CONGRESOS Y JORNADAS 56/00

FOURTH EUROPEAN NITROGEN FIXATION CONFERENCE

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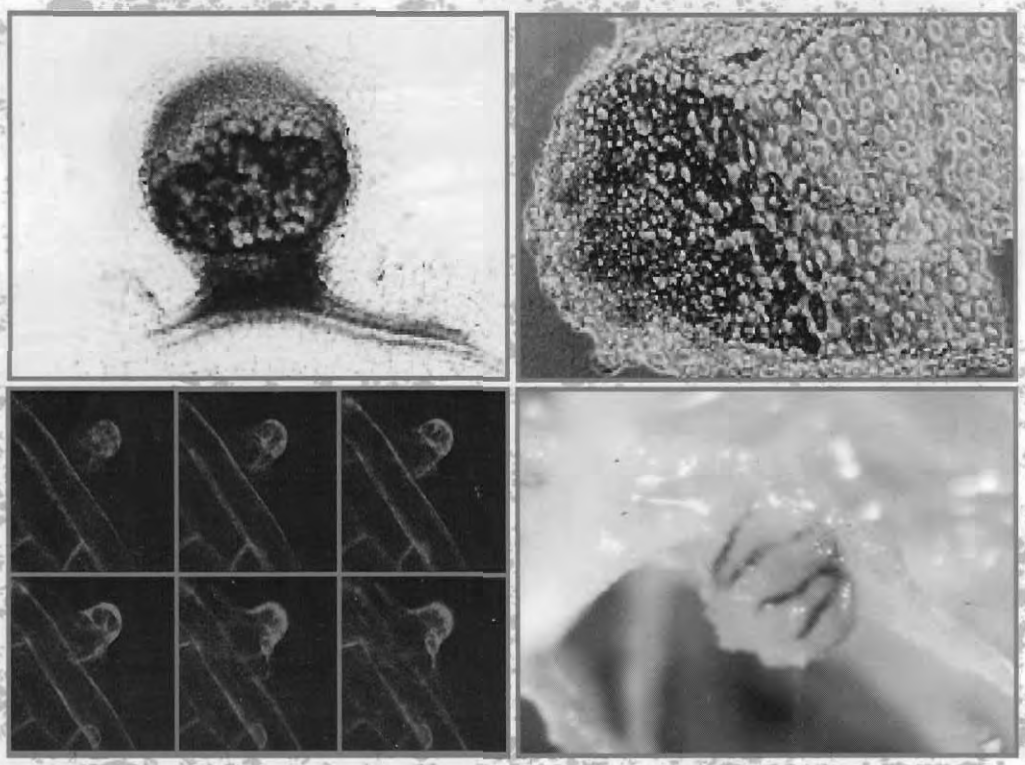

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CONTENTS

SPONSORS OF THE CONFERENCE 05

OPENING CONFERENCES 09

LECTURES ABSTRACTS 13

POSTER ABSTRACTS 49

AUTOR INDEX 343

LIST OF PARTICIPANTS AND ADDRESSES 355

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OPENING CONFERENCES

GENOME ARCHITECTURE AND GENOMIC DESIGN IN Rhizobium

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Due to homologous recombination between reiterated DNA sequences, the bacterial genomes are subjected to different types of rearrangements, including deletions, amplifications, inversions, and cointegration of different replicons. Highly dynamic genomes have been proposed for microorganism that show a large amount of DNA reiteration such as *Rhizobium*. If the position and orientation of the reiterated DNA families of a genome or a region of a genome is known, the different types of potential rearrangements produced by homologous recombination may be predicted. As experimental model we studied the symbiotic plasmid of strain NGR234. The rearrangements predicted by the DNA sequence were identified and derivative populations that are pure for specific rearrangements, were obtained. We propose that knowledge of the DNA sequence of a genome allows to design alternative genomic structures that in turn may be experimentally obtained.

CONTROL OF ROOT NODULE DEVELOPMENT BY RHIZOBIA

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Under nitrogen-limiting conditions rhizobia induce the formation of nitrogen-fixing nodules on the roots of their host plants. Nodule development is governed by signal exchanges between the two partners. First, flavonoids exuded by the roots induce the expression of the *Rhizobium* nodulation genes, whose products are involved in the synthesis and excretion of a family of structurally related lipo-chitooligosaccharides. These Nod factors induce Ca^{++} influx in root hairs which act as secondary messenger, evoking root hair deformation, cortical cell division and nodule organogenesis. Recently, we have identified several plant genes controlling nodule development. It was shown that not only Nod factors but also cytokinins and ectopic expression of the *enod40* gene reactivated the inner cortical cells for division. Then, the cortical cells arrested in the G0-phase are able to reenter the cell cycle; several elements of this mechanism have been characterized. After several cycles the division is arrested, and cells may undergo differentiation, endoreduplication and enlargement, controlled by a novel plant gene, *ccs52*. Altering the expression level of *ccs52* by its overexpression in sense or antisense orientation allowed us to control the endoploidy level, cell size and nodule development. Differentiation of the central nodule zone and its invasion is controlled from distance by the gene *Mszpt2-1* coding for a Krüppel-like zinc finger transcription factor protein, expressed in the vascular tissues of the nodules and the inoculated part of the roots. These studies may contribute to the dissection of the different developmental stages of nodule organogenesis.

LECTURE ABSTRACTS

S1-L1

NITROGENASE: BIOCHEMISTRY AND REGULATION OF EXPRESSION

Ray Dixon.
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Considerable knowledge has accumulated on the structure, function and mechanism of nitrogenases in the last few years. I will briefly review these developments and then move on to describe recent findings concerning how the expression of nitrogenase is regulated at the transcriptional level. The NIFL: NIFA two-component regulatory system integrates metabolic signals for the redox, energy and nitrogen status to fine tune regulation of nitrogenase synthesis in *Azotobacter vinelandii* and *Klebsiella pneumoniae*. PII-like signal transduction proteins, which respond to the nitrogen status via covalent modification and signal the carbon status through the binding of 2-oxoglutarate, have been implicated in the regulation of nitrogen fixation in several diazotrophs. The mechanism of signal transduction is best understood in enteric bacteria and involves covalent modification of the PII protein, encoded by *glnB*, by a uridylyltranferase/uridylyl removing enzyme (UTase/UR) encoded by *glnD*. The UTase/UR transduces the nitrogen signal through uridylylation of PII under conditions of nitrogen limitation and via de-uridylylation of PII under conditions of nitrogen excess. These interactions are not only influenced by the uridylylation state of PII, but are also allosterically modulated through binding of the effector 2-oxoglutarate to the PII protein in response to the carbon status.

We have investigated the response of the *A. vinelandii* NIFL-NIFA proteins to signal transduction by PII-like regulatory proteins using a purified *in vitro* system. Surprisingly, we observe that the NIFL-NIFA complex is itself responsive to the presence of 2-oxoglutarate, which relieves inhibition by NIFL in the presence of adenosine nucleotides. Hence the nitrogen fixation regulatory proteins are themselves exquisitely sensitive to the carbon status. Using the well-characterised PII-like proteins from *E. coli*, we find that the non-uridylylated form of the PII protein (hereafter referred to as Ec PII), but not the GlnK protein (Ec GlnK), is competent to activate the inhibitory function of NIFL in the presence of 2-oxoglutarate and adenosine nucleotides. The *glnK*-encoded PII regulatory protein from *A. vinelandii* (Av PII) also activates the inhibitory function of NIFL and this interaction is modulated by the uridylylation state of Av PII. These observations suggest a mechanism for signalling the nitrogen status in *A. vinelandii* through the interaction between PII-like proteins and the NIFL-NIFA system which is clearly different to that proposed for *K. pneumoniae*.

S1-L2

LONG-LIVED 1:1 COMPLEXES OF NITROGENASE COMPONENTS

Robert R. Eady, Thomas A. Clark, Silvana Maritano, Shirley A. Fairhurst, Richard W. Miller and Faridoon K. Yousafzai
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Mo-nitrogenase is made up of an Fe protein which functions as a MgATP-dependent electron donor to a MoFe protein which contains FeMoco, the active site of the enzyme where dinitrogen is reduced :
$N_2 + 8\ e + 8\ H^+ + 16$ MgATP→ $NH_3 + H_2 + 16$ MgADP + 16 Pi
When dithionite is used as electron donor after each electron transfer, the two proteins dissociate in a reaction that is rate-limiting. Following protein-protein dissociation, MgADP dissociates from the oxidized Fe protein which is then reduced to allow further electron transfer events. The transient electron transfer complex between the two proteins can be stabilised by trapping a putative transition state analogue with MgADPAlF_4^-. This complex is inactive and is thought to represent the hydrolytic MgADP.Pi conformation. The structures of the complexes of *Azotobacter vinelandii,* and *Klebsiella pneumoniae* nitrogenases have been determined by X-ray crystallography and small-angle X-ray scattering respectively. In these complexes which have a 2:1 stoichiometry of Fe:MoFe protein, the Fe protein has undergone a conformational change relative to that of the free protein.
We have recently reported the trapping of the MgATP-bound conformation of Kp-nitrogenase using MgADPBeF_x and shown it to be an equilibrium mixture of 1:1 and 2:1 complexes[1]. In both the AlF_4 and BeF_x complexes of Kp-nitrogenase the EPR spectra show the Fe protein to be oxidized despite the presence of excess dithionite. Tight complexes of Av-nitrogenase formed by mutated forms of the Fe protein locked in the ATP-conformation in the absence of MgATP also show the Fe protein to be oxidized. Similarly, the tight complexes formed by the Fe protein of *Clostridium pasteurianum* with Av or Kp MoFe proteins *in the absence of MgATP*, also contain oxidized Fe protein. Thus, this is a feature which characterises tight complexes of nitrogenase components.
We have shown that the formation of the heterologous nitrogenase complexes involving Cp Fe protein, form tight 1:1 complexes which can be isolated by gel filtration from a stable 2:1 complex formed under assay conditions. This provides evidence that the two Fe binding sites of MoFe proteins are not equivalent, and that the second Fe protein binds more weakly than the first.
These findings will be discussed in the context of normal nitrogenase turnover, where the predominant species is a complex containing oxidised Fe protein, and rapid-quench EPR data consistent with the initial formation of 1:1 complex.

1 Clarke, T.A., Yousafzai, F.K. and Eady, R.R. (1999) Biochemistry 31:9906

S1-L3

CATALYTIC DINITROGEN REDUCTION IN THE PRESENCE OF POLYNUCLEAR METAL COMPLEXES.

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Polynuclear vanadium(II) and molybdenum(III) complexes activate dinitrogen in protic media to further reduction to hydrazine and ammonia. Analysis of kinetics of N_2 reduction and EPR spectra of vanadium complexes confirms the formation of active tetra- and octanuclear complexes. Polynuclear molybdenum(III) complexes catalyse N_2 reductions to hydrazine and ammonia in the presence of such reductants as titanium(III) hydroxide, europium and sodium amalgams. The reaction rate strongly depends on the redox potentials of the reducing agents and the nature of the catalyst. Isolated FeMo cofactor of nitrogenase can catalyse the reduction of acetylene (as well as other nitrogenase substrates) and coordinate dinitrogen in the presence of the same amalgams. The comparison will be made of the synthetic catalysts and FeMoco.

S1-L4

RECENT PROGRESS IN SUPEROXIDE DEPENDENT NITROGEN FIXATION

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Nitrogen fixation in *Streptomyces thermoautotrophicus* involves three components, which are a Mo-dinitrogenase (St1), a Mn-superoxide oxidoreductase (St2) and a Mo-CO dehydrogenase (St3). Superoxide produced by CO dehydrogenase is the electron donor of the nitrogenase reaction. The superoxide is oxidized to O_2 by the superoxide oxidoreductase, and the electrons are delivered to the dinitrogenase where the reduction of N_2 to NH_3 takes place (1). All enzymes involved in nitrogen fixation are oxygen sensitive and oxygen is even an essential intermediate. The dinitrogenase is a heterotrimeric protein composed of subunits SdnL (90.980 Da), SdnM (31.536 Da) and SdnS (18.330 Da), and is a new member of the molybdenum-hydroxylase sequence family (2). Amino acid sequences of subunits of St1 protein have high similarity to the corresponding subunits of carbon monoxide dehydrogenase of *Oligotropha carboxydovorans*. SdnS shows to the corresponding CoxS 60.8% and 80.7% identity and similarity, respectively. SdnM shows to the corresponding CoxM 37.5% and 64.3% identity and similarity, respectively. SdnL shows to the corresponding CoxL 59.4% and 80.3% identity and similarity, respectively. The nitrogenase of *S. thermoautotrophicus* can not reduce ethin or ethene. In this study we report that cyanide and azide act as nonphysiological substrates for the nitrogenase of *S. thermoautotrophicus* in an atmosphere of pure helium. The optimum concentrations were 10 mM and 25 mM for KCN and NaN_3, respectively and the reaction product was ammonium. Compared to the specific activity of ammonium formation from N_2 the activities of ammonium formation from NaN_3 or KCN were 4.4-fold and 1.6-fold increased.

1 Ribbe M., Gadkari D., Meyer O. 1997. J. Biol. Chem. 272, 26627-26633.
2 Hofmann-Findeklee C., Gadkari D., Meyer O. 2000. In: Nitrogen fixation from molecules to crop productivity, Kluwer Academic Publishers, 23-30.

S2-L5

STRUCTURAL AND FUNCTIONAL GENOMICS IN LEGUMES

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Following the emergence of model legumes the molecular genetic analysis of symbiosis is now rapidly developing. Genetic maps are constructed for several legume species and different methods for positional cloning of symbiotic loci is currently applied in order to clone genes involved in nodule initiation, nodule function as well as autoregulation. Attempts towards map based cloning of the *Ljsym16* gene will be presented to exemplify these approaches.
Several novel methods for genetic analysis of gene function have been established in eukaryotes and a subset of these can be applied to investigate the function of plant genes. Insertion mutagenesis with T-DNA and transposable elements have for example been used in *Lotus japonicus*. A summary of the transposon insertion approaches and the future perspectives will be given. The cloning and characterization of the first transposon tagged symbiotic locus (*Nin*) will be presented. Gene inventories for soybean, *L. japonicus* and *M. truncatula* are accumulated from EST sequencing programs and functional analysis of these genes is now on the priority list. Approaches for converting this structural information into functional genomics will be discussed.

S2-L6

A FUNCTIONAL GENOMICS APPROACH TO EXPLORE *M. TRUNCATULA* SYMBIOTIC PROGRAMMES

Etienne-Pascal Journet[1], Hervé Crespeau[2], Diederik van Tuinen[3], Jérome Gouzy[1], Olivier Jaillon[2], Andreas Niebel[1], Véronique Carreau[1], Odile Chatagnier[3], Daniel Kahn[1], Vivienne Gianinazzi-Pearson[3] and **Pascal Gamas**[1]

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[3] UMR INRA/Université de Bourgogne, Biologie, Biochimie et Ecologie des Interactions Plantes-Microorganismes, CMSE/INRA Dijon, France

Large-scale EST sequencing is an essential tool for functional genomics studies, as it allows the direct identification of large gene collections in a given organism and makes the corresponding clones available for further molecular investigation. Moreover, random sequencing of cDNAs yields information about the relative expression levels of the corresponding genes in different tissue samples. We have applied this strategy to the molecular study of root symbioses in the model legume *Medicago truncatula*.

Three cDNA libraries have been constructed in a λZAP vector from young indeterminate root nodules (symbiosis with *Sinorhizobium meliloti*), endomycorrhizal roots (symbiosis with *Glomus intraradices*), and nitrogen-starved, uninoculated "control" root tips. After mass excision, 3 x 5,000 random plasmid clones from each of 3 libraries have been ordered and sequenced from 5' and 3' ends. A first global clustering operation has indicated that about 6,000 *M. truncatula* distinct genes are represented as EST singletons or clusters in our database. Systematic homology comparisons against the Swiss-Prot and TrEMBL databases have been performed and the annotation process is in progress, with the aim of providing the community with a biologically informative database accessible on a web site.

By using a number of known *M. truncatula* genes (mostly nodulin genes) as internal representation controls, we have been able to verify that the relative distribution of the corresponding ESTs within the 3 root libraries is consistent with the data obtained by Northern analysis. In addition, a search for genes showing a significant differential representation between the 3 libraries has revealed a number of candidate genes up-regulated in nodule and/or mycorrhizal tissues. Progress of this programme will be presented in the framework of anticipated developments for symbiotic transcriptome analysis.

S2-L7

TOWARD THE MAP BASED CLONING OF A GENE CONDITIONING NON-NODULATION PHENOTYPE IN ALFALFA (MEDICAGO SATIVA)

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The improved genetic map of diploid (2n=2x=16) alfalfa (*Medicago sativa* ssp. *coerulea* x *Medicago sativa* ssp. *quasifalta*) has been developed by analyzing the inheritance of more than 1500 genetic markers on 137 plant individuals of a F2 population. The genetic map of alfalfa in its present form contains 8 morphological, 2 SSCP, 10 isozyme, 26 seed protein, more than 400 RFLP, more than 700 RAPD and 200 specific PCR markers in 8 linkage groups. The genetic map covers 754 centimorgans with an average marker density of 0.8/cM. The correlation between the physical and genetic distance is about1000-1300 kilobase pairs per centimorgan.
The saturated linkage map of diploid alfalfa was used to compare with the genetic map of *M. truncatula*, pea (*Pisum sativum*) and *Arabidopsis thaliana*. High degree of macro and microsynteny could be demonstrated between *M. sativa* and *M. truncatula*, obvious similarity was found between the gene order of alfalfa and pea, while some degree of synteny between *Medicago* and *A. thaliana* was detected.
The location of a mutation (nn_1) conditioning non-nodulation phenotype was determined in a tetraploid alfalfa population. Using tightly linked markers BAC clones originating from a *M. truncatula* BAC library (D. Cook, TAMU, Texas, USA) were isolated. Chromosomal walking approach is taken to identify and isolate the genes conditioning the ineffective and the non-nodulation phenotype.

S3-L8

SEQUENCING THE CHROMOSOME OF *SINORHIZOBIUM MELILOTI*.

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[3]GATC, GmbH Fritz-Arnold-Str 23, D-78467 Konstanz, Germany.
[4]Université Catholique de Louvain, Place Croix du Sud 2, Bte 20, B-1348 Louvain-La-Neuve, Belgium.
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[6]Universität Bielefeld, 25, D-33615 Bielefeld, Germany.

The recognised importance of rhizobia in environment and agriculture and the prominent role played by *S. meliloti* have fostered a joint international effort towards sequencing its genome. Better understanding of the symbiotic nitrogen fixation process and reduced need of nitrate fertilisers - which are both expensive and polluting - may be derived from this sequence. Strain 1021 provided by S.R. Long was used as starting material to construct several BAC libraries. Following partial hydrolysis with *Sau*3A or *Hin*dIII, two whole genome libraries were made in pBeloBAC11. A third whole genome library was made with *Eco*RI fragments in the pBACe3.6 vector. BAC clones were organised in three contigs by placing numerous markers detected by PCR. A minimal set of BACs covering the chromosome were then selected and used for sequencing. Concomitantly to the sequencing which is in its final stage, a first level of annotation is being made using different tools. Protein coding genes are directly searched using the FrameD program (T. Schiex, unpublished). In addition, sequences are systema-ically compared with the SWISS-PROT and TrEMBL databases (Bairoch and Apweiler, 2000) using BLASTX.

Web sites: http://sequence.toulouse.inra.fr/meliloti.html and
http://www-recomgen.univ-rennes1.fr/meliloti

S3-L9

RHIZOBIUM NGR234 GENOMICS, TRANSCRIPTOMICS AND PROTEOMICS.

W.J. Broughton, G. Gottschalk, T. Hartsch, P. James, A. Johann, M. Münchbach, X. Perret, A. Rosenthal, C. Staehelin, R.A. Schmitz, W.R. Streit, and V. Viprey.
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The broad host-range *Rhizobium* sp. NGR234 contains three replicons – a 536 kb symbiotic plasmid (pNGR234*a*), a 2.2 Mb mega-plasmid (pNGR234*b*), and a 3.5 Mb chrmosome. pNGR234*a* has been completely sequenced [Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A. and Perret, X. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. *Nature* 387: 394-401], and random sequence data exists for the other two replicos. All open reading frames (ORFs) of pNGR234*a* [Perret, X., Freiberg, C., Rosenthal, A, Broughton, W.J. and Fellay, R. 1999. High-resolution transcriptional analysis of the symbiotic plasmid of *Rhizobium* sp. NGR234. *Mol. Microbiol.* 32: 415-425] as well as those random sequences which matched a data-base entry (922) were amplified and used in expression analyses. Only 3% of these latter sequences carried transcripts whose expression was clearly flavonoid dependent (compared to 37% on pNGR234*a*). Most ORFs transcribed under free-living conditions are repressed during symbiotic nitrogen-fixation since only 3.5 % of the selected clones were significantly expressed in nitrogen-fixing bacteroids. Several homologues of proteins containing Ca^{2+} binding domaines typical of the cycloysin/haemolysin family (which includes the host-range determinant NodO) were identified. Matrix-assisted laser desorption and ionisation time of flight (MALDI-TOF) mass spectra were accumulated from Coomasie blue stained spots of total proteins that had been separated by two dimensional electrophoresis. Ninety two percent of the daidzein inducible proteins mapped to pNGR234*a* confirming that this replicon carries most of the symbiotic genes.

S3-L10

PROTEOME ANALYSIS OF THE SYMBIOSIS BETWEEN *SINORHIZOBIUM MELILOTI* (STRAIN 1021) AND THE LEGUME *MELILOTUS ALBA*.

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Genomic Interactions Group, Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra ACT Australia, 2601.

Proteome analysis was used to investigate the metabolic changes that occur during the symbiotic interaction between *S. meliloti* strain 1021 and *M. alba* that leads to the formation of root nodules. We compared 2-dimensional arrays of total proteins from purified *S. meliloti* bacteroids to that of cultured cells and, in turn, to *M. alba* nodules and uninoculated *M. alba* roots to identify differentially displayed proteins and to tease apart the contributions of each of the two organisms to the nodule proteome. Protein abundance differences between bacteroids and cultured cells of *S. meliloti* were closely compared. These analyses show that there is a massive change in the metabolism of the bacteroids with over 130 moderate to high abundance proteins being up-regulated and over 350 moderate to high abundance proteins being down-regulated[1]. A comparison of bacteroid and nodule proteome patterns showed that the major proportion of the proteins in the nodule were of bacteroid origin. Nevertheless, about 70 proteins occurred in nodules that were not apparent in either purified bacteroids or uninoculated root tissue and these proteins are possible plant nodule proteins or "nodulins". N-terminal sequence and peptide mass fingerprinting analysis (using MALDI-TOF mass spectrometry) were used to assign identity to over 100 of 700 bacterial proteins. Bacteroids possessed down-regulated or repressed levels of proteins involved in the following processes: N-acquisition, polyhydroxy-butyrate (PHB) synthesis, cell division, and the acquisition of low molecular weight compounds including carbon. Proteins involved in nitrogen fixation and stress proteins involved in protein folding/stabilisation were up-regulated or induced in bacteroids. A more complete description of gene expression at the level of protein synthesis will be obtained by complementing peptide mass fingerprinting data with the entire DNA sequence of the *S. meliloti* genome[2]. This work shows that proteome analysis will provide an excellent strategy to bridge DNA sequence information and their protein products and provide a foundation for functional genomic analyses.

[1] Natera SHA, Guerreiro N and Djordjevic MA (2000) Molecular Plant Microbe Interactions. (in press).
[2] Barloy-Hubler F, Capela D, Barnett MJ, Kalman S, Federspiel NA, Long SR, and Galibert F. (2000) J. Bacteriol. 182: 1185-1189.

S3-L11

ANALYSIS OF THE SYMBIOTIC GENE REGION OF *BRADYRHIZOBIUM JAPONICUM* WITH EMPHASIS ON THE TYPE III SECRETION SYSTEM

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2 Institut für Mikrobiologie, Eidgenössische Technische Hochschule Zürich, 8092 Zürich, Switzerland

In the soybean symbiont *B. japonicum* more than 30 nitrogen fixation and nodulation genes have been characterized. The physical and genetic map of the chromosome revealed that they are clustered within a region of about 400 kb. Because of the complex interactions between the bacterium and the plant, we expected that this chromosomal sector contains additional genes that are involved in the maintenance of an efficient symbiosis. Therefore, we decided to determine its nucleotide sequence. To this end, 410 kb have been sequenced. The overall G+C nucleotide content is 59.1 %. The coding potential was analyzed with the program Glimmer (Salzberg et al. 1998. Nucleic Acids Res. 26:544-548). Using a minimum gene length of 150, almost 400 ORFs are predicted to encode proteins. 34 % of the predicted proteins show similarity to proteins of rhizobia. 16 % are similar only to proteins of other bacteria. No database match was found for almost 30 %. Repetitive DNA sequence derived ORFs account for the rest. The sequenced region contains all nitrogen fixation and besides *nodM* all nodulation genes that are known to exist in *B. japonicum.* Several genes were found that seem to encode transport systems for ferric citrate, molybdate or carbon sources. Some of them are preceded by –24/–12 promoter elements. A number of putative outer membrane proteins might be involved in the interaction with the host. Unexpected was the identification of a large cluster probably encoding a type III secretion system. Recently, such a cluster has been identified in *Rhizobium* sp. NGR234 (Viprey et al. 1998. Mol. Microbiol. 28:1381-1389). Both systems share considerable similarity that also includes a putative regulator. In *B. japonicum*, several secreted proteins were shown to be secreted independently of the type III export complex. NolX, which was shown to be secreted in *Rhizobium* sp. NGR234, does not exist in *B. japonicum*.

S4-L12

GENETIC CONTROL OF BOTH NITROGENASE SYSTEMS IN THE PHOTOTROPHIC PURPLE BACTERIUM *RHODOBACTER CAPSULATUS*

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The phototrophic purple bacterium *Rhodobacter capsulatus* is able to fix atmospheric nitrogen using either the molybdenum containing nitrogenase or an alternative iron-only nitrogenase. The expression and activity of both nitrogenase systems is regulated at least at three different levels:

1. Transcription of *nifA1*, *nifA2* and *anfA* is controlled by homologues to the enteric Ntr system. Under conditions of nitrogen limitation the phosphorylated form of NtrC activates not only promoters located in front of *nifA1*, *nifA2* and *anfA* but also the *glnK-amtB* operon and *amtY*, encoding a second ammonium transport system.
2. The activity of both NifA proteins is negatively controlled by the presence of ammonium. It was shown that this posttranslational control is released only in a *glnB/glnK* double mutant, whereas single mutants have no effect. Although the activity of AnfA is also controlled by ammonium this mechanism is not released in the *glnB/glnK* double mutant indicating that different mechanisms are responsible for NifA and AnfA control.
3. The activity of nitrogenase itself is regulated by DraTG dependent ADP-ribosylation. This control mechanism is relieved for both nitrogenase systems in the *glnB/glnK* double mutant.

Using a low copy yeast two-hybrid system the protein-protein interaction between the regulatory components known to be involved in control of nitrogen fixation was analyzed. Both, GlnB and GlnK showed a strong interaction with NifA1 and NifA2 indicating that the ammonia control of NifA activity might be achieved equally well by these two PII-like proteins. An *R. capsulatus* yeast two-hybrid expression library consisting of approximately 2.5 million clones was used to identify further interactors. Using GlnB as a bait, six independent clones carrying NtrB were identified to exhibit the strongest interaction. Using GlnK as a bait, a gene was identified encoding a protein, which exhibited a high degree of homology to *E. coli era* (*E. coli* ras-like protein). In *E. coli* this protein is involved in *E. coli* in cell division and might somehow link this process to the C/N status of the cell. The putative interaction with GlnK might indicate that the N-sensor for this process could be GlnK.

S4-L13

NITROGEN FIXATION WITH A *PSEUDOMONAS STUTZERI* STRAIN, A FACULTATIVE ENDOPHYTE OF RICE

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The *P. stutzeri* strain A1501 (formerly *Alcaligenes faecalis*) can fix nitrogen under microaerobic conditions at the free-living state and colonise rice endophytically. The nucleotide sequence and organisation of the nitrogen structural genes (*nifHDK*), *nifTY*, *nifNE* and associated ORFs are very similar to that of *Azotobacter vinelandii* A set of genes was found upstream of *nifH*, sharing identity with the *rnfCDGEFH* operon, involved in electron transport to nitrogenase in *Rhodobacter capsulatus*. A DNA region containing *rpoN* was identified. ORFs downstream from *rpoN* shared high identity with ORF2, *ptsN*, ORF4 and *ptsO* that are commonly found in other genera. Inactivation of *rpoN* led to a mutant strain with a Nif minus phenotype, that is also impaired in motility, histidine and nitrate utilisation and too a lesser extent in dicarboxylic acid utilisation. Inactivation of *RpoN* associated-ORFs led to a Nif positive phenotype, although nitrogenase activity of the *ptsO* mutant strain was decreased. Several regions encoding rpoN-dependent transcriptional activators were identified. The complete nucleotide sequencing of the regions carrying *ntrBC* and *dctBD* and *dctP/A* was established. Mutants in *ntrC* and *dctD* were obtained. Both are Nif positive, although a NtrC mutant does not display full nitrogenase activity. The *ntrC* mutant is impaired in nitrate and histidine utilisation, while the *dctD* mutant was partly impaired in dicarboxylic acid utilisation.

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S4-L14

IDENTIFIICATION OF *SINORHIZOBIUM MELILOTI* GENES INDUCED DURING SYMBIOSIS WITH ALFALFA AND CHARACTERISATION OF THE PYRUVATE DEHYDROGENASE GENES

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RNA fingerprinting by Arbitrarily-Primed PCR (RAP) was used with the aim of isolating *Sinorhizobium meliloti* genes up-regulated during symbiosis with alfalfa (*Medicago sativa*). Thirteen partial cDNAs were isolated whose corresponding genes were indeed differentially expressed between symbiotic and free-living conditions. Seven cDNAs corresponded to known or predicted *nif* and *fix* genes. Three exhibited no similarity with sequences present in databases whereas three presented high sequence similarity with genes coding for a component of the pyruvate dehydrogenase complex, a cell surface protein component, and a predicted argininosuccinate lyase (1).
The sequences predicted to encode the pyruvate dehydrogenase multienzyme complex (PDHc) were studied in further detail. We have found that the E1p component of pyruvate dehydrogenase of *S. meliloti* is encoded by two genes, *pdhA*α and *pdhA*β, a situation encountered in the α-proteobacteria *Rickettsia prowazekii* and *Zymomonas mobilis*, as well as in some Gram-positive bacteria and in mitochondria. *pdhA*α and *pdhA*β precede *pdhB* that encodes the E2p component, dihydrolipoamide acetyltransferase, of the PDHc. *pdhA*α, *pdhA*β and *pdhB* likely constitute an operon. The E3 component, lipoamide dehydrogenase, was not located in the immediate vicinity of *pdhA* and *pdhB* genes in *S. meliloti*.
We have found that *pdhA* symbiotic expression does not depend on the *fixLJ* regulatory cascade that regulates nitrogen fixation and respiration gene expression in response to oxygen availability in symbiotic *S. meliloti* cells. Instead, induction of *pdhA* expression could be obtained under free-living conditions upon the addition of pyruvate to the culture medium. Induction by pyruvate and symbiotic activation of *pdh* gene expression were shown to take place at the same promoter (2).

(1) Cabanes D, Boistard P and J. Batut (2000) *J. Bacteriol.*, in press.
(2) Cabanes D, Boistard P and J. Batut (2000) *Mol Plant Microbe Interact.* **13**: 488-493.

S5-L15

RECENT PROGRESS AND UPCOMING CHALLENGES IN RESEARCH ON ROOT-ASSOCIATED NITROGEN-FIXING BACTERIA.

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Nitrogen-fixing bacteria, such as *Azospirillum brasilense*, *A. lipoferum*, *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* have been repeatedly isolated in high numbers (> 10^6 CFU per plant) from crop plants. Moreover, recent studies with rice, cultivated species and landraces, indicate that the population of nitrogen-fixing bacteria associated with a given plant variety can be quite diverse.
Reinoculation studies of plants with any of these bacteria, however, fail to clearly demonstrate the added value for plant growth, a notably exception being *Azospirillum*. In that particular case, the observed plant growth stimulation is due to the bacterial synthesis of plant growth regulators (phytostimulation) rather than to biological nitrogen fixation.
Studies of the *nif/fix* regulatory cascades of the so-called root-associated nitrogen-fixing bacteria confirm the central role of NifA. Nevertheless, of particular interest are studies on the regulatory role(s) of the PII and PII-like proteins, the post-translational control of their activities, and the transcriptional regulation of the corresponding genes (*glnB*, *glnK* or *glnZ*). In *A. brasilense*, transcription of the *glnB-glnA* operon is not yet fully understood, with different promoters involved and an unknown regulator for transcription initiation under N-limiting conditions. Ammonium excretion under diazotrophic conditions can occur in *glnA* mutants with point mutations, while *glnA* knock-out mutants appear to be lethal. NtrBC control is exerted on the *amtB* gene (ammonium transporter), *nas* operon (nitrate assimilation) and *glnZ* gene (PII-like protein). Analysis of the *cytNOQP* operon, encoding the high affinity oxidase for growth under microaerobic conditions, suggests also redox regulation of the *nif/fix* cascade.
The plant growth promoting (PGP) effect of *A. brasilense* is most likely only indirectly linked to the nitrogen-fixation capacity (e.g. for survival in poor soils). Bacterial synthesis of phytohormones (IAA) on the other hand is directly involved in the PGP effect. IAA biosynthesis in *A. brasilense* is subject of complex regulation, with evidence that it is controlled by the plant. This so-called phytostimulation effect is now being experienced with other root-associated nitrogen-fixing bacteria. An overview of a selected number of root-associated nitrogen-fixing bacteria, taking *Azospirillum* as a reference, will be presented. Finally, a number of strategies on how to further exploit the potential of these bacteria will be suggested.

S5-L16

CYANOBACTERIAL NITROGEN FIXATION: NITROGEN CONTROL AND INTERCELLULAR TRANSFER OF METABOLITES

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Most nitrogen-fixing cyanobacteria separate temporally or spatially the processes of oxygenic photosynthesis and nitrogen fixation. Some filamentous cyanobacterial strains like *Anabaena* sp. PCC 7120 confine nitrogenase to heterocysts, differentiated cells specialized in nitrogen fixation. Heterocyst development is repressed by growth in the presence of combined nitrogen. The transcriptional regulator NtcA, which operates global nitrogen control in cyanobacteria, is required for heterocyst development to take place. Induction of the heterocyst development key regulatory gene *hetR* is impaired in an *ntcA* mutant, suggesting that NtcA links heterocyst differentiation to nitrogen deficiency. HetR has also been found to influence the expression of *ntcA* which is itself an autoregulatory gene. We have observed that NtcA is additionally required for activation of expression of other early heterocyst differentiation genes like *hetC* which, under nitrogen deficiency, is transcribed from an NtcA-type promoter located 571 bp upstream from the gene. Consistent with data showing that the *ntcA* gene is expressed in heterocysts, some genes which are expressed in the developed heterocyst have been found to be transcribed in an NtcA-dependent manner or from NtcA-type promoters. These include *glnA* encoding glutamine synthetase, *petH* encoding ferredoxin-NADP$^+$ reductase, and *nifHDK* encoding nitrogenase. The nitrogen-fixing heterocyst feeds fixed nitrogen, likely in the form of amino acids, to its neighboring vegetative cells. The mechanism of intercellular transfer of metabolites in nitrogen-fixing cyanobacteria is unknown, but we have observed that mutants of an ABC transporter for neutral amino acids, the Nat system which is normally expressed in vegetative cells, are impaired in diazotrophic growth, suggesting a role for this transporter in uptake by vegetative cells of amino acids transferred from the heterocysts. In order to make available their N for cellular metabolism, the transferred amino acids should be catabolized in the vegetative cells. Amino acids readily catabolized by *Anabaena* sp. PCC 7120 include arginine, aspartate, glutamine, and proline. Proline, which is a substrate for the Nat transporter, is accumulated extracellularly in nitrogen-fixing cell suspensions of the Nat mutants and might represent a nitrogen vehicle which, in the mutants, is lost from the filament.

S5-L17

INTERACTIONS OF *AZOARCUS* SPP. WITH RICE: ROLE OF TYPE VI PILI

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Grass-endophytic bacteria of the genus *Azoarcus* spp. were recently found to be naturally associated with rice. They were found most frequently and abundantly in wild rice species and land races of *Oryza sativa* (75% or 80% of flooded plants, respectively) mostly from Nepal, where a high genetic diversity of rice in rather undisturbed environments can still be found. Protection of these genetic resources may help to exploit the potential of novel endophytic diazotrophs. The new isolates allowed the reamendment of the genus *Azoarcus sensu lato*, leading to the description of three new genera: *Azovibrio restrictus*, *Azonexus fungiphilus* and *Azovibrio oryzae*, the latter being most frequent in rice.
Essential factors for successful colonization of rice roots by *Azoarcus* sp. BH72 are type IV pili. To study environmental factors influencing the expression levels of the structural gene *pilA*, transcriptional fusions with *gusA* were constructed. Equally high transcription levels were found under most culture conditions tested. However, when cells were exposed to starvation in a carbon-free medium, *pilA::gus* transcription levels increased 2.5 fold within 2 h. Induction occurred also by the non-metabolizable carbon source citrate, indicating that transcriptonal regulation was indeed dependent on the lack of an energy source. Upstream of the *pilAB* operon, a two-component regulatory system (*pilSR*) was encoded. Gene inactivation of *pilR* indicated that it is the transcriptional regulator of *pilAB*: gene inactivation of *pilR* led to a diminished transcription of *pilA::gus*. Deletion of the gene of the putative sensor kinase *pilS* resulted in an elevated *pilA::gus* transcription, which was highly induced (13 fold) in a culture-density dependent manner in comparison to the wild type (2.5 fold). *PilA::gus* induction by the supernatant of stationary-phase cultures indicated that the transcriptional activation was mediated by autoinducers. Thus, *Azoarcus* sp. BH72 showed an up to now unique transcriptional regulation of type VI pili. Elevated production of type IV pili after starvation and in dense cultures may lead to an improved colonization of a new host by microcolonies.

S6-L20

NOD FACTOR BINDING SITES IN LEGUMES: RECEPTORS OR ACCEPTORS?

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Nod factors are the rhizobial determinants of nodulation and host specificity in the legume-rhizobia symbiosis. These lipochitooligosacccharides (LCOs) are capable of eliciting various responses on the roots of legumes at pico-micromolar concentrations, suggesting that they are perceived by specific receptors in legumes.

By using radiolabelled Nod factors we have been able to characterise different types of binding sites in legumes. The relevance of these Nod factors binding proteins as putative receptors will be discussed.

S6-L21

MOLECULAR ANALYSIS OF SYMBIOSOME BIOGENESIS IN PEA ROOT NODULES.

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Symbiosomes are unique plant organelles – the product of a developmental pathway involving plant and bacterial cell differentiation. We have used a combination of biochemistry, molecular biology and genetics to investigate the biogenesis of plant components of the symbiosome. Using a "proteomics" approach, symbiosomes were isolated from pea nodule homogenates. A Golgi-derived glycoprotein component of the symbiosomal fluid (first identified using a monoclonal antibody) was subsequently characterised as a member of the legume lectin gene family following cDNA cloning. Nodule lectin (PsNlec) is a strongly expressed late-nodulin: it is also expressed in nodule-associated transfer cells and in cortical cells harbouring arbuscular mycorrhizae[1]. PsNLec1 is physically very closely linked to its orthologue the vegetative bud lectin (Blec). The apparent rapid turnover of PsNLEC in the symbiosome compartment encouraged us to search for a protease activity. Cysteine protease, Cyp15a was identified by RT-PCR as a transcript up-regulated in pea nodules. (A similar gene is up regulated in nodules of *Medicago* and *Lotus* spp.). Using a polyclonal antiserum raised against recombinant PsCYP15A protein, the antigen was localised in the symbiosome space: in uninfected nodule cells it was found in vacuoles and small cytoplasmic vesicles[2]. (Incidentally, this protease is also expressed in wilted shoot tissue).
We noticed that, in the symbiotically defective pea mutant Sprint-2 Fix$^-$ (sym31), the targetting of PsNLEC-1 was abnormal. In this mutant, symbiosome differentiation is apparently blocked at a very early stage and the baceroids remain undifferentiated[3]. Therefore we used this mutant to isolated transcripts of "late early nodulins" by suppression subtractive hybridisation. Among the clones isolated are a number encoding membrane transport proteins and others that might be involved in vesicle trafficking. We have investigated two novel transporters that could mediate the delivery of yet unidentified compounds to or from the differentiating bacteroids. Trans-membrane nutrient exchanges are probably modified during nodule development as part of symbiosome differentiation.

1. Balestrini R. et al. (1999) MPMI 12: 785-791
2. Dahiya P. et al. (1998) MPMI 11:915-923
3. Vincent J. et al. (2000) Plant Physiol 123: *In press*

S6-L22

NODULATION RESPONSES IN *VICIA* ROOTS

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Rhizobium leguminosarum biovar *viciae* (*Rl viciae*) secretes at least two types of saccharidic nodulation factors, (i) acidic extracellular polysaccharides (EPS), and (ii) lipochitin oligosaccharides (LCO). EPS are virulence factors, involved in the rhizobial infection process, whereas LCO are required for root hair curling, preparation of the infection pathway, and induction of the formation of nodule primordia in host plant roots.
Most EPS-deficient mutants of *Rl viciae* produce residual amounts of EPS. Recently, we characterized a novel completely EPS-deficient mutant. The gene concerned was homologous to the *rkpK* gene of *Sinorhizobium meliloti* which encodes a UDP-glucose dehydrogenase. Most probably, the gene product is responsible for production of glucuronic acid, specifically present in the EPS repeating unit of *Rl viciae*. The mutant was unable to infect *Vicia* roots, but induced formation of nodule primordia and autoregulation of nodulation (AUT). Interestingly, partial AUT in a split root system could be induced by non-mitogenic NodRlv-IV/V[18:1,Ac] LCO (18:1 LCO). The mechanism of AUT in this system may consist of two components, one dependent and one independent of induction of primordium formation. Studies on the transport capacity of *Vicia* roots for auxin, sucrose and zeatine showed that non-mitogenic 18:1 LCO were able to inhibit auxin and sucrose transport but, in contrast to mitogenic NodRlv-IV/V[18:4, Ac] LCO (18:4 LCO), unable to inhibit zeatine transport. This suggests that induction of nodule primordia requires local adjustment of auxin, sucrose and zeatine concentrations in host plant roots. Results consistent with this hypothesis have been obtained by use of pea root segments. The changes in auxin and sucrose transport capacity induced by 18:1 LCO may be responsible for the root growth phenomena related to AUT in a split root system.
The difference in mitogenicity between 18:4 LCO and 18:1 LCO suggested to us that the 18:4 acyl chain may specifically contribute to signaling in indeterminate-type nodulation. Our speculation that the 18:4 acyl chain is involved in oxylipin signaling was supported by the observation that indeterminate nodulation, dependent on activity of LCO carrying polyunsaturated fatty acyl chains, could be inhibited by salicylic acid (SA, known to inhibit oxylipin signaling), whereas determinate nodulation, dependent on activity of LCO carrying saturated or mono-unsaturated LCO could not be inhibited by SA.

S7-L23

RHIZOBIAL BIODIVERSITY IN CHINA

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The aims of the project "rhizobial biodiversity in China" (ERBIC18ct960103) was to characterize chinese rhizobial biodiversity resources, especially by using molecular methods for bacterial classification and identification; to look for specific genes involved in stress tolerance; to match rhizobial and plant genotypes by looking for molecular markers indicative of compatibility for high-yielding, efficient nitrogen-fixing symbioses; to perform inoculation trials with selected plants, rhizobial strains and other plant-growth promoting bacteria in several different regions and conditions, to measure inoculation success and effect on soil quality; to monitor the fate of inoculant bacteria and to improve inoculant quality, production and use. For these purposes, root-nodule bacteria (rhizobia) that nodulate the introduced peanut plant (*Arachis hypogaea*), rhizobia that infect the native legume *Astragalus sinicus*, and rhizobia from a wide range of wild native legumes growing in areas subjected to environmental stress (drought, salinity, extreme temperatures and pH), were sampled extensively, preserved and characterized taxonomically by using phenotypic and molecular methods. A large collection of putative inoculant strains were tested on plants in greenhouse and field conditions for assessment of yield, competitiveness, effect on soil, cross nodulation and osmotic stress tolerance. Genes for salt tolerance from two strains were characterized and several collected strains. Marker genes were introduced into rhizobia and used for field monitoring and inoculation methods development.
The work resulted in a collection of about 500 taxonomically well described rhizobia containing several new species, and in superior inoculant strains for use with *A. sinicus* and peanut in various soil and climatic conditions and with different peanut cultivars. Inoculant and reference strains can be obtained from the collection. A list of strains is available at our web site: (http://www.biocenter.helsinki.fi/groups/lindstrom/index.html). In addition to numerical taxonomy, ribosomal sequence RFLP and rep-PCR, the new AFLP fingerprinting method was taken into use for typing plant and bacterial genomes, making diversity and taxonomy studies more fast and reliable for large numbers of samples. Sequencing of genes with different kinds of phylogenetic information such as ribosomal 16S and 23S rRNA, *glnA*, *glnII*, *recA*, and the symbiotic *nod* genes yielded information about the evolution of the rhizobial genomes in relation to biogeography. Exchange of strains and of visiting scientists formed and important part of the work, leading to buildup of competence in the chinese laboratories especially with respect to molecular methods and bioinformatics.

S7-L24

RHIZOBIUM ETLI, A NATURAL MAIZE ENDOPHYTE

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Maize (*Zea maiz*) and bean (*Phaseolus vulgaris*) have been traditionally grown in association for thousands of years in Mesoamerica. *R. etli* strains which constitute the main symbiont nodulating beans are highly diverse and may harbor distinct symbiotic plasmids (e.g. bv. *phaseoli* or bv. *mimosae* [Wang et al., 1999]). We have isolated over 60 *Rhizobium* strains from within maize plants grown in association with beans. These strains have been characterized as *R. etli* bv *phaseoli* on the basis of 16S rRNA gene restriction patterns, metabolic enzyme electropherotypes (MLEE), organization of *nif* genes, and the ability to nodulate beans. The isolates were shown to recolonize maize plants in numbers 10-fold greater than those isolates normally recovered from bean nodules. We are currently studying the effects of *R. etli* inoculation on a large diversity of maize landraces. Additionally, from within maize grown in monoculture we have also recovered large numbers of *R. etli* isolates which lack *nif* genes and are incapable of nodulating bean. We therefore presume that these strains correspond to the previously described non-symbiotic *R. etli* recovered from bean rhizosphere (Segovia et al., 1991).

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S7-L25

RHIZOBIAL DIVERSITY IN THE BEAN PRODUCING AREA IN NW ARGENTINA

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The Andean region in the Northwest of Argentina (NWA) is considered as part of one of the two American centers of bean domestication. In addition, the NWA is, with about 250,000 Ha, the main bean producing region of Argentina. We have initiated a project granted by the European Union aimed to improve biological nitrogen fixation in beans by exploiting the diversity of bean nodulating rhizobia of NWA. The level of bean nodulating rhizobia is high all over the NWA, ranging between 10^4 to 10^5 rhizobia/g soil. Therefore, any project to introduce successfully strains that were probed to be more efficient in laboratory assays, have to take in consideration the competition of this quantitative significant indigenous populations as well as the complexity of such bean nodulating rhizobia.
In order to describe the diversity, we had applied molecular biology approaches to characterize the genomes of rhizobia isolated from common and wild beans, and also from other wild legumes that share the habitat with beans. The 16S rDNA allele of *R. etli* was found to be predominant in the populations isolated of common bean and wild bean *Phaseolus vulgaris* var.*aborigineus*. RFLP analysis of symbiotic sequences revealed diversity within this group. Other populations of bean nodulating rhizobia that were retrieved from soils, by using *Leucaena* as trapping host, but not found in nature associated to beans, have 16S rDNA alleles similar to *Sinorhizobium fredii* and to the species *Sinorhizobium saheli* and *S. teranga*. Since these genotypes were recovered from bean cropping soils that were earlier forest areas, it maybe possible that these isolates evolved from tree nodulating rhizobia.
Two out of five different genotypes found in the slow grower rhizobia populations isolated from *Phaseolus augusti* and *Desmodium uncinatum* and *D. incanum* were found closely related to *Bradyrhizobium japonicum* and *B. elkanii*, respectively. These isolates do not form nodules in soybean and beans although some genotypes isolated of *P. augusti* nodulates *Desmodium* spp. and viceverse.
Our studies reveal the complexity of the rhizobial community in NWA, in which the specific bean nodulating *R. etli* appears to be the predominant species among others populations which are also able to nodulate beans. The significance of these ecological studies and the potential use of diversity in bean inoculation will be discussed.

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S7-L28

IMPROVEMENT OF NITROGEN FIXATION AND GROWTH OF GRAIN LEGUMES BY INOCULATION WITH *AZOSPIRILLUM BRASILENSE*

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The increase in dry matter and nitrogen content in *Azospirillum* inoculated legumes, either co-inoculated with *Rhizobium* or naturally nodulated, in both greenhouse and field experiments has been attributed to early nodulation, increased number of nodules, higher N_2-fixation rates and general improvement of root development (Burdman et al 1998). The increase in nodulation could be explained by the promoting effects of *Azospirillum* on root hair formation and by an increased secretion of *nod*-gene inducing signals by the roots (Burdman et al 1998).
Inoculation of common bean with *A. brasilense* (10^7 colony forming units ml^{-1}) increased root length, root projection area, specific root length (m g^{-1}) and specific root area (cm^2 g^{-1}) as compared to non inoculated controls, resulting in root systems with longer and thinner roots. In water stressed potted plants, *A. brasilense* significantly increased the tap root length (German et al 2000). In inoculation experiments of natural pastures, it was found that *Azospirillum* caused an enrichment of legumes relatively to grasses and forbs (Itzigsohn et al 2000).
Cicer arietinum (chick pea, humous) and *Vicia faba* (fava bean, ful, broad beans) are consumed in large quantities by both the Israeli and Palestinian populations. They are winter-spring crops grown in semi-arid regions, that are often irrigated with supplemental saline water at critical reproductive stages.
In greenhouse and field experiments carried out during two seasons (1998-2000), we have observed positive effect of *Azospirillum* peat based inoculants on naturally nodulated plants, in increased nodulation, root growth and crop yield of chick peas and fava beans. In most experiments, the benefits (20-40% grain yield increases) are statistically significant when compared to non inoculated control plants.
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S7-L29

AN INTERDISCIPLINARY RESEARCH-STRATEGY TO IMPROVE SYMBIOTIC NITROGEN FIXATION AND YIELD OF GRAIN LEGUMES IN SALINIZED AREAS

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Results and interactions of a cooperative research group of agronomists, plant breeders, microbiologists, physiologists and molecularists to improve the symbiotic nitrogen fixation (SNF) and N_2-dependent yield of chickpea and common bean under moderate salinity in the Mediterranean basin will be presented. Agronomic survey in reference production areas show large spatial and temporal variations in plant nodulation and growth, and in efficiency of utilization of the rhizobial symbiosis. The later was associated with a large microbial diversity, including new bean nodulating species. Macrosymbiont diversity in SNF and adaptation to NaCl was found with the refernce *Rhizobium tropici* B CIAT899. However, interaction trials between plant lines and rhizobial strain revealed that contrasts between plant genotypes could be altered by some native rhizobia. Therefore variations in soil rhizobial population, in addition to agronomic practices and environmental constraints, may have contributed to erratic results observed in field inoculations. At the mechanistic level, nodule C and N metabolism, and abcisic acid content, were related to SNF potential and tolerance to NaCl. Their relation with nodule conductance to O_2 diffusion was addressed by in situ hybridization of candidate carbonic anhidrase and aquaporin genes. Moreover, a differential display between nodule cortex transcripts from P deficient versus P suficient common bean made it possible to clone putative new genes. Limits and prospects of the strategy will be discussed.

S8-L30

UPDATE ON NITROGEN-FIXATION IN NON-LEGUMES AND THE EFFECTS OF ENVIRONMENTAL STRESSES ON THE *RHIZOBIUM*-LEGUME SYMBIOSIS.

