic to make people happy.

Last but not the least, my deepest gratitude goes to my family specially to my mother who is the most important person in my life and deserve all my love for being with me always.