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Since this session deals with Nitrogen Fixation in Sustainable Agriculture and Environment, including aspects of microbial inoculant production and efficacy in the field when applied to both legumes and non-legumes, we will briefly review the two major topics listed in the title of our overview talk. The comments on progress in exploring nitrogen fixation with non-legumes will be based primarily on the results of a recent workshop on this topic organized by Dr. J.K. Ladha at IRRI in the Philippines (Laddha and Reddy, 2000), and some results from our own laboratory described therein. Since inoculant production and the persistence/competition /infection capacities of inoculant strains are highly dependent on environmental conditions, including dessication, nutrient deprivation, pH, heat and mutliple other factors, in the second part of our overview we will touch on genetic aspects of microbial tolerance and responses to these stresses, including the work from our laboratory and others based on the use of reporter genes to identify stress-induced loci and to study their role in survival and plant infection.

(J.K. Ladha and P.M. Reddy (2000) The quest for nitrogen fixation in rice. International Rice Research Institute Press, ISBN 971-22-0112-0).

S8-L31

AZOSPIRILLUM INOCULATION AND ITS AGRONOMIC APPLICATION IN MEXICO

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Over the past 25 years a lot of field inoculation experiments with *Azospirillum* were carried out worldwide. Positive responses to inoculation were frequently observed with significant yield increases ranging from 5 to 30% (1). A number of companies have registered *Azospirillum* inoculants for maize (1,2). In Mexico, maize, wheat, and sorghum field inoculation experiments with *Azospirillum* were successful with significant yield increases ranging from 20 to 70% (3; unpublished results). On the basis of these data, a large field-inoculation program was carried out in Mexico by the Ministery of Agriculture's Research Institute (INIFAP) in collaboration with the CIFN-UNAM. 450,000 ha of maize and approximately 150,000 ha of sorghum, wheat and barley were inoculated with a mixture of *A. brasilense* strains using sterilized peat as carrier. The inoculant was produced by the Fundación Mexicana para la Investigación Agropecuaria y Forestal A.C. Grain yields were evaluated in several tens of hectares with diverse soils and climatic conditions, different rates of N-fertilizers, and a lot of cultivars of maize, but also of sorghum and weat.
The preliminary results showed significant yield increases (average 25%) and are summarized below.

Plant species	Inoculation success	Grain yield increase
Maize (A)	95 %	11-98 %
Maize (B)	62 %	6-43 %
Barley (A/B)	86 %	17-65 %
Wheat (A/B)	83 %	10-58 %
Sorghum (A/B)	62 %	6-43 %

A) Crops without N-fertilization; B) Crops were fertilized with different rates of N

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S8-L32

GRAIN LEGUMES IN EUROPE: ENVIRONMENT-FRIENDLY CROPS AND SOURCE OF PROTEINS FOR FEED AND FOOD

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Europe, and especially the EU, is in serious deficit for vegetable sources rich in proteins for both humans and animals. Two thirds of the EU needs are imported, with great amounts of soya meals for animal feeds, and of common beans, chickpeas and lentils for human beings. Grain legumes are set to play an increasing important role in arable cropping.
The agronomic benefits of grain legume crops are widely recognised in cropping systems: contrary to other crops, grain legumes are able to fix the air nitrogen (thanks to the symbiotic fixation) and they do not need nitrate fertilisers. They are excellent break crops, providing a clear benefit in yield to the following crop of the rotation. Today, these crops are ones of the major arable crops in the European agriculture even if they represent only 2-3% of the areas. They have shown the most rapid expansion in Europe in a few years since the 1980's (from about 500 000 to 1400 000 ha for feed pulses).
In addition, the nutritional value of the legume seeds are appreciated both in animal feed and human food. Indeed, their seeds show a balanced composition (proteins, starches and fibres) and are both source of proteins and energy for humans and animals.
Pulses have positive impacts on human nutrition and health, high values for animal feeding, interesting functional properties for food ingredients, and potential for "green" raw materials for industrial non-food uses.
Therefore, there are an urgent need to combine efforts for targeting improved yield stability, high seed quality and favourable economic context, in order to increase the competitiveness of these quite recent crops which are under-exploited in Europe in comparison to their potential.

S8-L33

MICROBIAL INOCULANTS FOR ENVIRONMENTALLY FRIENDLY, SUSTAINABLE AGRICULTURE

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Mounting public concern regarding the environmental consequences of the continued use of chemical fertilizers, fungicides and pesticides is fuelling the search for more environmentally friendly alternatives to agri-chemicals. Given these concerns, the use of microbial inoculants for food production is now seen as a real alternative to chemical treatments and has the potential to contribute substantially to the goal of environmentally friendly, sustainable agriculture. For microbial inoculants to be accepted as commercially viable products, however, proven efficacy under field trial conditions is required. In addition, public acceptance of microbial inoculants, and in particular inoculants genetically modified for enhanced efficacy, requires the assessment of their ecological impact, including their effect on indigenous microbial populations. These key issues were addressed in the EU funded IMPACT programme (1993-1999). Among the inoculants extensively assessed during the course of the IMPACT programme was the biocontrol agent *Pseudomonas fluorescens* F113, which effectively controls damping-off disease of sugarbeet seedlings, caused by *Pythium ultimum*, via the production of the anti-microbial metabolite 2,4-diacetylphloroglucinol (Phl). In order to increase the efficacy of *P. fluorescens* F113 inoculants under agronomic conditions, several strategies were adopted to enhance Phl production at the transcriptional and post-transcriptional level. Specifically, strategies included increasing *phl* gene dosage, inactivation of the Phl biosynthetic repressor gene *phlF* and alteration in the regulation of secondary metabolite production. Paramount to the acceptance of GM *P. fluorescens* F113 strains is the demonstration of lack of negative impact on indigenous microbial populations (including beneficial rhizobia and arbuscular mycorrhizal fungi) and confirmation of compatibility with other microbial inoculants. This is of particular importance, given that the new wave of microbial inoculant preparations will most likely consist of consortia of GM bacterial inoculants (biofertilizers, phytostimulators and biocontrol agents) with beneficial fungi (e.g. AM fungi). Commercial scale field trial evaluations at several sites throughout Europe demonstrated that *P. fluorescens* F113 did not interfere with the nodulation process by indigenous rhizobia in soybean, clover and alfalfa, nor had any observable negative impact on crop yield parameters. Furthermore, when co-inoculated with a commercial strain of *Bradyrhizobium japonicum*, increased nodulation of soybean was observed compared with un-inoculated plants and plants singly inoculated with *B. japonicum*. Results from field trial evaluations demonstrating the lack of negative impact of *P. fluorescens* F113 inoculants on indigenous microbial populations and compatibility with other microbial inoculants suggest its suitability for inclusion in microbial inoculant consortia.

POSTER ABSTRACTS
SYMPOSIUM 1

S1-P1

ISOLATED FEMOCO CATALYTIC REACTIVITY: N_2 AND CO AS INHIBITORS OF C_2H_2 REDUCTION

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Studies of the substrate-reducing capabilities of the isolated FeMoco in non-enzymatic surroundings showed that FeMoco could reduce acetylene in some aprotic solvents (DMF, NMF, THF) using PhSH as a proton source and Zn amalgam (-0.84 V vs NHE) **(I)** or Eu amalgam (-1.4 V vs NHE) **(II)** as reducing agents [Bazhenova, T.A. et al. 1997, 2000]. FeMoco was isolated from the MoFe protein of *Azotobacter vinelandii* using the published method [McLean, P.A. et al. 1989]. The quality of the FeMoco extracted was evaluated from its ability to reconstitute the activity of FeMoco deficient MoFe protein in crude extracts of *Kp* 5058 [Hawkes T. R. & Smith B. E., 1983]. It has been found that the enzyme reconstitution activity of FeMoco before and after the reaction in non-enzymatic surroundings was the same, so during the reactions the structural integrity of FeMoco was retained. Steady-state kinetic investigation of found reactions has been carried out. CO was shown to be a potent uncompetitive inhibitor of acetylene reduction. CO does not react with FeMoco without reductant. The *Ki* for CO varied with the potential of the reducing agent indicating that different FeMoco oxidation levels interact with CO differently. For **(I)** CO inhibition of C_2H_2 reduction was reversible; *Ki* = 0.05 atm. For **(II)** the inhibition constants for C_2H_4 and C_2H_6 formation were different: *Ki* = 0.004 atm for C_2H_4 and 0.009 atm for C_2H_6. This means evidently that distinct acetylene binding sites are inhibited by CO differently. It was found for system **(II)** that N_2 also inhibits acetylene reduction at low unsaturated C_2H_2 pressure [Bazhenova, T.A. et al. 1999]. The type of inhibition was shown to be a competitive and reversible, K_i = 0.45 atm N_2. The constant value found for the reaction catalyzed by isolated FeMoco are very similar to those for wild-type and α-195^{Gln} nitrogenases [Dilworth M. J. et al. 1998]. So we can conclude that it is possible to obtain in non-enzymatic conditions such a state of isolated cofactor which is capable to bind N_2 molecule.

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S1-P2

AZORHIZOBIUM CAULINODANS MICROAEROBIC ENERGY TRANSDUCTION AND ELECTRON TRANSFER TO DINITROGENASE

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Certain microaerophilic bacteria, including the *Rhizobiaceae*, share the remarkable ability to efficiently fix N_2 and carry out oxidative phosphorylation under extremely low prevailing O_2. From molecular genetic, physiological, and biochemical studies on microaerobic energy transduction in the microaerophile *Azorhizobium caulinodans*, we conclude that aerobic and microaerobic energy transduction are qualitatively different. Because acetyl-CoA is a non-oxidizable substrate for microaerobiosis, by inference, the citric acid cycle, the core process of aerobic energy transduction, is then inactive. Alternatively, endogenous acetyl-CoA is diverted to synthesis of *poly*-β-hydroxybutyrate, a metabolic sink, as end-product of microaerobic energy transduction. We have also inferred the *A. caulinodans* pathway of electron transfer to dinitrogenase during both microaerobic, and symbiotic, N_2 fixation.

S1-P3

ISOLATED FEMOCO CATALYTIC REACTIVITY: STEADY-STATE KINETICS OF ACETYLENE REDUCTION

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The catalytic reactivity of the isolated FeMoco with respect to the reduction of C_2H_2 was investigated. These reactions were carried out in non-aqueous aprotic solvents (DMF, NMF, THF) with the addition of PhSH to provide protonation of coordinated substrate. Zinc amalgam (-0.84 V vs NHE) **(I)** or europium amalgam (-1.4 V vs NHE) **(II)** were used to reduce FeMoco for substrate binding and reduction [1, 2]. FeMoco was isolated from the MoFe protein of *Azotobacter vinelandii* using the column method [3]. The quality of the FeMoco extracted was evaluated from its ability to reconstitute the activity of FeMoco deficient MoFe protein in crude extracts of Kp 5058 [4]. It was shown that the activities for enzyme reconstitution of cofactor before and after the catalytic reaction in non-enzymatic surroundings were the same, so during the reaction the structural integrity of FeMoco was retained.

Steady-state kinetics of the reactions found was investigated. It was shown that under the influence of different reducing agents (**I** or **II**) the unlike redox levels of FeMoco were achieved and therefore distinct numbers and types of binding site for substrates and inhibitors were detected. For **(I)** we observed a normal hyperbolic response in Michaelis-Menten plot. For **(II)** the FeMoco catalyzed C_2H_4 and C_2H_6 production as a function of the C_2H_2 pressure showed sigmoidal kinetics which is consistent with substrate-induced cooperativity among 3 sites for C_2H_2 reduction. The K_m values for **(I)** and **(II)** differ from each other indicating that distinct FeMoco redox levels react with C_2H_2 differently. The specific activity was found to be 3÷5 nmol of C_2H_4 min^{-1} $nmol^{-1}$ of Mo for **(I)** and 40÷60 nmol of C_2H_4 min^{-1} $nmol^{-1}$ of Mo - for **(II)**. The bell-shaped profile was observed for the dependence of the rate of acetylene reduction on the source of protons concentration indicating a complicated protonation mechanism of coordinated substrate [5]. Summarizing all results obtained we can conclude that catalytic behavior of isolated FeMoco with respect to C_2H_2 reduction is very similar to this one within the protein.

Supported by RFBR (№ 98-03-32291), INTAS (№ 96-1503), Haldor Topsoe Post Graduate Grant.

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S1-P4

DINITROPHENOL REDUCTION IN *RHODOBACTER CAPSULATUS* IS REGULATED BY NifR AND DraTG

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Rhodobacter capsulatus grows by fixing N_2 in the presence of 2,4-dinitrophenol (DNP) due to the previous photoreduction of this uncoupler to 2-amino-4-nitrophenol (ANP). DNP reduction was switched-off by glutamine or ammonium, but this short-term regulation did not take place in a *draTG* deletion mutant. As the ammonium shock did not inactivate the nitrophenol reductase, an alternative target to DraTG has to be proposed. In addition to the short-term regulation, ammonium or glutamine represses the nitroreductase, thus suggesting that the long-term regulator *nifR1* may also be involved in the control of DNP reduction. As a matter of fact, the mutants of *Rhodobacter capsulatus* B10 affected in the *nifR1* or *nifR4* genes exhibit a 5-fold decrease in nitroreductase activity. The mutants lacking some flavoproteins (*nifF*, ORF14) involved in the electron transport to nitrogenase, photoreduced DNP to the same extent as the wild-type strain. By contrast, the mutants affected in the *rnfA* or *rnfC* genes, which are also under NifR1 control and that have been proposed to catalyze a reverse electron flow to nitrogenase, are unable to metabolize dinitrophenol. These results indicate that dinitrophenol reduction in *R. capsulatus* is controlled by NifR and DraTG either by affecting the expression or the activity of the Rnf proteins. Therefore, the Rnf complex seems to supply electrons for both nitrogen fixation and DNP reduction and for this reason some *rnf* mutants, probably affected in the reverse electron transport to $NADP^+$, are unable to phoreduce DNP.

S1-P5

ANTIOXIDANT ENZYMES IN LEAVES AND NODULES OF TRANSGENIC ALFALFA OVEREXPRESSING SUPEROXIDE DISMUTASES

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The superoxide dismutase (SOD) family of enzymes represents a primary line of defense against the superoxide radical and derived reactive oxygen species in all organisms. We have studied in detail the antioxidant composition of nodulated alfalfa plants (*Medicago sativa* L. x *Sinorhizobium meliloti* 102F78) of the elite genotype N4 and three derived transgenic lines overexpressing SOD.
SOD activity staining revealed that alfalfa leaves contain the three types of SOD (differing in the metal at the active site) that can be found in plants. Leaf extracts of untransformed alfalfa produced a single band of mitochondrial MnSOD activity. In lines transformed to overexpress *Nicotiana* MnSOD in mitochondria, the MnSOD activity band was considerably more intense than in the nontransgenic line. In contrast, two MnSODs were resolved in lines expressing *Nicotiana* MnSOD in the chloroplasts. Lines transformed to overexpress *Arabidopsis* FeSOD in the chloroplasts show three extra bands of FeSOD activity. Nodules of all alfalfa lines contained similar MnSOD and FeSOD isozymes to those found in the leaves. In addition, four CuZnSOD isozymes and a major band showing SOD activity, as yet unindentified, were observed. Only lines overexpressing MnSOD in the mitochondria of leaves overexpressed the transgene also in the nodules. This may be due to a weak activity of the 35S promoter in alfalfa nodules, although other possibilities such as gene silencing cannot be excluded.
All four lines had low CuZnSOD activities and an abundant FeSOD isozyme in leaves and nodules, indicating that FeSOD performs important antioxidant functions other than the scavenging of superoxide radicals generated in photosynthesis, as generally assumed. The entire sequence of the cDNA encoding alfalfa nodule FeSOD, the first of this type to be cloned from a nonphotosynthetic tissue, is reported. The deduced protein contained a plastid transit peptide and this localization was confirmed by subcellular fractionation.
Transgenic plants showed a compensatory effect in the expression of MnSOD (mitochondrial) and FeSOD (plastidic) in the leaves, implying that a mobile signal molecule is involved in coordinating SOD expression and activity. Because the product of SOD activity is H_2O_2, we also investigated whether the overproduction of MnSOD and FeSOD in the leaves enhanced the activities of the peroxide-scavenging enzymes. However, there were no significant differences in the activities of ascorbate peroxidase, guaiacol peroxidase, and catalase in the leaves or nodules for any of the four lines, indicating that these activities are sufficient for peroxide scavenging under normal conditions.

S1-P6

AZOSPIRILLUM BRASILENSE GLUTAMATE SYNTHASE: A COMPLEX IRON-SULFUR FLAVOPROTEIN.

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Glutamate synthase (GltS) catalyzes the reductive synthesis of L-glutamate from L-glutamine and 2-oxoglutarate (2-OG). GltS is known to play with glutamine synthetase a key role in nitrogen assimilation processes in microorganisms and plants. The NADPH-dependent GltS of *Azospirillum brasilense* (Ab-GltS), a nitrogen-fixing microaerophil bacterium, is the prototype of the bacterial enzymes and may serve as a model for the ferredoxin-dependent GltS found in photosynthetic organisms and for the NADH-dependent eukaryotic GltS found in plants, yeast and lower animals (1). Ab-GltS is a complex iron-sulfur flavoprotein: its $\alpha\beta$ protomer contains 1 FAD, 1 FMN, 1 3Fe/4S cluster and 2 4Fe/4S centers. Studies on the GltS holoenzyme and on its recombinant α (162 kDa) and β (52.3 kDa) subunits led us to propose a model for the GltS reaction: the β subunit is a FAD-containing NADPH oxidoreductase, which functions to transfer reducing equivalents from NADPH to the α subunit where the actual glutamine-dependent synthesis of glutamate takes place. The binding sites of NADPH and FAD within the GltS β subunit have been identified by characterizing the G298A mutant form of the protein (2). Characterization of site-directed mutant forms of the β subunit and of the GltS holoenzyme is in progress, in order to identify the ligands of the 4Fe/4S centers of GltS. These centers are found in the $\alpha\beta$ holoenzyme but not in either one of its isolated α and β subunits, and they may play both a role in redox communication between the sites of NADPH oxidation and glutamate synthesis and in stabilization of the GltS $\alpha\beta$ heterodimer. Each one of the conserved Cys residues located at the N-terminus of the β subunit is being substituted by an Ala residue. Preliminary results indicate that Cys-47, -50, -55 and -59 are essential for GltS activity and may be involved in $\alpha\beta$ protomer stabilization. The recent obtainment of recombinant Ab-GltS holoenzyme has opened the way to detailed studies of its functional and structural features. Towards this goal, the mid-point potential values of the redox centers of the enzyme cofactors are being measured and crystals suitable for x-ray diffraction analysis have been obtained.

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POSTER ABSTRACTS
SYMPOSIUM 2

S2-P1

GENETIC MAPPING OF THE MODEL LEGUME *MEDICAGO TRUNCATULA*

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Our objective is to construct a concensus genetic map of the diploid, autogamous model plant *Medicago truncatula* to identify and clone genes and QTLs involved in agronomical traits of interest (symbiotic genes, cold tolerance, resistance genes). From natural populations, homozygous lines have been selected and crossed to obtain several F2 populations and F6 Recombinant Inbred Lines (RILs). Introgression lines are in progress. The actual genetic map is based on a single F2 cross between an algerian population and the Jemalong variety. This map comprises 348 markers (RAPD, AFLP, isoenzymes, known genes, microsatellites and morphological markers) on 8 linkage groups (2n=16) and covers 1400 cM (about 400 kb/cM). In collaboration with G. Kiss (Institute of Genetics, Szeged, Hungary) we have established that diploid alfalfa (*Medicago sativa*) and *M. truncatula* are highly syntenic. The screening of *M. truncatula* natural populations has allowed us to identify plant lines having different requirements for Nod factors structure and/or displaying a strain x cultivar specificity. The monogenic determinism of some of these characters have been demonstrated and the corresponding genes have been mapped. *Mtsym3* gene is involved in the resistance to nodulation (Nod^-) by *S. meliloti* strains not producing O-acetylated Nod factors; it maps on linkage group VII. *Mtsym4* and *Mtsym5* genes are involved in strain-specific nodulation (Nod^-). *Mtsym6, Mtsym7a and 7b, Mtsym8* are genes involved in strain-specific nitrogen fixation (Fix^-). Plant lines showing a different nitrogen fixation efficiency have been identified and will be used for a genetic analysis of quantitative traits involved in symbiosis.

S2-P2

STUDY OF GENE EXPRESSION DURING ACTIVATION OF DORMANT ADVENTITIOUS ROOTLET MERISTEM.

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Sesbania rostrata is an annual leguminous plant from the Sahel region of West-Africa. As an adaptation to growth in waterlogged conditions, *Sesbania rostrata* carries dormant adventitious root primordia on several vertical rows along its stem. These preformed primordia can follow different developmental pathways depending on the growth conditions or external stimuli. At the distal part of these primordia a dormant root meristem is located that becomes activated to form adventitious roots upon immersion in water. This system presents distinct advantages to study the breaking down of dormancy in the meristems because of the special topology, abundance, and predetermined nature of the root primordia (MPMI 8, 861-824, 1995).
The aim of our work is to study gene expression upon water stimulation in order to isolate new functions that are involved in the transition from a dormant to an active root meristem in *Sesbania rostrata*.
A search for genes that are induced or enhanced by water was started using the differential display technique. A pool of root primordia RNA and a pool of root primordia RNA previously painted with water was compared. Different cDNAs were cloned and analyzed. Northern blot analysis confirmed a water-induction of these genes. Some of the differential display products shared significant homology to DNA/protein sequences in databases while other products revealed no identity to known sequences.
Another approach has been initiated to search for water-induced genes: the suppression subtractive hybridization (SSH) technique We have used a pool of root primordia RNA and a pool of root primordia RNA previously immersed in water for 4 hours (time after which the cells are supposed to reenter the G1/S phase). A subtracted cDNA library has been obtained. To identify the individual clones that represent differentially expressed genes, a differential screening has been done. Different cDNAs have been cloned and sequenced. Quantitative PCR was used to check the water-induction of the genes.
By the differential display technique we have isolated water-induced genes that seem to be rather abundantly expressed, whereas the genes isolated using the suppression subtractive hybridization (SSH) technique seem to correspond to low transcripts. Combining both techniques will allow us to work with a larger set of water-induced genes, and, investigating their expression pattern might give a broader insight in how those genes are involved in the dormancy break down in meristems.

S2-P3

GENOMIC ORGANIZATION AND EXPRESSION PROPERTIES OF THE MTSUCS1 GENE, WHICH ENCODES A NODULE-ENHANCED SUCROSE SYNTHASE IN THE MODEL LEGUME *MEDICAGO TRUNCATULA*

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We isolated and sequenced a sucrose synthase (SucS) cDNA from the model legume *Medicago truncatula*. This cDNA (MtSucS1) contained a reading frame of 2418 bp coding for a protein of 805 amino acids with a molecular mass of 92.29 kD. Northern blot experiments revealed that the corresponding gene is at least ten-fold higher expressed in root nodules in comparison to uninfected root, stem and leaf tissues. On the protein level, SucS was detected in root nodules from a variety of legumes including *M. truncatula*. Interestingly, an additional sucrose synthase of slightly larger molecular weight was detected in uninfected root, stem and flower tissues but not in root nodules of *M. truncatula*. From our expression and sequence data we infer that the MtSucS1 gene encodes a nodule-enhanced sucrose synthase from *M. truncatula*. An analysis of a genomic MtSucS1 sequence revealed that the gene consists of 14 exons. As common for SucS genes, the MtSucS1 gene contains a large intron of 747 bp in the 5' untranslated region. The transcriptional start of MtSucS1 was mapped and putative regulatory elements in the MtSucS1 promoter were identified [Hohnjec *et al.* (1999), Mol Gen Genet 261: 514-522]. We analysed the activity of different MtSucS1 promoter-*gus*Aint fusions in transgenic roots and nodules induced on hairy roots of *M. truncatula* und *Vicia hirsuta*. In particular, we compared the activity of the MtSucS1 promoter covering the region -2050/-19 with a promoter that was extended by the exon I and intron I sequences of the MtSucS1 gene (-2050/+800). Interestingly, the -2050/-19 promoter gives an expression of the *gus*Aint gene only in the vascular tissues of roots and nodules and not in any other tissue tested. In contrast, the MtSucS1 promoter extended by exon I and intron I (-2050/+800) gives additional expression in the complete inner region of the root nodule including the meristem as well as in the root tip. Hence, as shown for SucS promoters of cereals, the exon I and intron I region plays a role in regulating the activity of the MtSucS1 promoter. In contrast to the situation in cereals, we detected not only a stronger promoter activity when including exon I and intron I sequences, but a different expression pattern in tissues where the -2050/-19 promoter is not active. We currently analyze the whole-plant expression pattern of the MtSucS1 gene by analysing transgenic plants containing MtSucS1 promoter-*gus*Aint fusions.

S2-P4

EXPRESSION OF WATER-DEFICIT RESPONSIVE cDNA CLONES IN BEAN NODULES

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Several water-deficit induced cDNA clones had been previously isolated from *Phaseolus vulgaris* (Colmenero-Flores *et al.*, 1997). In order to study their possible involvement in the maintenance of the nitrogen-fixing activity under water stress, we investigated their expression in nodules of water-deprived plants and compared it with the expression levels in leaves.
Rhizobium etli inoculated plants were grown for three weeks in a growth chamber and water deprivation was imposed for one week. At this time plant material from water-deficient and control plants was collected for RNA extraction. Northern blots were performed with various probes and different expression patterns were observed. Clone rsP24, whose sequence shows similarity with lipid transfer proteins from different plants showed high expression levels in stressed leaves but no significant expression was observed in nodules from water-stressed plants. Differently, clone rsP37 wich encodes a proline-rich protein showed a much higher expression in water-stressed nodules than in leaves. The results indicate an organ-specific expression of the genes when the plant is subjected to water deficit, suggesting that the proteins encoded by those genes showing a higher expression in the nodule might be involved in nodule functioning under water stress.

Colmenero-Flores *et al.* (1997) Plant Mol. Biol. 35, 393-405.

S2-P5

DEVELOPING THE GENETIC MAP OF DIPLOID ALFALFA (*MEDICAGO SATIVA*) AND PERFORMING ITS COMPARATIVE GENOME ANALYSIS

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A detailed linkage map of diploid alfalfa (1) has been constructed developing the basic genetic map of alfalfa (2). Several markers on the genetic map of alfalfa showed distorted segregation ratios, in most cases the overwhelming number of heterozygous genotypes were detected. The markers displaying distorted segregation ratio hampered the determination of the linkage groups. The disturbing effect of the artificial linkage could be eliminated by novel mapping procedures namely by colormapping (3) and by using special maximum-likelihood formulas (4) to calculate the linkage values. The detailed linkage map contains about 1500 markers, including more than 200 genes with known functions. The 8 linkage groups span more than 750 centimorgans genetic distance. The correlation between the physical and genetic distance is about 1300 kilobase pairs per cM taking the haploid genome size of diploid alfalfa. The sequence of the 90 cDNA clone detecting 110 RFLP markers on the linkage map were determined to increase the number of genes with known function. The map position of different genes in alfalfa were compared to that of the orthologous genes already placed onto the genetic maps obtained for *M. truncatula*, pea and *A. thaliana*. The map comparison - performed in collaboration with Dr. D. Cook and T. Huguet - revealed high degree of synteny between the alfalfa and *M. truncatula* genomes, while lower level of synteny, but still significant conservation of the gene order in five linkage groups could be demonstrated between alfalfa and pea. The synteny between alfalfa and *A. thaliana* was also studied by comparison the map position of the genes coding ribosomal proteins. The comparative genome analyses allow us to extend the genetic information obtained from the analysis of the model plants, such as *M. truncatula* and *A. thaliana*.

1. Kaló, P et al. (2000) Construction of an improved linkage map of diploid alfalfa (*Medicago sativa*). TAG 100:641-657
2. Kiss GB et al. (1993) Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. MGG 238:129-137.
3. Kiss GB et al. (1998) Colormapping: a non-mathematical procedure for genetic mapping. Acta Biologica Hungarica 49:47-64.
4. Lorieux M et al. (1995) Maximum-likelihood models for mapping genetic markers showing segregation distortion. 2. F_2 populations. TAG 90:81-89.

S2-P6

TOWARDS THE MAP-BASED CLONING OF THE *MSNN1* GENE REQUIRED FOR AN EARLY STEP OF SYMBIOTIC NITROGEN FIXATION

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The non-nodulating (Nod$^-$) tetraploid MnNC-1008(NN) *Medicago sativa* mutant is defective in early plant responses, that is neither root hair curling nor cortical cell division occurs after bacterial infection, therefore, this plant is thought to be impaired in the perception of rhizobial signal molecules or in one of the first steps of their subsequent signal transduction pathway. A tetraploid F2 population segregating the non-nodulation phenotype was used in Bulked Segregant Analysis to identify RAPD markers co-segregating with the mutation. Some of these markers were mapped on the linkage group (LG) five of the diploid *Medicago* genetic map. Several markers from this region of the diploid map were tested also on the tetraploid population and proved to be linked to the non-nodulation trait. Using different individuals from the diploid mapping population which are maternal or paternal homozygous in this region, Bulked Segregant Analysis was carried out to isolate almost 50 RAPD markers for this part of the genome. A number of them were selected to be transferred to the tetraploid map and we could show that the order of the markers in the two populations was the same. Meantime, we extended the tetraploid population to more than 4000 individuals in order to start the fine mapping of the mutation. All individuals of the extended population were genotyped with selected markers to identify plants carrying recombination on either side of the mutation. With the help of the recombinant chromosomes the order of markers could be established around the mutation. Tightly linked markers showing no, or 1-2 recombinations to the mutation were used to isolate primary BAC clones from the genomic library of *Medicago truncatula* (kindly provided by Dr. D.R. Cook; Nam et al. 1999, Theor. Appl. Genet. 98:638-646). To test the microsynteny between *M. sativa* and *M. truncatula* in this region some of the markers were also mapped in *M. truncatula* and their location and order were the same. The isolated end-fragments of the primary BAC clones allowed us to identify overlapping clones and to build a contig in the region. We have started the systematic sequencing of the BAC clones to identify candidate genes. The RT-PCR technique was used to examine the expression of these genes. The sequence of the corresponding mRNAs from the mutant and the wild type *Medicago sativa* were analyzed to find possible mutations. Transformation experiments using *Agrobacterium rhizogenes* carrying different subclones/genes are under progress to achieve genetic complementation.

S2-P7

PEA (*PISUM SATIVUM* L.) GENETIC CONTROL OVER NODULE TISSUE DEVELOPMENT.

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To date more than 40 genes involved in control of symbiosis with rhizobia have been identified (Borisov et al., 1999) in pea (*Pisum sativum* L.). The most part of them has been classified in accordance with beginning of functioning at certain stage of infection process and bacteroid differentiation (based on own data and that published in literature). As a result 8 discrete stages have been defined for now (Borisov et al., 1999). In this study a set of pea symbiotic mutants was analysed to determine the block of nodule initiation and nodule tissue differentiation. It was found that some of the genes identified and classified take part in nodule initiation and nodule tissue formation. As a results this to some extent parallel process was subdivided into 4 developmental stages: (i) Ccd (cortical cell divisions) is dependent on the function of the genes *sym7*, *sym8*, *sym9 sym10*, *sym14*, *sym19*, *sym30* and *sym35*, (ii) Npd (nodule primordium development) is controlled by genes *sym5* and *sym34*, (iii) Nde (nodule emergence) is under control of the genes *sym2*, *sym36* and *sym37*, Ndm (nodule tissue maturation) is controlled by genes *sym21* and *sym39*. Identification of the gene *sym37* mutations in which block infection just after root hair curling at the stage of infection thread initiation (Iti) and nodule tissue development at the stage (Ndm) has special interest because it is implicated in signal perception governing the beginning of infection process but not involved in early steps of nodule tissue differentiation.

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Borisov et al., 1999. Pisum Genetics. 31: 40-44.

S2-P8

FUNCTIONAL GENOMIC ANALYSIS OF EARLY SYMBIOTIC DEVELOPMENT IN *MEDICAGO TRUNCATULA*

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In association with rhizobia, legume species form nodules in which the bacteria fix atmospheric nitrogen. Despite its widespread occurrence, relatively little is known of the molecular details of the plant's role in establishing this symbiosis. Using the model legume *Medicago truncatula*, we are using a functional genomic approach to identify genes expressed during these early interactions. Our objectives are to (1) construct an EST resource, based on expressed sequences from *Rhizobium*-induced cDNA libraries, (2) select and characterize a unigene set from the sequenced clones, and (3) use DNA microarrays to analyze *Rhizobium*-induced patterns of gene expression in *Medicago truncatula*. This is part of a larger, NSF-sponsored effort to investigate the function of the *Medicago* genome in nutrient acquisition and in response to microbial pathogens and symbionts. Together with colleagues, we have constructed 12 cDNA libraries from *M. truncatula*, including four from roots prior to inoculation and during early events of *R. meliloti* infection and nodule induction. Current efforts emphasize bulk sequencing and sequence analysis. As of May 2000, over 17,000 5' sequences have been obtained from the project's libraries. Sequences and BLAST results are publicly available at the project web site: http//chrysie.tamu.edu/medicago, and are updated frequently. BLAST and PHRAP analyses are being used to characterize sequences and to identify ESTs for further study. Current analysis emphasizes two groups of genes: a set of ESTs that may be legume-specific, which we call "leguminosins," and candidate genes that may be involved in cellular processes related to nodulation, based on their homology to genes of known function. Representatives of these two groups, plus controls including clones with known tissue-specific expression patterns, are being used to construct a small test set for microarray analysis. A long term goal is to analyze gene expression in EMS mutants of *M. truncatula* that are defective in nodulation, in order to identify genes important for rhizobial infection, nodule organogenesis, and autoregulation of nodulation.

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S2-P9

CYTOGENETICS FOR THE MODEL LEGUME *MEDICAGO TRUNCATULA*

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Medicago truncatula Gaerth. (barrel medic) have been selected as a model legume for studies on plant-microbe interactions (Barker et al., 1990; Cook, 1999). An important tool for model systems is a molecular cytogenetic map established by using FISH (fluorescence *in situ* hybridization) technique. Such maps provide information about the positions of molecular markers with respect to functional chromosomal elements (centromere, telomere, heterochromatin), and they are valuable in the constructions of physical maps and in positional cloning strategies.
In this study the ideogram of the pachytene karyotype of *M. truncatula* cv. Jemalong A17 is presented. The quantitative analyses (chromosome length, centromere index, size and distribution of heterochromatic regions) in combinations with FISH with 5S rDNA, 45S rDNA and pericentromeric repeat *Mt*R1 allowed the identification of all eight pachytene bivalents.
The feasibility of pachytene chromosomes for physical mapping is demonstrated by using BAC clones of different size (20-100 kb) as probes for FISH and by estimation of mapping resolution for the euchromatic part of chromosome 5. Consequently, hybridization with BAC clones made possible to assign chromosomes to genetically defined linkage groups.

Barker D., Bianchi S., Blondon F., Dattee Y., Duc G., Essad S., Flament P., Gallusci Ph., Genier G., Guy P., Muel X., Tourneur J., Denarie J. and Huguet T. *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*- legume symbiosis (1990). Plant Mol Biol Rep, 8 (1): 40-49
Cook D. *Medicago truncatula* – a model in the making! (1999). Current Opinion in Plant Biology, 2: 301-304.

S2-P10

CLASSICAL GENETIC AND PHENOTYPIC ANALYSIS OF NODULATION IN TRADITIONAL LEGUMES LIKE PEA (*PISUM SATIVUM* L.) IS STILL SCIENTIFICALLY SELF-VALUABLE AND FRUITFUL FOR FURTHER MOLECULAR ANALYSIS OF IDENTIFIED SYMBIOTIC GENES.

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Up to date about 80 legume plant mendelian symbiotic genes (usually termed *sym* genes) have been identified and for many mutants their phenotypes have been described in detail (Borisov et al., 1999; Phillips, Teuber, 1992). The most numerous collection of symbiotic mutants (more than 200 independently obtained and genetically classified ones) is known for pea (*Pisum sativum* L.) and more than 40 its *sym* genes have been identified to date (Borisov et al., 1999).
Despite the fact that pea is not a very suitable plant for molecular biology, the existence of a collection of phenotypically well characterised mutants (mutant genes) makes it possible to use the achievements of molecular biology in model legumes (e.g. *Medicago truncatula* Gaertn. and *Lotus japonicus* (Regel.) K. Larsen) to study pea genes identified to date at the molecular level. For example comparative phenotypic analysis of the mutants in the recently cloned and sequenced gene *Nin* of *L. japonicus* (Schauser et al., 1999) allowed the identification of pea mutants with a similar phenotype and opened up a possibility to clone the corresponding pea gene. This demonstrates the importance (along with self-valuable data on genetic dissection of pea root nodule morphogenesis) of continuation of classical genetic and phenotypic analysis of pea mutants obtained to date.

This work was supported by grants of Volkswagen-Stiftung, Germany (I/72 935) and RFBR (98-04-49883).

Borisov et al., 1999. Pisum Genetics. 31: 40-44.
Phillips, Teuber, 1992. In: Biological Nitrogen Fixation. New York; London. pp. 625-647.
Schauser et al., 1999. Nature 402 (11): 191-195.

S2-P11

EARLY EVENTS IN THE *AZORHIZOBIUM CAULINODANS–SESBANIA ROSTRATA* INTERACTION

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The tropical legume *Sesbania rostrata* is nodulated by its microbial symbiont *Azorhizobium caulinodans* at bases of lateral roots and stem–located adventitious root primordia. The primary bacterial entry of the host tissues is intercellular with the formation of infection pockets and symptoms of cell death. The intercellular invasion as well as the induction of nodule primordia are triggered by Nod factors. Stem nodulation presents particular advantages for transcriptome analysis of early nodulation events by the topology, abundance, and synchronous development of the predetermined nodulation sites. Differential display and suppressive subtractive hybridization have been used to collect a series of genes involved in the early events of nodulation. The present state of the analysis on the role in nodule initiation of a non-hydrolytic chitinase, a gibberellin–20–oxidase, a peroxidase will be discussed. The role of secondary messengers, such as ethylene and hydrogen peroxide, in nodule initiation has been studied. Bacterial and plant functions for intercellular invasion will be examined.

S2-P12

INSERTION MUTAGENESIS AND *IN VIVO* GENE FUSIONS IN *M. TRUNCATULA*

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M. truncatula has been proposed as a model plant to study rhizobial and mycorrhizal symbiosis because it is well suited for the genetic analysis of these processes (Cook D.R., 1999, Cur. Op. in Plant Biol., 2, 301-304). To facilitate the characterization of genes participating in the symbiotic process, we have initiated an insertion mutagenesis program in *M. truncatula* using either derivatives of the *En/Spm* maize transposon provided by Dr. J. Jones (John Innes Center, UK) or various T-DNAs as tags.
Transgenic plants carrying transposon-derived constructs have been analyzed to know whether the transposon can effectively excise from the original T-DNA construct. We have previously shown that the transposon can excise somatically at a low frequency from the T-DNA, but no germinal excision could be found. We also showed that the transposase locus is expressed at very low level in these plants, in agreement with the observed excision frequency. To circumvent this expression problem, we are now studying transgenic plants that express the transposase locus at higher level. We will present our results concerning the behaviour of this transposon *in M. truncatula*.
In parallel to the transposon mutagenesis, we also initiated a T-DNA mutagenesis approach. We are currently screening for the presence of symbiotic (Nod^-, Fix^- and Myc^-) and developmental (colorless, architecture) mutant progenies of the transgenic plants (more then 600) produced in the laboratory. 250 of these plants containing a T-DNA that can generate *in vivo gus*-gene fusions (Bouchez et al., 1993, C. R. Acad. Sci. Paris, 316, 1188-93) were in addition screened for *gus* expression in roots and nodules. 18 plants showing such pattern of expression were selected and the T-DNA borders were recloned to identify the tagged genes. The analysis of the *gus* expression patterns, the T-DNA border sequences and the various phenotypes obtained during this work will be presented.
This insertion mutagenesis program may give us the first *M. truncatula* tagged genes and will help us in the future to dissect the different molecular steps of nodule development leading to the establishment of nitrogen fixing symbiosis.

S2-P13

ISOLATION AND PARTIAL CHARACTERIZATION OF A HIGHLY REPEATED ELEMENT PRESENT IN *M. SATIVA* AND *M. TRUNCATULA* GENOMES.

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Studying the sequences downstream the polygalacturonase gene *MsPG3* we have found the presence of a sequence highly repeated in *Medicago sativa* and *Medicago truncatula* genomes. We have studied the difference in homology and/or copy number of this sequence between both species by Southern-blot analysis. This element named "Scotty" is represented by more than 705 clones in a *M. truncatula* cv Jemalong BAC library, indicating that more than 280 copies of the element are present per diploid genome in this plant, and around 16800 copies in *M. sativa*. The absence of related sequences in other plant genomes strongly suggest that this element is restricted to *Medicago* spp. By using a "Scotty" containing BAC in FISH experiments we have shown the ubiquitous distribution in *M. truncatula* genome of this repeated element and/or other sequences represented in the BAC. The presence of plant retrotransposons homologous sequences in the same BAC and its possible relationship to "Scotty" are discussed.

This work was supported by grants DGESIC PB96-1268 and PB98-1158.

S2-P14

EXPRESSION OF *fnrN*, *nifA* AND *fixN* GENES OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICEAE* AT LATE STAGES OF PEA (*PISUM SATIVUM* L.) ROOT NODULE DEVELOPMENT.

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Expression of rhizobial (*Rhizobium leguminosarum* bv. *viceae*) symbiotic genes *fnrN*, *nifA* and *fixN* was studied in nodules of pea (*Pisum sativum* L.) Fix$^-$ mutants blocked at different developmental stages: SGEFix$^-$2 (*sym33*) [1], RBT3 (*sym33*, *sym40*) [2], SGEFix$^-$1 (*sym40*) [1], Sprint-2Fix$^-$ (*sym31*) [3], RBT (*sym13*, *sym31*) [3] and E 135f (*sym13*) [4].

It has been revealed that expression of these rhizobial symbiotic genes takes place only in plant cells containing bacteria, which have been endocytosed. At the same time genes *fnrN*, *nifA* and *fixN* do not express in nodules of mutant SGEFix$^-$2 (*sym33*) and double mutant RBT3 (*sym33*, *sym40*). Nodules of these mutants are characterized by "locked" infection threads surrounded with abnormally thick plant cell walls and do not provide endocytosis of bacteria into host plant cell cytoplasm [1, 2].

Results of this work show that, first, rhizobial symbiotic genes *fnrN*, *nifA* and *fixNc* are not expressed in growing infection threads and, second, expression of these genes occurs only in bacteria endocytosed into plant cell cytoplasm.

1. Tsyganov et al., 1998. MGG 256:491-503
2. Borisov et al., 1997a. Proceedings of 11th International Congress on Nitrogen Fixation, Paris, France, July 20-25, p.345.
3. Borisov et al., 1997b. MGG 254:592-598
4. Knee at al., 1990. Plant Physiol. 94:899-905.

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POSTER ABSTRACTS
SYMPOSIUM 3

S3-P1

PROTEINS SECRETED THROUGH THE TYPE THREE SECRETION SYSTEM OF *RHIZOBIUM* SPECIES NGR234 PLAY DIFFERENT ROLES IN VARIOUS PLANTS SPECIES

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Rhizobium species NGR234 nodulates more than 112 plant genera which are distributed among 26 tribes in all three subfamilies of the Leguminosae. Bacterial signals involved in the symbiotic process include nodulation factors, polysaccharides as well as secreted proteins. The symbiotic plasmid of NGR234 carries genetic determinants called *rhc* genes that are involved in the formation of a type three secretion system (TTSS). In both animal and plant pathogens, the TTSS is an essential component of pathogenicity and delivers proteins into eukaryotic cells. In the presence of plant-produced flavonoids and in conjunction with NodD1, *rhc* genes are induced. Mutation of the *rhcN* gene, which encodes a putative ATPase providing the secretion machinery with energy, abolishes the secretion of at least five proteins. They are called Rsr proteins (for *Rhizobium*-signal response): RsrX (formerly NolX), RsrL (formerly y4xL) and three new ones, Rsr19, Rsr9 and Rsr7. These proteins have an apparent molecular weight of 64, 37, 19, 9 and 7 kDa, respectively.
The phenotype of the *rhcN* mutant varies with the host tested. On some plants, nodule formation was similar to that observed with the wild-type strain. On others, however, Rsr proteins appear to play a role in the nodulation process since nodule number obtained with the *rhcN* mutant was either significantly lower or higher. The various phenotypes observed do not depend upon nodule structures. All three phenotypes were observed on plants forming determinate or indeterminate nodules. Of the 232 plant species nodulated by NGR234 about 20 were tested for nodulation by the *rhcN* mutant. So far, no correlation could be drawn between effect of Rsr proteins on nodule formation and the tribe of the plant tested.
The genetic determinants of leguminous plants responsible for the various effects of Rsr proteins on the symbiotic process remain unknown. It is possible that some plant species do not have the proper receptor to recognise the bacterial secreted proteins. Alternatively, a component of the signal transduction pathway involved in mediating Rsr perception might be modified in some plants. Secreted proteins might also be recognised as virulence factors, provoking defence-like responses rather than symbiotic processes. The goal of our future research is to determine where and how the plant perceives the Rsr proteins.

S3-P2

CLONING AND GENOMIC LOCALIZATION OF CONJUGATIVE AND MOBILIZABLE ELEMENTS IN *RHIZOBIUM ETLI*.

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We have previously shown the existence of multiple mobilizable (Mob$^+$) regions in *Sinorhizobium meliloti*. Here we report on the cloning and genomic location of several mobilizable (Mob$^+$) or self-transferable (Mob$^+$/Tra$^+$) elements in another species, *R. etli* CFN42. This strain carries several large plasmids, in addition to the chromosome, but only one of these plasmids have been shown to be self-conjugative (1). The procedure described previously was followed (2). Several *R. etli* CFN42 derived strains were used as donors in matings with at least two different recipients: *R. etli* and *E. coli*. Donors were merodiploid populations carrying a genomic library from *R. etli* CFN42. Recipients acquiring the cosmid supporting the gene library were selected, and the identity of the transferred hybrid cosmids was determined by restriction analysis. About 10 non-overlapping cosmids containing putative mobilizable elements were isolated, which were confirmed to be either mobilizable from *R. etli* strains, or self-transmissible, as being able to promote self-transfer from other species unrelated to *R. etli*. One of the clones identified contains the Mob/Tra region from the only self-transmissible plasmid so far identified in strain CFN42 (see poster by Tun-Garrido and Brom), which further confirms the validity of our methodology to select for Mob$^+$/Tra$^+$ elements. The genomic localization of all these elements will be shown, and the possible relationship among Mob$^+$/Tra$^+$ elements in *S. meliloti* and *R. etli* will be discussed.

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S3-P3

THE *nir*, *nor*, AND *nos* DENITRIFICATION GENES ARE DISPERSED OVER THE *BRADYRHIZOBIUM JAPONICUM* CHROMOSOME

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Denitrification is an alternative form of respiration in which bacteria reduce sequentially nitrate (NO_3^-) or nitrite (NO_2^-) to nitrogen gas (N_2). This process implies the presence and expression of the enzymes nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos). The *nir*, *nor*, and *nos* genes coding functions for nitrite respiration, nitric oxide respiration and nitrous oxide respiration, respectively, are assembled in clusters in *Pseudomonas stutzeri*, *P. aeruginosa*, *Paracoccus denitrificans*, *Sinorhizobium meliloti* and *Rhodobacter sphaeroides*. Whether this organization is representative of other denitrifiers is not known. *Bradyrhizobium japonicum* species are Gram-negative soil bacteria which form N_2-fixing symbiosis with soybeans (*Glycine max*). In free-living conditions, *B. japonicum* cells assimilate and denitrify $^{15}NO_3^-$ simultaneously to $^{15}NH_4$ and $^{15}N_2$ when oxygen concentrations are limiting. In this work we report on the localization of the *B. japonicum* USDA110 *nirK*, *norCBQD* and *nosRZDEFYLX* genes on the correlated physical and genetic map of the *B. japonicum* 110*spc4* chromosome. Cleavage of genomic DNA from *B. japonicum* USDA110 by *Pme*I, *Pac*I, and *Swa*I has been used together with pulsed field gel electrophoresis and Southern hybridization to place the *nir*, *nor*, and *nos* denitrification genes on the chromosomal map of 110*spc*4. Gene-directed mutagenesis was performed by insertion of plasmid pUC4-KIXX-*aph*II-PSP into the *B. japonicum* selected genes. Mutation of the genes resulted in the integration of additional recognition sites for the enzymes *Pac*I, *Pme*I and *Swa*I, and the *aph*II gene, into the chromosome of USDA110 mutant derivatives *nirK* GRK13, *norCB* GRC131, and *nosZ* GRZ25. Restriction of *B. japonicum* USDA110 genomic DNA with the mentioned enzymes yielded three, five and nine fragments, respectively. Pulsed-field gel electrophoresis of restricted mutant DNAs resulted in an altered fragment pattern that allowed determination of the position of the selected genes.
Complementary mapping data were obtained by hybridization using digoxigenin-labelled *B. japonicum* USDA110 *nirK*, *norCB* and *nosZD* as gene probes. Denitrification genes were dispersed over the entire chromosome, being *nirK*, *norCB* and *nosZD* located close to $groEL_2$, *cycH* and *cycVWX*, respectively, on the 110*spc*4 genetic map.

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S3-P4

RHIZOBIAL GROUP II INTRONS AS POTENTIAL TOOL FOR STRUCTURAL AND FUNCTIONAL GENOMIC ANALYSES

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Group II introns are ribozymes (catalytic RNAs) that splice via a lariat mechanism resembling that of nuclear premRNAs. We have identified the *S. meliloti RmInt1*, the first group II intron within the family *Rhizobiaceae*. This genetic element is abundant in the genome of *S. meliloti* and appears to be mostly associated to IS*Rm2011-2*, a particular group of insertion sequences belonging to the IS*630*-Tc1/IS*3* retroposon superfamily. *RmInt1* is also an independent mobile genetic element capable of inserting site-specifically at high frequency into intronless alleles (homing), as well as into heterologous sites at lower frecuency (transposition). Both processes occur in the absence of homologous recombination and probably *via* an RNA intermediate. The target for *RmInt1* mobility requires a DNA region encompassing 25 nucleotides in which 14 nt are recognized primarily by base pairing with the intron RNA. *RmInt1* and its host IS element are being used as molecular tools to asses the genetic diversity within native subpopulations of *S. meliloti*. In addition we are currently dissecting the homing pathway and the rules for DNA target site recognition by *RmInt1*. This knowledge will be applied for retargeting of the intron and delivering foreign sequences to specific sites in both prokaryotic and eukaryotic genomes. This strategy could have widespread applications in functional genomic analyses and genetic engineering.

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S3-P5

THE GENETIC VARIABILITY IN A NATURAL POPULATION OF *SINORHIZOBIUM MELILOTI*

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Sinorhizobium meliloti is a soil bacterium able to infect *Medicago sativa* roots and to elicit the formation of nitrogen-fixing nodules. The occurrence of multiple ISs and in general of Mobile Genetic Elements could have a strong impact on the structure and stability of *S. meliloti* genome which is known to be highly polymorphic in natural populations. Up to now, 13 distinct ISs have been identified in the genome of *S. meliloti*. Some of them have been identified inside or flanking the symbiotic genes.
In this study the molecular characterization of 4 polymorphic genotypes of the 20 kb *nod* region is reported. These genotypes were found to be generated by the insertion of transposable elements such as Insertion Sequences and Mobile introns of group II in the intergenic regions. In particular we discovered, in the intergenic region between *nodQ* and *nodJ*, IS*Rm3*, IS*Rm10*, *Rm*Int1 and a new insertion sequence of 2804 bp. In the intergenic region between *nodD1* and *nifN* we discovered a 5500 bp insertion. This large insertion seems to be a complex of two or more insertion sequences. We concluded that in this particular region the genetic basis of the polymorphism is due to the activity of Transposable Elements. We also analyzed by ISs fingerprinting and RAPD technique the relationship between the bacterium life inside the nodule and the generation of genetic variability.

S3-P6

THE NODA SEQUENCE HELPS PREDICTS STRUCTURAL NOD FACTOR FEATURES OF RHIZOBIA

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The nodulation gene *nodA*, which is present in all rhizobia, is involved in the specific transfer of an acyl chain to the chito-oligosaccharide backbone of Nod factors (NFs). We sequenced the entire *nodA* gene of rhizobial strains, whose NFs have been characterized and which belong to several different genera. Phylogenetic analysis of all the available sequences, indicated that the NodA proteins form clusters correlating with some NF structural features such as fucosylation and/or arabinosylation at the reducing end, and the presence of polyunsaturated fatty acids at non reducing end. Thus we developped a statistical tool based on NodA sequence to predict NF types. These results indicate that the *nodA* gene could be used as a tool to search for novel NF structures and as markers for studying *Rhizobium*-legume coevolution.

S3-P7

RepA IS A NEGATIVE TRANSCRIPTIONAL REGULATOR OF THE *repABC* REPLICATOR GENES OF THE pSym OF *RHIZOBIUM ETLI*

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The symbiotic plasmid of *Rhizobium etli* (p42d), strain CE3, is a member of the RepABC family. The members of this family contain three conserved genes (*repA*, *repB* and *repC*) and, also a conserved intergenic sequence between the *repB* and *repC* genes. Recently was demonstrated that: a) The *repABC* genes are organized in an operon; b) RepC is essential for replication; c) RepA and RepB participate in the stability of the plasmid; d) RepA and RepB participate in the regulation of plasmid copy number; e) RepA is a *trans* incompatibility factor and f) contain two incompatibility regions, one located between *repB* and *repC* (*inca*), and the other, downstream of *repC* (*incb*) essential for replication.
The transcriptional analysis of *repABC* operon is presented in this work.
The results indicated that the minimal promoter and operator region is contained within 127 bp upstream of *repA*. The expression of the operon is negatively regulated by the *repA* product and the putative binding site for RepA is one inverted repeat sequence of 14 nucleotides located in the region 61 to 84 upstream of *repA*.
The transcription start site of the *repABC* operon was found 57 bp upstream of the initiation codon of *repA*. Upstream of this site, a sequence similar to the –35 and –10 boxes of the *Escherichia coli* sigma 70 promoter consensus were recognized. A mutagenesis analysis of the putative -35 and –10 boxes will be presented.
To obtain more information about the promoter structure, 18 sequences upstream of previously determined transcription start sites of different *Rhizobiaceae* genes were aligned. The results reveled a substantial degree of similarity between all sequences with the consensus sequence of the *Escherichia coli* sigma 70 promoter, however, the *repABC* operon is not expressed in this bacteria.
Finally, an alignment between the promoter regions of the RepABC family did not show homologies to -35, -10 and operator sequences, however, a small region is conserved upstream the –35 sequence, between *Agrobacterium* and *Rhizobium etli*.

S3-P8

COMPLEX INTERACTIONS BETWEEN REPLICONS, REGULATORS AND LUTEOLIN ARE DEMONSTRATED BY PROTEOME ANALYSIS IN *SINORHIZOBIUM MELILOTI*

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Sinorhizobium meliloti nodulation requires induction of nodulation (*nod*) genes by the presence of the NodD activator and plant exuded flavonoid compounds. Negative regulation is effected by the NolR protein, which has a known repressor function. Here we report on proteomic analyses to investigate the contribution made by plasmid-encoded functions on the intracellular regulation of this bacterium, and on the effect of mutation of the *nolR* gene.
Protein profiles from mutant strains which were either cured of their pSyma plasmid (strain 818), or contained an extensive deletion of this plasmid (strain SmA146), were compared. Plasmid pSyma has an estimated coding potential of 1400 proteins. However, under the growth conditions used we could detect 60 differences between the parent strain and its pSyma-cured derivative, strain 818, which were classified into 21 sub-groups, A to U, based on protein levels when the cells were grown in the presence or absence of luteolin. Comparisons made between the different strains to assess the possible interactions of the different proteins of the subgroups and plasmid pSyma suggest that pSyma has a role in the regulation of the expression of genes from the other replicons.
Proteomic maps from a wild-type *S. meliloti* strain and EK698 (a Tn*5*-induced *nolR*-deficient mutant), grown with or without the addition of the plant signal luteolin, were compared. Silver stained gels showed that the *nolR* mutant had 189 proteins that were significantly altered in their levels (101 protein spots up- and 88 down-regulated). Coomassie-stained preparative 2-D gels or PVDF membranes blotted from preparative gels showed that at least 52 of the altered proteins could be reproducibly detected and isolated from the *nolR* mutant. These were classified into five groups based on protein abundance and the effect of the addition of the inducer luteolin. The identity assigned to 38 proteins shows that a broad spectrum of cellular functions are affected by the loss of the NolR function. We propose that NolR is a global regulatory protein responding to environmental factors to fine tune intracellular metabolism, and having positive as well as negative regulatory affects.

S3-P9

THE COMPLETE SEQUENCE OF THE *SINORHIZOBIUM MELILOTI* STRAIN 1021 PSYMB MEGAPLASMID

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The genome of *Sinorhizobium meliloti* is 6.8 Mb in size and consists of a 3.7 Mb chromosome and two megaplasmids, pSymA (1.4 Mb) and pSymB (1.7 Mb). In an international effort the complete sequence of the genome of *S. meliloti* strain 1021 will be established. The chromosome is currently sequenced in the framework of an EU project coordinated in France. The sequence of pSymA is established in the US. The sequence of pSymB is under investigation by groups in Canada and in Germany.
The French group of F. Galibert established minimal sets of BAC (bacterial artificial chromosome) clones covering each of the three replicons of the genome of *S. meliloti* [1, 2]. The complete pSymB was covered by a minimal set of 24 BAC clones. The individual BAC clones of the minimal set were sequenced commercially in a shotgun approach.
Here we present the sequencing strategy and the annotation of the pSymB sequence. Sequence assembly was done using the Staden software package. Annotation was performed using the GENDB package developed at our university. GENDB is an UNIX-based integrated environment and is designed to automatically assign as many different features to the gene sequence data as possible. GENDB can gather and analyze evidence created by WWW or e-mail servers in combination with locally installed tools. Clearly, the final decision about what an open reading frame encodes can be made only by human researchers. Therefore, an interface that allows users to support or refute GENDB results is implemented.

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S3-P10

REGULATORY MECHANISM OF THE TRANSFER OF pCFN42a OF *RHIZOBIUM ETLI* CFN42.

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Rhizobium etli strain CFN42 is able to make nodules on roots of *Phaseolus vulgaris* (bean). This strain contains six plasmids;only one of these plasmids (pCFN42a) is able to perform self-transfer at a high frequency ($1x10^{-2}$). pCFN42a is also required for the transference of the pSym (pCFN42d). Due to these reasons we are interested in understanding the mechanism which regulates the transfer of pCFN42a.
We have isolated a cosmid clone of approximately 30 kbs (C-13).This clone contains all the genes required for the self-transfer of pCFN42a; C-13 is also able to promote the mobilization of the pSym. We have sequenced some regions of C-13. We have found genes that present a high homology with the *tra* and *trb* genes of pNGR234a (pSym of *Rhizobium* sp. NGR234), and lower homology with *tra* and *trb* genes from pTiC58 of *Agrobacterium tumefaciens*. We also have detected that strain CFN42 is able to produce several homoserin-lactones. We have identified the putative *tral* gene responsible for the synthesis of homoserin-lactone involved in the transfer process; since a mutation in this gene abolishes the transfer of pCFN42a. On the other hand we have made some experiments to determine the effect of *traM* gene from pNGR234a on the pCFN42a trasfer. The results show that this gene produces a decrease in the transfer frequency of pCFN42a.
Overall, we think that the mechanism of regulation of pCFN42a transfer is basically similar to the one described for pTiC58, although there seem to be variations in regard to the negative regulation.

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S3-P11

TOWARDS A MOLECULAR MECHANISM FOR RECOMBINATION ENHANCEMENT BY REPLICATION (RER) IN *RHIZOBIUM ETLI*.

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We have demonstrated previously that a region of the symbiotic plasmid (pSym), carrying most of the *nod-nif* genes, presents a high frequency of rearrangements such as amplifications and deletions. Both events have important consequences on the symbiotic effectiveness of *R. etli*. The rearrangements are fostered by the presence of reiterated genetic elements and an active recombination machinery. Recently, we have shown that induction of additional replication in the pSym leads to a thousand-fold increase in the frequency of rearrangements, provoking the deletion of the symbiotic region (Genetics, 154:971-983, 2000). We named this phenomenon as Recombination Enhancement by Replication (RER). RER is clearly due to an enhancement in homologous recombination; among its most interesting features are that i) it is restricted to the symbiotic zone of the pSym of *R. etli*, not affecting other endogenous plasmids, ii) RER is maintained even if the whole symbiotic region has been translocated into another plasmid, and (iii) RER also operates on the symbiotic region in the pSyms of other rhizobia, such as *R. tropici*.
Since the requisites for operation of RER (a region on the symbiotic plasmid, additional rounds of replication, the presence of a recombination system and reiterated sequences) are widespread among the rhizobia, we believe that RER might contribute to the understanding of several problems, such as the generation of new symbiotic plasmids (S. Brom, unpublished) and to explain some instances in the literature regarding pSyms instability. In nature, RER might be activated through processes such as cointegration between pSyms and other replicons during bacterial conjugation or under perturbations of plasmid copy number control.
Therefore, we want to analyze which structural features of the symbiotic region and which genes are responsible of RER, in order to generate new strains with modified symbiotic abilities. Some preliminary data towards this end will be presented.

S3-P12

DYNAMICS OF GENOME ARCHITECTURE IN *RHIZOBIUM* SP NGR234

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The *Rhizobium* sp NGR234 genome consists of three replicons. The previously identified Sym plasmid pNGR234a and the megaplasmid pNGR234b as well as a large DNA molecule of ~3500 kb. Southern hybridization with 16S rRNA and *recA* genes suggest the largest replicon was the chromosome. In order to analyze the dynamics of the *Rhizobium* sp NGR234 genome, siblings were obtained by plating on nutrient rich medium. Organization of their genomes was compared by a combination of Eckhardt, pulse-field gel electrophoresis (PFGE) and PCR methodologies. Analyses of 2500 individual clones demonstrated the occurence of large-scale DNA rearrangements consisting of fusions between two plasmid replicons and the chromosome. Excision of entire integrated plasmids was also observed. In the case of pNGR234a, integration and excision was shown to be via a Campbell mechanism mediated by insertion sequence elements. The observed changes in the genome architecture apparently did not alter the growth and symbiotic proficiencies of *Rhizobium* derivatives.

S3-P13

PHYLOGENETIC ANALYSIS OF THE *REPC* FAMILY OF PLASMID REPLICATION GENES

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There are more than 35 different *repC* sequence types (showing >20% sequence diversity) identified in rhizobia and two other α-proteobacteria (*Paracoccus versutus* and *Rhodobacter capsulatus*). Up to five different sequence types, each associated with a different plasmid, have been identified in a single *R. leguminosarum* strain, suggesting that the different *repC* groups constitute different compatibility groups.
This family of homologous, compatible replication sequences is, so far, unique in plasmid biology. The high level of variation within the rhizobial sequences suggests that *repC* pre-dates the divergence of the fast growing rhizobial genera (*Rhizobium, Sinorhizobium* and *Mesorhizobium*). We have recently shown that these three genera diverged before legumes existed, agreeing with an earlier assertion that the symbiotic genes, which are largely plasmid-borne in these genera, had a single origin and spread among the different genera. Horizontal spread of symbiotic genes is supported by the observation that identical symbiotic genes are associated with different *repC* sequences.
We will present phylogenetic analyses of *repC* and chromosomal and symbiotic gene sequences. These will address whether *repC* predates the divergence of the fast-growing genera and if there is evidence for inter-species or inter-genus exchange of *repC*.

S3-P14

LONG EXACT REPEATS IN SEQUENCED MICROBIAL GENOMES

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Despite its importance for genome plasticity, the annotation of DNA repeats in sequenced microbial genomes is frequently neglected. Long exact repeats (i. e. identical in sequence) are particularly important, because these are ideal substrates for intragenomic homologous recombination. Recently, we did a study (Res. Microbiol., 150:735-743, 1999) about the location and extent of exact repeats in the collection of sequenced bacterial genomes available at GenBank, using the program Miropeats. Our data reveal a striking breadth in the abundance of exact reiterated sequences (larger than 300 bp) between bacterial genomes, with *Helicobacter*, *Mycobacterium*, *Synechocystis* and some archaeal genomes being highly reiterated. This uniqueness is reinforced when only exact reiterated sequences larger than one kb are considered. In this case, the genomes of *Helicobacter*, *Mycobacterium* and *Synechocystis* stand out as highly reiterated.
We have extended this analysis to cover sequenced plasmids larger than 100 kb. It was expected that the abundance of exact repeats in molecules of this size will be representative of the degree of reiteration in complete genomes. Moreover, this approach allows to include other bacterial genomes, such as *Rhizobium* sp. NGR234, *Agrobacterium tumefaciens*, *Bacillus anthracis* and *Halobacterium* sp., whose chromosomes have not been sequenced yet. As expected from previous experimental data, the pSym of *Rhizobium* sp. NGR234, as well as the large plasmid of *Halobacterium* sp. show an abundance of exact repeats. Interestingly, a tumor-inducing plasmid from *A. tumefaciens* (pTi-Sakura) displays a scarcity of exact repeats.

S3-P15

NUCLEOTIDE SEQUENCE OF THE SYMBIOSIS ISLAND OF *MESORHIZOBIUM LOTI*

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The *Mesorhizobium loti* symbiosis island is a 501.8-kb mobile, chromosomally-integrated element which transfers to nonsymbiotic mesorhizobia in the environment converting them to *Lotus* symbionts. It integrates into a phe-tRNA gene, reconstructing the gene at the left end and producing a 17-bp direct repeat of the 3' end of the tRNA at the right end. Integration is mediated by a P4 integrase located at the left end of the element. The island is representative of a class of elements termed fitness islands which when acquired confer an advantage on the host under specific environmental conditions. We have completed the nucleotide sequence of the island. The island has a mosaic structure suggesting that it evolved in a step-wise fashion via multiple recombination events. As expected it contains common nodulation and nitrogen fixation genes including some which are spread across several replicons in other rhizobia. In addition the sequence revealed a wide range of other genes. Such genes include those likely to be involved in transfer of the island, genes of unknown function found on symbiotic replicons in other species, genes with no homologues in current databases, several putative regulatory genes, genes encoding cell membrane-associated components including porins, and an unexpected array of metabolic genes which may contribute to "fine tuning" of nodule metabolism. The nucleotide sequence provides a foundation to study previously uninvestigated aspects of the symbiosis using a functional genomics approach.

S3-P16

PROTEIN PATTERN FROM BACTEROIDS OF *RHIZOBIUM ETLI* DURING SIMBIOSIS.

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During the symbiosis between the bacterium *Rhizobium etli* and plants such as beans, the bacteria elicit the formation of nodules on the roots of host plants. The bacteria infect the nodule, enter the cytoplasm of plant cells and differentiate into a distinct cell type called a bacteroid, which is capable of fixing atmospheric nitrogen. This highly regulated process begins with the invasion of plant root cells by symbiotic bacteria leading to nodule formation, which culminates in the establishment of a fully developed N_2-fixing nodule. To understand how the bacteroids work, involves understanding the mechanisms of carbon and hydrogen metabolism in microaerobic conditions required for nitrogenase function, control of flow of compounds between plant and bacteroids, the mechanism of nitrogen export to the plant, etc. The N_2-fixing bacteroids, in determinate nodules like *R. etli*, have a typical behavior, principally constituted by 3 stages, induction, a peak of the highest nitrogen fixation activity and senescence; these process involve the fully regulation of the genome expression
Using Proteome analysis we have identified protein patterns that are essential for *Rhizobium* during nitrogen fixation in association with legume plants. Instead of the1200 proteins expressed in free life we found only 600 proteins were observed during the different stages of nitrogen fixation in association with *Phaseolus vulgaris*. To discover bacterial patterns of proteins involved in the infection and differentiation stages of symbiosis, we obtained 2D-gels of proteins expressed at the appropriate time in the nodule. Our protein-maps will help to understand the complex metabolism release during the nitrogen fixation in *R. etli* in association with legume plants to molecular analysis.
Proteome analysis provides a sensitive tool to examine the functional organization in order to evaluate the changes in *Rhizobium* gene expression between *R. etli* and *P. vulgaris* during the nitrogen fixation.
Some of proteins are being examined by Mass spectrometry (MALDI-TOF) and amino acid composition analysis to determine their possible functions.

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S3-P17

DIVERSITY OF BRADYRHIZOBIA ISOLATED FROM A WIDE RANGE OF FOREST LEGUMES NATIVE OF GUYANA AND AFRICA BY ANALYSIS OF PARTIAL 16S-23S rDNA INTERGENIC SPACER SEQUENCING

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Tropical rain forests are characterised by a high diversity of plant species. Only 30% of the trees in the world have been investigated for their capacity of developing nitrogen fixing symbioses and only a limited number of *Rhizobium* strains from leguminous trees have been isolated and characterised. The rhizobial diversity in Brazil has been recently studied by Moreira el *al.* (1993, 1998) using partial 16S rDNA gene sequences. We isolated 100 slow-growing bacterial strains from nodules of 7 forest legumes native of Guyana belonging to the genera *Andira, Dalbergia, Michaerium, Indigofera, Erythrina, Clitoria* and *Desmodium*, from 17 legumes natives of Guinea belonging to the genera *Penthaclethra, Aubrevillea, Mimosa, Desmodium, Piptadenium, Calpogonium, Centrosema, Mucuna, Milleria, Pterocarpus, Erythrina, Abrus, Samanea, Arthrosamanea, Piptadenia and Albizia*, and from 7 generes of Madagascar, *Dalbergia, Albizia, Desmodium, Crotalaria, Chadsia, Cadia* et *Mundulea*; representing 13 tribes belonging to either of the three subfamilies of the Leguminosae. The isolates were examined by analysis of partial 16S-23S rDNA Intergenic Spacer (IGS) sequences, a technique described by Willems el al. (in press) as a rapid tool to evaluate the diversity of bradyrhizobia isolated from tropical trees.

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POSTER ABSTRACTS
SYMPOSIUM 4

S4-P1

IDENTIFICATION AND EXPRESSION ANALYSIS OF A PUTATIVE BACTEROID TRANSPORTER IN *RHIZOBIUM ETLI* CNPAF512.

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Rhizobium etli CNPAF512 is a soil bacterium that interacts symbiotically with *Phaseolus vulgaris*, thereby inducing the formation of root nodules. Screening of a *R. etli* CNPAF512 mutant library resulted in the isolation of *btrA*, bacteroid transporter A, a gene from which the product shows homology with ABC exporters. The *btrA*-gene codes for a 715 amino acids protein with a calculated molecular mass of 79 kDa. BtrA can be divided into two domains: a N-terminal part that probably functions as a permease and a C-terminal part that contains the conserved Walker A and Walker B regions of ATPases. A *btrA-gusA* fusion was constructed and the expression was tested in different *R. etli* CNPAF512 regulatory mutants. This fusion was strongly expressed in bacteroids and could be induced under free-living microaerobic conditions. Our results indicate that expression of *btrA* is dependent on NifA and RpoN and also on the two-component system FixLJ. This is the first identified target gene of the FixLJ system in *R. etli* CNPAF512.

S4-P2

DIFFERENT CONDITIONS AFFECT THE EXPRESSION OF IRON-REGULATED OUTER MEMBRANE PROTEINS IN *SINORHIZOBIUM MELILOTI* 242.

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It is generally recognized that iron is a key element for cellular metabolism. Despite its abundance in the earth crust, its bioavailability is normally low. Microbes have evolved different strategies to obtain nutritional iron which include: (i) production and uptake of diverse siderophores, (ii) utilization of host iron-binding proteins, (iii) reduction of Fe^{3+} to the most soluble form (Fe^{2+}), (iv) enzymatic degradation of iron-binding compounds and (v) production of toxins that may eliminate competitors for iron. A common fact in the first two strategies is the production of iron-regulated outer membrane proteins (IROMPs). Like most other microbes, rhizobia have more than one iron acquisition mechanisms. They can use many compounds as iron sources: including their own, or exogenous Fe^{3+}–siderophore complexes, citrate, hemin and leghemoglobin. All of these Fe^{3+}– transport systems may require specific outer-membrane receptors. The aim of this work was to study conditions that affect IROMPs expression in *Sinorhizobium meliloti* 242.
Outer membrane fractions were analyzed by SDS-PAGE (8.5% w/v polyacrylamide). The outer membrane profile of iron-limited cells showed at least four IROMPs with apparent molecular masses of 200kD, 180kD, 153kD and 95kD. Prior growth history of cells affect IROMP expression. When iron-sufficient cells were transferred an grown in low-iron medium all four IROMPs could be detected. But when iron starved cells were transferred and grown in low iron medium, only the 95kD IROMPs was present. Differential expression was observed on media with different iron sources. The 95 kD protein and siderophore production could not be detected on iron-limited medium supplemented with hemoglobin/leghemoglobin as iron source, where as all other IROMPs where still present. The induction time of the IROMPs also varied. When washed iron-sufficient cells were transferred to iron-limited media, the 180kD IROMP was present after 120min, while the 200, 153 and 95 kD IROMPs were not expressed until 150 min.

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S4-P3

CARBON PARTITIONING IN NITROGEN-FIXING ROOT NODULES

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Nitrogen-fixing root nodules represent strong carbon sinks. Sugar partitioning was studied in three different symbiotic systems, namely a legume, *Medicago truncatula* and two actinorhizal plants, *Casuarina glauca* and *Datisca glomerata*. The expression levels of sucrose synthase and genes encoding sugar translocators in nodules and roots were compared, and the expression patterns of these genes in nodules were analysed using *in situ* hybridization. In all cases, sucrose synthase expression was induced in nodules compared to roots. Expression levels of sugar translocators in *M. truncatula* nodules were much higher in hydroponically cultured (stressed) plants than in aeroponically cultured plants. Sucrose translocator expression was very low in *C. glauca* and *D. glomerata* nodules, but *D. glomerata* nodules showed high expression levels of hexose translocator which could be shown to be confined to the infected cells. Enzyme activity of three invertase isoformes (vacuolar, apoplastic and cytosolic) and sucrose synthase was determined for roots and nodules. For *M. truncatula*, no significant differences in invertase activity were found determined between roots and nodules. However, in *D. glomerata* nodules, activities of all three isoforms were dramatically reduced in nodules compared to roots. In *C. glauca*, the most striking effect was the high activity of soluble acidic (vacuolar) invertase in nodules compared to roots. The interpretation of these data with regard to sugar partitioning mechanisms will be discussed.

S4-P4

COORDINATED REGULATION OF C_1 ASSIMILATION IN *SINORHIZOBIUM MELILOTI*

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In the diazotrophic α-Proteobacteria several key biochemical processes including photosynthesis, C_1 assimilation, CO_2 fixation, and N_2 fixation are transcriptionally regulated by a highly conserved two-component signal transduction system. The ActS/ActR system of *Sinorhizobium meliloti* belongs to this group, and is known to regulate numerous and diverse systems including substrate transport, nitrogen assimilation, C_1 assimilation and CO_2 fixation in response to an unknown environmental signal. In the current work we show that ActR transcriptionally regulates four key operons required for C_1 assimilation, namely those encoding methanol dehydrogenase, formaldehyde dehydrogenase, formate dehydrogenase and ribulose-1,5-bisphosphate carboxylase/oxygenase. Genes encoding each of the enzymes have been cloned and sequenced, and show strong similarity to previously sequenced genes from organoautotrophic and photoautotrophic bacteria. Transcription-level regulation of each operon by ActR was verified by demonstrating changes in mRNA levels resulting from different copy numbers of *actR*. Each of the enzymes were also assayed directly. Inactivation of *actR* resulted in a derepression of enzyme levels relative to the wild-type situation, whereas the provision of multiple copies of *actR* (plasmid-borne) resulted in repression of the activities.

S4-P5

REGULATION OF *nif*- GENE EXPRESSION, NITROGENASE ACTIVITY AND DINITROGENASE REDUCTASE (NIFH) MODIFICATION BY MULTIPLE P_{II}-LIKE PROTEINS IN *AZOARCUS* SP. BH72

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P_{II}-like signal transmitter proteins are known to be involved in the regulation of nitrogen assimilation and N_2-fixation in many prokaryotes. In *Azoarcus* sp. BH72 we reported for the first time the identification of three functional P_{II}-like proteins, named GlnB, GlnK and GlnY. Mutant analysis revealed that the three proteins are differentially involved in the regulation of nitrogen fixation.
Expression of structural nitrogenase genes was measured by transcriptional *nifH::gusA* fusions. Repression of *nif* gene expression in the presence of combined nitrogen (ammonia, nitrate) was observed in the wild type and the *glnB*$^-$ and *glnK*$^-$ mutant strains, whereas in the *glnBK*$^-$ double mutant (expressing GlnY) nitrogenase expression was not repressed. This indicates that GlnB and GlnK are able to sense the cell's nitrogen status, resulting in inhibition of the transcriptional activator NifA. In contrast, the novel third paralogue GlnY, which is always present in its modified (uridylylated) form, leads to constitutive *nif* gene expression.
We also observed differential roles of the P_{II}-like proteins in posttranslational regulation of nitrogenase activity. Fast and complete inactivation of nitrogenase activity after ammonium addition was dependent on GlnK and AmtB, a putative ammonium transporter/sensor protein. Ammonium "switch-off" was shown to correlate with a modified form of dinitrogenase reductase (NifH) in wild type cells but not in the *glnB*$^-$ and *glnK*$^-$ mutant strains, indicating that nitrogenase modification after ammonium addition is dependent on both, GlnB and GlnK. These results showed that NifH modification is not a prerequisite for the observed physiological ammonium "switch-off" effect and moreover, that another yet unidentified mechanism controling nitrogenase activity is present. Furthermore, we detected a modification of NifH induced by anaerobiosis which occurred only in the single *glnB*$^-$ and *glnK*$^-$ mutant strains but not in the *glnBK*$^-$ double mutant strain expressing GlnY.
These results indicate that the P_{II}-like proteins are involved in different signal transduction cascades controling nitrogenase activity and posttranslational modification in *Azoarcus* sp. BH72.

S4-P6

EFFECT OF BACTERIAL RESPIRATION ON THE REGULATION OF CARBON AND NITROGEN METABOLISM IN *PHASEOLUS VULGARIS* NODULES.

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The aim of the present study was to understand how bacterial respiration affects ammonia and carbon assimilation in bean nodule, by using *Rhizobium etli* strain CE3 and its derivative mutants CFN037 with increased respiratory capacity, and IFC01 with a lower respiratory capacity and a Fix- phenotype. Transcript levels, activities, protein abundance of plant enzymes involved in carbon and nitrogen metabolism and ureide content in the plant xylem exudates were compared in nodules induced by the *Rhizobium* wild type strain CE3 and its derivative respiratory mutants. *Rhizobium* respiration was correlated mainly with the activities of nodule sucrose synthase (SS) and glutamate synthase (NADH-GOGAT). SS activity was greatly increased from day 11 while NADH-GOGAT activity increased later and gradually. Early induction of phosphoenolpyruvate carboxylase (PEPc) activity was observed in day 11 but from day 13 PEPc activity was not significantly affected by the bacterial respiration. NADH-GOGAT and PEPcproteins were consistently high in nodules with increased bacterial respiration. However increased bacterial respiration promotes an early induction of NADH-GOGAT and PEPc transcripts. Xylem exudate of plants inoculated with the *Rhizobium* mutant CFN037 had lower ureide and higher amino acids content than in plants inoculated with the parental strain CE3.
Supporting this result, reduced activities of ureide synthesis enzymes were detected in nodules with increased bacterial respiration. The results presented here indicate that an increase in nodule O_2 consumption favor the synthesis and the transport of amide rather than ureide and support the idea that N_2 fixation in legumes might be regulated by a feedback mechanism involving N compounds.

S4-P7

METABOLISM IN *PHASEOLUS VULGARIS* INOCULATED WITH MUTANTS OF SALT TOLERANT *RHIZOBIUM* STRAINS

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Seeds of common bean (*Phaseolus vulgaris*) are widely used as a source of protein for human nutrition in developing countries. This grain-legume is an important crop in many of the irrigated areas where waters are frequently of low quality (Abbas *et* al., 1991). Salinity stress may dramatically limit plant growth and crop productivity. However it is necessary to identify the involved traits on the adaptation of tolerant plant to salt (Waissel, 1991). Also in previous work, we have shown that *Rhizobium* genes involved in salt tolerance under free-living conditions are also important for the establishment of efficient symbiosis with *Phaseolus vulgaris*.
In our experiment plant were grown in a controlled environment chamber and a nutritive solution without nitrogen source was applied. Plants were harvested at the beginning of the flowering. Growth was evaluated by shoot and root dry weight, total plant dry weight, number and dry weight of nodules. Nitrogenase activity was monitored by acetylene reduction activity (ARA) method (Hardy *et* al., 1968).
We studied carbon metabolism as well as enzymes related to oxidative metabolism and senescence in *Phaseolus vulgaris* nodules infected by salt-tolerant or salt-sensitive isogenic strains, which differ in their ability to grow in saline media. Our results suggest that *Rhizobium* uses the same genetic circuits for adaptation to stress inside and outside the root nodules.

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S4-P8

DOES NODULE SPECIFIC MODULATION OF GLUTAMINE SYNTHETASE (GS) IN TRANSGENIC *MEDICAGO TRUNCATULA* AFFECT PLANT NITROGEN METABOLISM?

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Higher plants play an essential role in the assimilation of inorganic nitrogen into organic compounds. Glutamine synthetase (GS) occupies a key position in plant nitrogen metabolism, since all inorganic nitrogen is, in a first stage, reduced to ammonium and then is assimilated by the GS enzyme. Moreover, GS is also responsible for the assimilation of the ammonium released in several metabolic reactions.
Our goal is to study the regulation of nitrogen assimilation in the legume-*Rhizobium* symbiosis where dinitrogen is fixed by the bacterium and then assimilated by the plant GS in root nodules. By modulation of GS gene expression specifically in nodules we aim to investigate whether these changes alter the expression of rhizobial nif/fix genes and the overall productivity of the plant. In this work, we present the study of the over-expression or partial inhibition of GS in transgenic *M. truncatula* root nodules. Transgenic plants were produced harbouring chimeric gene constructs of the GS cDNA clones (*GSa* or *GSb*) from *M. truncatula* fused in sense or antisense orientation to the nodule-specific leghemoglobin promoter.
Molecular characterisation (GS enzyme activity and protein levels) of the transgenic plants, indicate a 2 to 3-fold altered GS expression.
The altered GS gene expression observed in some of the transgenic plants, might influence the overall nitrogen metabolism of the nodule. To address this problem, further analysis, including measurements of other plant nitrogen metabolic enzymes (eg aspartate amino transferase and asparagine synthetase) and bacterial nitrogen fixation are being carried out.
These studies will provide the means to evaluate the role of plant GS in controlling the flux through the nitrogen assimilation pathway in root nodules.

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S4-P9

THE FIXK$_2$ PROTEIN IS INVOLVED IN REGULATION OF NITRIC OXIDE REDUCTASE IN *BRADYRHIZOBIUM JAPONICUM*

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Denitrification is an alternative form of anaerobic respiration in which nitrate (NO_3^-) or nitrite (NO_2^-) are reduced sequentially to nitric oxide (NO), nitrous oxide (N_2O), and, utltimately, dinitrogen gas (N_2). The reduction of nitric oxide to nitrous oxide is catalyzed by the cytochrome *c*-and *b*-containing nitric oxide reductase enzyme. The *norCBQD* genes from the N_2-fixing *Bradyrhizobium japonicum* strain USDA110 was isolated and sequenced. The deduced amino acid sequence exhibited a high degree of similarity to nitric oxide reductase from *Pseudomonas stutzeri*, *Paracoccus halodenitrificans*, *P. denitrificans*, and *Rhodobacter sphaeroides*, and with the FixN subunit of the cbb_3-type terminal oxidases from *B. japonicum* and *Rhizobium leguminosarum*. Analysis of the sequence upstream of the structural *norCB* genes revealed the presence of an anaerobox (tgcTCGATgcgcGGCAAc), located 87 base pairs from the putative translational start codon. Mutagenesis of each *norC* and *norB* genes resulted in strains GRC131 and GRB993, respectively, that were unable to grow when cultured microaerobically with either nitrate or nitrite.
Membrane proteins from wild-type USDA110 and mutants GRC131 and GRB993 cells were separated by SDS-polyacrylamide gel electrophoresis and stained for covalently bound heme proteins. A membrane-bound *c*-type cytochrome with M_r 16,000 was not detected in membranes from the mutant strain GRC131, which identifies this proteins as the NorC component of *B. japonicum* USDA110 membrane-bound nitric oxide reductase enzyme.
Maximal expression of a *norC-lacZ* fusion in USDA110 required both low level oxygen conditions and the presence of nitrate. The lack of expression of the *norC-lacZ* fusion in *B. japonicum fixLJ* and *fixK*$_2$ mutants indicated that *norC* expression depends on the low-oxygen-responsive two-component regulatory system FixLJ and on the Fnr/FixK-like DNA binding protein FixK$_2$. This conclusion was further corroborated by the succesful complementation of the *fixK*$_2$ mutant strain *B. japonicum* 9043 with the *fixK*$_2$ gene.

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S4-P10

FURTHER EVIDENCE FOR SUCROSE SYNTHASE AS THE KEY ENZYME CONTROLLING NODULE NITROGEN FIXATION UNDER DROUGHT.

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Nitrogen fixation in legume nodules has been shown to be very sensitive to drought and other environmental constraints. It has been widely assumed that this decline in nitrogen fixation was a consequence of an increase in the so-called oxygen diffusion barrier and a subsequent impairment to bacteroid respiration. However, we have recently shown that nitrogen fixation is highly correlated with nodule sucrose synthase (SS) activity under drought and other environmental stresses. Whether this correlation reflects a causative relationship or not and whether sucrose synthase activity decline leads to a decreased nitrogen fixation or this is the opposite have not been proven yet. If SS is controlling nitrogen fixation one would expect bacteroids to be limited by organic acids availability, whilst the decline in nitrogen fixation precedes this of carbon flux, malate levels should increase in nodules.
Malate and adenylate levels were determined in nodules of pea plants subjected to a progressive and mild water stress. Nodule water potential declined to –0.6 MPa in slightly stressed nodules and –0.8 MPa in moderately stressed nodules, being control water potential –0.4 MPa. Under these conditions, nodule malate concentration was reduced by 40% and 60%, respectively. Moreover, levels of ATP, ADP and AMP also decreased by 35% as water stress reached –0.6 MPa, with no further reduction in adenylate levels at lower nodule water potential. Thus, it is concluded that, under drought, nitrogen fixation in pea nodules is limited by organic acids shortage, as a consequence of sucrose synthase down-regulation.

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S4-P11

AN INCREASED CARBON FLUX IN PEA NODULES DOES NOT LEAD TO AN ENHANCED NITROGEN FIXATION

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It has been recently shown that nodule nitrogen fixation and sucrose synthase (SS) activities are highly correlated. In this way, the down-regulation of SS seems to cause a decline in nitrogen fixation under a wide range of environmental circumstances and also in different species. Recent evidences seems to point out that SS may control nitrogen fixation and not the opposite (see Gálvez *et al.*, this Conference). Thus, it is quite temptimg to speculate on the possibility of enhancing nitrogen fixation through an increase in nodule SS activity. Although, several engineered plant lines with increased SS activity may be available soon, an alternative and simple method to increase both SS and carbon flux in nodules is to enhance photosynthate availability. This can be achieved by growing plants at elevated CO_2 concentration (1000 µmol mol^{-1}). Under these conditions, nodules have an increased content of sugars and organic acids together with enhanced activities of a significant number of enzymes of carbon and nitrogen metabolism, including SS. When nitrogen fixation activity was assessed by the classical acetylene reduction assay, nodules grown at elevated CO_2 had an activity six-times higher than nodules grown at ambient CO_2 concentration. However, nitrogen fixation determined as H_2 evolution was virtually identical in both sets of plants. These results strongly suggest that nodule nitrogen fixation is limited both by carbon and oxygen and, therefore, perspectives for enhancing legume nitrogen fixation should take into account both aspects of nodule physiology.

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S4-P12

ANALYSIS OF HAEM (*hmu*) UPTAKE GENES AND A *tonB*–LIKE GENE IN *RHIZOBIUM LEGUMINOSARUM*.

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Bacteria use various strategies to acquire the important metal, iron. One of those used by animal pathogens is to sequester haem from the host, the haem being internalised by specialised transport systems. There is one report that rhizobia can use haem as an Fe source, but to date the genes for such an uptake system have not been identified in *any* non-pathogenic bacterial species.
In *Rhizobium leguminosarum*, we found an operon of four genes, *hmuSTUV*, which specifies haem uptake. These genes are adjacent to a fifth gene, termed ORF5, which specifies an extracytoplasmic factor (ECF) σ factor for RNA polymerase. The role of ORF5 and other transcriptional regulators (including *fur* and a different ECF gene, *rpoI*) in the expression of *hmuSTUV* will be described, as will be the effects of exogenous Fe and haem in the growth media.
Knockout mutations in the *hmuSTUV* operon were made. Doubly defective strains that could not import haem or the siderophore vicibactin were also constructed. The effects of these mutations on symbiotic N_2 fixation will be described.
Upstream of *hmuSTUV* is a gene with significant homology to *tonB* which, in other bacteria, is needed to transduce energy to drive the uptake of many siderophores. Mutations in this Fe-regulated gene over-produce vicibactin. The roles of various transcriptional regulators on the expression of this *tonB*-like gene and the symbiotic phenotypes of *tonB* mutants will be described.

S4-P13

THE SIDEROPHORE SYNTHESIS AND UPTAKE vbs AND fhuA GENES OF RHIZOBIUM LEGUMINOSARUM: THEIR STRUCTURE, THEIR REGULATION AND THE GENES AND SIGNALS THAT CONTROL THEIR EXPRESSION.

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Nitrogen-fixing bacteria have a particularly high demand for the metal iron. .Strains of *Rhizobium leguminosarum* bv. *viciae* make an unusual trihydroxamate siderophore, vicibactin, the vicibactin-Fe complexes being imported by a dedicated transport system (1) We had identified the *fhuACDB* genes that are needed for vicibactin *uptake* (2,3).. Here we describe the *vbs* genes that are involved in the *synthesis* of vicibactin. A large *vbsS* gene, which encodes the multi-functional synthase, and *vbsO*, which specifies a hydroxylase are in the same operon, closely linked to *rpoI*, a gene for an iron-sensitive extra-cytoplasmic (ECF) σ factor (4) and to *fhuA*, which specifies the vicibactin outer membrane receptor. Like the *fhu* genes, *vbsSO* are expressed in free-living cells only if Fe is scarce. Interestingly, both *vbs* and *fhu* are actually switched off in bacteroids and both *vbs* and *fhu* mutants can fix N_2, apparently normally in pea nodules. Thus, vicibactin does not appear to be a significant source of Fe in the nodules, despite the very heavy demand for the metal by the N_2-fixing bacteroids. Either directly or indirectly, the ECF σ factor RpoI is needed for the transcription of *vbsSO*. In turn, *rpoI* itself is regulated by a complex set of *cis*- and *trans*-acting regulators. The roles of the ECF σ factor RpoI and the well known "global" transcriptional regulator Fur on *vbs* expression will be described. We will also present progress on the structural characterisation of the RpoI protein and of other Fe-dependent transcriptional regulators on *R. leguminosarum*.
The reasons why hydroxamate uptake mutants of *R. leguminosarum* are Fix$^+$ on peas, whereas the equivalent mutants of *Bradyrhizobium japonicum* are Fix$^-$ on soybeans (Mary-Lou Guerinot, Pers. Comm.) is of interest and possible reasons for this difference will be discussed.

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S4-P16

NtcA-DEPENDENT TRANSCRIPTIONAL REGULATION IN HETEROCYST DEVELOPMENT AND FUNCTION

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Some filamentous cianobacteria differentiate, under conditions of nitrogen deficiency, cells specialized in nitrogen fixation called heterocysts. Heterocyst development in *Anabaena* sp. PCC 7120 depends on the global nitrogen regulator NtcA and requires, among others, the products of early heterocyst differentiation genes *hetR* and *hetC*. The *hetR* gene is transcribed from several promoters, one of which is dependent on NtcA and HetR, but it seems to bear no NtcA binding site. The *ntcA* gene is also transcribed from three promoters, one constitutively used under different nitrogen conditions and two which are induced in cells subjected to nitrogen stepdown and whose expression is dependent on NtcA and HetR. Thus, *hetR* and *ntcA* are autoregulatory genes that also influence the expression of each other. We have observed that NtcA is also required for activation of expression of *hetC* which, under nitrogen deficiency, is transcribed from an NtcA-type promoter located 571 bp upstream from the gene. NtcA-mediated activation of *hetR* was not impared in an *hetC* mutant, indicating that HetC is not an NtcA-dependent element required for *hetR* induction. On the other hand, some genes like *glnA*, *petH* and the *nifHDK* operon, which are expressed in mature heterocysts, have been found to be transcribed in an NtcA-dependent manner or from NtcA-type promoters. The *glnA* gene, which encodes glutamine synthetase, is expressed from three transcription start points, two of which (PI, PIV) are used in cells subjected to nitrogen stepdown while the other one (PII) is used independently of the nitrogen regime. The PI promoter shows the structure of the NtcA-activated promoters. Transcriptional fusions of different fragments of the *glnA* promoter to a *lacZ* reporter showed that the presence of the sequences corresponding to the PI promoter is essential for *glnA* expression, and that mutations which destroy the palindromic sequence of the NtcA binding site in the PI promoter result in overexpression of the *lacZ* gene. The *petH* gene, which encodes ferredoxin:$NADP^+$ reductase, is transcribed from two promoters, one (P1) constitutively used and the other one (P2) used in cells subjected to nitrogen stepdown and in nitrogen-fixing filaments. The P2 promoter, whose use is NtcA-dependent but HetR indepedent, is functional in mature heterocysts. Finally, the *nifHDK* operon, which encodes nitrogenase, is expressed in the heterocyst from a putative NtcA-dependent promoter. Although neither the P2 promoter of *petH* nor the *nifHDK* promoter have good NtcA binding sites, NtcA is able to bind weakly to fragments of DNA bearing the promoter regions of these genes. These promoters might be recognized more efficiently in vivo by a modified NtcA protein or by NtcA assisted by another regulatory element.

S4-P17

IDENTIFICATION AND CHARACTERISATION OF A HEME UPTAKE SYSTEM IN *BRADYRHIZOBIUM JAPONICUM*

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Rhizobia are able to use heme as the sole iron source (1). We have identified a gene cluster involved in heme uptake in *Bradyrhizobium japonicum*, the nitrogen-fixing soybean symbiont. Predicted products of these genes include an outer membrane protein homologous to the TonB-dependent heme transporter HasR from *Serratia marcescens*, a periplasmic heme-binding protein and an ABC transporter described as HemT and HemUVW, respectively, in *Yersinia enterocolitica*. The *hasR* locus is directly linked to genes, encoding proteins with highest similarity to a TonB-ExbBD system in *Pseudomonas putida*. Unlike the wild type, *B. japonicum hasR* or *exbD-tonB* null mutants are unable to use hemoglobin, leghemoglobin or heme as sole iron source during free-living growth. By contrast, the symbiotic phenotype of the *hasR* mutant (nodulation, nitrogen fixation) is indistinguishable from the wild type when assayed four weeks after infection of soybeans. Currently, we are investigating a potential role of the heme acquisition system under iron-limited symbiotic conditions. Regulation of the newly identified heme uptake system was studied with the help of transcriptional *hemT*- and *hasR-lacZ* fusions. Expression of both genes was induced (≥ 6 5-fold) at low iron conditions (≤ 1 µM) in free-living cells. Iron control was dependent on Irr but not on Fur, two recently identified iron-regulatory proteins in *B. japonicum* (2). Transcript mapping experiments led to the identification of putative -10/-35–type promoters and a common palindromic motif located around position -62 and -52, upstream of the transcription start sites of the divergently orientated *hemT* and *hasR* genes.

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S4-P18

TWO RESIDUES IN THE T-LOOP OF *KLEBSIELLA PNEUMONIAE* GLNK DETERMINE NIFL-DEPENDENT NITROGEN CONTROL OF *NIF* GENE EXPRESSION.

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X-ray crystallographic analysis of the *Escherichia coli* P_{II} protein paralogues GlnB and GlnK has shown that they share a superimposable structural core but can differ in conformation of the T-loop; a region of the protein (residues 37-54) that has been shown to be important for interaction with other proteins. In *Klebsiella pneumoniae* GlnK has been shown to have a clearly defined function in regulating NifL-mediated inhibition of NifA activity in response to the nitrogen status and GlnB, when expressed from the chromosome, does not substitute for GlnK (1,2). As the T-loops of *K. pneumoniae* and *E. coli* GlnB and GlnK differ at just three residues: 43, 52 and 54, we have used a previously constructed heterologous system, in which *K. pneumoniae nifLA* is expressed in *E. coli*, to investigate the importance of GlnK residues 43, 52 and 54 for regulation of the NifLA interaction. By site-directed mutagenesis of *glnB* we have shown that residue 54 is the single most important amino acid in the T loop in the context of the regulation of NifA activity. Furthermore a combination of just two changes, in residues 54 and 43, allows GlnB to function as GlnK and completely relieve NifL inhibition of NifA activity.

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S4-P19

INCREASED EFFICIENCY OF NITROGEN FIXATION AND ALTERED CARBON METABOLISM IN ALFALFA NODULES ELICITED BY *SINORHIZOBIUM MELILOTI ntrR* MUTANT

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In the nitrogen-fixing root nodules of *Medicago sativa* induced by its symbiotic partner *Sinorhizobium meliloti* a very close interaction is formed between the bacterial and plant metabolic pathways. Under conditions of nitrogen limitation, bacteroids convert atmospheric nitrogen to ammonia and provide nitrogen also for alfalfa. In turn, the host plant supplies the bacteroids with carbon source and energy. The symbiosis formed between the *S. meliloti ntrR* mutant and alfalfa was used to study the interdependence of these processes in nodules.
The *ntrR* mutant of *S. meliloti* displays increased nodulation and nitrogen fixation efficiency especially in the presence of fixed nitrogen. To explain the molecular mechanisms resulting in a higher dry weight and nitrogen content of alfalfa when inoculated by the *ntrR* mutant, the expression of *nifH* and *nifA* was determined. Both genes showed an elevated level of expression compared to the wild type nodules, both in the presence and absence of exogenous nitrogen. Contrary to previous data, ammonium in the medium resulted in a reduced *nif* expression both in wild type and mutant nodules. Our results suggest that the higher dry weight of alfalfa is due to the elevated *nif* gene expression in the mutant nodules, and indicate that *ntrR* may control *nif* genes via NifA. The higher rate of fixed nitrogen production may require a higher rate of carbon supply from the plant. The main source of reduced carbon for nodules is sucrose that is converted to malate and succinate, representing the utilizable forms of carbon for bacteroids. We examined whether the expected higher carbon requirements of the *ntrR* mutant induced an altered expression of sucrose synthase, PEP-carboxylase and malate dehydrogenase genes encoding enzymes of the pathway leading from sucrose to malate. Carbonic anhydrase involved in the refixation of CO_2, released during intensive respiration, was also tested. All examined genes except sucrose synthase were expressed at an increased level in the mutant nodules.

S4-P20

STUDIES ON THE REGULATION OF EXPRESSION OF THIAMIN BIOSYNTHETIC GENES IN *RHIZOBIUM ETLI*.

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Thiamin pyrophosphate (TPP) is made by the enzymatic coupling of two independently synthesized precursors, 4-amino-5-hydroxymethyl-2-methyl pyrimidine pyrophosphate (HMP-PP) and 5-(2-hydrohyethyl)-4-methyl thiazole monophosphate (THZ-P). Although TPP is the cofactor of very important enzymes of carbon metabolism like pyruvate and α-ketoglutarate dehydrogenases, little is known about the molecular mechanisms involved in the regulation of expression of the genes involved in its biosynthesis.
In *Rhizobium etli*, bacteria able to nodulate bean plants, we have characterized a cluster of four genes (*thiCOGE*). *thiC*, *thiG* and *thiE* are homologous to *E. coli* genes. *thiC* is involved in HMP-PP synthesis, *thiG* and probably *thiO* (which is a new gene that codes for a predicted flavoprotein with homology to D-aminoacid oxidases) are implicated in THZ-P production and the *thiE* product catalyzes the coupling of HMP-PP and THZ-P. We have also mapped the transcript initiation site of *thiC* gene of cultures grown in media without thiamin. It lies 210 bases upstream of *thiC* ATG codon. This leader contains a 39 bases long sequence named *thi* -box that is highly conserved in the upstream region of *thi* genes of organisms as diverse as *E. coli*, *Synechocystis*, *Bacillus subtilis* and *Mycobacterium tuberculosis* (*J. Bacteriol.* 1997. 179: 6887-6893.).
In this work we present the analysis of transcriptional fusions to the *lacZ* gene carrying various portions of the leader region and of the initial part of the coding region of *thiC* . We show that thiamin-dependent control of these fusions is a post-transcriptional event and that it requires the integrity of the leader and unexpectedly, of the initial part of the coding region of *thiC*. In addition, we will show that a fusion with a deletion of the *thi*-box does not express at all neither in media with or without thiamin, suggesting that the *thi*-box is very important for expression of thiamin-controlled genes. Also, we have found that a deletion of the region surrounding the Shine-Dalgarno sequence or the initial part of the coding region causes a less stringent control by thiamin of *thiC* expression under repressive conditions, proposing a role for these sequences in thiamin control. In order to guess the role of the *thi*-box in *thiC* expression, we have made a prediction of its secondary structure. The likelihood of its existence was substantiated by a covariational analysis with the *thi*-box sequences found in other organisms. A model of *thiC* expression consistent with the data obtained will be presented.

S4-P21

REGULATION OF GENE EXPRESSION IN RESPONSE TO OXYGEN IN *RHIZOBIUM ETLI*: ROLE OF FNRN IN *FIXNOQP* EXPRESSION AND IN SYMBIOTIC NITROGEN FIXATION.

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The genes which code for a bacteroid terminal oxidase, denoted as the cytochrome terminal oxidase cbb_3 have been identified as the *fixNOQP* operon. In *Sinorhizobium meliloti*, the expression of the *fixNOQP* genes is mainly regulated in response to O_2 availability, through the oxygen sensing cascade *fixL*, *fixJ* and *fixK* gene products. *fixL* codes for a membrane bound haem-containing protein which in response to low O_2 phosphorylates itself and FixJ, activating the capacity of FixJ to promote the transcription of *fixK*. In turn FixK activates the transcription of the *fixNOQP* operon and negatively regulates its own promoter. *fixK* codes for a transcriptional activator homologous to Fnr and Crp. In *R. leguminosarum* biovar *viciae*, an homologous but different transcriptional activator, FnrN, activates the two *fixNOQP* copies. In contrast with FixK, FnrN conserves the N-terminal domain involved in O_2 sensing present in Fnr from *E. coli* suggesting that in *R. leguminosarum* FnrN transcriptional activity is negatively modulated by oxygen. Analysis of the transcription of the *fixNOQP* operon in different *R. etli* mutants of the purine-thiamin biosynthetic pathway, suggested that a molecule closely related to AICAR is a negative effector of the expression of these genes. In this work we will present the cloning and characterization of two *R. etli fnrN* genes. We will describe the isolation of *R. etli* mutants in both genes, the study of their symbiotic phenotype as well as that of triple mutants affected also in *fixL*. Also, the participation of *fnrN* genes in the expression of *fixNOQP* and in their own expression will be presented. Finally, we will describe the isolation of mutations in one *fnrN* gene which are affected in the regulation of *fixNOQP* expression in response to oxygen and AICAR.

S4-P22

MOLECULAR PHYSIOLOGY OF TRANSGENIC ALFALFA PLANTS WITH QUANTITATIVE REDUCTION IN THE FLUX CONTROL OF THE SYMBIOTIC AMMONIUM ASSIMILATION PROCESS.

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In the *Rhizobium*-legume symbiosis the ammonium fixed by the bacteroid is assimilated by the plant nodule cells through the concerted action of the enzymes glutamine synthetase (GS) and glutamate synthase (NADH-GOGAT). Our research is aimed towards defining the precise regulation and function of this key metabolic pathway, therefore we have generated transgenic alfalfa plants with quantitative variations of the flux control of the process resulting from the nodule-specific decrease of NADH-GOGAT gene expression.
We will present data on the molecular and physiological characterization of five different transgenic alfalfa lines with a different level of nodulespecific down-regulation of NADH-GOGAT, which resulted in different alterations of their symbiotic phenotype. The transgenic lines have stableintegrations of a chimeric gene composed by the nodule specific soybeanleghemoglobin promoter and the alfalfa NADH-GOGAT cDNA gene, in theantisense orientation. These have normal low levels of NADH-GOGAT activityin the roots and they grow optimally in non-symbiotic conditions (plus N).
When inoculated with *Sinorhizobium meliloti*, each of these five transgenic lines show a different level - from 35% to 90% - of NADH-GOGAT gene expression in the nodules, evidenced by specific RNA concentration, protein concentration and enzyme activity. These nodule NADH-GOGAT antisense plants show deleterious symbiotic phenotypes that are more drastic in those plants that have less nodule NADH-GOGAT remaining activity. Plants from the transgenic line GA89, which has the least remaining NADH-GOGAT gene expression (around 35%), in symbiosis with *S. meliloti* show: less nitrogenase and normal GS activities, and lower amino acid content in the nodules, increased number of nodules with altered phenotype, very slow growth and chlorosis (lower chlorophyll content) which are clear symptoms of nitrogen deficiency. Our results point towards confirming that NADH-GOGAT is the limiting step of the ammonium assimilation process in the nodule, and a diminution in this enzyme activity result in the reduction of the metabolic flux and the consequent physiological nitrogen deprivation symptoms when plants establish symbiosis with *Rhizobium*.

S4-P23

NITROGEN REGULATION IN *KLEBSIELLA PNEUMONIAE*: PROTEIN-PROTEIN INTERACTIONS AMONGST NTRB AND NTRC DOMAINS.

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In *Klebsiella pneumoniae*, signal transduction in response to nitrogen availability is mediated by the two-component regulators NtrB and NtrC. NtrB, a bifunctional histidine-kinase, modulates the activity of the response regulator NtrC by phosphorylation. NtrB is a tight dimer capable of switching between opposing kinase and phosphatase activities. This switch, mediated by PII according to the N-status, requires the integrity of the amino-terminus of NtrB, which is not conserved among histidine kinase proteins. The carboxy-terminus of NtrB (transmitter module) posses similarity to other histidine kinases and has been shown to be responsible for NtrC regulation. Function of NtrC as a transcriptional activator depends on phosphorylation of its N-terminal domain by NtrB.

To contribute to the understanding of signal transduction by NtrB and NtrC proteins, we have used the yeast two-hybrid system to probe interactions between full length and individual domains of NtrB and NtrC from *Klebsiella pneumoniae*. Protein fusions of NtrB, NtrC, and derived polypeptides to GAL4 activation and DNA-binding domains were generated. Domain boundaries for NtrB truncated derivatives were chosen to separate sensor (S), phosphotransfer (H), N box (N) and kinase (G) domains. Domain boundaries for NtrC truncated derivatives separate receiver (R), catalytic (C), and DNA-binding (D) domains. To determine the ability of two given polypeptides to interact, we determined expression of both *GAL1:lacZ* and *GAL1:HIS3* reporters in strains of *Saccharomyces cerevisiae* Y190.

Results confirm previous data on NtrC association states and indicate that the sensor domain of NtrB provides the dimerisation interface, which is at variance with results in other systems. In addition, our data indicate a strong interaction between NtrB and NtrC, which maps to the phosphotransfer domain of NtrB and to the receiver domain of NtrC, indicating that recognition specificity in this two-component system does not involve interactions between surfaces outside the transmitter and receiver domains. In addition, the data suggest that dimerisation of the receiver domain is important for transmitter recognition.

S4-P26

SUBCELLULAR LOCALIZATION OF THIOL SYNTHETASES IN LEGUME NODULES

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In plants, the tripeptide glutathione (GSH; gGlu-Cys-Gly) is a versatile antioxidant that can directly scavenge reactive oxygen and participate in the ascorbate-GSH cycle for peroxide removal in the chloroplasts. The pathway for GSH synthesis involves the enzymes g-glutamylcysteinyl synthetase (gECS) and GSH synthetase (GSHS). In plants gECS and GSHS are present in the chloroplasts and cytosol of leaves. Legumes may contain homoglutathione (hGSH; gGlu-Cys-bAla) partially or fully replacing GSH. The synthesis of hGSH from g-glutamylcysteine and bAla is catalyzed by a specific hGSH synthetase (hGSHS).

We have localized the three enzymes in nodules of bean (hGSH-producing legume) and cowpea (GSH-producing legume) by subcellular fractionation using marker enzymes to assess cross-contamination of organelles. Most gECS activity of bean nodules was localized in the plastids and bacteroids, with somewhat less activity being present in the cytosol; in contrast, hGSHS activity was found in the cytosol, with no measurable activity in the mitochondria, plastids, peroxisomes, or bacteroids.

Crude extracts of cowpea nodules exhibited gECS and GSHS activities but not hGSHS activity. The majority of gECS activity was localized in the plastids and bacteroids, and the majority of GSHS activity in the cytosol and bacteroids. Unlike bean nodules, however, we found gECS and GSHS activities in the mitochondria of cowpea nodules. Mitochondria preparations showed negligible contamination with bacteroids or plastids, and therefore we decided to purify leaf organelles to verify results. Subcellular fractionation of cowpea leaves revealed that mitochondria, but not chloroplasts, contain GSHS. This study also confirmed the presence of low levels of gECS in cowpea mitochondria. Bacteroids purified on Percoll gradients were essentially free of contamination with other nodule compartments and showed high specific gECS and GSHS activities, but no hGSHS activity. The high potential of bacteroids for GSH synthesis prompted us to measure the thiol content of bacteroids. As expected, cowpea bacteroids had no hGSH, but bean and soybean bacteroids contained, respectively, 1.5 and 4.5 nmol hGSH per mg of protein. This significant hGSH concentration was not due to adsorption of the thiol to the bacteroid surface since it remained constant after repeated washings of bacteroids. Because bacteroids do not express hGSHS, we conclude that the hGSH found in the bacteroids was synthesized by the host plant and taken up through the symbiosome membrane.

S4-P27

ISOLATION OF FULL-LENGTH cDNA CLONES ENCODING THIOL SYNTHETASES OF LEGUME NODULES

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Glutathione (GSH) performs a multiplicity of important functions in plants and is synthesized by two sequential reactions catalyzed by γ-glutamylcysteinyl synthetase (γECS) and GSH synthetase (GSHS). Legumes may contain homoglutathione (hGSH), which is synthesized by a specific hGSH synthetase (hGSHS). We have proposed the hypothesis that GSH plays a critical role in N_2 fixation. As an essential step to elucidate this role, we have initiated the molecular study of γECS, GSHS, and hGSHS of legume nodules. In a previous work we showed that the precursor γECS proteins of pea and bean nodules contain a putative cleavage site motif (Ile-X-Ala-Ala) for plastid targeting. We have now isolated two GSHS cDNAs from a pea nodule library and a single GSHS cDNA from a bean nodule library. All three cDNA sequences have two stop codons and identical polyadenylation sequences. The pea cDNAs (*GSHS1* and *GSHS2*) shared 74% identity and were approximately 65% identical with those of other plants. The bean cDNA (designated *GSHS2* because of its higher homology with pea *GSHS2*) showed approximately 63% identity with other GSHS cDNAs. The three derived proteins have 55.9-61.5 kDa and 495-552 amino acid residues. Prediction programs indicated that pea GSHS2 and bean GSHS2 are localized in the cytosol, whereas pea GSHS1 has a mitochondrial transit peptide. The complete deduced GSHS sequences were used to construct an unrooted phylogenetic tree, which revealed that legume GSHS proteins cluster together with respect to the non-legume proteins and that there are two clusters within the legume sequences: pea GSHS2 and bean GSHS2 are more closely related to each other than either sequence is to pea GSHS1.
RT-PCR analysis using gene-specific primers revealed that pea *GSHS1* is equally expressed in leaves and nodules, but that pea *GSHS2* is expressed only in nodules. Pea *GSHS1* encodes a GSHS because pea leaves only express GSHS, whereas the product of *GSHS2* can be tentatively identified as a hGSHS because pea plants express hGSHS in nodules but not in leaves. Bean *GSHS2* is expressed at the same level in leaves and nodules and encodes a hGSHS since bean plants only express hGSHS. These assignments of enzyme activity were reinforced by the cluster analysis and were confirmed by the subcellular localization of the enzymes in pea and bean nodules, which is reported in another abstract.

S4-P28

INFLUENCE OF THE FORM OF NITROGEN NUTRITION REGIME ON SHOOT, NODULE AND ROOT GROWTH OF PEA: RELATIONSHIP BETWEEN NITROGEN FIXATION AND SOIL NITROGEN AVAILABILITY.

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The contribution of symbiotic fixation to overall N acquisition by legumes is affected by mineral nitrogen availability in the soil, which inhibits nodule formation and nitrogenase activity and determines the amount of mineral N assimilation that may complement fixation. Although plants relying on different N nutrition regimes seem to develop similar shoot systems in terms of shoot biomass accumulation (C and N) symbiotic fixation is thought to have higher carbon cost than mineral N assimilation. Therefore, the N nutrition regime might influence mainly below ground parts of the plant, a possibility which has never been explored. Moreover, there has never been any attempts to establish a quantitative relationship between symbiotic fixation activity and mineral N availability in the soil.
The influence of nitrogen soil availability and nitrogen nutrition regime on growth and symbiotic fixation under adequately watered conditions was investigated in a field experiment with Pea, *cv Baccara*. The level of symbiotic fixation inhibition was varied using 4 applications of N–fertilizer : 0, 100, 200 or 400 NH_4NO_3 – N kg/ha at sowing. Throughout the growth cycle (1) inorganic N in the soil was measured regularly, (2) plants were harvested for measurement of C and N accumulation, (3) root biomass was determined on excavated soil blocks and (4) the number of roots was counted along soil profiles accessed by excavating a trench adjacent to a row of plants. Nitrogen fixation was estimated using the ^{15}N isotope dilution technique with barley as a reference crop.
The applications of fertilizer resulted in differences in N nutrition regimes ranging from 80 % N_2 fixation (0 N) to 15 % N_2 fixation (400 N). There was no effect of the N nutrition regime on shoot development, including phenology and yield, but strictly N_2 fixing plants had significantly less biomass partitioned to the roots as compared with treatments that received mineral N. Hence, additional carbon was made available for root growth and exploration, probably resulting from lower respiratory costs associated with symbiotic fixation inhibition. The level of symbiotic fixation activity was linearly related to soil N availability up to 65 kg N/ha but above this level of soil mineral N symbiotic fixation was completely inhibited. This relationship was valid regardless of growth stage or treatment.
These results are used to build a model of C partitioning from shoots to roots which integrate the effects of nitrogen soil availability on morphogenesis and activity of the root compartments (ie roots and nodules).

S4-P29

EXPRESSION OF PLASTID-LOCATED GLUTAMINE SYNTHETASE IN ROOT NODULES OF *MEDICAGO TRUNCATULA*.

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Glutamine synthetase (GS) is an essential enzyme in nitrogen metabolism of higher plants. In legumes, GS plays a key role in root nodules being responsible for the assimilation of ammonia that is released at high rates by nitrogen-fixing rhizobia. In the model legume, *M. truncatula*, gs is encoded by only three expressed genes, one that codes for a plastid-located polypeptide (*mtgsd*) and two encoding polypeptides localised in the cytosol (*mtgsa* and *mtgsb*) (Stanford et al, 1993). It is generally accepted that the ammonium excreted from the bacteroids is assimilated by the cytosolic GS which is abundantly present in the infected cells. However, plastidial GS isoenzymes represent a significant part of GS activity in nodules of *M. truncatula* (Carvalho et al, 1997). The aim of this study is to clarify the importance of nodule plastidial GS in the legume-*Rhizobium* symbiosis.
A full-length 1.55 kbp GSd cDNA clone was isolated from a nodule cDNA library. The cDNA encodes a 428 amino acid precursor to the plastid GS polypeptide, including a transit peptide of 49 aa. In order to determine the kinetic properties of the plastid GS we have expressed the cDNA, without the transit peptide in *Escherichia coli*. The *E. coli* produced enzyme was catalytically and physiologically active as it was able to rescue a *glnA* deletion mutant by complementation. The expressed polypeptides were of the correct size, as revealed by western blots, and the isoenzyme behaved similarly to their native homologues on ion-exchange chromatography.
Northern and western blot analysis showed that expression of GSd is enhanced during effective nodule development. By contrast ineffective nodules express *MtGSd* at much lower levels. These results strongly suggest that the plastid isoform of GS performs some important role for nitrogen fixation.

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S4-P30

PURIFICATION AND CHARACTERISATION OF ANFA FROM *AZOTOBACTER VINELANDII*

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Azotobacter vinelandii is able to synthesise three genetically different nitrogenases, which are characterised by the presence of either a molybdenum, vanadium or iron atom in their respective co-factors. Each of the nitrogenases requires an activator to express the structural genes required for the enzyme. AnfA is the activator protein for the iron only nitrogenase.
AnfA has the characteristic three-domain structure of a σ^N–dependent transcriptional activator, where the N-terminal domain is thought to have a role in environmental sensing. Previous work has shown that AnfA has a requirement for the iron-protein (VnfH) of the vanadium nitrogenase *in vivo*[1]. An amino-terminally truncated AnfA has been shown to remain constitutively active *in vivo*[2] and *in vitro*[3], implying regulation of AnfA is via the N-terminus. A conserved cysteine motif is present at the N-terminal domain, which is vital for its activity[4]. This motif could be involved in the formation of a metallocluster, which may regulate the activity of the protein in a redox sensitive manner.
We have purified the native full-length AnfA and also the isolated N-terminal domain. The N-terminal domain has been shown to contain a red/brown chromophore. The chromophore exhibited similar spectral features to Fe-S clusters identified in other proteins. Data will be presented on the biochemical characterisation of the N-terminal domain.
In vitro experiments to assess the transcriptional activity of full-length AnfA will also be described.

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S4-P31

THE *fixN* REITERATED GENES IN *RHIZOBIUM ETLI* CFN42 ARE DIFFERENTIALLY REGULATED BY AN UNUSUAL FixL-FixK CASCADE

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For most *Rhizobium* species, two regulatory cascades (the FixL-FixJ-FixK and the NifA-RpoN cascades) have been described. The FixLJ and FixK proteins are the first effectors for regulation of nitrogen fixation gene expression. A common function of the FixLJ-FixK cascade is the regulation of the *fixNOQP* genes Although the overall characteristics of this regulatory system constitute an useful conceptual framework to understand the regulation of nitrogen fixation in *Rhizobium*, there are important differences between species. In particular, it has been recognized that the connectivity between the different cascades, the specific regulatory role of each element as well as its target genes may vary among the different nitrogen fixers (Fischer, Microbiol. Rev. 58: 352-386, 1994). Further differences are imposed by the presence of reiterated DNA sequences.

In the type strain of *R. etli* (CFN42) most of the *nif* and *fix* genes already described are localized on the symbiotic plasmid (Girard et al. J. Bacteriol. 173: 2411-2419, 1991). However, important regulatory elements described in other *Rhizobium* species, such as *fixL* and *fixJ*, are not encoded on this plasmid. In this work, we show that the only copy of *fixL* in this strain is located on a plasmid hitherto considered as cryptic (pCFN42f). In this plasmid, there is also a *fix* reiterated region containing copies of *fixK*, *fixNOQP* and *fixG* genes. Detailed expression analysis and mutation of the regulatory elements present in each reiteration reveals that the FixKf protein is needed as a positive element for expression of both *fixN* reiterations, whereas the FixKd protein appears to be dispensable. Notably, both *fixN* copies are regulated by a FixK protein encoded in a plasmid (pCFN42f) other than the pSym. Interestingly, expression of the *fixN* reiterations show a diferential dependence for FixL. Microaerobic expression of *fixNf* is totally suppressed in a *fixL::loxSp* mutant background, while expression of *fixNd* still shows microaerobic induction, albeit at a reduced level. This suggests the existence of a FixL-independent mechanism for expression of the fixNd reiteration.

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S4-P32

RESPONSE OF *MESORHIZOBIUM CICERI* TO SODIUM CHORIDE STRESS

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The characterization of salt-tolerant strains which also show efficient symbiotic performance under salinity may constitute a strategy to improve *Cicer arietinum-Mesorhizobium ciceri* symbiosis in stress environments. In this work the changes induced by salt stress in protein profiles have been investigated. *M. ciceri* responded to increased sodium chloride concentration en the medium by elevating the intracellular concentration of amino acids, glutamate and proline. The results confirm that *M. ciceri*, strain ch-191 is a moderately salt-tolerant strain (up to 200 mM NaCl) although higher salt dosages limited its growth. The protein profile showed major alterations at salinity levels which inhibited growth, mainly the lack of some low molecular weight polypeptides and an increase in the relative abundance of several high molecular weight polypeptides. Salt significantly increased the total amino-acid pool, this increase may be a consequence of protein degradation, although the accumulation of high-molecular-weight polypeptides may suggest the opposite, acording to this results. On the other hand, the accumulation of compatible solutes were evident from the lowest levels, suggesting that these changes represent adaptative responses to salt, allowing normal growth. In *M. ciceri*, in response to salt stress, the accumulation of proline surpasses that of glutamate.

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S4-P33

FUNCTIONAL STUDY OF THE *RHIZOBIUM LEGUMINOSARUM* BV. VICIAE UPM791 FnrN MICROOXIC REGULATOR

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Two copies of the *fnrN* gene control microoxic expression of both nitrogenase and hydrogenase activities in symbiotic cells of *Rhizobium leguminosarum* bv. viciae UPM791 (Hernando *et al.* 1995; Gutierrez *et al.* 1997). In addition, FnrN induces the expression of a large number of genes in microaerobic free-living cells (Sidler *et al.* unpublished results). FnrN belongs to the Crp/Fnr family of transcriptional regulators. The anaerobic regulator Fnr of *E. coli* is the best studied member of this family. Fnr contains a three cysteine arrangement at the N-terminus and a fourth conserved Cys in the central domain that are involved in redox sensing. These cysteines are essential for Fnr functionality. Additionally, several Fnr mutants insensitive to oxygen have been isolated. In contrast, FnrN of *R. leguminosarum* bv. viciae UPM791 posesses a cysteine motif that slightly differs from that of Fnr of *E. coli*. An additional fifth conserved cysteine is found in FnrN proteins from different rhizobia and amino acid residues that confer oxygen resistance in Fnr are not conserved in FnrN. To further characterize FnrN functionality we have generated both site-directed and random mutagents of *R. leguminosarum* bv. viciae UPM791 FnrN. Functional analyses of FnrN proteins mutated in each cysteine residue as well as in the C-terminal helix-turn-helix domain have been carried out by measuring activation of FnrN-dependent promoters in *R. leguminosarum*. The results obtained show that all the cysteine residues in the motif C17-X2-C20-X7-C28 and C116 are required for FnrN functionality, likely by coordinating [Fe-S] clusters. It was expected that activation of FnrN-dependent promoters of *R. leguminosarum* in *E. coli* would simplify the screening of randomly generated mutants in FnrN. However, no expression of these promoters has been obtained in *E. coli* so far, probably due to an inefficient interaction between σ^{70} from *E. coli* and FnrN from *R. leguminosarum*. This situation prompted us to clone the σ^{70} factor of *R. leguminosarum* bv. viciae UPM791. Expression of FnrN-dependent promoters of *R. leguminosarum* in *E. coli* as a function of σ^{70} and FnrN of *R. leguminosarum* is now underway.

Hernando et al. (1995) J. Bacteriol. 177: 5661-5669.
Gutierrez et al. (1997) J. Bacteriol. 179: 5264-5270.

S4-P34

POSITIVE AND NEGATIVE AUTOREGULATION OF *FNRN* GENE IN *RHIZOBIUM LEGUMINOSARUM*

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The *fnrN* gene from *Rhizobium leguminosarum* UPM791 controls microaerobic expression of both nitrogen fixation and hydrogenase activities in symbiotic cells. Two copies of *fnrN* are present in this strain, one chromosomal (*fnrN1*), and the other located in the symbiotic plasmid (*fnrN2*). A double mutant (DG2) affected in both copies induced ineffective nodules lacking both hydrogenase and nitrogenase activities. In this mutant the *hypBFCDEX* operon is not expressed, and this accounts for the hydrogenase-deficient phenotype. Regarding the possible control of FnrN over nitrogen fixation, in other rhizobial species the *fixNOQP* operon is subject to microaerobic control through an anaerobox recognized by FixK. This operon encodes components of a microaerobic cbb_3 terminal oxidase essential for bacteroid respiration. Analysis of the regulation of *fixNOQP* in *R. leguminosarum* UPM791 (Gutiérrez et al., 1997) revealed that it contains a functional anaerobox activated by FnrN, thus explaining the Fix-deficient phenotype of the *fnrN*-null mutant. In contrast to the situation described in other rhizobia, no functional evidence has been found for the existence of a *fixK* gene in *R. leguminosarum* UPM791.
By cloning the regulatory regions in *lacZ* promoter-probe vectors and testing their expression in wild type and fnrN mutants, the *fnrN* genes were found to be autoregulated (Colombo et al, 2000). Meanwhile in wild-type strain both *fnrN1* and *fnrN2* genes were expressed only at basal levels under aerobic conditions and at high levels under microaerobic conditions, they are expressed at basal levels in the double mutant DG2 (*fnrN1 fnrN2*) under any condition. The promoters of both genes contain two FnrN-binding sequences (anaeroboxes), centered at positions -12.5 (proximal anaerobox) and -44.5 (distal anaerobox). Expression analysis and gel retardation experiments with *fnrN1*-derivative promoter mutants altered in key bases of the anaerobox sequences demonstrated that binding of FnrN1 to the distal anaerobox is necessary for microaerobic activation of transcription, and that binding of FnrN1 to the proximal anaerobox results in transcriptional repression. The apparent affinity of FnrN1 for the proximal anaerobox was 5-fold lower than for the distal anaerobox, resulting in repression of transcription of *fnrN1* only at high FnrN1 concentrations. This positive and negative autoregulation mechanism ensures an equilibrated expression of *fnrN* in response to microaerobic conditions.

Gutierrez, D. *et al.* (1997) J. Bacteriol. 17: 5264-5270.
Colombo, M.V. *et al.* (2000) Mol. Microbiol. 36: 477-486.

S4-P35

A COMMON ORIGIN FOR *NIFV* AND *LEUA* GENES

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The enzymes a-isopropyl malate (a-IPM) synthase (EC 4.1.3.12) and homocytrate (HC) synthase (EC 4.1.3.21) catalyze very similar condensation reactions. a-IPM synthase, encoded by *leuA is* responsible for the condensation reaction between aceyl coenzyrne A and ot-ketoisoyalerate, and represents the first enzyme of the leucine biosynthetic pathway in both bacteria and fungi. HC synthase, encoded by *nifV* catalyses the condensation reaction between Acetyl-CoA and a-ketoglutarate. In prokaryotes the product of this reaction (homocytrate) is involved in nitrogen fixation, whereas in fungi is involved in lysine biosynthetic pathway. Homology between a-IPM synthase from *Salmonella typhimurim* and *Saccharoimyces cerevisiae* and the product of the *nifV* gene from *Azotobacter chroococcum, Azotohacter vinelandii* and *Klebsiella pneumoniae* was already observed. Recently the sequence similarity between ORF 1298 of *S. cerevisiae* and *nifV* of *A. vinelandii* led to the identification of LYS20, the major gene encoding HC synthase in S. *cerevisiae.* The availability of complete sequenced archaeal, bacterial and eukaryal (*S. cerevisiae)* genomes enabled a detailed comparative analysis of the *leuA* and *nifV* gene products. This analysis confirmed the high degree of sequence similarity between the two proteins, and suggested a common ancestry by paralogous gene duplication and subsequent evolutionary divergence. The presence of multiple copies of these genes in several microorganisms belonging to the three cell domains suggested that the ancestral gene, or one of its copies, underwent additional paralogous duplication events, leading to the formation of paralogous gene families. Moreover the presence of *leuA* and *nifV* in organisms belonging to the three cell domains also suggested that at least one copy of each *leuA* and *nifV* was present in the genome of LUCA (Last Universal Cormnon Ancestor), supporting the idea of an early origin of nitrogen fixation.

POSTER ABSTRACTS
SYMPOSIUM 5

S5-P4

NITROGEN FIXATION IN *ACETOBACTER DIAZOTROPHICUS*.

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Acetobacter diazotrophicus is a gram-negative diazotrophic bacterium, endophytic in sugar cane and other economically important crops. It has been proposed to be the main supplier of fixed nitrogen to the sugar cane. Two central issues in the understanding of this endophytic system are the processes of fixation, assimilation and export of nitrogen compounds from the bacterium to the plant, and secondly the mechanisms by which the bacterium utilizes carbohydrates from the plant as an energy and reductant source under aerobic conditions. The results of our studies concerning the latter will be presented.
In order to study the energetic requirements of *A. diazotrophicus* for the nitrogen fixation process, nitrogenase activity was monitored with pyruvate or glucose as energy/reductant source under different oxygen levels. From these experiments we show that in the presence of pyruvate and high oxygen levels, nitrogenase is initially inactivated and thereafter degraded, probably as consequence of oxidative damage. Glucose on the other hand can support nitrogenase activity even under high oxygen levels. In addition glucose can support recovery of nitrogenase activity after inactivation under pyruvate and high oxygen, in a translation-independent manner .
We have detected a protein cross-reacting with antibodies raised against the FeSII Shetna protein of *Azotobacter vinelandii*, which could indicate the transient formation of a complex between this protein and nitrogenase when cells are exposed to high oxygen in the presence of a poor energy/reductant source. Studies are in progress to identify this protein and investigate whether it in fact does protect *A. diazotrophicus* nitrogenase.

S5-P5

PRODUCTION OF AUXINS BY FREE-LIVING AND SYMBIOTIC CYANOBACTERIA

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Phytohormones play a crucial role in regulation of development and physiological proceses in plants. The possible phytohormone production by prokaryotes such as the large and structurally diverse group of cyanobacteria still remains an unexplored research area. Screening symbiotic and free-living strains for their ability to produce auxine-like substances was therefore performed using monoclonal indole-3-acetic acid (IAA) antibody in a competitive enzymelinked immunosorbent assay. The effect of additions of tryptophan, the main precursor for the biothynthesis of (IAA) was also investigated. Comparative analyses of selected free-living and symbiotic strains demonstrated that IAA levels in cell extracts and being secreated from the cells were considerable and more in the symbiotic isolate *Nostoc PCC* 9229. Tryptophan stimulated IAA synthesis as did the age of the culture. The biosynthesis phytohormones and their role in the cyanobacterial plant symbiosis, like the *Nostoc-Gunnera* symbiosis (1) is discussed.

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S5-P10

PCR-AMPLIFICATION, SEQUENCING, CLONING, SITE-DIRECTED MUTANTS, ACTIVITY AND ROLE OF LEVANSUCRASE FROM *ACETOBACTER DIAZOTROPHICUS*

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Levansucrase enzymes from gram negative and gram positive bacteria have a hydrolytic and a polymerase activity. The hydrolytic activity releases glucose and fructose from sucrose and the polymerase activity adds fructose to different acceptors.
Wild type polymerase levansucrase activity isolated from culture supernatants of *A. diazortrophicus* Pal 3 was characterized. In addition to sucrose (Glu-Fru), we previously detected polymerase activity with raffinose (Gal-Glu-Fru) and stachyose (Gal-Gal-Glu-Fru) as substrates but not with melezitose (Glu-Fru-Glu). The ratio of Glu/Fru products obtained from sucrose as well as the amount of fructose released from raffinose were determined using an enzyme assay (1). With both substrates, the formation of a product of higher degree of polymerization than the substrates could also be detected.
The levansucrase gene from *Acetobacter diazotrophicus* Pal 3 was PCR-amplified, se-quenced and cloned. The sequence obtained was the same as that of a different strain SRT4 of *A. diazotrophicus* (2). Two site-directed mutants of the wild type gene in the Histidine residue that has been proposed to have a role in substrate specificity (3) were obtained and their activity is being expressed and compared with the wild type activity.
Acetobacter diazotrophicus is a nitrogen-fixing endophyte of sugar cane. These mutants will be useful tools to determine the role of levansucrase polymerase activity as a means of survival in the sugar cane host, a natural environment with extremely high sucrose concentration and osmotic pressure as well as in nitrogen fixation by *Acetobacter diazotrophicus*.

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S5-P11

RHIZOBIUM PROMOTION OF SORGHUM GROWTH AND P NUTRITION WITH ROOT INFECTION AND LOCALIZATION IN THE XYLEM.

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Sorghum seedlings grown aseptically in Leonard jars with one-half strength Hoagland nutrient solution adjusted to contain 1 mM KNO_3 were inoculated with 4 different strains of root-nodule bacteria (i.e. rhizobia), namely *Bradyrhizobium japonicum* strain TAL 110, *Sinorhizobium meliloti* strain 1, *Rhizobium leguminosarum* bv. *viceae* strain Cn6 and *Rhizobium leguminosarum* bv *viceae* strain 30. The use of scanning and transmission electron microscopy established the presence of the bacteria on the surface and inside the xylem of inoculated roots. Applying rhizobial live cells to roots of the sorghum seedlings increased root length and biomass compared to uninoculated control. The difference in root length was significant ($P < 0.05$) at 94 d after planting, which in turn resulted in markedly higher ($P < 0.05$) P uptake by inoculated plants relative to uninoculated controls. Bacterial inoculation of sorghum seedlings also promoted shoot growth (on both fresh and dry weight basis) possibly due to the improved P nutrition. Bioassays of the test strains for indole acetic acid (IAA) showed that they produce biologically active amounts of this growth-promoting molecule. Our data thus demonstrate that 1) some rhizobia can naturally infect roots of non-legume cereal plants, and 2) if they produce biologically active levels of these auxins, they are more likely to promote plant growth through increased root development and nutrient uptake.

S5-P12

AN ENDOSYMBIOTIC *BURKHOLDERIA* STRAIN HARBOURS NITROGEN FIXATION GENES

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The widespread association between soil-born fungi and plant roots is known as mycorrhizal symbiosis. An arbuscular mycorrhizal fungus (*Gigaspora margarita* BEG 34) has been demonstrated to harbour a homogeneous population of endosymbiont *Burkholderia* (Bianciotto et al., 1996). A genomic library of total DNA extracted from the fungal spores was also representative of the bacterial genome, and was used to investigate the prokaryotic genome (van Buuren et al., 1999). The aim of the presentation is to demonstrate that the endosymbiotic *Burkholderia* posses a DNA region containing putative *nif* genes. Screening of the library with *Azospirillum brasilense nif*HDK genes as the prokaryotic probes led to the identification of a 6413 bp region. Analysis revealed three ORFs encoding putative proteins with a very high degree of sequence similarity with the two subunits (NifD and NifK) of the component I and with component II (NifH) of nitrogenase from different diazotrophs. The three genes were arranged in an operon similar to that shown by most archaeal and bacterial diazotrophs.The *nif*H ORF is preceded by an Ntr-A dependent promoter and two elements homologous to the Upstream Activator Sequences (UAS) required for NifA-mediated activation of *nif* genes in diazotrophs. PCR experiments with primers specifically designed on the *Burkholderia nif*HDK genes and Southern blot analysis demonstrate that they actually belong to the genome of the *G. margarita* endosymbiont. RT-PCR experiments with primers designed on the *Burkholderia nif*HD genes and performed on total RNA extracted from spores demonstrate the gene expression was limited to the germination phase.
A phylogenetic analysis performed on the available *nif*K sequences placed the endosymbiotic *Burkholderia* closer to *Azospirillum brasilense* than to free-living *B. vietnamiensis* strain TVV75, known to fix N_2.

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Bianciotto, V., Bandi, C., Minerdi, D., Sironi, M., Tichy, H.V., and Bonfante, P. (1996b). Appl. Environ. Microbiol. 62: 3005-3010.

S5-P13

AMPLIFICATION AND SEQUENCING OF A REDUCTASE-LIKE PROTEIN FROM A NITROGEN-FIXING ACTINOMYCETE USING UNIVERSAL *nif*H PRIMERS.

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Various actinomycetes were isolated from nodules of *Casuarina equisetifolia*. These isolates are able to reduce acetylene to ethylene, do not develop typical *Frankia* vesicles and are not able to induce root nodules on Actinorhizal Plants. A phylogenetic study based on a partial sequences of the 16S rRNA gene showed that they form a clade different from the Frankiaceae[1]. In order to amplify the *nif*H gene of these actimomycetes, a set of degenerate universal *nif*H primers[2] were used. The PCR products were transferred to a membrane and hybridized with the *Rhizobium etli* CFN42 *nif*H gene. A 396 bp band from the isolated 7501 was cloned. The vector utilized was pBlueScript SK- and *E. coli* DH5α was transformed by electroporation. Sequencing was done utilizing an ABI-PRISM automatic system. This sequence has 38% identity to *Frankia nif*H sequences, and the deduced amino acid sequence is 18% identical. A Blast-P search showed 50% identity and 70% of positives with an hypothetical reductase of *Streptomyces coelicolor* and significant identity with other proteins belonging to the aldo/keto reductase family. The role of this gene in the nitrogen-fixing system of this actimomycete is under investigation.

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S5-P14

ANALYSIS OF *argL*, A GENE INVOLVED IN ARGININE AND CYANOPHYCIN METABOLISM IN CYANOBACTERIA.

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In order to identify mutants of cyanobacteria in which nitrogen fixation is affected, we mutagenized the nitrogen-fixing heterocystous cyanobacterium *Nostoc ellipososporum* with a derivative of transposon Tn5. In one of such mutant strains, denoted NE1, the transposon was found within a gene whose translation product is similar in amino acid sequence to the arginine-biosynthetic protein N-acetylglutamate semialdehyde dehydrogenase encoded by *argC* of *Bacillus subtilis*. The *argC* reported from *Anabaena* sp strain PCC7120 (1) hybridized to a sequence different from the one interrupted by the transposon in NE1. The newly identified gene from *N. ellipsosporum* was denoted *argL* (2). The *argL* mutation renders certain processes in strain NE1 conditionally dependent on provision of L-arginine. Heterocysts and apparent akinetes that formed in the absence of added L-arginine failed to fix dinitrogen or to germinate, respectively, and lacked granules of cyanophycin, a copolymer of arginine and aspartic acid unique to cyanobacteria that is generally assumed to be a nitrogenous reserve material.
Under nitrogen fixing conditions, overexpression of *argL* in the wild type strain resulted in cells that were larger and profusely granulated. Measurements of cyanophycin contents showed that overexpressed cells contained, on average, 25% more cyanophycin than wild type cells. The presence of multiple copies of *argL* also resulted in a significant stimulation of the nitrogenase activity. The involvement of *argL* in arginine and cyanophycin metabolism as well as its relevance in the process of nitrogen fixation in cyanobacteria will be discussed on the basis of the presented results.

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S5-P15

NEW *AZOSPIRILLUM* AND *HERBASPIRILLUM* SPECIES FROM C4-FIBRE PLANTS AND 16S rRNA TARGETED OLIGONUCLEOTIDE PROBES

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Diazotrophic bacteria were isolated from the C4-fibre plants *Miscanthus sinensis* and *Pennisetum purpureum* grown in Germany and Brasil using semisolid, nitrogen free media. Appling the MPN-approach, up to 10^6 diazotrophic bacteria per gram root fresh weight were estimated. Among other known diazotrophs, like *A. lipo-ferum* and *Herbaspirillum seropedica*, new bacteria could be identified on the basis of 16S rDNA sequence analysis, DNA-DNA hybridization results, physiological tests and specific 16S rRNA-targeted oligonucleotide probes. The new diazotrophic *Azospirillum* sp. is closely related to the *A. lipoferum*, *A. largomobile* and *A. brasilense* and is named *Azospirillum doebereinerae* sp. nov. in honour of Dr. Johanna Döbereiner (Eckert et al., 2000). Until now, a PGPR-effect was observed, when *A. doebereinerae* was inoculated to *Miscanthus* plantlets. The new *Herbaspirillum frisingense* isolates were also characterized in a polyphasic approach (Kirchhof et al., 2000). After inoculation of *Miscanthus* plantlets with *H. frisingense* Mb11, a rapid colonization of the root interior could be demonstrated using mono-specific polyclonal antibodies, as well as a PGPR-effect on root development.
A hierarchical set of in situ binding 16S rRNA directed oligonucleotide probes at genus and species level was developed for the whole *Azospirillum-Skermanella-Rhodocista* cluster and the genus *Herbaspirillum* (Stoffels et al., 2000).

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S5-P16

A SURFACE PROTEIN OF *AZOSPIRILLUM BRASILENSE* SP245 AND INTERACTION OF THE BACTERIUM WITH WHEAT LECTIN

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Wheat lectin (wheat germ agglutinin, WGA) was found to cause significant changes in the *Azospirillum brasilense* Sp245 metabolism, including enhancement of IAA production, N_2 fixation and ammonia excretion. The lectin is suggested to serve as a signal molecule in the wheat - *A. brasilense* Sp245 symbiosis (Antonyuk *et al.*, 1995). The surface glycopolymer of *A. brasilense* responsible for the reception of WGA and the subsequent signalling remains unknown. Basing on the data on other azospirilla (Nikitina *et al.*, 1996; Karpati *et al.*, 1999) we proposed that *A. brasilense* Sp245 has a surface glycoprotein involved in WGA reception and signalling. A surface protein was isolated from strain Sp245 and purified using treatment with ethanol-acetone mixture, ammonium sulphate fractionation and chromatography on DEAE-Toyoperl 650 M. The protein showed hemagglutination and was thermally stable; its MW was found to be ca. 40 kDa. The structure of the hemagglutinin (HA) was studied using Fourier transform infrared spectroscopy. It was found that growth of strain Sp245 under N_2-fixation conditions in the presence of WGA caused induction of HA activity of the cells. Conversely, when the cells were grown under aerobic conditions in the presence of ammonium, this effect was not observed. We detected hemolytic activity of *A. brasilense* Sp245 that was observed in suspensions of very high densities only. When *A. brasilense* Sp245 was grown under N_2-fixation conditions, the presence of WGA in the growth medium brought about a sharp increase of hemolytic activity of the cells. It remains unknown yet whether hemolytic activity is an intrinsic property of the *A. brasilense* Sp245 HA or maybe the strain produces its own separate hemolysin like those produced by hemolytic strains of *E. coli* and some other pathogenic bacteria. This and some other questions (e.g. whether HA of *A. brasilense* Sp245 is a lectin; whether induction of hemolytic and hemagglutination activities of *A. brasilense* Sp245 under the influence of WGA is dependent on quorum sensing) are currently under study.

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S5-P17

MOLECULAR MECHANISMS OF DIAZOTROPHIC *KLEBSIELLA OXYTOCA* ENTER INSIDE THE PLANT TISSUE: THE ROLE OF POLYGALACTURONASE AND PECTATE LYASE

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Diazotrophic bacterium *Klebsiella oxytoca* VN13 is able to depolymerize a polymer of polygalacturonic acid – pectin, a compound of the plant cell wall, and possesses polygalacturonase (PG) and pectate lyase (PL) activity. Both activities are low and can be recognized well after induction with any substrates. To decipher molecular mechanisms of *K. oxytoca* enter inside the plant tissue we started with study of role of PG and PL in this process. Both activities were cloned in *E. coli* JM 109 that did not possess it naturally. Nucleotide sequences of the *peh* and *pel* genes were determined. Levels of homology of both DNA and aminoacid sequences of proteins coded for PG and PL of *K. oxytoca* and appropriate sequences of different bacteria deposited in GenBank were analysed, and no homology of DNA found for both the *pel* and *peh* genes. However, 63-77 % homology seen between aminoacid sequences of both pectinases of *K. oxytoca* VN13 and appropriate sequences of exopolygalacturonase (exoPG) and exopectate lyase of *Yersinia enterocolitica*, *Erwinia chrysanthemi* and *Ralstonia solanacearum*.
The *K. oxytoca* VN13 *peh* and *pel* gene mutants have got by recombination *in vivo* between normal copies and mutated ones. The mutants have been examined in interactions with the plant host (*Triticum aestivum* L.), using designed model system. It was defined that exoPG of *K. oxytoca* VN13 appeared did not play role in process of penetration into the plant interior, in contrast to exoPGs of phytopathogenic bacteria *E. chrysanthemi* and *R. solanacearum*. Pectate lyase activity of *K. oxytoca* VN13 positively correlated with penetration of bacterial cells inside the root tissue.

S5-P18

POPULATION OF ENDOPHYTIC BACTERIA IN THE STEMS OF SUGAR CANE GROWN IN TEMPERATE CLIMATE

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Population density of endophytic bacteria in the stems of sugar cane grown from the cuttings and the seeds in temperate climate has been studied. We showed that for complete sterilization removing of bark is a very important and crucial step. Total number of endophytes in the stems of sugar cane grown from the cuttings varied from 1.62 to $56.9x10^6$ cells g^{-1} of fresh stem. Population was uniformly distributed along the stem. Population of nitrogen-fixing bacteria varied from 3.60 to $5.37x9x10^6$ cells g^{-1} of fresh stem which comprised 0.94-30.1% from total endophytic population and was uniformly distributed inside the stem. Total number of endophytes in the stems of sugar cane grown from the seeds was very variable and has been changed from $0.47x10^2$ to $5.97x10^7$ cells g^{-1} of fresh stem. Nitrogen-fixing bacteria were not detected inside the all three sugar cane stems, which have been tested. Study of the other five stems showed presence of nitrogen-fixing bacteria inside the only two of them. Seeds of sugar cane and nursery soil possessing ARA could be a source of nitrogen-fixing endophytes for sugar cane grown from the seeds. Ten strains of endophytic nitrogen-fixing bacteria have been isolated and studied. Four of them showed higher ARA then the reference strain *Herbaspirillum seropedicae*.

S5-P19

CELLULASE PATTERNS IN *FRANKIA* STRAINS BELONGING TO DIFFERENT CROSS-INOCULATION GROUPS.

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Carboxymethyl cellulase activities were evaluated by different methods in eight strains of *Frankia* from diverse host specificity groups and geographical origins. Cellulase activity was found in culture supernatants of all strains in the absence of CMC (Carboxymethylcellulose) as inductor and was detected using both double-layer plate and reducing sugar assays. These findings indicate a constitutive production of CM-cellulases by *Frankia*. CM-cellulase isoenzymes were examined using activity gel electrophoresis of concentrated culture supernatants. Different electrophoretic patterns of CM-cellulases were observed among the eight strains tested, which can be relationed with previous taxonomic groupings obtained in this genus by different molecular techniques. Therefore, our results suggest that the analysis of cellulase isoenzyme patterns could be an additional and useful tool for the differentiation among strains of *Frankia* belonging to different cross-inoculation groups.

S5-P20

NEW ASPECTS OF NITROGEN FIXATION RESEARCH: INTERACTION OF *RHIZOBIUM LEGUMINOSARUM* bv. *TRIFOLII* STRAINS WITH THE NON-LEGUME RICE

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Very soon we shall have available a complete genome and proteome of a *Rhizobium* strain along with a complete genome of the non-legume rice. Several new technologies enable the researcher to now rapidly study global regulation of whole genomes in response to various stimuli and this represents a paradigm shift in genetic analysis. These new areas of analysis are underpinning current molecular genetics and are setting the pace of activity and re-stimulating new possibilities for old dreams of generating a reliable nitrogen-fixing interaction between a microbe and the non-legume.
Hence, we have investigated the colonisation and growth of rice seedlings using rice-rhizobia strains R4 and E4 (Yanni et al., 1997; Prayitno et al., 1999), the characterised *Rhizobium leguminosarum* bv. *trifolii* clover strain ANU843 and its plasmid cured derivatives (Rolfe et al., 1980). In addition, we have analysed rice seedlings for potential plant signals that can be sensed by rhizobia. Strain R4 stimulated rice seedling growth in a cultivar specific manner, while strains E4 and ANU843 inhibited the growth of rice seedlings (Prayitno et al., 1999). Using the Green Fluorescent Protein (GFP) as a constitutive marker, we found that strain R4 associated only with the first anchor root of rice seedlings and exhibited a particular colonisation pattern by forming long intercellular lines in lateral roots. In contrast, strains E4 and ANU843 infected at the lateral root junctions on different main roots. Various plasmid-cured derivative strains of ANU843 were investigated for their interaction with rice and the global regulation of gene expression in these strains was characterised by proteomic analysis. Our results showed that strain ANU845, cured of its Sym-plasmid, pa, inhibited rice seedling growth. Other derivative strains, however, lacking one of the pb, pc, pd or pe plasmids, or both plasmids pa and pc, or pa and pb, did not inhibit the seedling growth. Furthermore, a strain ANU843 derivative, cured of pc, showed a stimulatory effect with rice seedlings similar to that seen with strain R4 and had 10 protein spot differences to its parent strain. This result suggests that the presence of certain plasmids of strain ANU843 influences the effect of strain ANU843 on rice. Current experiments implicate the involvement of the phytohormone auxin in this complex rice-*Rhizobium* interaction.

S5-P21

DIRECT BOMBARDMENT OF NITROGEN FIXING BACTERIA INTO THE PLANT TISSUES

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Biological nitrogen fixation is a very effective form of nitrogen supply in nature, and is mostly realised in symbioses and associations between plants and diazotroph bacteria. The problem is that these bacteria form symbioses or associations mainly with wild species and not with economically important plants. Several attempts have been made to widen the range of the natural symbioses in the last two decades. One of the solutions is creating artificial symbioses.
Our aim is to establish artificial associations by in vitro methods using strawberry (*Fragaria x ananassa*) as host plant and Azotobacters as diazotrophic partners and the prolonged cultivation of these systems. The realization of such type of symbiosis may result considerable reduction in production and utilization of nitrogen fertilizers.
Incorporation of bacteria into the plant tissues were carried out by biolistic gun which is used for genetic transformation. *Azotobacter vinelandii* cells adhered to tungsten particles were shot directly into young leaves and regenerating shoot tips. Bacterium cells could be landed in two positions: in the intercellular spaces of the target tissue or inside the cells.
The presence of bacteria in the developing callus tissues and regenerating plants was detected by light and electron microscopy, the nitrogen fixing capacity could be detected by acetylen reduction assay.
For easier detection of the incorporated bacteria inside the plant tissues, *Azotobacter vinelandii* was genetically transformed: GUS marker gene was electroporated into the bacterium genome. This allows for safer selection of the succesful symbioses.
This was the first time to use living bacteria as microprojectiles for bombardment of plant tissues. The results undoubtedly show that this new method, developed by us, makes possible the introduction of nitrogen fixing bacteria into the economically important plants.

S5-P22

IDENTIFICATION AND CHARACTERIZATION OF GENES INVOLVED IN THE AMMONIUM SENSING MECHANISM IN *ACETOBACTER DIAZOTROPHICUS*

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The PII-like proteins, encoded by the *glnB* and *glnZ/glnK* genes, and the uridylyltransferase, product of the *glnD* gene, play a major role in the ammonium sensing mechanism and ammonia assimilation in bacteria.
DNA sequence analysis of the corresponding gene loci of *A. diazotrophicus* revealed a *glnA* downstream of *glnB* and an *amtB*-like gene adjacent to *glnZ*, while the *glnD* gene is cotranscribed with a *mutS*-like gene.
In order to understand the physiological role of the gene products and their involvement in the regulation of nitrogen fixation, a marker exchange mutagenesis was carried out. While interposon mutants of *glnZ* are not impaired in N_2-fixation, *glnB* mutants as well as the corresponding double-mutants are able to express nitrogenase even under high N-conditions. Thus, the PII protein could be involved in regulation of *nif* gene expression via interaction with transcription activator NifA (*nifA* expression is independent from NtrC (1)). In addition, the growth rate of double-mutant (*glnB*::kan; *glnZ*::*gusA*cat) is slightly decreased in rich medium compared to the wild-type strain Pal5 and drastically reduced in minimal medium with arginin as the sole nitrogen source.
Furthemore, *glnD* mutants of *A. diazotrophicus* exhibit a severe reduction of the growth rate even in rich medium and are unable to grow in minimal medium with ammonium as N-source.
To understand the function of the *amtB* gene product in *A. diazotrophicus* chromosomal interposon mutants of *amtB* were constructed. These mutants are able to grow on the limiting amounts of ammonium asnd uptake of ^{14}C-methylammonium is only slightly reduced. Thus, *A. diazotrophicus* is probably carries second transport system for ammonium.
Work is in progress to understand the regulation of expression of *glnb* and *glnz* genes as well as the postranslational modification pattern of the corresponding gene products.

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S5-P23

INCREASE IN FREE NITROGEN FIXATION IN THE RHIZOSPHERE OF *PINUS PINEA* SEEDLINGS INOCULATED WITH PGPRs IN A BURNED FOREST SOIL

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Free living diazotrophic bacteria are highly dependent on availability of organic substrates since free nitrogen fixation is a high energetic cost process. Organic matter content in the rhizosphere is mainly due to exudation (Glick, 1995).
The use of PGPRs as biofertilizers is one of the most promising biotechnologies to improve primary production with low inputs in fertilizers, through any of the many mechanisms possible: biocontrol, nutrient mobilization, phytohormone production and nitrogen fixation.
The aim of this study was to determine the effect of inoculating the PGPRs *B. licheniformis* (CECT 5106) and *B.pumilus* (CECT 5105) and the ectomycorrhiza *Pisolithus tinctorius*, on free nitrogen fixation in the rhizosphere of *P. pinea* seedlings grown on a burned forest soil, after 4 months of inoculation. Nitrogen fixation was determined by the Acetylene Reduction Activity (ARA) according to McNabb and Geist (1979) in the conditions proposed by Gutiérrez Mañero *et al.* (1994).
Results show an increase in nitrogen fixation under the influence of PGPRs probably due to the "sink effect" of bacteria, that stimulate exudation of sugars. An increase in ammonium is also detected in the rhizosphere, although this increase may be partially due to exudation. The increase in the nitrifying activity detected reflects in an increase in nitrate levels in the rhizosphere, although levels are below inhibitory limits of nitrogen fixation.

S5-P24

THE GS-GOGAT PATHWAY IS NOT OPERATIVE IN THE HETEROCYSTS FROM THE CYANOBACTERIUM *Anabaena* sp. PCC 7120. CLONING AND EXPRESSION OF *glsF* GENE.

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The gene encoding the ferredoxin-dependent glutamate synthase (Fd-GOGAT), *glsF*, from the heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120, has been cloned and sequenced. The *glsF* gene encodes a protein of 1549 amino acid residues. Comparative analysis of the deduced amino acid sequence shows a strong identity with the counterpart Fd-GOGATs from the cyanobacteria and with the corresponding Fd-GOGATs from higher plants. Unlike other cyanobacteria, *Anabaena* 7120 contains only Fd-GOGAT, lacking NADH-GOGAT. The amount of *glsF* transcript and Fd-GOGAT activity were similar under all the nitrogen growth conditions tested. Enzyme activity, western and northern blot analyses indicated that Fd-GOGAT is absent in the heterocysts, while glutamine synthetase (GS) and NADP-isocitrate dehydrogenase (IDH) were present in these specialised cells. Our results clearly indicate that the GS-GOGAT pathway is not operative in the heterocysts, and hence glutamate must be imported from the adjacent vegetative cells, to sustain GS activity. Heterocysts probably export glutamine or another nitrogen rich compound like arginine to the vegetative cells.

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S5-P25

TRANSGENIC AEQUORIN REVEALS CALCIUM TRANSIENTS IN RESPONSE TO NITRATE REMOVAL IN THE NITROGEN-FIXING HETEROCYSTOUS CYANOBACTERIUM *ANABAENA* sp. PCC 7120

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In order to investigate calcium-mediated processes in cyanobacteria, we constructed a recombinant strain of *Anabaena* sp. PCC7120 that constitutively expresses the Ca^{2+} binding photoprotein apoaequorin (1). Firstly, the homeostasis of intracellular Ca^{2+} was studied in this strain, finding that *Anabaena* has the capacity to regulate its intracellular Ca^{2+} levels. The resting level of free Ca^{2+} was found to be very similar to that reported for eukaryotic cells, between 100 and 200 nM.
Anabaena sp. PCC7120 differentiates heterocysts, specialized cells where aerobic nitrogen fixation takes place, upon removal of bound nitrogen (nitrate or ammonium). We used the recombinant strain expressing cytosolic apoaequorin to study whether Ca^{2+} signalling could be involved in the differentiation process. We found that a Ca^{2+} transient was induced shortly (from 30 to 90 min) after removal of nitrate. The transient lasted from 3 to 4 hours and reached a magnitude of approx. 400 nM. However, such a transient was not observed upon removal of ammonium. Other relevant nutrient deficiencies such as phosphate and iron deficiencies did not induce the transient either. The transient was never observed when cells were shifted from nitrate to ammonium. Thus, the observed Ca^{2+} change seems to be a specific response to nitrogen deprivation conditions following nitrate removal. We are currently investigating the biological significance of the observed Ca^{2+} transient.

Acknowledgements: this work was supported by DGESIC grants PB96-0487 and PB98-0114-C02-01.

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S5-P26

EFFECT OF STRUCTURAL ALTERATION OF THE POLYSACCHARIDE PORTION OF LIPOPOLYSACCHARIDES ON THE BIOLOGICAL ACTIVITY OF *AZOSPIRILLUM*

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The role of lipopolysaccharides (LPS) in plant-microbial associations is just beginning to be studied. The best way of studying the functions of individual structures is the use of mutants defective in the synthesis of the component of interest. In this work, we used the bacterium *Azospirillum brasilense* Sp245 and its Omegon-Km mutants KM252 and KM018. The mutants differ from the wild strain in the structure of the polysaccharide (PS) portion of LPS, as found by immunochemical analysis (Katzy et al., 1998)

We performed comparison studies of adsorption of *A. brasilense* Sp245 and KM252 to isolated roots of 3-day-old seedlings of the wheat cultivar Saratovskaya 29. The number of root-attached bacterial cells was determined by plating the root homogenate with adsorbed microorganisms on to solid nutrient media. In both cases, the number of seedling root-adsorbed cells increased with time, and by 3h of exposure it reached a value of (2.63±0.48) x 10^6 for the wild strain and a value of (3.35±0.94) x 10^5 for the mutant strain. The adsorption ability of *A. brasilense* Sp 245 was about seven times greater than that of *A. brasilense* KM252 over the entire period (0.25 to 48 h) of coincubation of roots and microorganisms.

LPSs were isolated from the outer membrane of *A. brasilense* Sp245, KM018 and KM252 and chromatographically purified. Using the slide technique, we found that LPS was able to induce root hair deformation in 2-day-old wheat seedlings. The LPS of the wild strain increased by 60% the number of deformations per 1 cm of root length as compared with the control. The activity of the LPS from the wild strain was two times greater than that of the LPSs from the mutant strains.

Using chromatographic techniques, we showed the presence of two O-specific polysaccharides (O-PSs), a neutral one (O-PS1) and an acidic one (O-PS2), in the LPS from *A. brasilense* Sp245. The LPS of the mutant strain KM252 contained only the neutral O-PS1*, and that of KM018 contained only the acidic O-PS2*. The data obtained were confirmed by the results of electrophoretic and immunochemical analysis of the LPS preparations.

Thus, our investigations showed that the mutation-induced impoverishment of the PS portion of the LPSs leads to a decreased biological activity of both intact *Azospirillum* cells and their LPSs.

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S5-P27

PRODUCTION OF VITAMINS BY *AZOTOBACTER* WITH PHENOLIC COMPOUNDS AS SOLE CARBON SOURCE.

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Production of water soluble vitamins has been related to the ability of *Azotobacter* spp. to enhance crop yielding and positively influence the *Rhizobium*-legume symbiosis, as exogenous supply of these biologically active substances affects plant growth and rhizosphere interactions (1,2,3). Qualitative and quantitative production of B-group vitamins by *A. vinelandii* in laboratory media supplied with 0.5 to 5% glucose is influenced drastically by the available concentration of carbon substrate (4). However, glucose is hardly the major carbon source available for *Azotobacter* in the rhizosphere, where survival and persistence of these bacteria is believed to be highly dependent on their ability to metabolize simple phenolic compounds, which are commonly present in soils at concentrations of 1-2 mM.

A. vinelandii ATCC 12837 and *A. chroococcum* strain H23 (CECT 4435) were able to grow on N-free or NH_4Cl-amended chemically-defined (Burk's) media, with protocatechuic acid (1 to 2 mM) or sodium *p*-hydroxybenzoate (1 to 10 mM) as sole C sources. At a concentration of 2 mM, both substrates supported nitrogen fixation (acetylene reduction assay) at similar or higher rates than in control media amended with 2 mM sodium succinate as C source. The two strains produced the B-group vitamins niacin, pantothenic acid, thiamine, riboflavin and biotin after 72 hours of growth in chemically-defined media with 2 mM protocatechuic acid, sodium *p*-hydroxybenzoate or sodium succinate as sole carbon source, either in N-free or in media amended with 0.1% NH_4Cl. Quantitative production of all vitamins was affected by the use of the different C and N substrates.

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POSTER ABSTRACTS
SYMPOSIUM 6

S6-P1

FURTHER STUDIES OF THE PHYTOHORMONE INVOLVEMENT INTO THE NODULE DEVELOPMENT DURING PEA-*RHIZOBIUM* SYMBIOSIS.

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We continued our investigationes of the functions of phytohormones in the pea (*Pisum sativum* L.)–*Rh. leguminosarum* bv. *viciae* symbiosis by the including of some new pea genotypes. We studied the sensitivity to exogenous auxin of two hypernodulating pea lines P64 (*sym28*) and P88 (*sym29*), obtained on cv. Frisson (Sagan, Duc, 1996). The differences between P64 plants and parental cultivar were revealed: on the medium containing 20 mg/l of auxin the root explants of cv. Frisson (and P88) formed calluses whereas P64 roots died. From the other side we investigated the nodule forming abilities of two allelic pea *ageotropum* lines (Blixt, 1970), which are probably defective in root auxin transport system. Both mutants had significantly decreased amount of nodules in compare with parental cv. Weibull's Weitor.
From studied earlier pea forms, which did not differ from parental genotypes on phytohormonal traits, we chose Nod$^-$ lines of different origin with mutation in gene *sym8* (line R25, obtained on cv. Sparkle, from the collection of T. LaRue, Boyce Thompson Institute, USA; lines Sprint2 Nod-1 and Nod-2, obtained on the line Sprint2, from the collection of Russian Research Institute for Agricultural Microbiology, Russia). It was proposed, mutations in *sym8* gene led to the structural and functional changes in plant cell membrane and/or cell wall (Borisov et al, 1994). To test it we began the experiments with nystatin, the polyene antibiotic, targeted plasma membrane of eucariotic cells (Koller, 1992). The results of the experiments with *sym8* lines and also with *sym9* line R72, which has symbiotic phenotype similar to the *sym8* lines (Markwei and LaRue, 1992), will be presented.

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S6-P2

CHARACTERIZATION OF *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII pssB* GENE PRODUCT AS INOSITOL MONOPHOSPHATASE WHICH INFLUENCES THE EXOPOLYSACCHARIDE SYNTHESIS.

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Rhizobium leguminosarum bv. *trifolii* produce a large amount of the exopolysaccharide (EPS) that is required for establishment of the symbiosis with clover. The EPS is a polymer composed of repeating octasaccharide units, which consist of five glucoses, two glucuronic acids and one galactose with acetyl, pyruvyl and hydroxybutanoyl modifications. The assembly of the repeat units begin by transfer of glucose-1-phosphate to isoprenylphosphate (IP) carrier by *pssA* gene product. The *pssDE* and *pssC* genes are involved in the later steps of the synthesis (Becker and Puhler 1998, review). The *pssB* gene located upstream of the *pssA* encodes protein homologous to a family of inositol monophosphatases (IMPases). Mutation in the *pssB* gene caused increased EPS synthesis and non-nitrogen-fixing phenotype of *Rhizobium leguminosarum* bv. *trifolii* mutant on clover (Janczarek et al. 1999). Extra copies of *pssB* gene introduced to the wild type of *Rhizobium leguminosarum* bv. *trifolii* strain TA1 resulted in the reduced EPS production. To understand the function of PssB in the EPS biosynthesis and the symbiosis with clover, we overexpressed and affinity-purified the PssB as N-terminally His_6-tagged protein. The purified PssB showed inositol monophosphatase activity and hydrolyzed *myo*-inositol 1-monophosphate to *myo*-inositol and phosphate. The catalytic properties of the PssB (e.g. K_m 0,23 mM, V_{max} 3,2 µmol/min/mg, requirement for Mg^{2+}, uncompetitive inhibition by Li^+) indicate that rhizobial phosphatase is a typical enzyme belonging to the IMPase family. The role of the PssB in the synthesis of EPS is not clear. Our studies suggest that PssB may play a regulatory function in this process.

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S6-P3

NEW GENES INVOLVED IN THE BIOSYNTHESIS OF CAPSULAR POLYSACCHARIDES IN *SINORHIZOBIUM MELILOTI*.

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The production of exopolysaccharide (EPS) was shown to be required for the infection process by rhizobia that induce the formation of indeterminate nodules on the roots of leguminous plants. In *Sinorhizobium* (formerly called *Rhizobium*) *meliloti* Rm41, a capsular polysaccharide (CPS) analogous to the group II K antigens of *Escherichia coli* can functionally replace EPS during nodule development.The presence of the CPS requires the unusual fatty acid synthase-like gene cluster *rkpABCDEFGHIJ* in the chromosomal rkp-1 region. The role of the *rkpABCDEFGHIJ* gene products in CPS biosynthesis is unclear so far. After we failed to restore the CPS biosynthesis of rhizobial mutants by trans-complementation we reanalysed the rkp-1 region. Two new genes involved in the CPS biosynthesis were identified and a new genetic organisation in the rkp-1 region was determined. The new gene *ctrA* shows homologies to the lipoprotein CtrA of *Neisseria meningitidis*. This lipoprotein belongs to the same complementation group as the *rkpABCDEFG* genes and is therefor a likely acceptor of the ß-ketide synthesised by the RkpABCDEF proteins. It is thought that CtrA is involved in the transport of CPS across the outer membrane of Gram-negative bacteria. The second new gene is homologous to the *kpsF* gene from *Escherichia coli* but ist exact biochemical function is unknown so far.

S6-P4

NOD FACTORS OF INDIGENOUS *RHIZOBIUM LEGUMINOSARUM* BIOVAR *VICIAE* ISOLATES THAT VARY IN HOST SPECIFICITY

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Rhizobium leguminosarum bv. *viciae* (*Rlv*) is by definition capable of nodulating legumes of the Vicieae tribe. This rhizobial host specificity is determined by the exchange of signal molecules between the plant and bacteria.
The potential for analysing Nod factors (NF) by a novel method was examined in this study. Bigge *et al* (1995) devised a method of efficient fluorescent labelling of glycans using 2-amino benzamide (2-AB). 2-AB can be incorporated through reaction with the reducing terminus of glycans, without detectable degradation of the glycan, allowing molar quantification of individual glycans. This method was adapted to the fluorescent labelling of the reducing terminus of the GlcNAc residue in the NF backbone.
Rlv isolates were obtained from nodules of native and cultivated *Pisum, Vicia* and *Lathyrus* legume species. Cross-inoculation experiments, determining host range, demonstrated variable host specificity, particularly for isolates obtained from *V. faba*. Selected isolates showing both genetic diversity and host specificity were subsequently analysed for NF production (Mutch, L. A. 2000). Naringenin was used as the *nod* gene inducer (Spaink *et al.* 1995).
Preliminary results indicate that the *Rlv* strains tested with varying host range produce identical NF profiles, however, there are quantitative differences for these. Isolates capable of effectively nodulating *V. faba* produce relatively more NFs.
Future work aims to include the characterisation of plant flavonoid inducers for each host plant studied.

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S6-P5

TYPE III SECRETION SYSTEM AND FLAVONOID RESPONSIVE EXTRACELLULAR PROTEINS OF *BRADYRHIZOBIUM JAPONICUM* 110*SPC4*

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Sequencing of the symbiotic genome region of *B. japonicum* revealed a gene cluster probably encoding a type III secretion system. This cluster with similarity to those found in *Sinorhizobium fredii* and *Rhizobium* sp. NGR234 consists of at least four transcription units that comprise genes coding for structural core components of the secretion system. In addition, one gene is likely to encode a transcriptional regulator (Y4xI) that has similarity to members of the two-component regulatory systems. Upstream of *y4xI* a *nod*-box promoter was found pointing out that flavonoids could be involved in the regulation of this gene cluster.
To elucidate the role of type III secretion during symbiosis, four different deletion mutants have been constructed. Deletions of structural core components affected symbiosis with *Glycine max* and *Vigna unguiculata* only marginally whereas functionality of *Macroptilium atropurpureum* nodules improved. Therefore, type III secretion is not essential for establishment of symbiosis but seems to be involved in fine-tuning of the interaction. Deletion of the gene region encompassing *y4xI* delayed nodule development with all three host plants.
Stimulation of protein secretion in *B. japonicum* by flavonoids appeared to be complex. Comparison of the electrophoresis pattern of extracellular proteins isolated from induced and non-induced cultures revealed a set of proteins whose synthesis or export seems to be induced by flavonoids. One protein accumulated to a smaller amount in induced cultures. All flavonoid-responsive proteins were secreted by a type III-deletion mutant indicating that these proteins are exported by different systems.

S6-P6

FUNCTION ANALYSIS OF THE ACETYL TRANSFERASE NOLL DURING SYMBIOSIS WITH *LOTUS JAPONICUS*

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Leguminous plants can have a symbiotic interaction with bacteria of the family *Rhizobiaceae*. The bacteria that naturally infects the leguminous plant *Lotus japonicus* is *Mesorhizobium loti*. The success of this symbiosis is dependent of an interchange of specific signal molecules between plant and bacteria, the molecules secreted by the bacteria are called nod factors. The nod factors synthetized by *M. loti* are formed by a chain of N-acetyl glucosamine units with different substituents at both reducing and non reducing terminus. One of these substituents is an acetyl fucose residue at the reducing terminus. We focus on the function of the acetyl group on the fucose in host specific nodulation of *L. japonicus*. It was already described that NodZ is a transferase with the function of adding a fucose residue to the reducing terminus of the nod factors. NolL had been shown to have homology with some acetyl transferases, and it was proposed to be in charge of the transferring of the acetyl group to the fucose residue, added by NodZ. To know the exact function of NolL we transferred only the *nodZ* gene or both *nolL* and *nodZ* gene to *Rhizobium leguminosarum*, that makes nod factors without the acetyl fucose group. After the transferring of these genes, *R. leguminosarum* became able to infect *L. japonicus* in both of the cases, with only *nodZ* and with *nodZ* and *nolL* genes. Although the nodulation never reached the efficiency of the infection by *M.loti*, the nodulation of the plants infected with the bacteria with *nodZ* and *nolL*, was more efficient than the nodulation of the plants infected with the bacteria onl y carrying *nodZ*. These results brought us to the conclusion that NolL has an important role in host specificity in the symbiosis with *L. japonicus*. After isolation of the nod factors produced by the two strains, we saw that the strain with *nodZ* and *nolL* was producing a higher amount of nod factors than the strain with only *nodZ*. After mass spectrometric analysis, it was seen that this bacteria had an acetyl-fucose group at the reducing terminus (that before was not present). From these results we deduced that the NolL protein functions as an acetyl transferase on the *M. loti* nod factors (Pacios Bras *et al.*, vol.13,No. 4, 2000, pp.475 - 479).
Infection and competition experiments with *Rhizobium* bacteria labelled with autofluorescent proteins will also be presented.

S6-P7

SINORHIZOBIUM MELILOTI LIPOPOLYSACCHARIDES (LPS) PROMOTE THE FORMATION OF ROOT NODULES IN THE SYMBIOSES WITH *MEDICAGO* SPP.

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Current evidence indicates that the symbiotic requirement for an intact LPS is more stringent in those rhizobia-legume interactions where root nodules are of determinate type. In *Medicago sativa* (alfalfa), a legume that develops indeterminate nodules, most *S. meliloti* LPS mutants are able to establish fully compatible associations. However, *S. meliloti lpsB* mutants derived from strain 2011 have an altered competitiveness to nodulate *M. sativa* (alfalfa), and do not fix nitrogen in *Medicago truncatula*. These characteristics present *lpsB* as a key mutation to study the LPS requirement in the symbioses between *S. meliloti* and *Medicago* spp.
To investigate possible roles of *S. meliloti* LPS in signaling to the plant, we carried out nodulation assays in which *M. truncatula* roots were pretreated with electrophoretically purified wild type LPS and then inoculated with either an *lpsB* mutant or a wild type rhizobia (10 exp+4 cfu/plant). The LPS was added on the emergent root hairs in a one microliter spot of sterile water containing from 20-200 ng LPS. None of these LPS amounts allowed nitrogen fixation by *lpsB* mutants. Surprisingly, an unexpected high number of nodules was observed in the plant roots that had been pretreated with 20 ng of LPS (approx. 2 picomol/plant) and inoculated with the wild type strain, compared to the control roots that had been pretreated with water prior to the inoculation (in average double number of nodules, P=0.05). A similar effect was also observed when *M. sativa* was used as host plant. The enhanced nodulation of the wild type rhizobia by LPS was dose dependent in the range of 0.2 to 20 ng, and was not observed either at lower or higher LPS amounts. The stimulatory effect was abolished when the active LPS fractions were treated with 0.7% sodium metaperyodate, or boiled in 2% acetic acid. No stimulation was observed with 10 ng of chromatographycally purified LPS of the unrelated bacteria *Escherichia coli* strain O111:B4.
Results taken together indicate that the addition of wild type LPS did not correct the Fix$^-$ phenotype of *lpsB* mutants, showing that the exogenous polysaccharide is not able to correct the defective infection of the mutant (i. e. the mutant may have a disturbed envelope incompatible with a normal infection, and/or a sustained requirement for the presence of LPS all along the infection may exist). However, a biological effect of soluble LPS was evident in promoting the nodulation by the wild type rhizobia on *Medicago* spp., suggesting an early signaling role for this molecule since the bacteria is outside the plant.

S6-P8

MOLECULAR CHARACTERIZATION OF THE *SINORHIZOBIUM MELILOTI* STATIONARY GROWTH PHASE EXPRESSED 'SURVIAL OPERON'

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Recently we have identified a cluster of genes on the *S. meliloti* chromosome which is involved in processes important for stationary growth phase survival [1]. The *S. meliloti* stationary growth phase expressed survival operon encompasses four open reading frames: *surE*, *pcm*, *bioS* and the *nlpD* gene. Since the unique biotin-regulated locus *bioS* [1, 2 ,3] was identified within the survival operon we were interested in the function and regulation of the *surE, pcm* and *nlpD* gene. All the genes were subject to a molecular analysis and were overexpressed in *E. coli*. The *surE* gene codes for a protein with a molecular mass of 29 KDa, the *pcm* and *nlpD* gene for proteins with molecular masses of 23 and 57 kDa, respectively. Several insertion and deletion mutants of the different genes were constructed. Work in progress analyzes the phenotypes associated with these mutations and employs reporter fusions to analyze the expression of the genes under different environmental conditions.

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[2] MPMI, 1999, 12, 803-812
[3] FEMS Microbiol. Lett. 2000, 182,41-44

S6-P9

enod40 IN LEGUMES AND IN ACTINORHIZAL PLANTS

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In both *Rhizobium*/legume- and actinorhizal symbioses, plants form root nodules to host nitrogen fixing soil bacteria. Plant *enod40* genes have been implicated in the induction of cortical cell divisions in reaction to rhizobial Nod factors during legume nodule development and are expressed also in non-symbiotic plant development. We have cloned *enod 40* homologs from two different actinorhizal plants, *Alnus glutinosa* and *Casuarina glauca*. Although both of them displayed the two conserved sequence regions characteristic for *enod40* genes, they did not contain the ORF for the short peptide of 10-13 amino acids of which the first conserved region represents the 3' half in all other *enod40* sequences known thus far. DNA Gel Blot hybridization analysis suggested that *enod40* is encoded by a single gene in both plants. The expression patterns of *enod40* in actinorhizal nodules was studied using in situ hybridization. To analyse *enod40* expression during actinorhizal nodule development, the *C. glauca enod40* promoter was cloned and fused with ß-glucuronidase (GUS) and brought into *Allocasuarina verticillata*. In order to compare the regulation of *enod40* expression in legumes versus actinorhizal plants, GUS staining patterns of *C. glauca enod40* promoter-GUS and soybean *enod40* promoter-GUS fusions were compared in transgenic *A. verticillata* and *Lotus japonicus*, respectively. Studies on *enod40* function were performed using transgenic tobacco and Arabidopsis plants expressing 35S- *enod40* constructs.

S6-P10

COMPARISON OF EARLY STEPS IN NODULATION IN THE *FRANKIA*–ACTINORHIZAL AND *RHIZOBIUM*-LEGUME SYMBIOSES

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In order to compare nodule induction mechanisms in legume and actinorhizal plants we used marker genes whose expression is induced by the microsymbiont in early stages of nodule development in both systems.
The *enod12* and *enod40* genes are expressed early in legume root nodule development. It has been shown that signal factors of rhizobia (Nod factors) are responsible for the induction of *enod40* and *enod12* expression (Vijn et al., 1995). Recently, *enod40* cDNAs were isolated from three actinorhizal plants, *Alnus glutinosa*, *Datisca glomerata* and *Casuarina glauca* (K. Pawlowski, unpublished) The expression pattern of actinorhizal enod40 shows similarities with the expression of *enod40* in course of legume nodule development (K. Pawlowski, unpublished). Meanwhile, up to now, attempt to isolate enod12 like gene in actinorhizal plants were unsucessfull.
To obtain information on the conservation of signal transduction pathways of these two early symbiotic genes, we constructed transgenic *Allocasuarina verticillata* plants with a *Casuarina enod40* promoter-GUS, a soybean *enod40* promoter-GUS and a Pea enod12-GUS fusion.
We are currently studying the β-glucuronidase gene expression in symbiotic and non symbiotic tissues of trangenic *A. verticillata*. Spot inoculation is used to apply either *Frankia*, *Rhizobium* NG234 strain or *Rhizobium* Nod factors on transgenic roots. Results of the expression of the *enod40*- and *enod12*-GUS chimeric genes will be discussed in view of the suggested common origin of root nodule symbioses.

Vijn et al. 1995. Plant J. 8:11-119.

S6-P11

MOLECULAR CHARACTERIZATION OF THE *MEDICAGO SATIVA* RESPONSE TO INFECTION WITH *SINORHIZOBIUM MELILOTI ILVC* MUTANTS.

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S. meliloti ilvC mutants are auxotrophic and unable to establish a nitrogen fixing symbiosis with *M. sativa* plants. The *ilv*C mutation blocks a metabolic branch chain of the amino acids biosynthetic pathway as consequence of the lack of isomeroreductase activity. Interestingly, it is not possible to explain the defective symbiotic phenotype displayed by these mutants as a nutritional deficit for the amino acid products of the metabolic pathway. Early plant responses to inoculation with *ilvC* mutants are characterized by the presence of root hair curling and cortical activation but the absence of infection threads. No nodules were observed four weeks after inoculation, only thickenings in certain restricted areas of secondary roots. In this zone, the outer cortical cells appear hypertrophied. It was also observed that the pattern of inner cortical cell activation, assessed by starch accumulation, is characteristic and differs from those induced for EPSI or *nodFL* mutants.
Nodule development can be arrested at different stages by means of rhizobial mutants impaired in the production of putative signaling molecules required to establish an effective symbiotic association. Our observations suggest that an anomalous signal exchange between the symbiotic partners may be responsible for the *ilvC* phenotype and induce a modified spatial or temporal pattern of nodulin gene expression in the plant host. Hence, expression of a selected group of nodulin genes was monitored to compare at a molecular level the *ilvC* response with other symbiotic structures. Transcript levels for nodulin genes, assessed by RT-PCR, were compared with those detected in nodules induced by exopolysaccharide mutants or spontaneously (in NAR+ alfalfa plants, able to form nodule-like structures in the absence of *S. meliloti*). Expression levels of the early nodulin genes *MsEnod40* and *MsNod93* (homologous to the soybean *GmEnod93* gene) in the *ilvC*-structures were similarly induced as in the other nodule-like structures. Transcripts of other early nodulin genes were not detected in these structures suggesting that nodule formation is arrested at a very early developmental step. Our results demonstrated that structures induced by *ilvC* mutants are different of those induced by other symbiotic-defective mutants.

S6-P12

THE *SINORHIZOBIUM MELILOTI LEUA* GENE IS ESSENTIAL FOR NODULATION OF ALFALFA AND NODD1-DEPENDENT EXPRESSION OF *NOD* GENES.

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Mutations in the *S. meliloti leuA* gene determine a symbiotically defective phenotype. This gene encodes the enzyme 2-isopropylmalate (2-IPM) synthase which catalizes the first step in the leucine specific biosynthesis pathway. *S. meliloti leuA* strains are leucine auxotrophs, lack 2-ipm synthase activity and are unable to nodulate alfalfa roots. Supplementation of plant growth media with leucine or leucine precursors allow *leuA* strains to recover almost wild-type symbiotic capacity, regardless of the ability of these nutritional compounds to relief *leu* auxotrophy.
leuA mutants cannot achieve wild-type induction of a plasmid-borne *nodC:lacZ* fusion; in contrast, expression of *nodD1-lacZ* or *nodD3-lacZ* fusions, as well as the NodD3-dependent constitutive expression of a *nodH-lacZ* fusion proceed normally.
In strains overproducing NodD proteins, NodD1-dependent, but not NodD3-dependent, expression of a *nodA-lacZ* fusion was completely abolished in *leuA* strains. This NodD1-dependent induction of *nodA* was recovered after the addition of leucine or leucine precursors to the growth medium.
Thus, *leuA* mutants appear unable to direct wild-type NodD1-dependent induction of *nod* genes in response to luteolin. Ours results suggest that a leucine precursor, probably 2-IPM, is required for *nodD1* activity.

S6-P13

CHARACTERIZATION OF A SWARMING MUTANT IN *SINORHIZOBIUM MELILOTI*.

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Swarming is a form of active surface motility that is widespread among flagellated, Gram-negative bacteria (1). It has been described in species of the genera *Proteus, Vibrio, Serratia, Escherichia, Salmonella, Yersinia* and *Pseudomonas*. Swarmer cells are generally long and multinucleate, always hyperflagellated, and can move rapidly over the agar surface in a coordinated manner. Swarming might play an important role in the colonization of natural environments by microorganisms. In fact, there is evidence that the differentiated swarmer-cell stage facilitates pathogenic associations with the host tissue. Despite the importance of this mode of translocation on a solid surface, almost nothing is known about the molecular signaling mechanism involved.
During the screening of a Tn5-mutagenized population of *S. meliloti* GR4 in semisolid (0.7% agar) minimal medium supplemented with carboxymethyl cellulose (CMC), we identified a colony (QS77) showing a dendritic pattern characteristic of some colonies containing swarmer cells. This phenotype is not shown by the wild-type strain GR4 and in the mutant QS77 seems to be a cell density-dependent phenomenon. DNA hybridization studies revealed that the QS77 mutant carries a single Tn5 insertion within a gene that showed striking similarities with the *rpfB* gene of *Xanthomonas campestris* as well as with the *fadD* gene of several microorganisms which encodes a long-chain fatty acylCoA ligase. The *rpfB* gene has been described as belonging to a cluster of at least seven *rpf* (regulation of pathogenicity factors) genes involved in the control of the synthesis of extracellular enzymes and therefore the virulence of *Xanthomonas campestris* (2). RpfB is predicted to be a long-chain fatty acylCoA ligase that mediates regulation via a low-molecular-weight diffusible substance called DSF (diffusible signal factor). An *Escherichia coli fadD*-deficient mutant is unable to grow on agar plates containing oleic acid as sole carbon and energy source. Whereas *S. meliloti* GR4 can grow on minimal medium with only oleate, the QS77 mutant can not. This result along with the homology data suggest that the affected gene in QS77 encodes for a long-chain fatty acylCoA ligase. The characterization of this locus as well as its involvement in symbiosis will be discussed.

1. Harshey (1994) *Mol. Microbiol.* 13: 389-394.
2. Barber *et al.* (1997) *Mol. Microbiol.* 24: 555-566.

S6-P14

SINORHIZOBIUM MELILOTI GENES INVOLVED IN RESISTANCE TO ANTIMICROBIAL PEPTIDES

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The innate inmunity of animals and plants to microbial infection is mediated in part by small cationic peptides with antimicrobial activity. Succesful pathogens have evolved mechanisms to withstand the antibiotic activity of these molecules. Early studies reported that mutants, wich show increased sensitivity to antimicrobial peptides, have reduced virulence, suggesting that resistance to host peptides has a direct role in bacterial pathogenesis (1,2).
S. meliloti GR4 was mutagenized with the transposon Tn*5*, and 7 mutants were selected, all of them showing increased sensitivity to protamine, a 32-aa cationic peptide found in salmon spermatozoan nuclei. The site of action of protamine is the cytoplasmic membrane, where it causes membrane permeabilization, posibly by creating a large pore in the membrane. Characterization of the Tn*5* insertions showed that mutations had ocurred in i) genes involved in succinoglycan biosynthesis and transport, such as *exoU* and *exoT* and cyclic-β-1,2 glucan biosynthesis, *ndvB*, ii) a putative regulator of gene expression, and iii) a gene coding for a transmembrane protein that belongs to a putative ABC transporter.
Although there is no reported evidence of production of antimicrobial peptides by legume plants, our results indicate that similarly to pathogenic bacteria, *Rhizobium* genes involved in resistance to antimicrobial peptides, are also important for nodule infection.

1. López-Solanilla et al. 1998. The Plant Cell 10:917-924
2. Parra-Lopez et al. 1993. The EMBO Journal 12:4053-4062

S6-P15

SVQ120: A *SINORHIZOBIUM FREDII* STRAIN HH103 MUTANT IN THE *hrcQ* GENE LOCATED IN THE TYPE III SECRETION PROTEIN GENE REGION.

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Transposon Tn*5-lacZ* (Tn5-B20) mutagenesis on *S. fredii* HH103 allowed the isolation of HH103 derivatives that showed high β-galactosidase activity in the presence of flavonoids (naringenin). One of these mutants (SVQ120) was chosen for further studies. We have constructed plasmid pMUS442 to facilitate the clonation of the rhizobial DNA that is flanking transposon Tn5-B20. This plasmid is a derivative pSUP202 (Tc^R, Ap^R, Cm^R, suicide in *Rhizobium*) and has cloned the kanamycin-resistance gene of pUC4K into the *Pst*I site of the Ap gene. Conjugational transfer of pMUS442 into SVQ120 and selection of Tc^R transconjugants allows the isolation of SVQ120 Tc^R clones in which plasmid pMUS442 is specifically integrated inside transposon Tn5-*lacZ* by homologous recombination between the kanamycin-resistance genes. After genomic DNA digestion with *Eco*RI and further religation, a plasmid is generated. This latter plasmid must carry part of pMUS442 and the rhizobial DNA region flanking the inserted Tn5-B20. Sequencing this DNA region from a specific primer (designed into the right IS of Tn5) allows the identification of the mutated gene.
In the SVQ120 mutant, the sequenced region showed high homology with the *hrcQ* genes of *S. fredii* strain USDA257 and *Rhizobium sp.* NGR234. Using this fragment as a probe against the gene library of the parental strain HH103 a cosmid carrying the wild copy of *hrcQ* gene was isolated. This gene was sequenced and computer analysis showed that it is identical to that of *S.fredii* and highly homologous to the *hrcQ* gene of NGR234.
Although the *hrcQ* gene is located into the Type III secretion-protein gene region, we could not detect any difference in the profiles of extracellular, cytoplasmic or periplasmic proteins of flavonoid induced cultures in SDS-PAGE compared with those of HH103. Also SVQ120 showed normal Nod factors in TLC analysis and LPS profile in SDS-PAGE in relation with HH103.
In comparison with its parental stain HH103, mutant SVQ120 has no significant alteration in its capacities of nodulation, N_2-fixation, kinetics of nodulation and competitiveness for the nodulation with soybean cultivar Williams. We have found, however, that SVQ120 was able to fix N_2 in *Erythrina variegata*, while HH103 developed small fix- nodules.

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S6-P16

RELATIONSHIP BETWEEN BORON AND CALCIUM IN EARLY *RHIZOBIUM*-LEGUME INTERACTIONS AND NODULE INVASION

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Both boron (B) and calcium (Ca) have been shown to regulate the establishment of the symbiosis of rhizobia with legume plants (1,3), and an interaction between both nutrients in plants has been reported (4). We are studying a possible B-Ca relationship and its implication in molecular-plant microbe interactions that lead to the development of N_2-fixing root-legume nodules.
The activity of *nod* gene of *Rhizobium leguminosarum* bv *viciae* D24 (measured as *nodABCIJ* promoter activity) induced by pea root exudates was highly dependant on the level of B and Ca during plant growth. Boron deficiency led to root exudates with a 6-7 times lower inducing capacity than exudates derived from plants with a normal B nutrition. Such difference caused a decrease of root hair curling in B-deficient inoculated pea plants. The increase of Ca to the plants resulted in exudates with a higher *nod* gene activation capacity both in B-deficient or B-sufficient roots. Consequently, the addition of extra Ca during growth increased root hair curling of inoculated plants growing in the absence of B.
Because B has previously been implicated in *Rhizobium*-legume cell surface interactions during nodule development, and Ca was able to recovered symbiotic N_2-fixation (2), the relationship between both nutrients during infection and invasion steps has been studied using *R. leguminosarum* 3841 carrying a stable plasmid that constitutively expresses green fluorescent protein (GFP) activity. A supplement of Ca to B-deficient inoculated pea plants can restored infection thread development and tissue invasion that are aborted under B deficiency.

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S6-P17

GENETIC ANALYSIS OF THE SYMBIOTIC *BRADYRHIZOBIUM JAPONICUM* MUTANT 2-10

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The *Bradyrhizobium japonicum* strain 2-10 results from a Tn*phoA*-mutagenesis. The transposon is inserted presumably in a region encoding a transmembrane or periplasmic protein.
In symbiosis with *Glycine max* the nodules are weakly infected. Ultrastructural analysis of the infected tissue revealed a lot of starch granules and vacuoles in the plant cells, indicating that the mutated operon is required for an efficient symbiosis.
A 6.8 kb DNA fragment, containing the Tn*phoA*-insertion, was sequenced. Further analysis of this area indicated six open reading frames, including one showing high similarity to an integrase gene from *Bacillus subtilis*, and another ORF with similarity to putative inner membrane proteins from bacteria, e.g. *Buchneria aphidicola*. For the ORF *ub12*, carrying the transposon-insertion, no significant homologies were found in the databases of the National Center for Biotechnology Information (NCBI).
ORF *ub12* encodes an assumed gene product of 306 aa. Reporter gene fusion and computer-analysis predict a periplasmic loop between two transmembrane helices. This predicted periplasmic loop is the domain where the Tn*phoA* insertion took place.
An inframe TGA codon, followed by a potential *cis*-element, was obtained within the DNA sequence of ORF *ub12*. The UGA codon in addition with this stem loop structure, in the mRNA, could result in the translation of UGA as selenocysteine.
Based on genetic complementation tests, the symbiotic relevance of the ORFs located downstream of the insertion was revealed phenotypically.
According to this, ORF *ub12* seems to be part of an operon containing three open reading frames.
Further complementation tests and reporter gene fusion assays should clarify the operon structure of this DNA region and the symbiotic relevance of the ORFs.
Expression assays under controlled conditions are in progress to investigate, whether UB12 is the first selenoprotein reported in *Bradyrhizobium japonicum*.

S6-P18

CHARACTERIZATION OF A PURINE AUXOTROPHIC MUTANT OF *SINORHIZOBIUM FREDII HH103*.

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The phenotypes of different *Rhizobium* auxotrophs have provided important information to the physiology of nodule development. The symbiotic properties of *Rhizobium* auxotrophic mutants depend on the type of mutation. For example, leucine or riboflavin auxotrophs are Fix$^-$ while adenine are usually Nod$^-$. We have carried out transposon Tn5-mob mutagenesis of *S. fredii* strain HH103-1 and have isolated an auxotroph mutant (SVQ295) that requires adenine and thiamine to grow on minimal media. Mutant SVQ295 also grows in minimal media (MMB) supplemented with AICA-riboside (a precursor of adenine) and thiamine.
This mutant formed pseudonodules on *Glycine max* cv.Williams. This is also the case of other described *Rhizobium* purine mutants on their host plant. This mutant also fails to nodulate, or forms Fix$^-$ nodules on other legumes that are usually effectively nodulated by the *S. fredii* wild-type strain HH103-1.
The addition of AICA-riboside to the plant nutritive solution lead to the formation of small nodules. *Glycyrrhiza uralensis* was the only tested legume in which mutant SVQ295 formed nitrogen-fixing nodules (indeterminate type nodules). Bacteria could be visualized inside the plant cells.
Plasmid pSUP202 was integrated inside transposon Tn5-Mob of mutant SVQ295 (by homologous recombination between the Mob regions of the transposon and the plasmid). Recombinant strain SVQ295::pSUP202 allowed the isolation of a DNA fragment that contained the end of the IS50R of transposon Tn5–Mob and the adjacent rhizobial mutated DNA. The sequenced rhizobial fragment showed homology to the *purL* gene of *Lactobacillus* that encodes for an FGAR amidotransferase. To characterize the entire *purL* gene of *S. fredii* strain HH103, a gene bank of the total genome of *S. fredii* HH103 was crossed with a *purL* mutant (CE382) of *R. etli* CE3 to isolate a cosmid that contains the *S. fredii* gene. Cosmid pMUS509 complemented mutant CE382 of *R. etli* to grow on minimal media and to nodulate *Phaseolus* beans. As expected, cosmid pMUS509 enabled *S. fredii* SVQ295 to form nitrogen-fixing nodules on soybeans. Further genetic analyses allowed the sequencing of the *S. fredii purL* gene. This gene showed high homology to other *purL* genes from diverse microorganisms.

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S6-P19

THE *dpp* GENES ARE NEEDED FOR AMINO-LEVULINIC ACID UPTAKE AND INTERACT WITH SIDEROPHORE TRANSPORT IN *RHIZOBIUM LEGUMINOSARUM*.

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We identified an operon, *dppABCDF*, in *Rhizobium leguminosarum* whose gene products had a strong homology to the Dpp proteins; in other bacteria, these are involved in the uptake of dipeptides. As in other bacteria, there is a step-down in the levels of transcription within the *dpp* operon, this being due to a non-translated terminator region between *dppA* (which specifies the periplasmic binding protein) and *dppB*, (which encodes an inner membrane transporter).
We confirmed that *dpp* mutants of *R. leguminosarum* are defective in dipeptide transport and that they also fail to import amino-levulinic acid (ALA), a precursor of haem. The *dpp* knockout mutants induced N_2-fixing nodules on peas, showing that the plant does not supply ALA to the bacteria in the nodule.
For reasons that are not clear, the cloned *dpp* genes suppressed the phenotype of a number of mutants (in the *fhu* and *tonB* genes) that produced high levels of the hydroxamate siderophore vicibactin in the extracellular medium. On media containing the general siderophore stain chrome azural sulphonate (CAS), the presence of cloned *dppABCDF* caused the enlarged halo to "revert" to wild type. A speculative model to account for these observations will be presented.

S6-P20

A NOVEL TYPE OF KINASE INDUCED DURING THE SYMBIOTIC INTERACTION OF *SINORIZOBIUM MELILOTI* AND *MEDICAGO* SSP.

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The interaction between *Medicago* spp. and *Sinorhizobium meliloti* leads to the development of a novel organ, the root nodule. A gene, *Mtpk1*, encoding a novel protein kinase containing an ankyrin domain was identified as being induced during nodulation and in spontaneous nodules. We have developed immunological approaches using antibodies raised to the *E. coli* expressed Mtpk1 protein, as well as translational fusions to Histidine-tags and GFP to attempt to localise the protein in root tissues during symbiosis and in transfected protoplasts. The expression of this gene was characterized in different alfalfa organs and in early stages of the symbiotic interaction. *M. truncatula* mutants affected in symbiosis (monitored using GFP-labelled bacterial strains) were also tested for expression of this marker.
The *Mtpk1* gene was isolated by screening of a *M. truncatula* BAC library, and the sequence of its genomic region and certain adjacent clones was determined. Several genes showing homologies to previously identified sequences in data banks were identified in the vicinity of the *Mtpk1* gene. The distribution of exons and introns (10) was analyzed in detail for *Mtpk1*, and compared with three homologous genes identified in *Arabidopsis thaliana*. Moreover, in this model plant, a T-DNA insertional mutant in one of these genes was identified and is now currently being analyzed to search for the function of this kinase in development.
These data support that *Mtpk1* might be involved in the early steps of the symbiotic interaction although is not exclusively associated with nodulation. The developed tools will serve to analyse gene product function, and localisation for elaborating hypotheses about its role in the initiation of nodule organogenesis.

S6-P21

SELECTIVE TAGGING OF *SINORHIZOBIUM FREDII* SYMBIOTIC PLASMIDS WITH KANAMYCIN-RESISTANCE AND MOBILISATION FUNCTIONS.

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Little is known about the contribution of the symbiotic plasmid and/or the bacterial background (defined as chromosome plus non-symbiotic plasmids) to the effectiveness and competitiveness of *Rhizobium* strains. However, it is reasonable to expect that conjugal transfer of pSym plasmids between *Rhizobium* strains could result in new pSym/bacterial-background combinations showing enhanced competitiveness or nitrogen-fixation capacity.
Mobilisation of rhizobial plasmids requires that the plasmids have been previously marked with a selectable marker, such as transposon Tn5-Mob. Although Tn5-Mob is an extremely useful genetic tool, large screenings of Tn5-Mob carrying transconjugants have to be carried out to identify those bacterial clones that contain the transposon inserted into the symbiotic plasmid. Because of this, we have constructed a plasmid (pMUS573) that facilitates the specific marking of *S. fredii* symbiotic plasmids. This plasmid (a derivative of pK18-Mob, Km^R) is suicide in *Sinorhizobium* and it carries the promoter and the 5'-end of the *nodZ* gene of *Sinorhizobium fredii* HH103. Conjugal transfer of plasmid pMUS573 to *S. fredii* strains 042B(s) and USDA257 and selection of Km^R transconjugants allowed the isolation of clones in which plasmid pMUS573 has been integrated into the symbiotic plasmid of the recipient *S. fredii* strains. This integration occurs by homologous recombination between the cloned *nodZ* fragment of pMUS573 and the *nodZ* gene of the symbiotic plasmids and does not cause a nodZ- phenotype, since the profile (by TLC) of nodulation-factors produced by the recombinant strain 042B(s) pSym042B::pMUS573 and USDA257::pMUS573 are to those shown by the parental strains. Conjugational transfer of recombinant pSym::pMUS573 plasmids to pSym-cured derivative of *S. fredii* USDA193 produced Km^R transconjugants that formed nitrogen-fixing nodules on soybeans. These results demonstrate that plasmid pMUS573 is an effective tool for the specific marking of *S. fredii* plasmids.

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S6-P22

A *hes*B MUTANT OF *SINORHIZOBIUM FREDII* STRAIN HH103 SHOWS REDUCED NODULATION ABILITY

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The symbiotic properties of the strain SVQ124, a mutant derivative of *S. fredii* HH103-1 carrying a Tn*5-lacZ* (Tn*5*-B20) insertion into the *hes*B gene, were studied. Mutant strain SVQ124 and its parental strain HH103 showed similar kinetics of nodulation with soybean cultivar Williams. However SVQ124 shows a different nodulation capacity (a significant reduction in the number of nodules developed) and also a reduction in its nitrogen fixation ability in this soybean cultivar. SVQ124 developed fewer and smaller nodules than HH103 and the plants showed clear symptoms of nitrogen starvation. SVQ124 is less competitive than its parental strain HH103-1 to nodulate soybean c.v. Williams. We also assayed the symbiotic properties of SVQ124 with other legumes: Mungbean, *Macroptilium atropurpureum*, *Macrotyloma axillare* and *Cajanus cajans*. In all of them we obtained similar results to those obtained with HH103. The only exception was *Cajanus cajans*, where the plants were yellow and the nodules smaller.
Apparently surface polysaccharides of strain SVQ124 are not altered as indicated by the colony morphology and the LPS profile in SDS-PAGE. TLC analysis of the Nod factors produced by SVQ124 after induction with flavonoids are similar to those produced by strain HH103.
The complete gene mutated in SVQ124 was sequenced. Computer analysis showed that this gene is identical to the y4vC gene of *Rhizobium* sp NGR234 (homologous to the *hes*B gene of *Anabaena* sp. strain PCC 7120). Similarly to NGR234, the *S. fredii hes*B gene is also preceded by a *nif*A dependent σ^{54} promoter and by a more distant (780 pb) nod-box. The presence of the nod-box region explains the fact that mutant SVQ124 shows increased β-galactosidase activity in the presence of flavonoids.

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S6-P23

EROSION OF ROOT EPIDERMAL CELL WALLS AS RELATED TO PRIMARY HOST INFECTION IN THE *RHIZOBIUM*-LEGUME SYMBIOSIS

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Ultrastructural studies using transmission electron microscopy indicate that the portal of entry for rhizobial infection of the legume root hair is a completely eroded hole that is slightly larger than the width of the bacterial cell and is presumably created by localized enzymatic hydrolysis of the host cell wall. Consistent with these ultrastructural events, various rhizobia have been shown to produce cell-bound cellulases and pectinase, and the legume host produces a polygalacturonase in response to the rhizobial symbiont. In this study, we have used various types of microscopy and enzymology to further define the rhizobial modification of root epidermal cell walls in order to shed new light on the mechanism of primary host infection in the *Rhizobium*-legume symbiosis. Quantitative scanning electron microscopy indicated that the incidence of highly localized, partially eroded pits on legume root epidermal walls that follow the contour of the rhizobial cell was (i) higher in host than in non-host rhizobia-legume combinations, (ii) inhibited by nitrate supply, and (iii) not induced by wild type chitolipooligosaccharide Nod factors reversibly adsorbed to latex beads. More detailed TEM ultrastructural analysis of these partially eroded, epidermal pits indicated that the amorphous, non-crystalline portions of the wall were disrupted whereas the crystalline portions remained ultrastructurally intact. In microscopical studies on infection-related biological activities of rhizobial chitolipooligosaccharides, we found that their brief exposure to growing roots will extend the duration of growth elongation of root hairs, and significantly increase their infectibility while introducing localized disruptions in crystallization of their cell wall. We proposed that this Nod factor-induced alteration in wall architecture would promote its erosion by glycanases and thus facilitate rhizobial entry. Consistent with this hypothesis, we have recently found by a combination of phase contrast/polarized light microscopy and enzymology that (i) the structural integrity of white clover root hairs are dependent on wall polymers that are valid substrates for rhizobial cell-bound glycanases;(ii) these rhizobial enzymes can completely erode the root hair wall at a highly localized site on the isotropic, noncrystalline apex of the root hair tip and (iii) these cell-bound rhizobial glycanases can more extensively degrade clover root hair walls when grown with chitolipooligosaccharides from clover rhizobia. These results suggest a complementary role of rhizobial cell-bound glycanases and chitolipo oligosaccharides in creating the localized portals of entry for successful primary host infection in the *Rhizobium*-legume symbiosis.

S6-P24

CHARACTERIZATION OF *nodI* GENE OF *SINORHIZOBIUM FREDII* HH103

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Soybean [*Glycine max* (L.)] is considered one of the oldest crops in the world, and it actually provides the main source of proteins for millions of inhabitants in China and Brazil. *S. fredii* HH103 is a fast-growing soybean symbiont isolated from Hohghu (China).
There are a reduced number of nodulation genes described for *S. fredii*. In order to locate and describe *S. fredii* HH103 *nod* genes, Tn*5*-B20 mutants of *S. fredii* HH103 were made using suicide plasmid pSUP102:Gm::*lacZ*. Generated mutants showing increased β-galactosidase activity after flavonoid induction were selected for further studies. We studied one of these *nod* gene mutants (*S. fredii* SVQ282).
Location of Tn*5* insertion in *S. fredii* SVQ282 was carried out using *lacZ* as a molecular marker. Sequence analysis of region upstream Tn*5* transposon showed two ORFs presenting 98.6% identity with *nodI* gene, and 98.7% identity with *nodC* gene of *Rhizobium* NGR234 respectively. Data indicates that *S. fredii* SVQ282 is a mutant in *nodI* gene which is located downstream *nodC* gene.
NodI and NodJ have been described as ABC-transporters and they form a polysaccharide secretion system involved in LCO secretion. Genes coding for these proteins are usually organized in the same transcriptional unit as *nodABC*, and regulated by plants flavonoids in other rhizobia.
S. fredii nodI mutant was characterized for symbiotic properties. No significant nodulation delay was observed though *nodI* mutant was completely outcompeted when inoculated along with *S. fredii* HH103 wild type strain.
LCO secretion kinetics was performed for both *S. fredii* HH103 and *S. fredii* SVQ282. Nod factors could be detected in media 15 minutes after naringenin induction, and highest levels of secretion were observed 3 hours after induction. *S. fredii* SVQ282 did not show a significant decrease in the amount of LCO secreted, and no delay in LCO secretion was observed. Though TLC analysis showed that *S. fredii* SVQ282 Nod factors present the same number of spots as wild type strain, lower migration of them in the silica plate indicates presence of more hydrophobic compounds.

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S6-P25

EARLY EVENTS IN THE INFECTION AND NODULATION OF *LUPINUS ALBUS* VISUALIZED WITH GFP-TAGGED BACTERIA

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Green fluorescent protein (GFP) labelling of cells allows direct examination by confocal fluorescence microscopy and can be used as a tool to study the colonization of the legume root by rhizobia.
Lupinus albus is usually colonized by *Bradyrhizobium* sp. (*Lupinus*) and in order to study the early stages of the infection and nodulation processes in this symbiosis, which does not seem to follow the infection thread mechanism, we attempted the transformation of the bacteria with the *gfp* gene. However transformation of several *Bradyrhizobium* sp. strains by various methods resulted unsuccessful.
We transformed by electroporation a *Mesorhizobium loti* strain isolated from lupin nodules with a plasmid vector that constitutively expresses a *gfp* gene and that contains the stabilization fragment of plasmid RK2, which ensures that cells remain labelled throughout infection and nodulation without selective pressure (Cheng and Walker, 1998).
Lupin plants inoculated with the transformed bacteria formed nodules similar to those produced when the plant is inoculated with *Bradyrhizobium*. Fluorescence microscopy evidenced the presence of GFP-tagged bacteria inside the nodules, thus providing a powerful method to investigate the early events of infection and nodulation in lupin, a process that is controlled by the host plant.

Cheng and Walker (1998) J. Bacteriol. 180, 5183-5191.

S6-P26

CHARACTERIZATION OF A *nolT* MUTANT OF *SINORHIZOBIUM FREDII* HH103: IT'S ROLE IN TYPE III SECRETION PROTEIN.

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The mutant strain SVQ288 from *Sinorhizobium fredii* HH103 was obtained after Tn5-*lacZ* mutagenesis and selected for increased β-galactosidase activity in presence of flavonoid inducers. We cloned "in vivo" the mutation, obtaining plasmid pMUS554 that harbours the 3´-end region of the gene in which the Tn5-*lacZ* transposon is inserted. The sequence of this region, using a specific primer into the right IS of Tn5, indicates that SVQ288 is mutated in the *nolT* gene.
A 2.1Kb XhoI-EcoRI from plasmid pMUS554 was used in colony hybridization experiments to isolate a cosmid clone from a genomic library of HH103. The isolated cosmid (pMUS606) contains the entire *nol* region of HH103 (*nolXWBTUV*). The *nol* region is responsible of the Type III secretion protein system. Indeed, the *nolT* mutant strain lacks, at least, five proteins that are secreted by the wild type strain after flavonoid induction. The secretion of proteins by the Type III secretion system in HH103 is also depending of the *nodD1* gene as it has been previously described for NGR234 and USDA257.
Strain HH103 and its mutant derivative SVQ288 were able to induced nitrogen-fixing nodules in *Glycine max*, *Vigna unguiculata* and *Cajanus cajan*. Although SVQ288 induced less nodules, Acetylene Reduction Assay (ARA) of nodules formed by SVQ288 was not significantly different from that of nodules induced by HH103. In *Glycine max* the mutant strain was less competitive than the wild type strain HH103 and presented retarded kinetic of nodulation. Mutant SVQ288 also formed effective nodules on *Erythrina variegata*, a legume in which strain HH103 only forms ineffective pseudonodules.

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S6-P27

Sinorhizobium fredii HH103 HAS A NON FUNCTIONAL COPY OF *nolO*

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Transposon Tn*5-lacZ* mutagenesis was carried out to generate a set of mutant derivatives of *S. fredii* HH103. Mutant strain SVQ121 shows increased β-galactosidase activity in the presence of flavonoids suggesting that the promoterless *lacZ* is now under the control of a nod promoter. The mutated gene of SVQ121 was cloned and sequenced. Analyses of the sequence showed that this gene is highly homologous to the *nolO* gene of *Rhizobium* sp. NGR234. The NolO protein is responsible for the 3 (or 4)-O-carbamoylation of the non-reducing terminus of Nod factors. However, HH103 synthesizes non-carbamoylated Nod factors.

TLC analyses showed that SVQ121 Nod factors are altered in comparison to those produced by the parental strain HH103. Mutant strain SVQ121 is less competitive than its parental strain HH103 to nodulate soybean cv. Williams, although the kinetic of nodulation and the ability to fix nitrogen were not affected.

Nodulation factors produced by SVQ121 show a lower methyl-fucosyl/fucosyl ratio than that show by HH103 Nod factors. Because the O-methylation of nodulation factors is carried out by *NoeI*, we sequenced the complete *S. fredii noeI* gene which is located downstream of the *nolO* gene.

A HH103 *noeI* mutant (SVQ503) was constructed by insertion into the *noeI* gene of a spectinomycin-cassette. SVQ503 shares most of the SVQ121 characteristics except that Nod factors produced by the former were devoid of methyl groups.

When the wild copy of *noeI* was introduced into SVQ503 and SVQ121 mutants, both of them produced Nod factors showing the methylation level of those produced by HH103. Comparison between the *nolO* coding sequences of HH103 and NGR234 showed that the former should produce a truncated NolO protein due to the deletion of two separated bases that originates an early stop codon.

Therefore, it is possible to conclude that the SVQ121 phenotype is due to a polar effect on the *noeI* gene, and the methylation level of the Nod factors affects bacterial competitiveness for nodulation soybean c.v. Williams.

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S6-P28

REGULATION OF SULPHATION *nod* GENES DETERMINES *RHIZOBIUM TROPICI* NOD FACTOR STRUCTURE

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To trigger the nodulation process of host plants, rhizobia produces lipo-chito-saccharides called Nod factors. These molecules are constituted of 3 to 5 β1,4-linked N-acetylglucosamine which bear different substutient in its reducing and non reducing end. *Rhizobium tropici* produces a large variety of Nod factors which are metylated and may be sulphated or not. Sulphation *nodHPQ* genes present different organization in *R. tropici* and *S. meliloti*.
To study *nodHPQ* regulation we carried out different interposon insertions in its upstream region. One of these generated interruptions, *nodI* mutant, produces nonsulphated Nod factors suggesting a possible dependence of these genes on *nodABCSUIJ* operon promoter. Moreover, results analysis of *lacZ* transcriptional fusions with these genes in symbiotic plasmid showed dependence of these genes on NodD protein. In addition, interposon insertion in *nodHPQ* upstream region indicates that *nodHPQ* belong to *nodABCSUIJ* operon. On the other hand, comparison between *nodP::lacZ* β-galactosidase activity in symbiotic plasmid and in pHM500 plasmid (containing *nodHPQ* genes) suggested two kind of regulation: constitutive expression in free living conditions which may play a role in the bacterial house keeping metabolism, and flavonoid-inducible expression in symbiotic conditions.
Transference assays of *R. tropici nodHPQ* genes to other rhizobia, that do not present sulphated substitutions demonstrated sulphotransferase specificity of NodH protein to C6 of the reducing end.

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S6-P29

CLONING AND CHARACTERIZATION OF A *cobO* MUTANT OF *SINORHIZOBIUM FREDII* HH103.

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Sinorhizobium fredii is a fast-growing bacterium that nodulates soybean (*Glycine max* (L.) Merr.) and several other legume species.
The phenotypes of different rhizobia auxotrophs have provided important information about the physiology of nodule development. The symbiotic phenotype of rhizobia auxotrophic mutants varied with the type of mutation. For example, adenine auxotrophs are in most cases Nod^-, while leucine auxotrophs are Fix^-.
We have carried out transposon Tn5-B20 mutagenesis of *S. fredii* HH103-1 strain (=HH103str^R) and have isolated a methionine auxotroph mutant (SVQ336). The right Tn5-B20 flanking region from the SVQ336 mutant strain was subcloned and sequence analysis revealed a putative open reading frame highly homologous to several cob(I)alamin adenosyltransferase coded by the *cobO* gene. The highest similarity was obtained with the *cobO* gene of *Pseudomonas denitrificans*. This enzyme is involved in the cobalamin biosynthesis pathway. Cobalamin acts as a coenzyme of some enzymes, such us cobalamin-methionine syntase (MetH), which catalyzes the last step of the biosynthesis of methionine. Mutant strain SVQ336 is able to grow on minimal media supplemented with methionine or cobalamin. This mutant strain showed a larger generation time than the wild-type strain HH103-1 even in the presence of methionine or cobalamine. On TY rich-medium generation time was about 4 hours for mutant strain SVQ336 while it was of 2 hours for HH103-1. On minimal medium supplemented with cobalamin or methionine generation time was about 10 and 7 hours for SVQ336 and HH103-1, respectively.
Induction of the common *nod* genes was studied on SVQ336 mutant strain, using plasmid pMP240, which contains the *lacZ* gene under control of the *nodA* promoter. No difference on *nodA* promoter activity were found between HH103-1 and its mutant derivative SVQ336 when the flavonoid genistein was used as inducer.
Mutation in the *cobO* gene severely affected the symbiotic phenotype of HH103-1 strain, since the number of nodules on soybean plants inoculated with SVQ336 was reduced 96% in comparison with the number of nodules produced by the wild-type strain HH103-1.

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S6-P30

AN URACILE AUXOTROPH (*pyrF*) MUTANT OF *SINORHIZOBIUM FREDII* HH103 IS SEVERELY IMPAIRED FOR NODULATION AND SYMBIOTIC NITROGEN-FIXATION ABILITY.

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We employed suicide plasmid pSUP5011 carrying transposon Tn5-Mob to randomly mutagenize *S. fredii* HH103 Rif^R. Mutant SVQ292 requires the presence of uracile to grow in liquid minimal media. *Glycine max* cv. Williams plants inoculated with SVQ292 formed ineffective pseudonodules. Light-microscopy studies showed that plant cells did not contain intracellular bacteria. The symbiotic properties of mutant SVQ292 were also tested in other legume plants that are effectively nodulated by the wild-type strain HH103. Mutant strain SVQ292 failed to nodulate *Macrotyloma axyllare* and induced ineffective pseudonodules on *Macroptilium atropurpureum*, *Indigofera tinctoria*, and *Desmodium canadense*. In *Cajanus cajan*, SVQ292 induced small ineffective nodules. Exogenous addition of uracile to the plant-nutritive solution did not enhace SVQ292 to nodulate soybean cv. Williams.
The SVQ292 rhizobial DNA that is adjacent to the IS50R of transposon Tn5-Mob was cloned and sequenced. Computer analyses showed that transposon Tn5-Mob in SVQ292 is located inside an ORF that is homologous to the *pyrF* gene of *Pseudomonas aeruginosa*, a gene that encodes for an orotidine-5-phosphate-decarboxylase.
A genomic library of wild-type strain HH103 was transferred en masse into SVQ292 and Nm^R Tc^R SVQ292 transconjugants were used to inoculate soybean cv. Williams plants. Soybean roots developed many pseudonodules and a few nitrogen-fixing nodules. Bacteria isolated from the Fix$^+$ nodules were Nm^R Tc^R and contained a cosmid (pMUS344). Mutant SVQ292 carrying cosmid pMUS344 was able to grow in minimal medium without uracile and formed nitrogen-fixing nodules on soybean.
A 1.5Kb *Pst*I fragment of pMUS344 was sequenced. The analysis of the sequence revealed a putative ORF that encodes for a polypeptide of 232 amino acid homologous to the *pyrF* gene of different bacteria, including *Bartonella bacilliformis* (68% identity), *Hemophilus influenzae* (41% identity), *Pseudomonas aeruginosa* (40% identity) or *E. coli* (38% identity).

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S6-P31

EFFECT OF *nodD1* FITA MUTATIONS ON *SINORHIZOBIUM FREDII* NODULATION ON SOYBEAN.

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It has been described that *nodD* FITA (Flavonoid Independent Transcription Activation) mutations can alter several rhizobial symbiotic properties. Interestingly, some of the FITA mutants previously described are affected (positively or negatively) on their nitrogen fixation ability. We have developed a genetic system (based on the presence of a gene conferring tetracycline resistance under the transcriptional control of a *nod* promoter) that allows positive selection of *nodD* FITA mutants. By using this system, we have isolated ten FITA mutants of *Sinorhizobium fredii* HH103 Rif^R pSym::Tn*5*-Mob (strain SVQ270). FITA-SVQ251 is significatively less effective with soybean cv. Williams than its parental strain SVQ270. Instead, FITA-SVQ255 appears to be more efective than SVQ270 with this plant. Transfer of the symbiotic plasmids of SVQ270, SVQ251 and SVQ255 to the pSym-cured derivatives of *S. fredii* USDA192 and USDA193 allowed the study of FITA-mutations in different *S. fredii* backgrounds. Transconjugants carrying pSym-SVQ255 produced more nodules on soybean that those harbouring pSym-SVQ270 or pSym-SVQ251. Moreover, USDA192-pSym- or USDA193-pSym-transconjugants carrying pSym-SVQ270 or pSym-SVQ251 were equally effectives with soybean c.v. Williams. These results show: 1) the importance of the pSym/chromosomal background interaction and 2) the possibility to obtain different symbiotic phenotypes derived from *nod* gene constitutive expression. The *nodD1* genes of mutants SVQ251 and SVQ255 were isolated by PCR, sequenced and compared to the *nodD1* sequnce of HH103. In both cases we found punctual differences with regard to the wild-type sequence, affecting always to a leucine residue. Leucine at position 244 has changed to a isoleucine residue in FITA-SVQ251, whereas in FITA-SVQ255 the same leucine residue has changed to a valine residue and the leucine at position 288 has been substituted by a proline residue. Interestingly, most of leucine residues of NodD1 proteins are strongly conserved, suggesting that they could play an important role in the structure and/or function of this protein.

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S6-P32

THE *pssNOP* REGION OF *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII* TA1: TRANSCRIPTIONAL ACTIVITY.

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In the *exo* region of *Rhizobium leguminosarum* bv. *trifolii* TA1 (Król et al. 1998), we have identified three genes designated *pssN*, *pssO* and *pssP*. On the basis on sequence analysis and according to the nomenclature of Paulsen (1997), putative PssP protein may be included into MPA1 family (cyplasmatic membrane periplasmatic auxiliary protein). The PssN belongs to the OMA family (outer membrane auxiliary protein), and exhibits features typical for this family of proteins: presence of N-terminal hydrophobic sequence and the signal peptidase II cleavage site. Secondary structure and homology to surface proteins suggest that PssO could be integral outer membrane protein. PssN and PssP, together with PssO may be the part of type 1 system found in Gram-negative bacteria and could be involved in polymerisation and translocation of EPS across the membranes. We measured transcriptional activity of *pssNOP* region, as a level of β-galactosidase synthesis, using defined, restriction DNA fragments preceding each of the identified genes, cloned in the front of promoterless *lacZ* gene. In the wild type background the *pssN* promoter activity is weak and is not affected by tested inducers (flavonoids, EPS, phosphates, ammonia). On the other hand the *pssP* promoter shows greater, than *pssN* activity, that depended on the length of cloned fragments. Moreover, low concentration of flavonoids, phosphates and ammonia increased the level of *pssP* promoter activity twofold. The promoter of *pssO* gene revealed the highest activity and it was significantly induced with low concentration of flavonoids, phosphates and ammonia. Taking into consideration these results, and possible localisation of PssO in the outer membrane, we speculate that this protein can play role in the signal transducing from environment to bacterial cell.

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S6-P33

AUTOREGULATION OF NODULATION IN *VICIA SATIVA* SSP. *NIGRA*

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Rhizobium bacteria induce formation of root nodules on leguminous plants, such as *Vicia sativa* ssp. *nigra* (vetch). Only plants in a particular physiological state are able to form root nodules. At least two conditions render legume roots resistant to nodulation, (i) the presence of a large amount of combined nitrogen, for example nitrate, and (ii) the presence of already formed root nodules. The latter situation is called autoregulation of nodulation (AUT). On roots in which AUT is induced, root nodules arrested in various stages of development can be observed. The mechanism of AUT is unknown. We study AUT by using a so-called split root system, in which two roots on one plant, root A and root B, are separately grown and inoculated. The systemic influence of treatment of root A on root B can be analyzed. Results obtained with this system show that AUT in root B can be triggered by rhizobial nodulation of root A and, to a lesser extent, by inoculation of root A with a *Rhizobium* mutant strain that induces formation of normal nodule primordia but not of infection threads. Purified vetch-specific Nod factors (NodRlv-IV/V[18:4,Ac], mitogenic, and, to a lesser extent, NodRlv-IV/V[18:1,Ac], non-mitogenic) added to root A induced partial AUT in root B. These results indicate that induction of a partial AUT is not dependent on formation of infection threads and nodule primordia. Significantly, under unbuffered conditions, induction of AUT coincided with growth differences between root A and root B. Root A became much longer and thicker than root B, while the growth medium of root B became very acid in comparison to that of root A. Strong buffering of the plant growth medium normalized growth of root B, and equalized or diminished growth differences between root A and root B, while AUT remained. Interestingly, under unbuffered conditions, but not under buffered conditions, the AUT-related phenomena at root B could be induced by addition of zeatine to root A. Possibly, the mechanism of AUT in a split root system consists of two components, one dependent on nodule primordium formation in root A, and one dependent on the physiological state of root A, determined by its hormonal balance.

S6-P34

MUTATIONS WITHIN THE *RHIZOBIUM LEGUMINOSARUM BV* VICIAE *pssCFGHI* GENES ENCODING PUTATIVE GLYCOSYLTRANSFERASES DO NOT LEAD TO ALTERATIONS IN THE STRUCTURE OF ACIDIC EXOPOLYSACCHARIDE REPEATING UNIT BUT COURSE DIFFERENT CHANGES IN SYMBIOSIS

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The production of acidic exopolysaccharide (EPS) was shown to be required for the infection process by rhizobia that induce the formation of indeterminate nodules of leguminous host plants. Recent molecular genetic studies of EPS biosynthesis in *R. leguminosarum* bv *viciae* VF39 allowed us to identify and sequenced seven genes (*pss*EDCFGHI) which deduced amino acid sequences displayed significant homology to glycosyltransferases. Mutations in the *pss*E and *pss*D genes completely blocked the synthesis of EPS whereas mutations in other genes led to production of EPS although in the lowered amount (40-80% of wild type strain). The polysaccharide from the VF39 strain was found to be quite similar to the polymers identified in many other *R. leguminosarum* strains. The octasaccharide repeating unit of the EPS with carbohydrate backbone (GlcA: Glc: Gal/ 2: 5: 1) carried pyruvate ketals at OH-4 and OH-6 of Gal-*h* and Glc-*g*, and was partially substituted with 3-hydroxybutanoyl (at OH-3 and OH-2 of Gal-*h*) and acetyl (at OH-3 and OH-2 of Glc-*a* and GlcA-*c*) groups. This conclusion is based on results of component analysis, NMR ^{13}C of depyruvilated and deacylated EPS, and NMR ^{1}H and ^{13}C spectra of octasaccharide fragments obtained by EPS treatment with crude preparation of depolymerases induced by rhizobial bacteriophage 543. The same methods were applied to study structure of the polysaccharides from the PssCFGHI mutants. Rather unexpectedly, these polymers were found to be identical to the EPS from the parental strain. The influence of the mutations on nodulation of *V. faba* was also studied. It was shown that PssC,D,E, and PssF mutants failed to form nodules at all, whereas PssH mutant formed small ineffective nodules. No changes in symbiosis was observed with PssG and PssI mutants. We hypothesise that i) some glycosyltransferases can be interchangeable in EPS biosynthesis, and ii) the level of EPS synthesis and/or extent of EPS polymerization and modification are important for normal nodule formation.

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S6-P35

THE REGULATION OF EXOPOLYSACCHARIDE BIOSYNTHESIS IN *SINORHIZOBIUM MELILOTI*

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Sinorhizobium meliloti produces two structurally different exopoly-saccharides: EPS I (succionoglycan) and EPS II (galactoglucan). At least one of these EPSs is required for invasion of *Medicago sativa* nodules by *S. meliloti*. The biosynthesis of EPS I is directed by the *exo/exs* genes, whereas the *exp* genes are required for the biosynthesis of EPS II. *S. meliloti* wild type cells normally produce only EPS I. The biosynthesis of EPS II is induced by phosphate limitation (Zhan *et al.*, 1991) or in the presence of a mutation in either the *exp*R or *muc*R gene. An additional regulatory gene could be identified with the gene *expG* which is located in the *exp* gene cluster (Becker *et al.*, 1997). Using *exp-lacZ* transcriptional fusions a stimulating effect of either extra copies of this *expG* gene or the *mucR* mutation on the transcription of all *exp* complementation groups was determined. Phosphate limitation also resulted in increased expression of the *exp-lacZ* fusions. This increase was reduced in strains characterized by a deletion of *expG*. The *S. meliloti phoB* gene was required for the activation of *exp* gene expression under phosphate limitation, but not for induction of *exp* expression by MucR or ExpG. The *expA*, *expD*, *expG* and *expE* promoters contain sequences with similarities to the PHO box known as the PhoB-binding site in phosphate-regulated promoters in *Escherichia coli*. Additional analyses of these putative PHO boxes with the help of a new constructed promoter-probe vector supported the assumption that these PHO boxes might represent binding-sites for PhoB.

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S6-P36

DUAL CONTROL OF THE *NODA* OPERON OF *AZORHIZOBIUM CAULINODANS* ORS571 BY A *NOD* BOX AND A NIFA-σ^{54}-TYPE PROMOTER

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Earlier studies showed that the *Azorhizobium caulinodans nodA* promoter is controlled by a plant-derived flavonoid signal via a NodD transcription activator. Here we report that the transcription of the operon is also under the *A. caulinodans* NifA control. A NifA-σ^{54}-type promoter, P2*nod*A, is present upstream of the *nod* box consensus motif of the *nod*A gene and directs expression of a *nod*A-gus reporter gene both in free-living bacteria under nitrogen fixation conditions and in bacteroids. Mutation of P2*nod*A affected, under certain conditions, the efficiency of nodulation and the timing of nodule senescence, suggesting that the dual control may help to optimize nodule initiation and functioning in the natural habitat of the symbiosis.

S6-P37

MEMBRANE BIOGENESIS IN PEA NODULES

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The legume root nodule has many features that make it an excellent model in which to analyze the trans-Golgi network in plant cells. In the central tissues of pea nodules, each host cell is hyperinfected by endosymbiotic *Rhizobium* cells. Each bacterium is individually enclosed by a plant-derived "symbiosome membrane" (SM). The total surface area of this membrane is 30-100 times greater than for plasma membrane, making it a highly specialized, highly amplified membrane compartment. Although several SM specific proteins have been identified, the targeting of these components to the SM is completely unknown and no specific target information seem to be present in the different nodulins analysed to date. Due to the "mosaic" nature of the SM, which shares characteristics of both the plasma and the vacuolar membrane, it seems possible that the infected cell is able to re-direct more than one targeting route to the SM.
A symbiotically defective pea mutant, sym31, is arrested at an "early" stage of symbiosome development, probably as a result of an aberrant vesicle targeting pathway. Evidence from antibody probes suggests that in this mutant the composition of the SM is indistinguishable from that of plasma membrane. Moreover, the enclosed bacteroids fail to develop the capacity for nitrogen fixation and remain morphologically undifferentiated. Nodule-derived cDNA obtained from sym31 was subjected to suppression subtractive hybridisation (SSH), in order to enrich for those transcripts with enhanced expression compared to uninfected roots or wild type nodules. Using this approach, we have identified several novel transporters that could mediate the delivery of yet unidentified compounds to the differentiating bacteroids. It is likely that the nature of these nutrients changes during nodule development, together with the differentiation of the symbiosome membrane.

S6-P42

AUXIN, ETHYLENE AND NODULATION IN SOYBEAN ROOTS

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Nodulation control in legumes involves both endogenous and environmental factors. Several lines of evidence pointed to the involvement of plant hormones as secondary signals for nodule organogenesis. Some of these leads to the so-called auxin-burst-control (ABC) hypothesis[1], which states that once nodule initiation has occurred, the shoot responds with an increase in translocation of auxins to the root inhibiting further nodule initiation and maturation. Recently, a quantitative increase in root auxin concentration was observed in soybean cv. Bragg following inoculation with *Bradyrhizobium*, but not in its nts382 mutant (defective in autoregulation response)[2]. On the other hand, evidences also support ethylene as a mediator of nodulation control in soybean and other legumes under both inhibitory and non-inhibitory nitrate conditions[3, 4, 5].
In this work we have studied the relationship among auxin, ethylene and nodulation in roots of soybean. Auxin strongly inhibited nodulation in soybean cv. Bragg. When applied at inoculation, nodule number was decreased at concentrations of IAA as low as 1 µM (25% inhibition), this reduction increasing up to 45% at 10 µM. This effect was overcome with the simultaneous application of Ag^+ ions, indicating that ethylene might be involved in such an inhibition. As it is known that ethylene acts inhibiting nodulation in a short periode after inoculation[3, 4, 5], we tested whether auxin might elicit ethylene biosynthesis in a similar time scale. In roots grown in a culture system with vermiculite, IAA at concentrations from 1 to 20 µM did not induce a significant increase in ethylene production 24, 48, and 96 h after its application. However, in an *in situ* assay using aeroponically grown soybean seedlings, auxin significantly increased ethylene evolution, this effect being evidet at 1 µM and only 3 h after IAA treatment. This increase was dependent on the concentration of IAA and reached a maximun 12 h after treatment, ranging between 73% and 250% stimulation for 1 and 100 µM IAA, respectively. Overall, results agree with the ABC hypothesis, but with ethylene being the causal effect as a second messager for auxin.

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S6-P43

A *PHASEOLUS VULGARIS* NODULIN IS A HOMOLOG OF A CHLOROPLAST NUCLEOID DNA-BINDING PROTEIN FROM TOBACCO.

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Nodule organogénesis is a developmental program involving complex molecular signaling events between Rhizobium and leguminous plants. Nodules are specialized organs where many plant proteins (nodulins) are expressed in order to contribute to the establishment of multiple physiological conditions for nitrogen fixation. We recently purified to homogeneity a 41.5-kDa-bean nodulin by chromatographic methods. A polyclonal antiserum was raised in rabbits and used to probe immunoblots of protein extracts from several tissues. A single 41.5 kDa was exclusively detected in bean nodules with a kinetic pattern corresponding to a late nodulin (12-30 dai). By immuno-fluorescence confocal microscopy we observed that this protein is restricted to the infected cells of the nodule central tissue. In order to gain insight into its probable function, the amino acid sequence corresponding to two contiguous internal peptides was determined, the aligned sequence (BLAST program) gave a high identity match (36%) and similarity (68%) in 25 aa, to a protein found in tobacco chloroplast (CND41). This protein seems to bind to DNA and to negatively regulate photosynthetic genes in non-photosynthetic tissues. Currently we are cloning a corresponding cDNA and characterizing its possible nodule function.

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S6-P44

IDENTIFICATION OF FUNCTIONAL DOMAINS IN THE CCS52 PROTEIN REGULATING CELL DIFFERENTIATION DURING NODULE ORGANOGENESIS

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Recently it was demonstrated that a nodule-enhanced cDNA encodes a cell cycle switch protein CCS52 that regulates the transition from cell proliferation to differentiation (Cebolla et al., 1999). We showed that CCS52 is required for cell division arrest and transformation of mitotic cycles to endocycles. Southern analysis revealed that *ccs52* represent a gene family in Medicago. We have isolated two ccs52 genes in Medicago sativa and Medicago truncatula exhibiting different tissu-specificity and different expression pattern during cell cycle. The CCS52 proteins are characterised by 7 repeats of the WD 40 motif at the cental and C-terminal parts, several putative CDK phosphorylation sites and by the conservation of two oligopeptides in the N-terminal region. In order to test structure-function relationships a set of deleted forms of the protein lacking either one or both oligopeptide motifs and a series of point mutations affecting the phosphorylation sites were generated. The effect of these mutations on the biological activity of the CCS52 protein will be presented.

S6-P45

CLONING AND CHARACTERIZATION OF CELLULOLYTIC GENES OF *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII* ANU843

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Batteries of several enzymes are required for the microbial conversion of the cellulose that is an important constituent of the lignocellulosic wall materials of the legume host plant. At least three different classes of hydrolytic enzymes are thought to participate in this process: endo-1,4-β-glucanase, exo-1,4-cellobiohydrolase and β-glucosidase. During the infection of white clover roots by *Rhizobium leguminosarium* biovar *trifolii* leading to development of the root nodule the bacteria must pass across the root hair wall. On the way into the root, local cell wall degradation is required to allow passage of the rhizobia in the infection thread from one cell into the next. This cell wall degradation has been suggested to be caused by enzymes secreted by the bacteria, by the plant, or both rhizobial and plant enzymes.

In another poster we summarize the molecular characterization of the cell-bound cellulase C2 from wild-type ANU843 that we have purified, isolated and sequenced. A β-glucosidase gene was amplified from the genomic DNA of ANU843 with the Polymerase Chain Reaction (PCR) using primers designed from the sequence of two internal oligopeptides of this enzyme. The amplified fragment was mutated by insertion of an interposon and used for gene replacement in the wild-type strain. The β-glucosidase encoding gene fragment was labeled and used as a DNA probe in hybridization of the genomic DNA of ANU843 in order to obtain the adjacent genes. A small DNA library was constructed in Bluescript plasmid with fragments surrounding the hybridization signal. A 5 kb *Eco*RI DNA fragment from this library that includes our gene was isolated and sequenced. The resulting sequence was compared to the database bank and found to have high homology to cellulolytic genes. The chromosomal β-glucosidase mutant was used in studies on the role of this enzyme in the root-nodule symbiosis.

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S6-P46

birS, A NOVEL REGULATORY LOCUS IS INVOLVED IN THE *SINORHIZOBIUM MELILOTI* GROWTH RESPONSE TO BIOTIN.

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We are exploring the molecular and ecological roles of plant exuded compounds for alfalfa rhizosphere colonization by *S. meliloti*. Because *S. meliloti* growth in alfalfa rhizospheres strongly depends on exogenous biotin [1] we have begun to explore the molecular mechanisms the microorganism employs to sense and utilize plant exuded biotin. *S. meliloti* responds to external biotin signals through the *bioS* regulatory locus [2,3]. BioS carries traits of LysR type regulators and the *bioS* gene is transcribed in response to minute amounts of plant exuded biotin. In addition to the *bioS* gene, we have identified a second biotin-regulated DNA locus involved in the mobilization of poly-3-hydroxybutyrate [3]. The *bioS* and the *bdhA* genes are expressed in stationary growth phase and we hypothesize that both genes are co-regulated by a global regulator which has not yet been identified. Work in the lab focuses now on the isolation and characterization of an overall regulatory locus central for the *S. meliloti* biotin response and involved in the regulation of the *bioS* and the *bdhA* genes. For this purpose a *bioS-lacZ* fusion strain was mutated using a transposable promoter probe, which carries a promoterless *nptII* gene. Screening in the absence of biotin but in the presence of kanamycin for a possible repressor protein involved in *bioS* regulation, resulted in the isolation of three putative repressor mutants. The mutants showed a constitutive *bioS* expression on plates containing X-Gal and in defined medium. The flanking DNA regions of the transposon of one of the mutants (Rm1021-WS80-1) were cloned, subject to DNA sequencing and the mutated gene was termed *birS*. The *birS* gene codes for a novel regulatory protein and is located on the bacterial chromosome in close distance to the *phoU-B* locus. Growth studies in defined media support the hypothesis that the *birS* gene is involved in processes important for resuming growth after stationary phase starvation.

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S6-P47

CHARACTERISING THE COMPETITIVE ADVANTAGES OF BACTERIOCIN PRODUCTION AND RESISTANCE IN RHIZOBIA.

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Our culture collection contains a number of strains of rhizobia which have been shown to produce bacteriocins against a range of sensitive strains. Bacteriocins are often defined as narrow-spectrum antibiotics produced by bacteria and active against only closely related species or strains. It is logical to assume the purpose of producing bacteriocins is to enhance the competitiveness of producer strains. Bacteriocins may play an important role in improving inoculant survival against indigenous soil rhizobia.
We have developed an assay to measure the competitiveness of a bacteriocin-producing strain against a sensitive: This is fast and reliable alternative to the more traditional plant tests which screen for root nodules. The assay involves pipetting a 1:1 ratio of a producer and sensitive strain onto a nitro-cellulose membrane placed onto suitable media. The bacteria are allowed to grow for 3 - 5 days on the membrane, before they are washed off and plated onto to selective media to determine the final ratios.
Using this method we have been able to characterise how effective different bacteriocin producers are at killing a sensitive strain, what distance a sensitive strain needs to be to prevent inhibition by a certain bacteriocin producer and how quickly bacteriocin inhibits a sensitive strain.
This method is useful in characterising bacteriocin production of a particular strain and maybe useful to characterise other traits identified as useful to improve competitiveness, but plant tests will still be necessary to confirm symbiotic effectiveness as a final stage in development of superior inoculant strains with improved competitiveness.

S6-P48

AN *eglC* MUTATION IN POLYSACCHARIDE DEFICIENT *SINORHIZOBIUM MELILOTI* STRAINS RESTORES THE ABILITY TO FORM NITROGEN-FIXING ROOT NODULES

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Production of either of the bacterial exopolysaccharides (EPS) succinoglycan or galactoglucan is required for the establishment of a nitrogen-fixing symbiosis between *Sinorhizobium meliloti* 2011 and its host plant alfalfa. *S. meliloti* mutants unable to produce EPS induce non-infected nodules that display symptoms of plant defense and do not fix nitrogen. Such mutants regained the capacity to form nitrogen-fixing nodules if they carried a mutation in *eglC* within the *eff-482* gene region located on the megaplasmid 1 (Sharypova et al., 1999). The *eglC* gene encodes a putative endo-β-1,3-1,4-glycanase that is similar to the ExsH glycanase of *S. meliloti*. The ExsH and ExoK glycanases are involved in degradation of succinoglycan (York & Walker, 1997). A triple *S. meliloti exoK exsH eglC* mutant similarly to double *exoK exsH* mutant induced normal nitrogen-fixing nodules, implicating that *eglC* was not required for the generation of symbiotically active succinoglycan oligosaccharides. An *eglC-gusA* fusion was expressed at a very low level ex planta, whereas it was induced in nodules. Microscopic analysis of alfalfa nodules elicited by a *S.meliloti exoY eglC* mutant demonstrated that the *eglC* mutation allowed a successful nodule development, although it did not restore the normal structure of infection threads. Similarly to the *exoY*-induced infection threads which had been described elsewhere (Niehaus et al. 1994), infection threads formed by the *exoY eglC* mutant were much thicker than normal ones and contaied numerous densely packed bacteroids. The *eglC* mutation was able to suppress symbiotic defects of not only *exoY* mutant, but others including double *exoY expA1* mutant and *exoB* mutant.

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York G.M. & Walker G.C. 1997 Mol. Microbiol. 25: 117-134.

S6-P49

MucR AND MucS ARE ESSENTIAL FOR GALACTOGLUCAN PRODUCTION IN *SINORHIZOBIUM MELILOTI* EFB1.

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S. meliloti can produce two types of acidic exopolysaccharides, succinoglycan (EPSI) and galactoglucan (EPSII). Strain SU47 and derivatives under normal culture conditions produces only EPSI, and secretes EPSII under phosphate limitation conditions or in mutatants in either two regulator loci, *mucR* or *expR*. Conversely, strain EFBI simultaneously produces both EPSs resulting in the fromation of very mucoid colonies. Genes responsible for the production and secretion of both EPSs are organized in two clusters, the exo/exs cluster for EPSI and the exp cluster for EPSII. It has been proposed that MucR acts as a transcriptional repressor that blocks the expression of the exp genes responsible for galactoglucan production. Within the *exp* cluster a regulatory gene *mucS* has been found and is necessary for the expression of *exp* genes and for EPSII production under phosphate limitation conditions. We have previously shown that in strain EFB1, *mucR* is necessary for galactoglucan production and *mucS* expression. A *mucS* mutant produces non mucoid colonies which are fluorescent in calcofluor. Both mutants were functionally complemented by the cloned *mucR* and mucS genes respectively. These results indicate that both *mucR* and *mucS* are essential for galactoglucan production. LacZ transcriptional fusions of the *mucS* promoter have shown a high expression in EFB1, three times lower expression in a mucS mutant and no expression in both a mucR and a mucR/mucS mutant. These results strongly indicate that *mucR* is positively regulating the *mucS* gene in EFB1 and that *mucS* may regulate its own expression. Alternatively, *mucS* may not be auto regulated and the expression level observed in EFB1 might be a combination of *mucS* and the downstream gene *ExpC* expression. We have also investigated the effect of *mucR* and *mucS* on the expression of the *expE* promoter. The expression was about 95% lower in the *mucS*$^-$ background and gives no expression in the *mucR*$^-$ background, which is consistent with the role of both genes in EFB1. Deletion analysis of this promoter has revealed an important region for gene expression. This region consist in a 20 bp sequence situated 230 bp upstream the start codon of *expE1* and contains a palindromic region. Sequence analysis of the *exp* regulon has shown that similar regions are also present upstream the start codon of *expA1*, *expD1* and *expG*. The sequence is highly conserved and is located at similar distance of the start codon of the first gene of each operon , except in *expG* that is only 20 bp upstream. It is possible that this latter region forms part of the *expC* promoter. This hypothesis might explain the results on *mucS* expression in the *mucS*$^-$ background.

S6-P50

EXPRESSION SYSTEMS BASED ON RHIZOBIAL NOD PROMOTERS CAN BE USED TO EXPRESS FOREIGN GENES IN THE ALFALFA RHIZOSPHERE.

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Sinorhizobium meliloti and *Pseudomonas fluorescens* F113 are good colonizers of the alfalfa rhizosphere, which form microcolonies on the root surface. Colonization patterns are different for both bacteria as root hairs were not colonized by the *Pseudomonas*. Strain F113 was originally isolated from the sugarbeet rhizosphere and can control the fungal pathogen *Pithium ultimum* because of DAPG production. *bph* genes that allow this bacteria to grow in biphenyl and several PCBs have been introduced into this bacterium in order to create a bioremediation strain. The aim of our work was to design expression system that allows foreign genes expression (i.e. bioremediation genes) in the plant rhizosphere.
We have analyzed expression systems based on two *Sinorhizobium meliloti nod* promoters (nodbox1 and nodbox4) and their activator protein, NodD1. Different expression systems have been fused to the *lacZ* gene for measuring activity *in vitro* and to *gfp* for visualizing expression in the rhizosphere. The constructs were introduced into *S. meliloti* and into *Pseudomonas fluorescens* F113 and tested. Expression of nod promoters was different in each bacterium, although NodD1 activity was necessary in all the cases for full promoter expression. Nodbox1 was the strongest promoter in *S. meliloti*, and was inducible by luteolin (the major flavonoid inducer in alfalfa). Conversely, only nodbox4 was functional in *P. fluorescens* although expression was independent of luteolin. Bacteria harbouring these constructs expressed *gfp* activity in alfalfa rhizosphere, as judged by confocal laser microscopy, indicating that they can be used to induce foreign gene expression in the rhizosphere. We are presently fusing the expression system to the *bph* genes in order to engineer strains able to degrade biphenyl and PCB's in the alfalfa rhizosphere.

S6-P51

THE NODULIN VFENOD18 IS AN ATP-BINDING PROTEIN IN THE INFECTED CELLS OF *VICIA FABA* L. NODULES

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The broad bean nodulin gene VfENOD18 was identified following a differential screening of a nodule cDNA library. The corresponding 0,8 kb transcripts were exclusively detectable in root nodules, transcription started 5 days after inoculation and was localized by tissue-print hybridization in the nitrogen fixing zone III. By comparison, broad bean leghemoglobin gene expression commences one day later. Several VfENOD18 genomic clones were isolated. Surprisingly, only one clone contained the 5' UTR known from the isolated VfENOD18 cDNAs. All other clones had a different sequence instead. We conclude that two different VfENOD18 loci exist in broad bean, only one of them being responsible for the synthesis of the VfENOD18 transcripts detected in root nodules. The potential promoter sequence of the VfENOD18 gene was fused to the GUSint reporter gene and used to generate transgenic *Vicia hirsuta* roots via the *A. rhizogenes* transformation system. GUS staining in transgenic nodules was located exclusively in the nitrogen fixing zone III.To characterize VfENOD18 on the protein level polyclonal antibodies were generated using the purified recombinant VfENOD18 protein produced in *E. coli* by employing the pMAL-c expression system. These antibodies recognized immunoreactive proteins in tissue extracts from indeterminate nodules of different leguminous plants, but also from non-symbiotic tissues of *Glycine max* and from tissues of *Arabidopsis thaliana* and *Zea mays*. Using immunogold labelling the nodulin VfENOD18 was localized in the cytoplasm of infected cells in the nitrogen-fixing zone of broad bean nodules. The VfENOD18 amino acid sequence displayed homologies not only to amino acid sequences deduced from various plant ESTs, but also to the bacterial MJ0577 superfamily. The name-giving *Methanococcus jannaschii* protein can bind ATP. PCR experiments revealed that the amino acid sequen-ces of the putative C-terminal ATP-binding sites of the VfENOD18 homologues from *Lens culinaris*, *Vicia hirsuta*, *Vicia sativa* and *Vicia villosa* are conserved. Interestingly, most of the amino acids involved in ATP-binding are con-served in the VfENOD18 protein and its plant homologues. Using the recombinant VfENOD18 protein in conjunction with the biotin photo-affinity ATP analog $8N_3ATP[\gamma]$biotin it could be demonstrated that VfENOD18 is an ATP-binding protein. The recombinant VfENOD18 showed no ATPase activity by itself. We propose that VfENOD18 is a member of a novel family of ATP-binding proteins in plants probably showing ATPase activity only in combination with factors undetected so far.

S6-P52

MAP KINASES IN THE SIMBIOSIS *BRADYRHIZOBIUM-LUPINUS*

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The aim of our work is to investigate a possible involvement of MAPKs (mitogen- activated protein kinases) in the establishment of the symbiosis *Bradyrhizobium-Lupinus*. For this purpose different antibodies, M23, M24, H140 and H141, raised against synthetic peptides representing the COOH terminal 10 aminoacids of the alfalfa MAP kinases, MMK1, MMK4, MMK2 and MMK3 respectively (1) have been used to detect the correspondig MAPKs in *Lupinus* . MMK1 and MMK4, both stress-induced MAP kinases are renamed SIM and SAM kinases (2).
The immunoblottings of the SDS-PAGE crude extracts proteins of non innoculated and innoculated roots and nodules, were visualized by ECL showing distinct MAP kinase proteins. SIMK a kinase of 46 kD is involved in general hyper-osmotic conditions. Constitutively expressed proteins of SIMK were detected in lupin roots and young nodules of 10 and 15 days. SAMK, a 44 kD kinase induced by different forms of stress and activated rapidly and transiently by wounding, was detected in roots and nodules of *Lupinus*. MMK2 is involved in cell growth and was also detected in lupin roots and nodules. MMK3 is a kinase activated in late mitosis, but this protein could be found during all stages of the cell cycle. It was detected in apical meristemes and in young nodules of Lupin plants.
A gold-immunolocalization at ultrastructural level was carried out in young nodules and non inoculated roots. When M23 antibody was used labelling was mainly on the cytoplasm of cortex and infected cells. No immunoreaction was observed in cell wall, intercellular space, vacuole and another organelles, except nucleus in which gold particles were dispersed on the different regions. The constitutive nuclear localization of SIMK has been described (3). Some differences were observed between recently infected cells and completely infected cells. In latter, a lower labelling was observed on the cytoplasm. Immunolabelling with H141 antibody was mainly in nucleus, specially in nucleolus. It is interesting to notice that a clear inmunolabelling was found in the root vascular bundles when M24 was tested.
The phosphorylating activities in the immunoprecipitates of SIMK, SAMK and MMK3 were analysed using γ^{32} P-ATP, but further experiments have to be carried out to assure the mediation of a determined MAPK in the infection by *Bradyrhizobium*.

(1) Jonak et al. 1996. PNAS. 93: 11274-11279;
(2) Bögre et al. 1997. Plant Cell. 9: 75-83;
(3) Munnik et al. 1999. Plant J. 4: 381-388.

S6-P53

THE FUCOSE BIOSYNTHESIS LOCUS OF *RHIZOBIUM ETLI* IS CONSTITUTIVELY TRANSCRIBED AND IS REQUIRED BOTH FOR NOD FACTOR AND LIPOPOLYSACCHARIDE BIOSYNTHESIS

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In a previous report (Vinuesa *et al.*, 1999) we described the identification of a plasmid-borne locus of *R. etli* strain KIM5s involved in lipopolysaccharide (LPS) O antigen biosynthesis. Here we present an extension of that work, reporting the identification, mutagenesis and regulation analysis of two other genes involved in O antigen biosynthesis. We show that the *gmd-fcl* locus of *R. etli* KIM5s is presumably involved in the synthesis of a constitutive fucose pool, which is used by specific fucosyltransferases for LPS and Nod factor (LCO) fucosylation, respectively. *gmd-fcl* orthologues have been previously identified in other rhizobia (e.g. *A. caulinodans*, *S. fredii* and *Rhizobium* sp. NGR234), where they are flavonoid- inducible as part of *hsn* loci, being called *noeL* and *nolK*, respectively. However, in *R. etli* strains this locus forms part of LPS O antigen biosynthesis clusters, and is expressed constitutively, both under free-living and symbiotic conditions, as shown by transcriptional fusions to *gusA*. *R. etli* strains carrying mutations in the *gmd-fcl* locus display a rough LPS, eliciting empty pseudonodules on bean plants, the infection threads aborting in the root hairs or in the first layers of cortical cells, as revealed by microscopical analysis of *gusA*- and *gfp*-tagged cells. The LCOs secreted by such mutants show an altered mobility on TLC plates, as compared to those of wild-type strains, presumably because they are not fucosylated. This will be determined by GC-MS analysis of the LCOs produced by the *wt* and mutant strains. Using degenerate primers we could PCR-amplify and clone an internal fragment of the *nodZ* gene of *R. etli* strains. This fragment was used for mutagenesis of the *nodZ* locus. We are constructing *nodZ::lacZ* transcriptional fusions to study the regulation of this gene. We hypothesize that in strains producing fucosylated LPSs (and/or EPSs) and LCOs, the fucose biosynthesis locus is constitutively transcribed, representing a metabolic link in the biosynthesis of surface polysaccharides and LCOs. We expect that in *R. etli* strains only *nodZ*, the LCO-specific fucosyltransferase, is flavonoid regulated. We are currently testing this working hypothesis experimentally.

Vinuesa *et al.*, 1999. J. Bacteriol. 181:5604-5614

S6-P54

MOLECULAR CHARACTERIZATION OF CELLULASE C2 FROM *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII* ANU843.

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A central event in development of root nodule symbiosis is the erosion of a cellulosic plant wall through which the bacterial symbiont passes to establish a nitrogen-fixing, intracellular endosymbiotic state within the host. This process of wall degradation must be delicately balanced in order for the slow, localized penetration of the bacterial symbiont to occur without over destruction of the host cell. Several studies have reported on the presence of various enzymes produced by rhizobia that degrade plant cell wall polymers. However, the activities of these rhizobial enzymes are very low and at the limit of sensitivity of conventional reducing sugar assays; this has hampered research progress in this area. Recently, we have developped a method to purified the cellulase C2 from *Rhizobium leguminosarum* bv. trifolii ANU843. By using several chromatographic methods we got an apparently pure 1 ,4-ß-D-endoglucanase that only can degrade noncrystaline cellulose. The molecular mass of the enzyme was estimated to be 33.3 KDa. The optimal pH for enzyme activity was 5 and the maximal rate of CMC hydrolysis was reached at 40°C. The enzyme had an apparent Km of 89.4 mg/mL for CMC as substrate. An internal peptide sequence of this enzyme has homology to a sequence from the gene *celC* of *Agrobacterium tumefaciens*. This gene has homology to endoglucanase genes from *Acetobacter xylinus* (*EngX*), *Erwinia chrysanthemi* (*EGY*), *Cellulomonas uda* (*Cuda*) and *Escherichia coli* K12 (*bcsC*). These genes from *Agrobacterium* and *Acetobacter* are localized in a region of chromosome involved in cellulose synthesis. Cellulase C2 could be correlated to *the R. leguminosarum* bv. trifolii *cel* genes (1) rather than to PlyA and PlyB proteins (2,3). In another poster we will summarize the current status of studies on the biological activity of this *Rhizobium* cell-bound cellulase involved in *Rhizobium*-clover root hair interaction.

1. N. Ausmees et al. 1999. *Microbiology* 145: 1253-1262.
2. C. Finnie et al. 1998. *Journal of Bacteriology* 180: 1691-1699.
3. A. Zorreguieta et al. 2000. *Journal of Bacteriology* 182: 130-1312.

S6-P55

IN VITRO MUTAGENESIS OF *BRADYRHIZOBIUM JAPONICUM sipF* GENETIC REGION BY A NOVEL TRANSPOSON TnKPK2

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We have previously constructed symbiotic mutants of *B. japonicum* by introducing the transposon Tn*phoA* into its genome, thereby setting polar mutations. Two individual mutant strains turned out to be of particular interest, because they exhibited specifically disturbed phenotypes only in symbiosis with the host plant (*Glycine max*). As revealed by a detailed genetic analysis, Tn*phoA* was inserted in two different alleles of prokaryotic signal peptidases. Since both signal peptidase genes, *sipS* and *sipF*, have been demonstrated to encode functional enzymes (Bairl and Müller 1998), the concept was developed that in *Bradyrhizobium* the signal peptidases are involved in the signal transduction between the invading bacteria and the host plant, and the two different signal peptidases are responsible for the processing of different subsets of preproteins.
The adjacent DNA fragments of the *sipF* and *sipS* genes were cloned and sequenced hoping to identify genes encoding putative preproteins that have to be processed by one or the other signal peptidase. To identify periplasmic proteins, and to test their expression and functional importance for the symbiotic interaction with soybeans, an *in vitro* mutagenesis was carried out using a novel '*phoA*-Km^R cassette that is based on a modified version of Tn*5* (Reznikoff et al.1999). The method and the newly designed transposon TnKPK2 will be presented, transposition events will be introduced, and further options to use TnKPK2 as a versatile tool will be discussed.

Bairl A, Müller P (1998) Mol Gen Genet 260:346-356
Reznikoff W, Goryshin I (1999) Epicentre Forum 6:5-7

S6-P56

CLONING AND SEQUENCING OF THE GLUTATHIONE SYNTHETASE GENE, *gshA*, OF TWO DIFFERENT *BRADYRHIZOBIUM* STRAINS

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Unpublished work in our laboratory has led to the identification of a partial gene of *Bradyrhizobium japoniucm* 110*spc*4. As determined by nucleotide sequence analysis this DNA framgment exhibits a high similarity to glutathione synthetases of different other organisms. The partially sequenced gene therefore was designated *gshA*. When an internal 600 bp *Bgl*II-*Bam*HI fragment was used as a hybridization probe only one band was detected, indicating that this locus exists only once in the genome of *B. japonicum* 110*spc*4. When the 600 bp *Bgl*II-*Bam*HI fragment, inserted in the mobilizable vector pJQ501, was introduced into the wild-type strain, this rusulted in the generation of a gene disruption mutant, which exhibited a severe symbiotic phenotype.
Physiological studies in Argentina with *Bradyrhizobium* sp. SEMIA 6144, a recommended strain to inoculate peanuts, have demonstrated that acid pH and aluminium (Al^{3+}) have a strongly negative influence on the viability of *Bradyrhizobium* sp. SEMIA 6144. These results demonstrate that the bacteria respond to this particular stress with a significantly enhanced content of glutathione.
By heterologous Southern hybridization analysis the corresponding gene was identified as as single hybridization signal in the genomic DNA of *Bradyrhizobium* sp. SEMIA 6144. Using a pair of primers which were designed based on highly conserved regions in the *B. japonicum gshA* gene, a 950 bp fragment was obtained by PCR, both from strain 110*spc*4 and from strain SEMIA 6144. The SEMIA 6144 amplification product was finally cloned into pTOPO and the nucleotide sequence of the DNA insert was determined. A comparison of the two partial sequences revealed a high degree of similarity, indicating that both strains are closely related to each other. The construction of a reporter gene fusion in the SEMIA 6144 background, to monitor and quantify the *gshA* gene expression as a response to acid pH and/or high Al^{3+} concentrations, is in progress.

S6-P57

INTEGRATION OF CELL CYCLE IN THE NODULE DEVELOPMENTAL PROGRAMME OF *MEDICAGO*

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Symbiosis between *Rhizobium* soil bacteria and their leguminous host plants results in the formation of a novel plant organ, the root nodule, specialised for symbiotic nitrogen-fixation. Nodule development can be programmed in a well defined root zone by application of the *Rhizobium* lipochitooligosaccharide Nod factors acting as host-specific mitogenic signals.
In nodule organogenesis cell cycle plays key roles both i) at the formation of the *de novo* nodule meristem by activating the cell cycle in the differentiated cortical cells and ii) at cell differentiation by arresting cell division or transforming mitotic cycles to endocycles for development of large symbiotic cells.
Studying nodule organogenesis in the *Rhizobium meliloti-Medicago* symbiotic model system, we aim at the identification of cell cycle components switching the cell fate either from quiescent state to proliferation or from proliferation toward differentiation. In *Medicago* roots and cell cultures Nod factors activate the cell cycle in the G0-arrested cells. To elucidate the action of Nod factors on re-entry of cell cycle and cell proliferation we have been studying different cell cycle elements, particularly cyclins regulating the transition of cells through the G0/G1-S and G2-M phases. The cell cycle function of an A2-type cyclin and its involvement in nodule organogenesis will be presented. Moreover, we show that a cell cycle switch gene, *ccs52* is essential for differentiation of nodule cells. Our studies indicate that *ccs52* is a novel regulator of the plant cell cycle that controls growth arrest, endoreduplication and cell size.

S6-P58

MOLECULAR MECHANISMS INVOLVING *ENOD40* AND A KRÜPPEL-LIKE ZINC FINGER IN *Medicago truncatula* NODULE ORGANOGENESIS

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Under nitrogen limitation, rhizobia induce the formation of nitrogen-fixing root nodules on their plant host. Mechanisms regulating plant host differentiation of the nitrogen-fixing root nodules remain mostly unknown. Expression of the early nodulin gene *enod40*, coding for an RNA containing only short ORFs, is induced in all dividing cortical cells of the nodule primordium and in the neighbouring pericycle. Transgenic *M. truncatula* plants overexpressing *enod40* exhibited accelerated nodulation by *Sinorhizobium meliloti* due to increased initiation of primordia. Two transgenic lines with reduced levels of *enod40* transcripts (likely due to co-suppression) formed few and modified nodule-like structures. The Krüppel-like Zn-finger gene, *Mtzpt2-1*, strongly expressed in vascular bundles of roots and nodules, was also functionally analyzed. Sense overexpression of this gene caused abnormal flower development in *M. truncatula*. In contrast, antisense plants grew normally but developed Fix- nodules where differentiation of the nitrogen-fixing zone and bacterial invasion were arrested. These results indicate that a vascular bundle-associated Krüppel-like gene is required for the formation of the central nitrogen-fixing zone whereas *enod40* function regulates initial steps of nodule morphogenesis.

S6-P59

ION CHANNELS CURRENTS IN PLASMA MEMBRANE VESICLES FROM BEAN ROOTS.

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Soil bacteria of the genera *Rhizobium* interact with the roots of legumes and form nodules in which atmospheric nitrogen is fixed. The specificity of this symbiotic relationship is given by Nod Factors (NFs) which are morphogenic lipochitooligosaccharides (LCOs) secreted from *Rhizobium*. These molecules are able to elicit, at pico- or nanomolar concentrations symbiotic responses on the host plant. We have reported that one of the earliest responses of bean root hairs to specific NFs is a calcium increase in the cytosol which is due at least in part by Ca2+ influxes from the external medium [1]. Felle et al. [2] have proposed that in root hairs of alfalfa (Medicago sativa) a calcium influx and a plasma membrane depolarization could be caused at least in part by a Cl- efflux, followed by a K+ efflux to rectify the membrane potential. Kurkdjian et al. [3] recently reported that anion channels, K+ channels and H+-ATPase pump currents are involved in these responses.
In order to understand the nature of the ion fluxes involved in the NF-induced electrical response, we directed this study to examine the different ion channels present in the plasma membrane of root cells. To do this, plasma membrane vesicles purified from roots of the common bean (*Phaseolus vulgaris* L.) were fused with DiphyPC planar bilayers under voltage-clamp conditions. Following this approach, we have found at least two kinds of voltage dependent conductances: a high conductance anionic channel and a more frequently observed, cationic channel. An analysis of each channel type will be presented and its possible role on NF signaling will be discussed.

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2. Felle, H.H. et al. (1998). Plant J. 13 : 455-63.
3. Kurkdjian, A.C. (2000). Plant J. 22 : 9-17.

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S6-P60

GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF BIOFILM FORMATION IN *RHIZOBIUM ETLI*

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Biofilms can be defined as communities of bacteria, which are attached to biotic and abiotic surfaces. We are working with Rhizobium gram-negative bacteria, which fixing Nitrogen associates with leguminous plants.
On the symbiotic process, the bacteria attach and infect the roots of the plant and formed a structure known as nodule. When the nodule is established, the differentiated bacteria, reduce atmospheric nitrogen to ammonia which is excreted to the plant cell and this assimilated to organic nitrogen by the plant.
R.etli form biofilm on PVC when it is grown in different culture conditions. The first observation on the biofilm formation by *Rhizobium* was that it is made at the stationary phase. This quite distinct to the biofilm formation by other bacteria such as *E.coli* or *Pseudomonas* that made biofilms during exponential phase and in very short period of time the complete formation of biofilm is made. A key observation was that if glucose was present, the factor was not produced and also those additions of glucose prevent the biofilm formation. We screen for mutants with altered biofilm formation in PVC. In this screen we expected 2 kind of mutants. The down mutants that produce less biofim and the up mutants with increase the biofilm formation. Downs mutants, all mutants identified were in central metabolism. In *pckr*, negative regulator of piruvate carboxiquinase activity, *sucd* encoding to succinil coa synthase, *phbc* encoding for polibetahydroxybutyrate synthase, *fixg* positive regulator of *fixnoqp* operon which encoding to cytochrome oxidase with high affinity for 02. Also identified mutants with increase biofilm formation. The isolated mutants were affected in a *cya*, *aglf* and *malk*. Two of them *aglf* and *malk* affected genes that belong of disaccharides transporters. The third mutant was in *cya* homologous gene encoding adenylate cyclase, and the physiologic characterization showed deficient grow in all disaccharides tested as carbon source and the most important result was the deficient utilization of glucose. We suggest that the glucose depletion is the signal for biofilm formation in *R. etli*, and in this conditions the bacteria synthesizes glycanase for the utilization of external polysaccharide like glucose source and in conditions the cell have made more monomers, dimers o trimers of the polysaccharides, changing for a more sticky cell.

S6-P61

DIFFERENT TYPES OF NODULES INDUCED BY SURFACE POLYSACCHARIDE MUTANTS TR53 AND TR63 OF *SINORHIZOBIUM MELILOTI*.

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Two mutants Tr53 and Tr63 defective in surface polysaccharide synthesis were obtained by random Tn5-mutagenesis in *Sinorhizobium meliloti* CXM1-188. Tr63 showed inability to grow on LB medium with DOC but had the same lipopolysaccharide profile as the parent strain. Tr53 was resistant to DOC but demonstrated changed LPS profile. In sterile plant tests on *Medicago sativa* (bv. Vega) both mutants induced nodules of four types: (1) white small irregular in shape nodules; (2) white long curved nodules; (3) white-pink cylindrical ineffective nodules and (4) pink effective nodules. The nodules of two first types were hystologically and cytologically defective and contained undifferentiated bacteroids in plant cells. The nodules of 3 and 4 types were morphologically like to these of CXM1-188 strain. From the effective nodules of 4 type were isolated bacteria with altered polysaccharide phenotype, which were more mucous than Tr53 and Tr63 but sensitive to DOC.
Diversity of nodule types may be result of individual interaction between host plant and bacteria defective in synthesis of surface polysaccharides in attempt to establish an effective symbiosis.

S6-P62

COMPETITIVE NODULATION BLOCKING IN *PISUM SATIVUM* CV. AFGHANISTAN.

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Competitive Nodulation Blocking (CNB) is a term which has been used to describe the interaction between compatible and incompatible strains of *Rhizobium leguminosarum* biovar *viciae* on certain types of primitive peas, such as cultivar Afghanistan. This cultivar can be nodulated by *R.l* bv. *viciae* strain TOM, which produces Nod factors carrying an acetyl group on the reducing sugar. No nodules are formed by strains such as 8401 pRL1JI that lack the *nodX* gene required for the presence of the acetyl group.
Blocking of nodulation of cv. Afghanistan by strain TOM can be achieved by the addition 8401 pRL1JI or of purified Nod factors. Using strain TOM marked with constitutively expressed *lacZ*, we have demonstrated that CNB does not occur at the level of infection thread initiation but affects infection thread growth. The possibility that CNB is a defence reaction has also been investigated and experiments with split-roots show that CNB is a localised response and not translocated throughout the whole root.
The genetic locus that confers nodulation resistance in cv. Afghanistan is *sym2*^{A}. We have used pea lines in which the *sym2*^{A} locus (from cv. Afghanistan) has been introgressed into the western varieties Sparkle and Rondo. CNB did not occur in the introgressed lines, indicating that CNB sensitivity is not linked to the *sym2*^{A} locus.

This work was funded by the BBSRC.

S6-P63

RECENT CONCEPTS ON THE GENETICS AND ROLE OF BRADYRHIZOBIAL CYCLIC BETA-GLUCANS IN SYMBIOTIC NITROGEN FIXATION

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Cyclic beta-(1,3)(1,6)-D-glucans are essential for symbiotic nitrogen fixation by *Bradyrhizobium japonicum* in soybeans. These bacterial molecules function as periplasmic osmolytes in free-living bacteria and appear to have an additional role in nodule development and symbiosis. Our current hypothesis is that these molecules function in the suppression of a host defense response during nodule development.
Previously we identified two genes, *ndvB* and *ndvC* involved in glucan synthesis. Mutation in *ndvB* abolishes glucan synthesis and mutation in *ndvC* results in synthesis of structurally altered glucans. Recently, we identified a third gene, *ndvD*, by complete sequencing of the *ndvC,D* region, deletion, mutagenesis and complementation. *ndvD* is required for glucan synthesis, motility and effective symbiosis.
Evidence for the role of the cyclic beta-glucans in the suppression of the host defense response in soybean will be presented.

S6-P64

MsPG3 TRANSCRIPTIONAL REGULATION IN TRANSGENIC *MEDICAGO TRUNCATULA* PLANTS

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We have isolated and characterized a genomic clone of *Medicago sativa* containing the entire *MsPG3* polygalacturonase gene. Our expression studies (RT-PCR and *in situ* hybridization) suggest that this enzyme may be involved in the early stages of the symbiotic interaction *Medicago sativa-Sinorhizobium meliloti*. To characterize the cellular localization and the transcriptional regulation of *MsPG3*, *Medicago truncatula* transgenic plants containing a 2,7 Kb *MsPG3* promoter-β-glucuronidase (*gus*) fusion were obtained. These transgenic plants showed a nodule specific *gus* expression pattern. To determine the promoter regions responsible of this nodule specificity a deletional analysis of *MsPG3* promoter has been done. Five *MsPG3* promoter fragments (600, 413, 306, 205 and 92 bp) fused to *gus* reporter gene were used to transform *M. truncatula*. Although 205 bp of the *MsPG3* promoter region are able to drive *gus* expression in roots and flowers, 600 bp of *MsPG3* promoter region are required to drive gus expression in the nodule, suggesting the presence of nodule specific regulatory sequences between 600 and 413 bp upstream the AUG codon. Sequence analysis of this region show the presence of a conserved sequence present in the early nodulin *Enod12* promoter from different legumes (Christiansen, H., 1996, *Plant. Mol. Biol.*, 32, 809-821). The involvement of these regions in the *MsPG3* transcriptional regulation is discussed in this work. In addition, the presence of regulatory sequences in other untranslated regions of the *MsPG3* genomic clone (3´-UTR, introns) has also been studied in transgenic *M. truncatula* plants.

This work was supported by DGESIC grants PB96-1268 and PB98-1158.

S6-P65

DISSECTION OF NODULATION SIGNALLING USING PEA MUTANTS DEFECTIVE FOR NOD-FACTOR-INDUCED CALCIUM SPIKING IN ROOT HAIRS

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Intracellular calcium was monitored using a fluorescent dye in pea root hairs following the addition of *Rhizobium leguminosarum* bv. *viciae* Nod factors. About 10 min after Nod factor addition, spikes in fluorescence occurred at a frequency of about one per minute. These spikes, corresponding to approximately a 200 nM (maximal) increase in the calcium concentration, were localised around the nuclear region, and were similar to those induced by Nod factors in alfalfa (1). Calcium responses were analyzed in non-nodulating pea mutants representing seven loci that affect early stages of the symbiosis. Mutations affecting three loci (*sym8*, *sym10* and *sym19*) abolished Nod-factor-induced calcium spiking, whereas a normal response was seen in peas carrying alleles of *sym2*^{A}, *sym7*, *sym9* and *sym30*. The absence of Nod-factor-induced calcium spiking, suggests that this response is related to nodulation signalling and that at least three independent loci are required to transduce the Nod-factor recognition into a calcium spiking response. A model for the potential order of pea nodulation genes in nodulation and mycorrhizal signalling will be proposed based on our data on calcium spiking, root hair deformation and infection, along with previous observations by others on the lack of mycorrhizal infection in some of the *sym* mutants.

1. Ehrhardt, D. W., Wais, R. & Long, S. R. (1996) *Cell* 85, 673-681.

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S6-P66

DO NOD FACTOR BINDING APYRASES PLAY A ROLE IN LEGUME NODULATION?

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Our laboratory, in conjunction with a number of collaborators (too numerous to mention above), have been investigating the possible role of apyrases (nucleotide phosphohydrolases) in nodulation. These enzymes are encoded by a multigene family in both soybean and *M. truncatula*. Specific members of this family are rapidly induced (<3 hours) upon rhizobial inoculation. Interestingly, the inducible genes were mapped to a chromosomal region that is syntenic between soybean and *M. truncatula*. The apyrase proteins were localized either to the plasma membrane or to the golgi. Antibody against the inducible apyrase, located in the plasma membrane was found to block nodulation. We conclude from these data that apyrases are clearly involved in nodu ation but their specific biochemical role has yet to be identified.

S6-P67

INVOLVEMENT OF SIX DIFFERENT PECTIN METHYLESTERASE GENES SUGGEST THE COMPLEXITY OF PECTIN MODIFICATION DURING SYMBIOSIS

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Change in the components and architecture of the plant cell wall during the symbiotic *Rhizobium*-legume interaction are still poorly understood. We have been studying the role of plant pectin methylesterase (PME) genes expressed during the establishment of symbiosis between *Sinorhizobium meliloti* and *Medicago truncatula*. Recently, we developed several molecular approaches to study the expression of the *M. truncatula* PME gene family during nodule development. Two groups of genes were identified based on their pattern of expression. *MtPER*, is a PME gene which is specifically induced during the first steps of the interaction by the presence of rhizobia. This gene is located in a cluster containing two other highly homologous PME genes expressed only in the pollen, strongly suggesting that they have evolved by gene duplication and that their expression is restricted to a single organ. We show that five other *M. truncatula* PME genes constituting the second group are under a more general transcriptional control, resulting in different levels of expression in several organs of the plant and during nodulation. These results suggest that for recruiting PME gene expression during the infection process and during the nodule organogenesis two different evolutionary mechanisms were involved.

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S6-P68

MSPG3 *IN VIVO* LOCALIZATION USING GFP FUSIONS

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We have described a *Medicago sativa* polygalacturonase gene, *MsPG3*, specifically expressed during the symbiosis with *Sinorhizobium meliloti*. In order to determine the subcellular localization of MSPG3 as well as its trafficking pathway, translational fusions of *MsPG3* fragments to green fluorescent protein (GFP) reporter gene under the control of 35S CaMV promoter were constructed. Since mature GFP is intrinsically fluorescent, it can be used as reporter protein for applications *in vivo*. The constructs were tested in transient expression assays by particle bombardment of onion epidermal cells. The onion epidermis has large, living transparent cells in a monolayer, making them ideal for visualizing GFP. In addition to MSPG3 subcellular localization, using this system we have demonstrated *in vivo*, by epifluorescence microscopy and confocal scanning, the importance of the predicted *MsPG3* signal peptide in the trafficking pathway and also the role that different parts of the protein could play in this localization. To study MSPG3 subcellular localization and trafficking during root development, we generated *Medicago truncatula* transgenic roots transformed with the related *gfp* fusions via *Agrobacterium rhizogenes* infection. Some of these roots were inoculated with *S. meliloti* to try to identify MSPG3 subcellular localization during the first steps of the symbiotic interaction. Recent results using these *Medicago* roots are also presented in this work.

This work was supported by DGESIC grants PB96-1268 and PB98-1158.

S6-P69

SYMBIOSIS-SPECIFIC EXPRESSION OF *RHIZOBIUM ETLI CASA* ENCODING A SECRETED CALMODULIN-RELATED PROTEIN

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The symbiotic interaction between *Rhizobium etli* and its leguminous host *Phaseolus vulgaris* requires elaborate communication between the partners throughout the interaction process. We identified a calmodulin-like protein in *R. etli*. This protein was termed calsymin and constitutes the first example of a calmodulin-related protein in a gram-negative bacterium. Calsymin has a modular structure with three repeated homologous domains, each carrying two putative ef-hands. Expression of the gene encoding calsymin, *casa*, is induced during colonisation and infection of *R. etli* with the host and in the bacteroid stage. Expression of *casa* occurs independently of nodulation and nitrogen fixation genes, but is controlled by a repressor protein belonging to the tetr family of regulatory proteins. Inactivation of *casa* disturbs the development of bacteroids during symbiosis and strongly reduces symbiotic nitrogen fixation. The calsymin protein was purified and demonstrated to possess Ca^{2+} binding activity. *R. etli* secretes calsymin without amino-terminal cleavage of the protein. We propose that calsymin is an essential component of the communication between the plant and *Rhizobium* during the late stages of the interaction.

S6-P70

GROWTH INHIBITION OF *RHIZOBIUM LEGUMINOSARUM* DUE TO SMALL BACTERIOCIN IS MEDIATED THROUGH QUORUM SENSING GENE REGULATORS

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The bacterial genes required for the establishment of the symbiosis between *Rhizobium leguminosarum* bv. *vicia* and legumes are carried on the symbiotic plasmid pRLIJI. We have examined a region of pRL1JI carrying genes involved in plasmid replication and transfer. The replication genes, *repABC*, are adjacent to the *trb* operon involved in plasmid transfer. The *trbABCDEFGHI* genes are preceded by *traI*, which is predicted to encode an *N* - acyl-L-homoserine lactone (AHL) synthase. Downstream of the *trb* operon are two genes, *bisR* and *triR*, that encode LuxR-type regulators required for *traI/trb* expression
Expression of *traI*, in a strain of *Agrobacterium tumefaciens* which produces no AHLs of its own, showed that TraI is involved in the formation of at least seven AHLs as revealed by thin layer chromatographic analysis. In *R. leguminosarum* expression of *traI* is complex and dependent on BisR and TriR. It appears that BisR upregulates *triR* in response to 3-hydroxy, 7-cis-tetradecenoyl homoserine lactone (3OH,$C_{14:1}$-HSL). TriR then controls *traI* expression, resulting in a positive feedback loop that enhances *traI-trb* gene expression. Another component involved in the regulation of *traI* is TraM, a negative regulator of *traI* expression. Negative regulation of *traI* due to TraM is removed in the presence of 3OH,$C_{14:1}$-HSL. 3OH,$C_{14:1}$-HSL was formerly known as small bacteriocin and causes growth inhibition of strains of *R. leguminosarum* carrying pRL1JI. BisR and TriR, regulators of *traI* expression, are also involved in the sensitivity of pRLIJI - containing strains to 3OH,$C_{14:1}$-HSL. This sensitivity requires BisR and is enhanced by TriR. The bisR and triR genes confer sensitivity in the absence of pRLIJI, implying that growth inhibition is due to regulation of an unknown gene by BisR.

This work was funded by the BBSRC.

S6-P71

SYMBIOTIC CHARACTERISTICS OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE* AND *BRADYRHIZOBIUM* SP. (*LUPINUS*) HUP$^+$ AND HUP$^-$ STRAINS

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About 30% of *Rhizobium leguminosarum* bv. *viciae* and *Bradyrhizobium* sp. (*Lupinus*) nodule isolates collected from plants growing near Lviv (Ukraine) and Lublin (Poland) were Hup$^+$. *Vicia villosa, Vicia faba*, and *Lupinus luteus* after inoculation with respective Hup$^+$ microsymbionts showed 15-20% higher dry green mass than those bacterized with Hup$^-$ microsymbionts.
Using the transposon mTn5SSgusA21, two gus marked strains determined B18/13 and N23/12 were isolated from *R. leguminosarum* bv. *viciae* B18 Hup$^+$ and N24 Hup$^-$ strains, respectively. Obtained Gus$^-$ transconjugants were tested for nodulation ability, nodulation competitiveness, symbiotic effectiveness (dry green mass, nitrogenase activity), and uptake hydrogenase activity. It was found that strain B18/13 Gus$^-$ *R. leguminosarum* bv. *viciae* was more competitive in nodule formation on *V. villosa* than wild type strain B18 and acquired the uptake hydrogenase activity. Other examined Gus$^-$ *R. leguminosarum* bv. *viciae* strain N24/12 exhibited Hup$^-$ phenotype similar as the wild type strain N24.

POSTER ABSTRACTS
SYMPOSIUM 7

S7-P1

MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF BEAN RHIZOBIA ISOLATED FROM MOROCCAN SALINE SOILS

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Among 150 isolates collected from three different soils of Morocco, 30 were found to tolerate more than 2% NaCl in YEM media. Most of them were found to tolerate also acid conditions (4-4.5). In alkaline media all the strains grew well at pHs 7.5 and 8.5 except strains RP 3 and RP 62 which died at the later pH. At pH 9 all the strains, except one (RP 89), were moderately stressed (50% of growth). Strain RP 89 seems to be the more alkaline-tolerant strain in our collection. This strain was also very acido-tolerant, because it grows in buffered YEM medium at pH 4.5.
The fact that more than 60% of the strains tested until now can grow in media between pH 4-4.5 and 8.5 indicate that these strains are very plastic concerning their tolerance to common stresses in soils such as salinity, alkalinity or acidity. The high tolerance capacity to such different stresses rises the question of the mechanisms involved in the tolerance to each stress and possible cross protection phenomena.
Molecular characterization of these strains by REP and RAPD PCR methods has been used successfully to identify genomic groups in the Moroccan bean rhizobia collection. Three groups were identified: Group 1, includes strains close to *Rhizobium tropici* CIAT 899 type IIB, Group 2 contains 3 strains (RP 9, RP 10 and RP 41) and Group 3 also contains 3 strains (RP 174, RP 192, RP 111). The remaining strains did not fit in any of the groups defined above.
Until now *R. tropici* was currently found only in tropical soils of south America and Africa. For the first time, we describe some strains showing genomic similarities with *R tropici* in a Mediterranean soil.

S7-P2

GENETIC CHARACTERIZATION AND SYMBIOTIC EFFECTIVENESS OF FAST-GROWING RHIZOBIAL STRAINS ISOLATED FROM SOYBEAN NODULES IN BRAZIL

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This study aimed to characterize genetically, and to evaluate the symbiotic effectiveness of thirty fast-growing rhizobial strains (doubling times of 85 to 225 min in yeast mannitol medium) isolated from nodules of Asian and modern soybean genotypes that had been inoculated with soils from disparate regions of Brazil. Analyses by ERIC-REP-PCR and RAPD indicated a high level of genetic diversity: each isolate was a unique strain. By RFLP of PCR-amplified 16S rRNA genes and partial 16S rRNA sequencing analyses, it was possible to determine the relatedness of the strains to established rhizobial genera and species. None of the strains showed similarity with *Sinorhizobium fredii*, whereas fourteen were genetically related to *R. tropici*. For the strains isolated from undisturbed areas covered with native vegetation, the genetic analyses indicated similarity with *R. tropici*, *Rhizobium* sp. OR 191, *R. huautlense* and with *Agrobacterium* spp. In areas previously inoculated and cropped with soybean, *R. tropici*, *Bradyrhizobium japonicum* and *B. elkanii* were isolated from soils under no-tillage management systems, while in those under conventional tillage, characterized by stressful conditions such as high soil temperature and low moisture, the only species detected was *R. tropici*. The phospholipid fatty acids analysis (PFLAs) was less precise in delineating phylogenetic relationships, but some PFLAs groups were similar to clusters obtained by ERIC-REP-PCR and RAPD. The rhizobia fit into eight Nod-factor profiles that were related to species, but not to N_2-fixation capacity or competitiveness. Eight strains lost the effectiveness after fifteen replicates. Under axenic conditions, some of fast growers fixed as much N_2 as the *B. japonicum/B. elkanii* strains carried in Brazilian commercial inoculants. However, in a co-inoculation experiment, very few strains were able to compete against *B. elkanii* strain SEMIA 5019, but competitiveness increased in another experiment, when the pH of a non-sterile soil raised from 5.1 to 6.8 and 7.9. At this time, the low competitiveness of those strains limits their recommendation for the Brazilian acid conditions.

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S7-P3

ANALYSIS OF PHENOTYPIC RELATIONS,CELLULAR FATTY ACIDS AND CROSS NODULATION OF SOME ROOT-NODULE BACTERIA ISOLATED FROM WILD LEGUMES IN EGYPT

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Seventy two strains of root-nodule bacteria from 5 wild legumes (*Melilotus indicus*, *Trifolium resupinatum*, *Alhagi murarum*, *Sesbania sesban* and *Acacia nilotica*) grown in cultivated lands of the Nile Valley, were compared with 16 strains of rhizobia from *Trifolium alexandrinum* (a cultivated forage legume) and with 10 representative strains of the genera *Rhzobium*, *Sinorhizobium* and *Mesorhizobium*. The whole set of strains (98 strain)› were classified into 2 major groups at 85% similarity, and in 10 clusters at 90%› similarity, after numerical analysis of their phenotypic characterisics. Group (1) harbored 14 strains of wild rhizobia, 8 strains of *T. alexandrinum* and the references›(10 strains). The remaining strains of wild rhizobia (58 strains) appear to be with different traits, they are not related to the references and occupied group (2) with 8 strains of *T. alexandrinum*. Only 20 strains of wild rhizobia that formed nodules on roots of one or two of the cultivated legumes *Pisum sativum* (7 strains), *Medicago sativa* (7 strains), *Vicia faba* (4 strains) and *Vigna sinensis* (2 strains). Only 5 strains which formed effective symbiosis. The fatty acid profiles of 20 strains (the nodulating strains) of wild rhizobia were compared to those of 3 strains from *T. alexandrinum* and 10 reference strains. The 33 strains of rhizobia were classified into 2 major groups at 75% similarity after numerical analysis of the fatty acid profiles. However, data of fatty acid analysis were conflicted with data of phenotypic relations. Identification and classification of new strains of rhizobia appear to require both traditional (phenotypic) and modern (molecular) methods of analysis

S7-P4

TOLERANCE AND LIPOPOLYSACCHARIDES PATTERN OF RHIZOBIA NODULATING ACACIA SPP. IN MOROCCO.

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Ten representatives strains from a collection of rhizobia nodulating Acacia in morrocan soils and characterized for their phenotypic and genetic diversity have been tested in Yeast Extract Mannitol medium for tolerance to different salts, sulfates and chlorides of sodium, calcium and potassium. The most inhibitory salt was $CaCl_2$ followed by NaCl. As the last is generally the predominant one soils interested by the study, it was used to classify strains regarding degree of tolerance. 2 strains are highly resistant (more than 850 mM), 5 are middle resistant (500 to 680 mM) while 3 are less tolerant (250 to 340 mM). Growth inhibitory NaCl concentration affected strongly the cells viability of all strains. In YEM medium strains have a time generation from 1h 30 min to 4h 30 min. In NaCl added medium, this parameter was affected at different degrees. It doubled at 680 mM NaCl for the tolerante strains and at only 170 mM for the most sensitives.
When added to the YEM medium at the NaCl inhibitory concentration, aspartate, glutamate and glycine betaine increase the viability of strains and, furthermore, allowed growth of some of them. To confirm this, experiences have been conduced in synthetic medium with four strains presenting different salt tolerance degrees. Glutamate, aspartate and glycine betaine were effective with strain MSMC 45, MSMC 47 and MSMC 38 and permit their growth respectively at 420, 510 and 680 mM NaCl. However, if aspartate, glutamate and betaine were similarly effective for strain MSMC 38 the most tolerant, betaine was less efficient than the amino acids in the case of MSMC 45 and MSMC 47. No osmoprotectant effect have been observed on the sensitive strain MSMC 414. Therefore, it seems that salt tolerant strains involve more mecanisms in osmoprotection than less tolerant.
Furthermore, it is known that lipopolysaccharides of Rhizobium have an important role in the first steps of interaction between microsymbiont and the legume host. LPS alteration might impair bacteria-plant interaction. In our first experiments the SDS-PAGE pattern of LPS from bacteria cultivated in non added NaCl YEM medium showed differences between strains from the three clusters identified by analysis of 16S rDNA (Khbaya et coll., 1998). These results suggest that LPS profiles could be used to study the diversity of rhizobia. Actually, we are prospecting LPS profiles from salt stressed cells and the relation between LPS pattern and strains infective.

S7-P5

DIVERSITY AND PHYLOGENY OF RHIZOBIA NODULATING *ACACIA* SPP. GROWN IN MOROCCO

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Phenotypic characteristics of 48 isolates obtained from root nodules of four Acacia species (*Acacia cyanophylla, A. gummifera, A. horrida and A. raddiana*) growing in soils collected from the arid and saharan regions of Morocco, were studied. The rhizobia were very diverse with respect to their cross-nodulation patterns, as well as their physiological and biochemical properties (Zerhari et al. 2000). Dendrograms obtained through computer numerical analysis of 52 phenotypic characteristics showed that isolates could fit into four clusters below the boundary level of 0.85 average distance and that they were very distinct from the reference strains. Some interesting isolates for inoculation trials have been identified.

Rhizobia were compared by analyzing both the 16S rRNA gene (rDNA) and the 16S-23S rRNA spacer by PCR/RFLP. Analysis of the length of 16S-23S spacer and the RFLP analysis of the amplified spacer showed a considerable diversity within these microsymbionts.

Three clusters were identified when 16S rDNA analysis was carried out. Two of these clusters include somme isolates which nodulate, nonspecifically, the four *Acacia* species. These clusters A and B, fit whithin *Sinorhizobium* lineage and are closely related to *S. meliloti* and *S. fredii*, respectively. The third cluster appeared to belong to the *Agrobacterium-Rhizobium galegae* phylum and is more closely related to the *Agrobacterium tumefaciens* species. These relations were confirmed by sequencing a representative strain from each cluster (Khbaya et al. 1998).

The hybridization of the two groups A and B against reference strains of the different species of the *Sinorhizobium* shows that (i) the group A is clearly different from species *S.terangae, S. saheli, S. fredii, S. kostiense, S. arboris and S. medicae* and relatively close to the *S. meliloti* species, (ii) the strains of *Sinorhizobium* sp. (*Acacia* spp.) group B are more related, but clearly distinct from the *S. fredii*. Based on all these results we suggest the creation of a new species, *Sinorhizobium taliouini* (by reference to the Moroccan region from which was isolated the strain type of this species; Taliouine), for this group of rhizobia that nodulate *Acacia* spp. in arid and semi-arid areas of Morocco.

S7-P6

DIVERSITY OF CHICKPEA *RHIZOBIA* ISOLATED FROM MOROCCAN ARID AREAS.

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Phenotypic and molecular characteristics of fifty four strains isolated from chickpea growing in arid and semi-arid areas of Morocco were studied. The analysis of strains tolerance to salinity, high temperature, heavy metals, acid and alkaline pH, and to antibiotics as well as the study of the symbiotic and cultural characteristics allowed to identify interesting strains and to describe a physiological diversity of rhizobia strains nodulating chickpea. Through computer numerical analysis of these phenotypic characteristics, it has been shown that strains were grouped into five clusters. Furthermore, some interesting strains for inoculation trials have been identified.
On the other hand, molecular studies performed by RFLP/PCR of 16S rRNA genes confirmed the heterogeneity of these rhizobia. Analysis of restriction fragments obtained with three endonucleases (*Taq*I, *Hin*fI and *Msp*I) permitted to identify six 16S rDNA genotypes in fifty four chickpea rhizobia strains. Three major groups containing 42 strains are closely related to *Mesorhizobium ciceri* and *Mesorhizobium* sp, the other strains seem closer to the *Agrobacterium* (4/54) and to *Bradyrhizobium* (8/54).

S7-P7

HERBASPIRILLUM SEROPEDICAE PII PROTEIN RELIEVVES NIFA INHIBITION BY NIFL IN AN ENTERIC BACKGROUND

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In *Klebsiella pneumoniae* GlnK relieves NifL inhibition of NifA under ammonium limitation, whereas PII interacts with NtrB under nitrogen excess conditions resulting in the dephosphorylation of NtrC-P and diminished *nifA* expression. PII also controls glutamine synthetase (GS) activity by interacting with GlnE. In *H. seropedicae* PII is not involved in the control of GS activity but it apparently controls NifA activity. The *glnB* gene of *H. seropedicae* was able to relieve *Klebsiella pneumoniae* NifL inhibition of NifA in an *E. coli glnK* strain. Similar result was observed in an *E. coli glnD* strain, suggesting that uridylylation of *H. seropedicae* PII was not required for this activity. *H. seropedicae* PII is structurally similar to *E. coli* PII and GlnK proteins. However, the C-terminal region *H. seropedicae* PII has a helix 3_{10} motif that is observed only in *E. coli* GlnK. These results suggest that *H. seropedicae* PII is functionally similar to the enteric GlnK and raise the possibility that the C-terminal region of GlnK/PII proteins might be involved in nitrogen signalling to NifL/NifA.

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S7-P8

BRADYRHIZOBIUM JAPONICUM PRODUCES QUORUM-SENSING SIGNAL MOLECULES.

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Quorum-sensing in bacteria is a regulatory mechanism that involves the interaction of small signal molecules (autoinducers) such as N-Acylhomoserine lactones (AHLs), with transcriptional activator proteins. The accumulation of quorum-sensing molecules enables the organism to monitor cell density and to regulate gene expression in response to fluctuating population sizes.
AHLs and other autoinducers are normally detected using bacteria harboring a reporter gene, whose expression requires the addition of the exogenous signal compound. Quorum-sensing autoinducers have been detected, characterized and identified in a variety of different soil microbes, including members of the *Rhizobiaceae*. However, AHL-like activities have thus far not been detected in the soybean symbiont, *Bradyrhizobium japonicum*. AHL production is a process clearly dependent on environmental growth parameters such as temperature and carbon source and the bioreporters used to detect AHL activity have different specificities and sensitivities. Therefore, negative results have been dificult to interpret.
Here, we report the biodetection of *B. japonicum* AHLs using *Chromobacterium violaceum* CV026, a white violacein-negative mini-*Tn*5 mutant in which (purple) pigment production can be restored by different microbial AHLs. This sensitive bioassay can detect AHLs based on the induction or inhibition of the purple pigment. We will present data showing that *B. japonicum* possesses a biological activity that can activate (AHL-related) reporter gene expression, suggesting that *B. japonicum* produces (an) AHL-like molecules(s). Preliminary data on the number and tentative structure of putative AHL signal molecules were obtained by TLC. Further studies including analytical and preparative HPLC-MS were carried out and results will be reported.
Work is in progress in our laboratory to identify the gene/s involved in quorum-sensing molecules production and to elucidate its biological function.

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S7-P9

NEGATIVE CONTROL OF THE N-TRUNCATED NIFA PROTEIN ACTIVITY OF *HERBASPIRILLUM SEROPEDICAE* BY ITS ISOLATED N-TERMINAL DOMAIN *IN VITRO*.

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The *H. seropedicae* is a nitrogen-fixing bacterium found in association with economically important gramineae. The activity of the NifA protein of *H. seropedicae* is controlled by O_2 and NH_4^+. The NifA proteins comprises three domains: the DNA binding C-terminal domain, the central domain, responsible for the interaction with the σ^N subunit of the RNA polymerase, and the N-terminal domain, involved in inhibition of NifA activity by ammonium ions. Our previous results showed that an N-truncated NifA protein escapes activity control by NH_4^+ and activates *the K. pneumoniae nifH* promoter in *E. coli*. Control of NifA activity by NH_4^+ ions was, however, restored by the N-terminal domain expressed *in trans* suggesting an effective interaction between this domain and the N-truncated NifA protein *in vivo*.
In the present work we analysed whether this interaction could occur *in vitro*. Band-shift assays showed that the N-truncated NifA protein was able to bind to both *R. meliloti nifH* and *H. seropedicae nifB* promoters. The purified N-terminal domain caused a disruption of the DNA-protein complex indicating a negative effect of the N-terminal domain on the DNA binding activity of the N-truncated protein.
Protease protection assays showed that the purified N-terminal domain protected the N-truncated protein against digestion by Proteinase K and Trypsin. The rate of proteolysis was dependent on the N-terminal domain concentration.
These results suggest that the N-terminal interacts with the N-truncated NifA protein affecting negatively DNA complex formation and its transcription activation activity.

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S7-P10

FIELD EVALUATION OF N-FIXATION AND N-TRANSFER (^{15}N) IN SHRUB SPECIES FROM THE NATURAL SUCCESSION IN A MEDITERRANEAN ECOSYSTEM, AS AFFECTED BY MYCORRHIZAL INOCULATION

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Inthe context of an EU funded project (INCO DEC ERBIC 18 CT97 0197 – MYRISME) a number of experiments are being addressed for studying the management of rhizobial and mycorrhizal fungi, ecosymbionts with targettree and shrub legume species. The general objective is aimed at the sustainable recovering of desertified mediterranean ecosystems. Experiments were carried out in a representative area within a desertified semiarid ecosystem, located in Sierra de los Filabres, Almeria (southeast Spain). Present natural vegetation is a degraded shrubland where *Anthyllis cytisoides* and *Lavandula multifida* are woody species commonly associated in the natural succession in thisarea. One of our aims was to evaluate under field conditions, and by using ^{15}N isotope dilution techniques, N fixation and N transfer within the consortium*Anthyllis – Lavandula*. Two-month-old seedlings of both *Anthyllis* and *Lavandula* plants were transplanted to the target ecosystem. All *Anthyllis* seedlings were inoculated with a rhizobial culture previously selected. A completely randomized block design with two factors i. e. planting combinations and arbuscular mycorrhizal (AM) treatments was used. The planting combinations included (i) *Anthyllis* growing alone, (ii) *Lavandula* growing alone and (iii) a mixture of both species. The other factor, the AM treatments, included two components (i) AM inoculation, based on the natural taxa, and (ii) a non-mycorrhizal control (but becoming mycorrhizalin the field during the experiment). Upon transplanting all plants received a single soil applied dressing of the isotope ^{15}N as $(NH_4)_2SO_4$ with 10 % ^{15}N atom excess, at a rate equivalent to 5-kg N ha^{-1}, to measure N_2-fixation andN-transfer from the N-fixing to the non-N-fixing plants. After ten months growing under natural conditions, plants were harvested and shoot biomass were determined, shoot tissues analysed for isotopic N composition, and the root evaluated for nodulation and AMF colonization. Results showed remarkable ecosystem developments: (i) lavender plants benefited from growing in mixture with the N-fixing legume, with regard to both biomass accumulation and N acquisition, particularly from N-transfer, (ii) inoculation with native AM fungi benefited plant growth, N fixation and N-transfer,and (iii) the mycorrhizal legume enhanced the mycorrhizal level of lavender plants growing nearby. These facts, already described for agricultural crops, were here corroborated for natural plant communities in a semiarid ecosystem, and support an important role of shrub legumes as a source of mycorrhizal inoculum for the surrunding area and in improving N nutrition for non-N-fixing vegetation.

S7-P11

THE IMPORTANCE OF ARBUSCULAR MYCORRHIZAE FOR NITROGEN FIXATION IN ENVIRONMENTS WITH LIMITATIONS OF WATER AND NUTRIENTS

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Sustainable agriculture and revegetation are two challenges of increasing importance in our society. In the traditional agriculture, the soil has been subordinated to the plant production. However, nowadays, the soil is considered as a complex and fragile system that must be protected in order to guarantee its stability and productivity. In fact, the main objective of the sustainable agriculture is "maximum plant production with a minimum of soil loss". Consequently, sustainability demands that alternatives to nitrogen fertilizer are sought. Biological nitrogen fixation can offer this alternative as it uses the capacity of certain nitrogen fixing bacteria to convert atmospheric nitrogen into ammonia. Moreover, in Mediterranean climate regions, erosion and desertification have been enhanced as a consequence of both low precipitation and human impact. In these arid ecosystem tree crops, and especially woody legumes, are essential to maintain organic matter and soil fertility. The availability of phosphorus and the plant water status are some of the most limiting factors for nodule activity. The effect of mycorrhizal fungi on growth, nodulation and nitrogen fixation in legumes has been a subject of increasing interest since several parameters directly related to nitrogen fixation are positively affected by mycorrhizal association.
Our research has been devoted to studying the symbiosis of legumes with nitrogen fixing bacteria and mycorrhizal fungi under stress. The first works were focused on the effect of water stress on the functioning of *Medicago sativa-Rhizobium-Glomus* association. Results demonstrated that, under drought, the benefit of mycorrhization could not be only explained by increased phosphorus uptake by fungus. Subsequent studies indicated that positive effects of mycorrhizae were also related to the hormonal levels of plant host. At present, we are working on the *Anthyllis cytisoides-Bradyrhizobium-Glomus* association as a part of an INCO-DC Project in Biological Nitrogen Fixation (ERBIC 18CT970197). *Anthyllis cytisoides* is a woody legume widely distributed in arid areas of Southern Spain, and it has been used in revegetation programmes. The objective pursued in one of our recent studies has been to determine the effect of different phosphorus and nitrogen levels on the establishment and activity of nitrogen fixing bacteria associated with mycorrhizal *Anthyllis*. The general conclusion is that mycorrhizal infection in legumes is essential under stressed conditions, such as water or nutrient limitations.

S7-P12

HIGH RELATIVE HUMIDITY REDUCES THE EFFECT OF ELEVATED CO_2 IN N_2-FIXING ALFALFA BY ALTERING NODULE METABOLISM AND NUTRIENT STATUS

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Some factors like low temperature and insufficient nutrient supply limit plant responses to elevated CO_2. We hypothesize that high relative humidity (low vapour pressure deficit, VPD), could also reduce plant response to elevated CO_2, especially in Mediterranean plants, which are usually subjected to low VPD. In previous studies with alfalfa, it was found a lack of nitrogen fixation improvement in response to elevated CO_2 under low VPD conditions. Thus, the main purpose of this study was to investigate whether such effect could be related to a nutritional unbalance and/or to a negative effect on nodule metabolism.
Thirty-day-old alfalfa (*Medicago sativa* L. cv. Aragón) plants inoculated with *Rhizobium meliloti* 102F78 strain, were grown for one month in controlled environment chambers at 25/15°C, 14 h photoperiod, 600 μmol^{-2} s^{-1} photosynthetic photon flux (PPF), using a factorial combination of CO_2 concentration (400 μmol mol^{-1} or 700 $mmol$ mol^{-1}) and humidities (45% and 85% RH, corresponding to 1.74 kPa and 0.48 kPa VPD, respectively, at 25°C). Leaf and root nitrogen and carbon contents were measured at the end of the second month by using an elemental organic analyzer. The rest of nutrients were determined by using ICP (Inductively Coupled Plasma emission). Plant nodule dry weight, nodule leghemoglobin, soluble proteins and carbon metabolizing enzymes were measured in plant and bacterial fractions of the nodules.
Lower nutrient concentrations were found under low VPD and elevated CO_2 did not modify such effect. Moreover, independently of CO_2, nodules from low VPD plants showed the lowest carbon metabolizing enzyme activities in both plant and bacterial nodule fractions. It is suggested that the absence of CO_2 effect on plants grown under low VPD could be related to nutritional unbalance and decreased nodule carbon metabolism which will affect nitrogen fixation.

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S7-P13

THE EFFECT OF THE INTERACTION BETWEEN MYCORRHIZAL INOCULATION AND DIFFERENT PHOSPHORUS SOURCES ON GROWTH AND BIOLOGICAL NITROGEN FIXATION OF COMMON BEAN (*PHASEOLUS VULGARIS*)

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A pot experiment was conducted under glasshouse conditions to investigate the effect of the interaction between the inoculation with an arbuscular mycorrhizal (AM) fungus (*Glomus clarum*) and different phosphorus sources: Water soluble source (Super phosphate 46% P_2O_5) and Phosphate rock (Patos de minas 24% P_2O_5) using different doses, on the growth and contribution of biologically fixed N_2 to *Phaseolus vulgaris* cv Carioca in a P deficient soil (available P: 4.5µg g^{-1}). The ^{15}N isotope dilution technique and non-nodulating bean NOD125 (control crop) were used to quantify the contribution of N_2 fixation. All the pots were inoculated with a peat-based *Rhizobium* (2g per pot) containing 10^8 cell g^{-1} of a mixture of two species of *Rhizobium leguminosarum* biovar phaseoli and *Rhizobium tropici*. The mycorrhizal inoculum was applied in the form of spores (100 spores per pot). Inoculation with AM fungus significantly increased the dry matter production by 5 – 60% according to phosphorus treatments and to the bean variety (Carioca, NOD125). The non-mycorrhizal plants required much more phosphorus as did mycorrhizal plants irrespective of the solubility of the phosphorus source.
The non-nodulating bean variety is the best control crop we can use for the assessment of the BNF in bean crop. The fraction of N derived from the atmosphere increased significantly with AM fungus inoculation and P fertilisation ranging from 38% to 60%, showing the best response for the super phosphate at the equivalent dose of 200 kg/ha P_2O_5. In adverse, the addition of such source of P, reduced dramatically and gradually the percent of root colonisation by *Glomus clarum*, showing the inhibition of the occurrence and efficiency of plant- arbuscular mycorrhizal fungi symbiosis when the soil available P is high. The response was different according to the phosphorus source . For the rock P treatments the improvement of nitrogen fixation was enhanced by the *Glomus clarum* inoculation specially at the third dose (200kg P_2O_5/ha), showing that the mycorrhizal inoculation can greatly improve the utilisation of P from this unavailable P source which is favoured by the acidity of the soil (soil pH_{H2O}=5.5). thus the use of P rock could be a good alternative for P fertilisation in these conditions.
The results reaffirm the indirect and positive effect of mycorrhizal inoculation on P utilisation from different P sources with different solubilities and also on the N_2 fixing association between *Rhizobium* and *Phaseolus vulgaris*.

S7-P14

HETEROCYSTOUS CYANOBACTERIA IN URUGUAY RICEFIELDS: DIVERSITY, NITROGEN FIXING ABILITY AND TOLERENCE TO HERBICIDE AND COMBINED NITROGEN.

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Uruguay is the sixth world rice producer. This cereal is the first crop exported in our country with a cultivated area that nearly duplicated in the last ten years.
Fooded rice fields are a privileged niche for biological nitrogen fixation, as they posses a variety of N-fixing systems. It has been established that cyanobacteria play a vital role in the maintenance and buildup of paddy soil fertility. To evaluate the potential use of nitrogen-fixing cyanobacteria as natural biofertilizer for rice in our conditions, the diversity, abundance and nitrogen fixing ability of these microorganisms were studied in the field and in the laboratory.
The effect of urea fertilization on population density and diversity of heterocystous cyanobacteria was determined on a 3-years assay performed under field conditions. The highest number of cyanobacteria, 1,6 x 10^5 CFU/cm^2, was found at the control (non-fertilized) treatment 8 weeks after flooding. The application of urea led to a significant decrease of population density in two out of the three years analysed. In contrast, urea application had no significant effect on the diversity. About 90% of the heterocystous cyanobacteria found in both treatments belonged to the genera *Anabaena* and *Nostoc*. Other genera present, although at very low density, were *Calothrix*, *Cylindrospermum*, *Nodularia*, *Gleotrichia*, *Scytonema* and *Tolypothrix*.
Nitrogen fixation showed a positive correlation with the abundance of cyanobacteria. The nitrogenase activity measured at midday reached an average maximum of about 36 mmoles C_2H_2 reduced/m^2.h after 8 weeks of flooding in both treatments
In order to improve the understanding of the environmental factors that can be limiting nitrogenase activity in rice fields, two cyanobacterial native isolates, *Nostoc* .sp BI142 and *Anabaena* sp BI146, were tested for tolerance to combined nitrogen and two herbicides For the two isolates, 0.2 mM ammonium, a concentration usually found in the field, inhibited nitrogenase activity after 24 h culture. The addition of field-recommended doses of propanil and quinclorac, two herbicides widelly used in uruguayan rice fields, did not affect nitrogenase activity, but a 10-fold higher concentration led to a significan decrease of nitrogenase activity and also to alterations on cell morphology

S7-P15

RAPIDLY-GROWING GREEN-MANURE LEGUMES AS A SOURCE OF BIOLOGICALLY FIXED NITROGEN FOR A SUBSEQUENT CEREAL CROP IN A SINGLE GROWING SEASON

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In the semi-arid tropics the availability of rainfall often reduces the growing season to less than six months. While various species of leguminous green-manure crops have been shown to be able to accumulated large quantities of N from biological nitrogen fixation (BNF), in such regions farmers are loath to utilise these legumes as a N source for cereals as the time taken for these plants to accumulate fixed N reduces the available growing season for the subsequent cereal crop. In this study a field experiment was performed on a sandy (81 % sand) infertile acidic (pH 5.0) soil (Ultisol, 0.42 % C, 0.05 % N) near Rio de Janeiro to examine the progress of the accumulation of dry matter and total N and BNF by three green manure crops (*Crotalaria juncea*, *Mucuna niveum* and soybean [*Glycine max*]) and their benefit to a subsequent maize crop. All 3 legumes showed the maximum rate of dry matter and N accumulation between 40 and 60 days after planting (DAP). Although inoculated with *Bradyrhizobium* the soybean fixed only 5 –10 kg N due to the acidic and low fertility soil conditions. The ^{15}N natural abundance of the legume crops compared to 5 non-N_2-fixing reference species, showed that there was little further contribution of N_2 fixation after 60 days of growth and by that time the *Crotalaria* and *Mucuna* had derived 90 and 60 kg N ha^{-1} from BNF, respectively.
In a parallel pot experiment (12.5 kg soil and 2 plants pot^{-1}) with *Canavalia ensiformis* and *Mucuna*, the ^{15}N leaf labelling technique developed by McNeill et al. (1997 Austr J. Agric Res. 48: 295-304) was utilised to estimate the proportion of plant N deposited in the soil as root residues which are unrecoverable by sieving. The leaves were labelled over a period of 9 days from 30 DAP with a solution of urea labelled with 71 atom % ^{15}N. The plants were harvested at 70 DAP and all soil was removed and sieved to remove roots and nodules. For the *Canavalia* and *Mucuna*, respectively, 39 % and 48 % of the plant N was under ground. For the *Canavalia*, of this fraction 45 % was nodule N, 29 % in recoverable roots and 26 % in non-recoverable roots. The equivalent figures for the *Mucuna* were; nodules 19 %, recoverable roots 53 % and non-recoverable roots 28 %. These results confirm findings by Australian researchers that if the below-ground contribution of BNF is not considered then the true BNF benefit to the plant/soil system is seriously underestimated. While extrapolations to field results should be made with caution, if in the above field experiment, the proportional BNF contribution to the legume roots was the same as that in the shoot tissue the estimate of total BNF contribution to the soil plant system should be increased from 60 to 89 kg N ha^{-1} for the *Mucuna* and from 90 to perhaps 120 or 130 kg N ha^{-1} for the *Crotalaria*.
The results of this study show that very significant BNF inputs can be obtained from fast-growing green manures such as *Crotalaria* and *Mucuna* to benefit a subsequent cereal crop within a single 6-month cropping season. In the Brazilian Cerrado (central savanna) region this technology is now being tested on-farm by maize producers with considerable success.

S7-P16

PHYSIOLOGY AND SCREENING FOR DROUGHT TOLERANCE OF SYMBIOTIC N_2 FIXATION

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The legume-rhizobium symbioses are very sensitive to drought stress. Understanding the physiological mechanisms involved in the inhibition of N_2 fixation, as well as screening diverse plant germplasm are important steps in the improvement of legume drought tolerance. Carbon shortage, oxygen limitation, and feedback regulation by ureide accumulation have been considered in the present research as three potential mechanisms affecting N_2 fixation in response to drought stress. The carbon shortage hypothesis was investigated by ^{14}C labeling and by studying the combined effects of atmospheric CO_2 enrichment and water deficits on nodulation and N_2 fixation in soybean. CO_2 enrichment under drought resulted in an enhanced photo assimilation, an increased partitioning of carbon to nodules, whose main effect was to sustain nodule growth, and sustained N_2 rates under soil water deficits. The interaction of nodule O_2 permeability and drought stress on N_2 fixation led to the overall conclusion that the O_2 limitation seems to be involved only in the initial stages of water deficit stresses in decreasing nodule activity. The involvement of ureides in the drought response of N_2 fixation was initially suspected by an increased ureide concentration in shoots and nodules under drought leading to a negative feedback response between ureides and nodule activity. The basis for ureide accumulation is hypothesized to result from decreased ureide catabolism in the leaf. Experimental evidence for inhibition of nitrogenase activity by ureides supported the feedback hypothesis. Further, the role of manganese (Mn) as a cofactor in ureide catabolism was substantiated with Mn application and the consequent alleviation of the drought-induced decline of N_2 fixation. We conclude that all three mechanisms (carbon, O_2, ureide feedback) are important in understanding the response of N_2 fixation to soil drying. Based on these physiological data, a three-stage process was developed to screen over 3000 soybean plant introductions for germplasm with increased N_2 fixation tolerance to drought. Eight tolerant lines were ultimately identified as being tolerant. These lines are being studied to investigate the physiological basis of their tolerance, and in breeding efforts to incorporate this trait into commercial germplasm. In the light of this multidisciplinary approach for drought tolerance studies in soybean, future perspectives of N_2 fixation improvement will be discussed for both ureide and non-ureide grain legumes cultivated under dry conditions.

S7-P17

RHIZOBIAL PHOSPHATIDYLCHOLINE IS REQUIRED FOR THE ESTABLISHMENT OF THE ROOT NODULE SYMBIOSIS BETWEEN RHIZOBIA AND LEGUMES.

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Phosphatidylcholine (PC), a major membrane lipid in eukaryotes, is found in only some bacteria such as the members of the Rhizobiaceae family. Therefore it has been speculated for more than 3 decades that rhizobial PC might be important for the ability of rhizobia to establish a nitrogen-fixing root nodule symbiosis with legume hosts. The answer to this question has been delayed, however, because rhizobia seem to possess at least two pathways to synthesize PC. In Sinorhizobium meliloti for example, the phospholipid N-methyltransferase (PmtA) pathway and the newly discovered phosphatidylcholine synthase (Pcs) pathway of PC biosynthesis exist (de Rudder et al., 1999. J. Biol. Chem. 274: 20011-20016). In the PmtA pathway, PC is formed by three-fold methylation of phosphatidylethanolamine. In the Pcs pathway, choline is condensed directly with CDP-diacylglycerol to form PC and CMP in a single step. We have constructed mutants, deficient in PmtA or in Pcs and both types of mutants are still able to produce PC in normal amounts when grown on complex culture media. Also, both types of mutants can establish normal nitrogen-fixing symbioses on alfalfa. Double mutants, however, deficient in PmtA and in Pcs are unable to form PC and do not form any nodules on alfalfa. These results demonstrate that rhizobial PC is indeed required for the establishment of the root nodule symbiosis between S. meliloti and alfalfa. A more detailed characterization of the PC-deficient S. meliloti double mutant and its symbiotic behaviour will be presented.

S7-P18

DIAZOTROPHIC ENTEROBACTERIA IN GRASSES

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Diazotrophic acid-gas producing bacteria were isolated in acetylene-reduction positive semisolid N-deficient (with 100 mg L^{-1} yeast extract) glucose and sucrose cultures from surface-sterilized or unsterilized roots and stems of wild rice (*Oryza minuta*), sugarcane (*Saccharum officinarum*), and lahar-grown grasses (*Saccharum spontaneum*, *Rynchelytrum repens*, *Panicum maximum*). The isolates have desirable traits such as phosphate-solubilizing, siderophore-producing, indole acetic acid –producing, and polymer-degrading activities. Some of them have also been shown to be endophytic colonizers and capable of N_2-fixing or non-N_2-fixing growth under oligotrophic and facultative anaerobic (fermentative and nitrate-reducing) conditions. These properties in addition to fast growth rates most probably give them a great edge in successful colonization of plant tissues. Phylogenetic analysis based on partial sequence of 16S rRNA showed that the isolates were closer or similar to *Enterobacter cloacae*, *E. agglomerans (Pantoea agglomerans)*, *Klebsiella pneumoniae*, *K. oxytoca*, *K. planticola*, *Serratia marcescens*, *Citrobacter freundii*, *Escherichia coli*, and *Rahnella aquatilis*.

S7-P19

EXPRESSION OF YELLOW LUPINE PR10 GENES DURING SYMBIOSIS DEVELOPMENT

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The expression of PR10 encoding genes and accumulation of proteins have been detected after pathogen invasion, wounding and other environmental stress conditions, suggesting their involvement in plant defence mechanism.
These proteins are encoded by multigene families and differenctially expressed in various plant organs. Two gene subfamilies were identified in yellow lupine: *Llpr10.1* and *Llpr10.2.* The expression of lupine PR10 proteins is modulated during development of symbiosis with *Bradyrhizobium* sp. (Lupinus). They are constitutively expressed in roots, but their expression is significantly decreased in young and mature nodules, but not in senescent nodules. Immunocytochemical staining localised the proteins in parenchymatous tissues of root and senescent nodule, mainly in the cortex. The PR10 proteins were not detected in nodule bacteroid tissues. Expression in aerial part of the plants is generally lower and only LiPR10.1B protein is expressed constitutively in stem, leaf and petiole, while L1PR10.1A protein is accumulating in infected leaves, around the site of pathogen invasion.
Our data consistent with the proposed function of PR10 proteins in general plant defence mechanism. In the plant-microsymbiont interactions, which are beneficial to the plant bost (symbioss), the expression of defence proteins appears to be suppressed to enable symbiosis development.
The L1PR10.1A and L1PR10.1B proteins were overproduced in *E. coli* cells using T7 promoter/T7 RNA polymerase system. The recombinant proteins were purified to homogeneity by ammonium sulphate fractionation and two chromatographic steps on DE52 cellulose followed by size exclusion chromatography on Sephadex 75 Hiload 16/60 FPLC column.
To characterise their function, we have crystallised both recombinant proteins (L1PR10.1A and L1PR10.1B) and solved the structure by X-ray crystallography to resolution of 1.95A and 1.66A, respectively, using molecular replacement method with Betv1 (birch pollen allergen) molecule as probe. Both proteins have similar fold, consisting of seven-stranded β-sheet and three α-helices, but significant structure diference is observed, mainly within long C-terminal α-helix.
RNAse assay revealed hydrolytic activity of recombinant L1PR10.1B protein. In contrast to the inactive L1PR10.1A homologue, the L1PR10.1B specifically cleaved single stranded regions of RNA substrates, however, the level of its activity was about 2000 fold lowe comparing to S1 nuclease.

POSTER ABSTRACTS
SYMPOSIUM 8

S8-P2

EFFECT OF BEAN INOCULATION: FIELD TRIALS

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The experiment was conducted in field conditions on the black meadow soil in the location Nova Gajdobra (the Vojvodina Province). The objective of this study was to assess symbiotic effectiveness of eight bean genotypes (Slavonski; žuto-zeleni, Vukovarski težak, Dvadesetica, Royal Duch, Belko, Zlatko, Sremac, Panonski gradištanac). The genotypes Belko, Zlatko and Sremac were selected at the Institute of Field and Vegetable Crops, Novi Sad, Slavonsko žuto-zeleni and Vukovarski težak at the Agricultural Station, Vukovar, Croatia, Panonski gradištanac at the Vegetable Breeding Center, Smederevska Palanka. Royal Duch was selected in the Netherlands. The genotypes were inoculated immediately before seeding with a microbiological preparation (NS-Nitragin) containing highly effective strains of *R. leguminosarum bv. phaseoli*. NS-Nitragin for beans had been developed at the Institute of Field and Vegetable Crops in Novi Sad.
The seeding was done at the optimum date, in three replications. The soil cultivation was in conventional, without mineral fertilization.
Plant length, dry matter mass and nitrogen content in the above ground plant parts, roots, nodules and whole plants were determined at the stage of flowering and the results were processed using variance analysis and the values obtained were tested with the LSD at 1% and 5%.
The obtained results indicate that the varieties Vukovarski težak and Slavonsko žuto-zeleni had a higher symbiotic effectiveness than the variety Belko, i.e., that the effectiveness of symbiotic association depends on plant genotype.
The dry matter mass of whole plant was significantly increased in Vukovarski težak, Slavonsko žuto-zeleni, Royal Duch and Dvadesetica in relation to Belko wich had the lowest dry matter mass.
The tested genotypes had significantly increased nitrogen contents in relation to Belko. The highest increases in nitrogen content were achieved in Vukovarski težak and Slavonsko žuto-zeleni, the lowest in Zlatko. The experiment with two bean genotypes, conducted in another location, indicated that both genotypes (Zlatko and Slavonsko žuto-zeleni) formed more effective symbiotic associations than the non-inoculated plants.
Further study should clarify how inoculation effects bean yield.

S8-P3

EXPERIMENTS OF *AZOSPIRILLUM* BACTERIZATION OF MAIZE SEEDS (*ZEA MAIS* L) IN OPEN FIELD

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One of the main aims of the Agricultural Department of the Marche Region (Central Italy) is to reduce chemical nitrogen fertilization and environmental pollution acquiring, by field tests, useful knowledge on the use of nitrogen fixing microorganisms as biofertilizers for cereals. Field experiments of *Azospirillum* inoculation of maize were carried out during the spring-summer 1999. Twelve experimental plots of different surface, six as control and six with maize inoculated with a commercial preparation of *Azospirillum* were arranged in 6 farms, located in five areas of the region. Uninoculated treatments were supplemented with full dose of N-fertilizers, whilst inoculated treatments received only 70% dose of N-fertilizers. Inoculation with *Azospirillum* had little effect on yield of maize. Indeed the productivity was equivalent to that obtained with the full nitrogen fertilization. However, significant increase in plant height, maize cob numbers and maize smut resistance occurred in the inoculated plants. The present field experiments have shown, once again, that maize respond positively to inoculation with *Azospirillum*.

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S8-P4

NEGATIVE EFFECT OF N-FERTILISATION ON N-FIXATION IN *ALNUS-FRANKIA* SYMBIOSIS AS SHOWN BY ^{15}N INCORPORATION

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We present a ^{15}N incorporation study showing the effect of ammonium nitrate (NH_4-NO_3) fertilisation on nitrogen fixation activity of *Frankia* in symbiosis with *Alnus incana*. 35 3-4 year *A. incana* trees were transferred into the experiment pot with 5 soil-space partitions (7 plants each). After initial adjustment period the plants were fertilised with 5 different concentrations of NH_4-NO_3 which were equivalents of N-fertilising loads of 0, 25, 50, 100 and 150 kg N ha^{-1} y^{-1}, respectively. For the 10-day ^{15}N incubation the pot was sealed hermetically 2 cm above the soil surface, thus creating a conjoint air layer throughout the pot. 2 litres of ^{15}N N_2 gas was entered through a valve. As a result, about 2% of the gas volume in the pot comprised of ^{15}N. Control plants were not fertilised and received no additional ^{15}N. At 95% confidence limits, we found higher nodule dry masses in case of N addition rates of 0 and 50 kg N ha^{-1} y^{-1} than at 150 kg N ha^{-1} y^{-1}. This was most probably due to nodule decay as the fertilisation period was too short for any negative effect on nodule growth at higher nitrogen fertilisation rates to emerge. ^{15}N concentrations ($^{15}N‰$) in leaves, shoots and nodules were measured. As for the leaves and shoots the highest $^{15}N‰$ were found in case of no mineral nitrogen addition, 15,67‰ ± 3,45; 16,82‰ ± 3,14 in leaves and shoots, respectively (± indicates the 95% confidence limits). At 150 kg N ha^{-1} y^{-1} the $^{15}N‰$ of leaves was significantly lower than at any other treatment staying as low as 2,62‰ ± 1,12. ^{15}N concentration in shoots was also the lowest at 150 kg N ha^{-1} y^{-1} being 3,23‰ ± 1,78, but the difference with $^{15}N‰$ at 25 kg N ha^{-1} y^{-1} was not significant at 95% confidence limits. The activity of nitrogen fixation over the 10-day ^{15}N-incubation period was calculated (mg ^{15}N per gram of nodule dry weight). Fixation activity at N addition rate of 150 kg N ha^{-1} y^{-1} was significantly lower than at N addition rates 0, 50, 100 kg N ha^{-1} y^{-1}.

S8-P5

CASUARINA–FRANKIA SYMBIOSIS: THE IMPORTANCE OF NURSERY INOCULATION

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Casuarina are actinorhizal woody plants that can fix atmospheric nitrogen when nodulated with an actinomycete known as *Frankia*. Effect in nursery of inoculation with different strains of *Frankia* on *Casuarina glauca* and *Casuarina cuninnghamiana* seedlings is studied. All the four strains tested are infective and effective. The growth increases of inoculated *Casuarina glauca* and *Casuarina cuninnghamiana* seedlings are respectively between 32 and 126 % and 15 and 96 % compared to the non inoculated seedlings.
One year after transplantation in the field, *Casuarina glauca* nodulated seedlings have higher survival rates than non inoculted ones.

Key words: *Casuarina*, *Frankia*, Artificial inoculation, symbiosis.

S8-P6

ISOLATION AND CHARACTERIZATION OF BACTERIA FROM THE RHIZOSPHERE OF TAGASASTE (*CHAMAECYTISUS PROLIFERUS* SUBSP *PROLIFERUS* VAR *PALMENSIS*)

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The root system of higher plants is associated with a diverse community of microorganisms which exert their influence on plant growth through different mechanisms. Due to the beneficial effect on plant growth of many of these microorganisms, a great effort has been made in the last decades to use them to improve crop production. (Lambert & Joos, 1989)
The legume tagasaste is a multipurpose high yield fodder shrub, endemic to the Canary Islands, of agronomic interest due to its high nutritive value. About 16 kg of edible dry matter (EDM) per plant in the second year could be reached, with a content in crude protein ranging from 17-30% of the EDM and only around 18% of indigestible crude fibre. (L. Snook, 1996).
The aim of the present work is to select PGPRs from the rhizosphere of tagasaste. In order to do so, we are currently characterising the bacterial population of the rhizosphere of tagasaste, to which we collected plants from two locations in the Tenerife Island at 500 and 1000 metres above sea level. Rhizobacteria were recovered by agitation of main and lateral root segments in Amies solution. Aliquots were inoculated on different media from which 120 isolates were selected and 84 subjected to further identification. Diazotrophic bacteria were isolated in enrichment semisolid N-free medium.
Many authors support that each plant species select a characteristic bacterial natural population. In tagasaste, it showed a great stimulation of gram negative bacteria. In fact, only 3 of the 84 isolates studied were gram positive. The isolates were phenotipically characterised by API 20NE and IAA production and, when they correspond, confirmation of diazotrophic nature by biochemical tests was carried out. Genotypic characterisation of the isolates was done by ERIC-PCR fingerprint.

Lambert, B & Joos, H. Fundamental aspects of rhizobacterial plant growth promotion research. TIBTECH. 7:215-219. (1989)
Snook, L.C. Tagasaste. A productive browse shrub for sustainable agriculture. 2nd Ed. AGROVISION. Mansfield. QLD. Australia. (1996).

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S8-P7

16S ARDRA AND ERIC-PCR FINGERPRINTING OF BRADYRHIZOBIA ISOLATED FROM THE ROOT NODULES OF *LUPINUS* SPP. AND *ORNITHOPUS* SPP. IN SPAIN

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Sixteen strains of bradyrhizobia isolated from *Lupinus* spp. and *Ornithopus* spp. in different locations of Spain were characterised by 16S ARDRA and ERIC-PCR fingerprinting, and compared with reference strains from *Bradyrhizobium japonicum*, *B. elkanii*, *B. liaoningense* and some Canarian bradyrhizobia isolates from tagasaste (*Chamaecytisus proliferus*) and other endemic woody legumes from our collection. UPGMA cluster analysis of combined 16S rDNA RFLP patterns with four restriction enzymes showed most of the *Bradyrhizobium* sp. (*Lupinus*) isolates shared a 16S ARDRA genotype with our Canarian isolates, while only three of them could not be distinguished from *B. japonicum* and *B. liaoningense*. None were related to *B. elkanii*. These results confirm our previous studies (Jarabo-Lorenzo et al., 1999) where we found a unique 16S ARDRA genotype among some Canarian bradyrhizobia isolates and two *Bradyrhizobium* sp. (*Lupinus*) clearly distinguishable from the three currently reported *Bradyrhizobium* species. A random sample of *Bradyrhizobium* sp. (*Lupinus*) isolates tested on tagasaste for nodulation gave a positive result. Thus, it might indicate that these Canarian legumes, *Lupinus* spp. and *Ornithopus* spp. could constitute a cross-inoculation group nodulated by a distinctive group of bradyrhizobia strains. ERIC-PCR fingerprinting was used to discriminate isolates at strain level, showing a high genetic diversity among the isolates.

Jarabo-Lorenzo A, Velázquez E, Pérez-Galdona R, Vega-Hernández MC, Martínez-Molina E, Mateos PF, Vinuesa P, León-Barrios M. 1999. In: Pedrosa FO, Hungría M, Yates, MG, Newton WE, eds. *Nitrogen fixation: from molecules to crop productivity*. Kluwer Academic Publishers, Dordrecht, (2000).

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S8-P8

GENETIC ANALYSIS OF RHIZOBIA NODULATING CHICKPEA FROM THE SOUTHERN OF PORTUGAL

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Chickpea (*Cicer arietinum*) is the third most widely grown grain legume in the world and it is an important source of protein. Recently improved winter forms of chickpea made possible its use as a winter crop with higher yield potential when compared to the traditional spring varieties. However, recent results indicate that this higher yield potential is site dependent. One possible explanation concerns the different natural rhizobia populations in the distinct geographic areas. Rhizobia are soil bacteria with the ability to form nitrogen-fixing symbiosis with leguminous plants, like chickpea. The symbiotic genes, such as *nif*H, which encodes the nitrogenase reductase component of the nitrogenase complex, are usually located in large plasmids. Indigenous rhizobia isolates were obtained from nodulated chickpea grown in three different soils of the southern of Portugal (Beja, Elvas and Évora). To evaluate the differences between rhizobia isolates from the three distinct soils, their genetic characterisation was done by plasmid profiles, restriction analysis of PCR amplified *nif*H gene, and Southern hybridization of total DNA. The symbiotic efficiency of the isolates was also determined. Beja isolates showed higher genetic diversity indicated by plasmid profiles and *nif*H restriction analysis. Isolates showing a single plasmid revealed a significantly higher symbiotic efficiency.

S8-P9

DGGE/TGGE AS A TOOL FOR DETECTION OF 16S RDNA SEQUENCE VARIATION IN RIZOBIA AND BRADYRHIZOBIA POPULATION OF NORTH WEST OF ARGENTINA (NWA).

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Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) had been used in molecular microbial ecology to assess the genetic diversity of total bacterial communities or particular populations and to identify sequence variation in a number of genes from several different organisms. The aim of this work was to investigate the diversity of a collection of rhizobia isolates from NWA and reference strains by using the DGGE technique to resolve 16S rDNA PCR-products. Firstly, the alignment and comparison of 16S rDNA sequences of known species of rhizobia and bradyrhizobia were performed in order to identify the regions with high variability. A region of 260 bp that was described by Young et al was found to be more diverse and therefore exploited to design primers that were similar to those proposed by the authors. A GC-clamp was added in the 5'-end of forward primer. Secondly, we applied these primers in the study of reference strains and found DGGE-patterns that differentiated between related species *R. etli* and *R. leguminosarum* and also among others beans nodulating rhizobia such as *R. tropici*. Likewise, we found that the genus *Bradyrhizobium* and species of genus *Rhizobium* showed a significant difference in migration properties. This approach is applied to examine the degree of diversity in the native population of rhizobia isolated from wild beans *Phaseolus vulgaris* var *aborigineus* and from *P augusti* found in the NWA. These legumes are nodulated by strains of genus *Rhizobium* and *Bradyrhizobium* respectively (Aguilar et al. 1998; López et al. 1999). We found that 16s rDNA-products from *Rhizobium* isolates were resolved by DGGE and some isolates yielded specific migration bands that differ of reference strains. However, results of analysis of most isolates are correlated with the date obtained by applying other approaches such as ardra and dna fingerprinting.

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López et al. 1999. Proceeding of the 12th International Congress on Nitrogen Fixation, Parana, Brasil, p. 203.

S8-P12

PHYLOGENETIC ANALYSIS, HOST RANGE AND EFFECTIVENESS OF NATIVE RHIZOBIAL STRAINS ISOLATED FROM THREE SPECIES OF *SESBANIA* IN VENEZUELA

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Sesbania sericea (*Ss*), *S. exasperata* (*Se*) and *S. juncea* (*Sj*) are species natives of seasonally flooded areas in Venezuela, and are characterized by the presence of nodules exclusively on adventitious roots arising from the submerged stems and rhizomes. However, there are no information regarding the diversity of rhizobia involved in their nodulation. The present investigation was, therefore, undertaken to evaluate the rhizobial isolates (RI) from root nodules (RN) of three *Sesbania* species in terms of their effectiveness in their origianl hosts, host range, phylogenetic relationships, and their capacity to form true stem nodules (SN) in native *Sesbania* and in the foreign *S. rostrata* (*Sr*) under flooded conditions. The results indicate that all RI formed effective RN in their original host, but not in *Sr*, and that none of the RI induced the appearance of SN in the native *Sesbania* species. Most of the RI from Ss (RI*Ss*) formed ineffective RN in *Phaseolus vulgaris*, *Vigna unguiculata*, and *Leucaena leucocephala*, and did not nodulate *Macroptillium atropurpureum*. In turn, inoculation of *V. unguiculata* and *M. atropurpureum* with RI of *Se* (RI*Se*) did not produce RN in the former, but formed ineffective RN in the latter. Several RI*Se* and RI of *Sj* (RI*Sj*) formed effective RN in *P. vulgaris*, *L. leucocephala*, *V. unguiculata*, *M. Atropurpureum* and *Centrocema pubescens*. Host range analyses also revealed that the strain ORS571 of *Azorhizobium caulinodans*, formed ineffective RN in *Ss*, *Se* and *Sj*, but did not nodulate stems of native *Sesbania*. Effective SN were, however, detected in *Sr* inoculated with the RI*Ss* denoted as S121 as well as with the the RI*Se* S127 and S128. Dice/UPGMA analysis of combined *Cfo*I, *Dde*I, *Mbo*I and *Msp*I restriction patterns of PCR-amplified 16S rDNA regions from *Sesbania* isolates showed that RI*Ss* S121, the RI*Se* S127, S128, S130 and S145 as well as the RI *Sj* denoted as S49 were closely related to *Rhizobium huautlense*. Similarly, the RI*Sj* S42, S45, S53 and S56 and the RI*Se* S140 were identified as *Mesorhizobium* sp. *Mesorhizobium* RI*Sj* and RI*Se* were incapable to nodulate *Lotus corniculatus*.

S8-P13

ACETYLENE REDUCTION ACTIVITY IN MEDITERRANEAN GORSE SHRUBLANDS

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Fire commonly increases phosphorus (P) availability in relation to nitrogen (N). It has been suggested that leguminous plants establishing nitrogen (N_2)-fixing symbioses with members of the *Rhizobiaceae* family may have a competitive advantage after a wildfire. Obligate seeders such as *Ulex parviflorus* (gorse) and *Cistus albidus* (thicket) are frecuently dominant in post-fire communities in Western Mediterranean areas of Spain. *Ulex parviflorus* is a nodulated legume and its competitive success in front of non-fixing shrubs such as *C. albidus* could be favoured by the relative increase in P availability after fire.
Besides N_2 fixation by the legume-*Rhizobiaceae* symbioses, other non-symbiotic N_2-fixing microorganisms may be present in the rhizosphere of *U. parviflorus* and *C. albidus*, and in the cryptobiotic crusts covering the soil surface. These N_2-fixing systems may also contribute to a progressive increase of the N pool in soils devastated after a fire.
We wanted to assess the effect of changes in nutrient availability on seasonally N_2-fixation in an early-successional community dominated by *U. parviflorus* and *C. albidus*. We selected a experimental site in Moixent (Valencia, Spain) under Mesomediterranean climate conditions, and on highly carbonated soils developed from marls. Three years after a wildfire, N and P fertilization were applied to 1x1 m plots in a complete factorial design with two factors (N and P) and three replicates. Fertilization doses corresponded to 100 kg N ha^{-1} and 50 kg P ha^{-1}. At that time vegetation was almost completely dominated by *U. parviflorus* and *C. albidus* individuals. Five months after fertilization, and seasonally thereafter we measured acetylene reduction activity (ARA) in nodulated roots of *U. parviflorus*, in soil from the rhizosphere of *U. parviflorus* and *C. albidus* rhizosphere, and in the cryptobiotic crusts. From the samples obtained in the summer of 1999, our results indicated that there was a high heterogeneity in ARA of root nodules from *U. parviflorus* ranging from 5 to 200 nmol C_2H_4 $plant^{-1}$ $hour^{-1}$ corresponding to c. 150-6,000 nmol C_2H_4 m^{-2} h^{-1}). On a surface area basis, ARA was higher in cryptobiotic crusts (7.6 nmol C_2H_4 cm^{-2} h^{-1} corresponding to 76,000 nmol C_2H_4 m^{-2} h^{-1}). N_2-fixation activity could not be detected in the rhizosphere of *U. parviflorus* and *C. albidus* plants.

S8-P14

INTERACTIONS BETWEEN N FIXATION AND ECTOMYCORRHIZAS IN MIXED *ACACIA*/DIPTEROCARP PLANTATIONS.

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Acacia mangium is a nitrogen-fixing, fast-growing plantation tree which has been widely planted in S E Asia. There is now considerable interest in using *Acacia mangium* as a nurse species to facilitate the establishment of slower growing, but more valuable, indigenous hardwood species in the family Dipterocarpaceae.

All members of the Dipterocarpaceae form ectomycorrhizas, and it has recently been shown that *Acacia mangium*, unusually for an N-fixing species, may also form associations with ectomycorrhizal fungi. Our project examines the role of ectomycorrhizas in the establishment of dipterocarps under a canopy of *Acacia mangium*, and asks the following questions:

1. Do the same ectomycorrhizal fungi colonise both *Acacia* and dipterocarps?
2. Can *Acacia* act as a source of mycorrhizal inoculum to aid dipterocarp establishment?
3. Does ectomycorrhizal infection facilitate the transfer of nitrogen from *Acacia* to dipterocarps?

The poster will present preliminary data on mycorrhizal infection and foliar ^{15}N natural abundance of *Acacia* and dipterocarps in existing plantations in Malaysia, and describe the establishment of experimental plantations.

One of these experimental plantations has been established on degraded former tin-mining land (tin-tailings). These sites are widespread in peninsular Malaysia, and the combination of N-fixing and ectomycorrhizal symbioses offer particular advantages for their rehabilitation

S8-P15

PCR METHOD FOR RAPID IDENTIFICATION OF *SINORHIZOBIUM MELILOTI* FIELD ISOLATES.

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A PCR reaction using four primers has been tested as a reliable method for identification of *S. meliloti* strains. The use of four primers results in amplification of two loci located in different parts of the genome, *mucR* gene in the chromosome and nodbox 4 in one of the symbiotic megaplasmid. Both loci are specific of *S. meliloti* strains. This method is useful for rapid identification of field isolates when a high number of bacteria is collected and reliable when other saprophytic bacteria colonise the root system.

Sinorhizobium meliloti is a nitrogen fixing bacterium that forms symbiosis with plants of the genera *Medicago, Melilotus* and *Trigonella*, inducing the formation of a new organ, the root nodule. Isolation of *S. meliloti* from field is a relatively easy but time consuming process that requires nodule surface sterilization, maceration and reinoculation in axenic conditions. Additional problems are the lack of a selective culture medium and the pleiomorphism of the species with mucoid and dry strains.

PCR has been successfully used for the identification of bacteria. However some of the techniques are focused in the differentiation of strain within an species, such as ERIC and REP-PCR. We have investigated a method for *S. meliloti* identification based in the use of four primers for the amplification of two regions located in different replicons. One pair for the amplification of *mucR* gene located in the bacterial chromosome and the other pair targeted for nodbox 4 promoter located in one of the symbiotic megaplasmid. The simultaneous detection of both regions would increase the specificity compared to other methods based in the use of a single primer pair. We have tested the method with laboratory strains and field isolates and we have used it for generating a collection of *S. meliloti* strains from hydrocarbon-polluted soils.

S8-P16

MIXED PLANTATIONS OF *EUCALYPTUS GRANDIS* AND *DALBERGIA NIGRA* INOCULATED WITH *BRADYRHIZOBIUM* AND MYCORRHYZAL FUNGI

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The tropical seasonal Atlantic forest in Brazil was cut down for expansion of *Eucalyptus* plantations, and that substitution produced some environmental impacts such as the reduction of soil porosity. However, in some states of Brazil, the maintenance of riparian forest with native species are foreseen in law. Nitrogen fixing leguminous trees are particularly interesting for the revegetation of ecosystems. However the major portion of woody legume species of the Atlantic Forest in Brazil, still remain unexamined regarding the contribution of rhizobia and mycorrhizal inoculants for the plant growth. *Dalbergia nigra* (Vell.) Fr. All. ex Benth, (brazilian rosewood) is a native species which produces the most valuable timber among the timber species in Brazil. Nevertheless, that is a species which was included in the official list of Brazilian endangered species, vulnerable category. The purpose of the present study is to evaluate the effects of dual inoculation with bradyrhizobia and mycorrhizal fungi on growth and nitrogen content of *Dalbergia nigra*, as a strategy for the revegetation of Rio Doce riparian forest. *Dalbergia nigra*, was cultivated in mixed plantation with *Eucalyptus grandis* trees. During 24 months, the treatment of complete nitrogen fertilization and inoculation with selected bradyrhizobia strains BHICB-Dn 15 or BHICB-Dn 53, associated or not to arbuscular mycorrhizal (AM) fungi were compared. In spite of the high levels of index of precipitation of (1.200 mm) in the summer of 1997, which resulted in the overflowing of the Rio Doce , the inoculation with the strain BHICB-Dn 15 alone increased the height (2.44 m) and diameter (7.6cm) growth and nitrogen content (3.0 mgN/g plant) in relation to the other treatments. Such treatment may substitute the nitrogen fertilized plants. In contrast, the inoculation of strain BHICB-Dn 53 alone was the worst treatment and this inoculated strain improved the plants growth only when associated to mycorrhizal fungi. However, the dual inoculation of BHICB-DN 15 and mycorrhizal fungi did not increase the plant response in relation to height, and diameter growth. These results suggest that BHICB-DN 15 strain did not show a synergical relationship with mycorrhizal fungi but the strain BHICB-Dn 53 does. On the other hand, the strain BHICB-DN 15 showed to be a competitive strain, able to occupy the majority of nodules as indicated by serological tests. The BHICB-DN 15 strain differ from BHICB-Dn 53 and those native strains by an additional band of low molecular weight on their lipopolysaccharide (LPS) electrophoretic profile.

S8-P17

USE OF *nod* GENES TO IDENTIFICATE RHIZOBIA STRAINS ISOLATED FROM SOILS

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Rhizobium is a genus of soil bacteria belonging to the family Rhizobiaceae, whose members are able to establish symbiotic relationships with a range of legume plants with agricultural and environmental importance. These bacteria induce the formation of nodules on the roots of leguminous plants in which they fix atmospheric nitrogen. In most *Rhizobium* species genes essential for symbiosis, collectively called *nod* genes are carried on a symbiotic plasmid. These genes are classified in two sets. The first constituted by common *nod* genes present in all rhizobia described such as *nodA*, *nodB* and *nodC* genes. The second set constituted by specific *nod* genes present only in some rhizobia such as *nodZ*, *nodEF* and *nodH* genes.
In this work we tried to use specific *nod* genes (*nodH* and *nodZ*) for quick identification of rhizobia isolated of soils. We used thirty strains from Argentine, Brazil and Spanish soils. These strains were trapped with *Phaseolus vulgaris*. We used *nodH* primers combined with *nodC* and *nodZ* as positive and negative control respectively, to identify *R. tropici* strains. Eight strains classified as *R. tropici* corresponded with classification done by other phylogenetic tools, such as RNA 16S sequencing.
RFLP analysis of *nodH* PCR products of these strains identified as *R. tropici* showed concordance with Nod factors produced, since T29N32 and T44N22P strains that were clearly differentiated produce a completely sulphated LCO. In contrast the rest of strains produce a Nod factor with a mixture of sulphated and non sulphated LCO. We also studied RFLP of *nodQ* PCR products of these strains in order to confirm the fidelity of *nod* genes in phylogenetic study.

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S8-P18

EFFECT OF GROWTH pH CONDITIONS IN FATTY ACIDS COMPOSITION IN BEAN RHIZOBIA.

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Common bean (*Phaseolus vulgaris*) is the most important legume in human nutrition. Symbiotic interaction between *Rhizobium* strains and leguminous plants leads to the formation of nitrogen-fixing root nodules. Establishment of symbiosis and formation of nitrogen fixing nodules are influenced by many environmental factors, such as acid and alkaline soil. These factors affect the host plant, the population of rhizobia and the symbiotic interaction itself. Cellular envelopes are the first barriers that protect the bacterium of different environmental stresses. Therefore, changes in cellular envelope composition may represent adaptive mechanisms against the stress caused by acid and alkaline pH. In this way, changes in the cellular fatty acids are involved in the regulation of membrane fluidity and permeability.

Rhizobium strains differ in their pH tolerance. In some cases, rhizobia pH sensitivity is the limiting factor in the symbiosis process under stress conditions. There is a great variety of rhizobia nodulating *Phaseolus vulgaris*, like *R. etli*, *R. gallicum*, *R. giardinii*, *R. leguminosarum* bv. phaseoli and *R. tropici*. A broad collection of rhizobia isolated from bean producing areas was analized in this study. We have tested their ability to grow on acid and alkaline buffered culture medium. Bean nodulating rhizobia show variety of bacteria response under stressfull pH conditions. *R. tropici* strains were the most tolerant to acid pH.

We have also studied the cellular fatty acids of bean nodulating rhizobia in response to acid, neutral and alkaline growth pH in order to study whether there are changes in their composition. This study shows that fatty acids composition is related to the belonging specie and it is influenced by the growth pH conditions. Principal component analysis showed that strains could be divided into clusters corresponding to their species and, for each specie, into clusters corresponding to the growth pH. The fatty acid composition of strains studied was markedly affected under growth stress conditions. These results give novel information about the adaptative changes that happen in the composition of the rhizobia cellular envelope on stress conditions.

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S8-P19

ARACHIS PINTOI LITTER DECOMPOSITION INTERCROPPED WITH FORAGE GRASSES IN "CERRADO"AND "VARZEA" SOILS UNDER SEASONAL DROUGHT.

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In tropical pastures which are generally underutilized, nutrients are returned to the soil mainly via litter decomposition. However little is known about the decomposition of tropical forage species in pastures. This work studied *A. pintoi* litter decomposition dynamic, pure and intercropped with grasses: *Cynodon nlenfluensis* (in "Cerrado"soil) and *Hyparrhenia rufla* (in "Varzea"soil) in Sete Lagoas region in the state of Minas Gerais, Brazil, under seasonal drought. Litter bags contained the legume, grass or mixture of both species were incubated to estimate the decomposition rate and microorganisms colonization. Decomposition constants and litter half-lives were estimated by exponential model and microorganisms number in specific medium by plate dilution. The cellulolytics population increased in "Cerrado"soil when *Arachis* was added to *Cynodon*. The grass decomposition rate was also increased when the legume was present in all plots, specially in those with pure grass plots. There were high correlation (r = 0.79) between remaining mass and P total on mix litter in intercropped plots. The decomposition rate in "Varzea" soil was lower than "Cerrado" soil. There were more nutrients retention and lower microorganisms number.

S8-P20

SOYBEAN INOCULANTS PRODUCTION USING PEAT WITH DIFFERENT CHARACTERISTICS.

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Due to economical reasons, inoculant production using peats with different water retaining capacity, 35 to 120%, is being used in different countries. The humidity content of peat can influence Bradyrhizobium japonicum's survival for large periods of time. The aim of this work was obtaining soybean inoculants using peat with different characteristics and impregnated with variable cellular concentrations adding different concentrations of xanthan gum (Somasegaran, 1985). A strain of Bradyrhizobium japonicum 5019 was used. The selected medium contained (g/l): glycerol, 30; yeast extract, 6; KNO3, 2,4 and salts (Balatti et al. 1991). The volume of inoculum was 5-10% of the volume used in the fermentation process. Bacterial growth and survival were determined by viable cell counts. The experiments were performed in a rotary shaker at 250 rpm and 2.5 cm stroke, and in a mechanically stirred fermentor (New Brunswick type). The obtained cellular concentration was in the order of 6-7 x 1010 cell/ml. The peat was impregnated with different cellular cultures adjusted on the carrier at initial concentrations of 1 x 107 and 3 x 1010 cell/ml. In some cases, xanthan gum was added to the bacterial suspensions as stabilizing agent in a concentration of 0,1-1 g/l. The results obtained showed that the peats with low water absorption and retention capacity had a good survival rate when xanthan gum was added in 1 g/l. It was shown that the peats with reduced water retaining capacity impregnated with 107 cell/ml after 180 days presented a survival rate higher than 109 cell/g. Besides, the other experiments impregnated with a higher concentration culture 6 x 1010 cell/ml showed high survival rates. It is important to emphasize that the addition of xanthan gum had a favourable effect on cell stabilization and humidity content of the carriers with low content of water, reaching values higher than 8 x 109 cell/ml at 180 days and keeping their symbiotic properties.

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S8-P21

CONTRIBUTION TO THE STUDY OF THE NITROGEN FIXATION IN LUPIN

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The Lupin is regarded as a leguminous plant of great importance seen its high content in protein and out of oil. This plant represents a potential to forage poorly exploited, having the possibility of developing where the other leguminous plants are not. However, the culture of the Lupin in Morocco is under various agronomic constraints.
A study was undertaken in order to know the optimal conditions for development of this leguminous plant which remains in perfect symbiosis with the specific *Rhizobium*. We carried out the follow-up of the vegetative cycle of the leguminous plant *Lupinus luteus* (aged six months). Various parameters were measured: the average height, the number of sheets, the number of inflorescence and the number of pods.
In our study we have select some stock efficient and powerful which present a significant capacity to fix the atmospheric nitrogen.
The site of coring at *Lupinus luteus* is located on the level of the principal root. The significant development of this plant makes difficult its culture in vitro.
Thus, worked out a farming technique at the laboratory in order to follow in vitro at the same time the development of the plant and the phenomenon of coring following the test of infectivity. The test of infectivity enabled us to select a powerful stock nodulante reference BL6. An optimization of culture of BL6 is followed by the study of the influence of certain physico-chemical parameters (temperature, pH and carbon source).
The inoculation of *Lupinus luteus* by the potentially symbiotic stock shows promising results compared to its culture witnesses.

S8-P22

INOCULATION OF SOYBEAN CULTIVAR OSUMI WITH *SINORHIZOBIUM FREDII* UNDER FIELD CONDITIONS IN THE GUADALQUIVIR VALLEY (SEVILLE, SPAIN).

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Soybean is an interesting crop in irrigated areas of South Spain (Andalusia and Extremadura), where it can be rotated with other crops like corn, cotton, sunflower, potatoes or wheat. The most suitable soybean cultivars for this area are those of maturity groups II and III, like the traditional soybean cultivars Amsoy and Williams. Grain yields can reach 3,000-5,000 kg/ha, depending of diverse agronomical and environmental factors. Inoculation with selected strains of *Sinorhizobium fredii* or *Bradyrhizobium* is necessary because Spanish soils are devoid of native populations of soybean-rhizobia.
New soybean cultivars, showing superior productivity, are already available to the farmers. One of these new cultivars (Osumi, maturity group II) could be better adapted than cultivar Williams to our agronomic conditions. A field trial was carried out in a fertile alluvial soil (silty loam, pH 8.2) in the Guadalquivir Valley, near Seville, to study the response of cultivar Osumi to inoculation with 8 *Sinorhizobium fredii* strains and *B. japonicum* USDA110. Three non-inoculated treatments with 0, 200, and 400 kg/ha of mineral nitrogen fertiliser were used as controls.
The highest yield (7,001 kg grain/ha) was obtained in the control fertilised with 400 kg Nitrogen/ha, although it was neither significantly different (P=0.05) from those yields obtained with 200 kg Nitrogen/ha (6,396 kg grain/ha) nor with *B. japonicum* USDA110 (6,514 kg grain/ha) or *S. fredii* SMH12 (6,246 kg grain/ha) inoculants. Inoculation with *Sinorhizobium* strains A8318 or HH29 produced yields over 5,000 kg grain/ha. The non-inoculated and non-fertilised control (NINF-control) yielded 4,400 kg grain/ha. Grain nitrogen content was also significantly higher in fertilised controls and in plots inoculated with *B. japonicum* USDA110 or *S. fredii* SMH12 (5.3-5.1 % N) than that scored in the NINF-control (4.4 % N).
New soybean cultivars, like Osumi, can reach high grain production in fertile soils of South Spain. Soybean yield obtained by using selected *Sinorhizobium* or *Bradyrhizobium* inoculants are similar to that obtained by applying high doses of nitrogen fertilisers.

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S8-P23

THE NADP-DEPENDENT GLUTAMATE DEHYDROGENASE FROM *TUBER BORCHII* VITTAD. MYCELIUM: PURIFICATION, CHARACTERIZATION AND CLONING OF THE ENZYME GENE.

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Glutamate dehydrogenase catalyzes either the reductive amination of 2-oxoglutarate to glutamate, using NAD(P)H as electron donors or the oxidative deamination through the reverse reaction. NADP-dependent glutamate dehydrogenase from the ectomycorrhizal fungus *Tuber borchii* was purified throghout two purification steps. The first was carried out on a Toyopearl DEAE 650M column and a 203-fold purification was achieved. The subsequent chromatographic step, on the 2',5'-ADP Sepharose 4B affinity column, allowed us to obtain the enzyme in the pure form. The purified enzyme resulted to be an hexamer of apparent molecular weight of 310 kDa on a Progel TSK G3000 SWXL column and a subunit molecular weight of 54 kDa as obtained by SDS-PAGE. The K_ms for 2-oxoglutarate, NADPH and ammonium are 3.32 mM, 30 μM and 35.8 mM respectively, and the purified enzyme has a broad pH optimum of 7.6. The purified enzyme was tested with several compounds known to be allosteric modifiers of GDHs to determine their effect on enzymatic activity. Nucleotides such as AMP, cAMP, ADP, GTP and ATP, divalent cations such as Zn^{++}, Ca^{++}, Mn^{++} and Mg^{++} and compounds like oxalic acid, nitrilotriacetic acid, phenantroline, EDTA and p-HMB did not remarkably affect either the aminating or deaminating reaction. On the other hand, thiol modifiers like DTNB, NEM and iodoacetamide led to the complete inactivation of the enzyme after few minutes of incubation, thus suggesting that *T. borchii* GDH, like those of other species, contains free –SH in the active site. The subsequent addition of β-MSH to the reaction mixture allowed the immediate reactivation of GDH. The activity of the enzyme was lost even when pyridoxal 5'-phosphate was added, indicating that one or more lysil residues are also involved in the active site. Furthermore we performed several PCR analyses on cDNA and genomic DNA of *T. borchii* mycelium with a pair of degenerate primers. In this way we were able to clone the central part of the coding region and the correspondic genomic region. Using the first clone as a probe, we cloned the complete gene from a 30 day-old mycelium cDNA library, and we obtained the corresponding genomic region by PCR. In this way, we characterised the gene and we could obtain the complete aminoacid sequence of GDH. Further studies will be performed by Northern blot analyses in order to evaluate the expression of the enzyme changing the mycelium growth conditions and during truffle life cycle.

S8-P26

SUBCLONING AND SEQUENING OF DNA FRAGMENT RELATED TO SALT TOLERANCE IN *SINORHIZOBIUM FREDII* RT19

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S. fredii RT19 is a salt tolerant strain and can grow on TY plate containing 0.6M NaCl. A 23Kb DNA fragment related to salt tolerance was obtained from the gene library of RT19, and *Bam*HI was selected to digest 23 Kb DNA fragment. The resulting fragments were ligated with plasmid pML122,and the recombinant plasmids were transformed to *E. coli* S17-1 on selective medium. Three transformants were obtained. Then, two parental mating experiments were carried out with these transformants as donor and strain RC3-3 as recipient, which is salt sensitive mutant of RT19. The transconjugant was selected on FY plates containing gentamycin and 0.4M NaCl. A 4.4Kb DNA fragment related to salt tolerance was obtained. Sequence analysis of this DNA fragment showed that *fix*O, *fix*N genes and three ORFs were found. Basing on physical map of this fragment and using pBBRIMCS-2 as vector, five recombinant plasmids were constructed. They were transformed to *E.coli* S17-1 and conjugated into RC3-3 strain respectively. The results showed that only ORF2 could recover the salt tolerant ability in RC3-3. The DNA fragment containing ORF2 was sequenced and its length was 1062 bp. The nucleotide and deduced amino acid sequence of ORF2 were compared with other bacteria using BLAST algorithm. The result showed that the ORF2 putative protein sequence was 36% identify to exoO protein of *Sinorhizobium meliloti* 2011, and exoO protein is a glucosyl transerase, which required for secretion of several proteins, some of which influence exopolysaccharide modification. So, the ORF2 putative protein might be connect to exopolysaccharide production. This DNA fragment will be cloned into the expression vector to study the function of fusion protein.

S8-P27

TRITERPENOID LIPIDS IN *BRADYRHIZOBIUM JAPONICUM*: OCCURANCE OF NEW HOPANOID AND GAMMACERAN DERIVATIVES

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Strain *Bradyrhizobium japonicum* USDA110 is a soil bacterium that can form symbiotic root nodules on soybeans and fixes nitrogen inside these nodules. It has been shown that these bacteria contain triterpenoid lipids, such as hopanoids [1], with as yet unclear function(s). As a preliminary to functional analysis, we used standard lipid extraction methods together with GC and GC/MS techniques to investigate further the lipid content of these bacteria. Additional hopanoid derivatives, e.g. aminobacteriohopanetriol and adenosylhopane, were identified hitherto unknown to occur in bradyrhizobia. Furthermore, gammacerane derivatives were also identified, namely tetrahymanol and, for the first time, methylated derivatives thereof, such as 2β-methyltetrahymanol, 20α-methyltetrahymanol, and 2β,20α-dimethyltetrahymanol. The hopanoid and gammacerane skeletons are synthesized from squalene. Mutational inactivation of the squalene synthase identified in *Bradyrhizobium* [2] showed that it is an essential gene in this bacterium.

1. E.L. Kannenberg et al. 1995 FEMS Microbiol. Lett. 127: 255-262, [2] Perzl et al., 1998, Biochim. Biophys. Acta 1393, 108-118.

S8-P28

CHARACTERIZATION OF RHIZOBIA OF LEGUME TREES FROM URUGUAY BY GENETICAL, FAME AND ENVIRONMENTAL RESISTANCES APPROACHES.

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Legumes are symbiotically associated with microorganisms that promote their growth as nitrogen-fixing rhizobia and mycorrhizal fungi. Native legumes in Uruguay are studied with the purpose of including them in agricultural or agroforestry systems.The objetives of this work were: 1) to characterize 61 rhizobia isolates from 8 species of native legume trees. Their growth characteristics, antibiotic, salinity and acidity resistances were determined; 2) after analyzing results of applications of rep-PCR and FAME techniques, the NTSYS program was applied in order to group the isolates. 3) to study taxonomic relationships between native isolates and characterized species of rhizobia.
The results were analized with the UPGMA method of grouping (NTSYS-PC).
The rhizobia from native legume trees were represented basically by fast-growing strains, that may be included in the *Rhizobium* genus. The generation time varied from 2,0 to 5,5 h and produced acid in YEM agar with bromothymol bleu. The isolates from *Prosopis, E. crista-galli, E. contortisiliquum* grown as a typical fast-growing rhizobia, and those from *Acacia* did it a intermediate growing rate
Isolates from *S. virgata, P. dubium and L. nitidus* showed more sensitivity to antibiotics. More than 60% isolates were resistant to 50 µg/mL of Ery and 40% to 50µg/mL of Str, but were more suceptible to Rif and Neo. Isolates from different trees showed different behavoir: more than 50% of *Prosopis* and *E. crista-galli* isolates resisted more than 50 µg/mL of tested antibiotics, while those from *P. dubium* and *L. nitidus* showed to be more suceptible. Sixty two percent of strains grew in YEM with 2% NaCl and 16% did it with 3%. All strains grew up to pH 5,5; 93% grew at pH 4,6 and 76% did at pH 4,4. In general this rhizobial population showed to be resistant to acidity. The strains from commercial inoculants were more suceptible to these levels of antibiotic, salinity or pH. The rep-PCR analysis allowed to recover the strains from nodules after inoculation and showed a great homology between isolates from the same tree and marked differences between isolates from different hosts, in this way showing to be a good tool to follow bacterial strains in natural ecosystems. The characterization of this population by ecological approaches showed 7 groups. *A. caven* isolates showed high homology and the same results were obtained by the rep-PCR determination. Grouping the population with FAME tecbnique allowed to make 5 groups. In general, each group contained isolates from the same host. FAME determinations showed to be a very sensitive method for the characterization of diversity in a microbial populations.

S8-P29

NITROGEN FIXATION IN SOME SHALLOW AND STRATIFIED LAKES IN ESTONIA

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The fixation of molecular nitrogen (Nfix) was measured in 1998-99 in large shallow eutrophic Lake Võrtsjärv and in four small and deep stratified lakes (Kooraste Linajärv, Verevi, Vellavere Külajärv and Holstre Linajärv).
In L. Võrtsjärv Nfix occurred in 1998 from July 21st to September 15th. The total amount of nitrogen fixed in this period was 60.44 µgN l^{-1}, mainly cyanophyte *Aphanizomenon skujae* was responsible for that. Nfix rate was the highest in July 21st (2.177 øgN l^{-1} day^{-1}). Nfix started when Ntot/Ptot mass ratio in lake water was 20, this value is much higher than expected from the Redfield´s ratio (N/P= 7 mg/mg). In enrichment experiments where phosphates were added to lake water (0.1 mgP l^{-1} final concentration in experimental vessel) Nfix started a couple of weeks earlier and was also more intensive than in the lake. In lake Võrtsjärv planktic Nfix seems to be limited by both N and P availability. There occurs a lag between the set-up of favourable conditions for Nfix in the lake (deficiency of mineral N, increased amount of nitrogen fixing cyanobacteria) and the Nfix itself. The number of heterocysts in algal filaments coupled with the dynamics of measured Nfix intensity. In 1999 Nfix did not occur in lake Võrtsjärv. The main reason for this was very low biomass of *A. skujae* through the year and low concentration of soluble reactive phosphorus (SRP) during the summer months.
In stratified lakes Nfix was detected only in L. Kooraste Linajärv, both in 1998 and 1999. In this lake Nfix occurred in 27.07.98 and 26.07.99 at 2-2.5 m depth (1.076 µgN l^{-1} day^{-1} and 4.056 µgN l^{-1} day^{-1}, respectively). Ntot/Ptot mass ratio in these water layers was around 15 which is lower than in L. Võrtsjärv but still more than twice higher than Redfield´s ratio. Main Nfix cyanophytes in this lake were *Anabaena sp.* and *Aphanizomenon sp.* High Ntot/Ptot mass ratio (from 21 to 40) was evidently the main reason for the lack of Nfix in other stratified lakes.

S8-P30

GENERAL CHARACTERISTICS OF PHAGES SPECIFIC FOR *ASTRAGALUS CICER* RHIZOBIA

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Three newly isolated phages, K1, K2, and C1, specific for *A. cicer* rhizobia were characterized by their morphology, host range, rate of adsorption, DNA patterns, and DNA molecular weights. All three phages were classified to the morphological group B of Bradley's (*Siphoviridae* family) on the basis of the heads hexagonal in outline and long noncontractile tails. The dimension of phage heads and tail lengths were in the ranges between 61.6 – 69.8 nm and 146.3 – 164.1 nm, respectively. Phages K1, K2,and C1 exhibited wide host range. Phage C1 and K2 lysed not only microsymbionts of *A. cicer* but also *Mesorhizobium ciceri* Ca7^T, and *M. mediterraneum* CP 92, whereas phage K1 revealed a broader lytic activity and additionally lysed also *Rhizobium galegae* HAMBI 1141, *R. galegae* HAMBI 1185, and *Sinorhizobium meliloti* 13. The plaiting efficiency of phage K1, K2, and C1 on native bacterial hosts was higher than on other rhizobia sensitive to them and changed from 99.6% to 69.8%. All examined phages are closely related to each other regarding not only morphology and host range but also of their DNA patterns obtained after digestion with restriction enzymes.

S8-P31

SYMBIOTIC CHARACTERIZATION OF BRADYRHIZOBIA NODULATING *SAROTHAMNUS SCOPARIUS*

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S. scoparius (Scotch broom) is the leguminous shrub with nitrogen-fixing capabilities suitable for temperate climate. It grows in Europe extending northwards to Sweden and eastwards to Ukraine but also in other countries where *S. scoparius* was introduced as an ornamental plant. Scotch broom root nodules exhibited typical indeterminate nodule histology with apical, peristent meristem, age gradient of nodule tissues, open vascular bundles, and also with some particular features such as: the presence of mitotic activity in the infected meristematic cells, lack of infection threads, distribution of bacteria by process of host cell division, and occurrence of a large bacteroid zone with infected cells only. The results of cross-inoculation tests have shown a broad host range for *S. scoparius* microsymbionts including not only the native host but also species such as: *Lupinus luteus, Ornithopus sativa, Lotus corniculatus, Genista tinctoria, Chamaecitisus ratisbonensis, Macroptilium atropurpureum,* and *Phaseolus vulgaris*. In addition, the close symbiotic relationship of *S. scoparius* nodulators to *Bradyrhizobium* sp. (*Lupinus*) was established at the molecular level by comparison of the partial sequence of *nodC* gene of the strain specific for the Scotch broom, to those from *Bradyrhizobium* sp. (*Lupinus*) strain D1 and contained in the public databases.

S8-P32

HIGH MOLYBDENUM CONTENTS SEEDS FOR INCREASING N_2 FIXATION EFFICIENCY

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Studies performed in Brazil showed the importance of molybdenum (Mo) and cobalt (Co) to biological nitrogen fixation (BNF) in many tropical acid soils, increasing soybean yields up to 44.2%. Although, previous work reported that Mo and Co application on the seeds, together with the inoculant, reduced bacterial survival on the seeds, nodulation and BNF efficiency. The aim of this study was to evaluate whether the use of soybean seeds with high Mo content can supply Mo to plant without reducing nodulation and increasing BNF. Soybean cultivars (BR 37 and BR 48) with different Mo contents in the seeds were obtained by spraying different levels of Mo as sodium molybdate during the pod filling stage. It was possible to produce seeds with increased Mo contents ranging from 2.3 to 28.4 µg Mo.g seed^{-1} in BR 37 and from 9.6 to 61 µg Mo.g seed^{-1} in BR 48. Soybean cultivation using seeds with high Mo contents increased yield by 15.4% for BR 37 and 22.7% for BR 48. An additional supply of 20 g of Mo to the seeds with normal and high Mo contents increased yield, by 6.3% for BR 37 and by 9.2 for BR 48. Additional increases in yield of 6.3% for BR 37 and 10% for BR 48 were also obtained when the addition of Mo was made through spraying on the leaves instead of applying on the seeds. These results have shown that seeds with high Mo content have higher BNF than those with low content.

S8-P33

DNA: DNA HYBRIDIZATIONS REVEAL AT LEAST ELEVEN GENOSPECIES IN *BRADYRHIZOBIUM*.

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DNA-DNA hybridizations were performed between *Bradyrhizobium* strains from *Faidherbia albida*, *Aeschynomene* species, and several small legumes from Senegal, as well as from *Bradyrhizobium* reference strains. We found at least eleven genospecies, I to XI, among the strains included. These genospecies form four subgeneric groups of genospecies in the genus *Bradyrhizobium*. Genospecies inside each subgeneric groups were more closely related to each other (>40% DNA hybridization) than to other genospecies (<40% DNA hybridization). They consisted of (1) genospecies I (*Bradyrhizobium japonicum*), III (*Bradyrhizobium liaoningense*), IV and V; (2) genospecies VI and VIII; (3) genospecies VII and IX; and (4) genospecies II (*Bradyrhizobium elkanii*), X and XI. Photosynthetic *Aeschynomene* isolates were found to belong to at least 2 distinct genospecies in one subgeneric group. DNA-DNA hybridization data will be compared with AFLP analysis, 16S rDNA sequence and 16S-23S rDNA spacer sequence analysis.

S8-P34

INFLUENCE OF APPLICATION OF SEWAGE SLUDGE ON N_2-FIXING POPULATION AND SOIL MICROBIAL ACTIVITY

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The effect of application of irradiated sewage sludge to agricultural soils have been investigated. Populations of aerobic heterotrophic bacteria, fungi and free living nitrogen fixing bacteria was evaluated. Response of N_2-fixation and dehydrogenase activity was also measured.
The experiment was conducted in pots filled with composite soil samples taken from 0-20 cm and added with sludge at the rates: 10, 50 and 80 $t.ha^{-1}$. As control we used a soil sample without amendment added with a standard mineral fertilization. Half of the pots were seeded with *Lolium multiflorum* and the other half with *Trifolium subterraneum* and maintained in a greenhouse. Nitrogenase activity (ARA) was evaluated using clover plants inoculated with soil suspensions obtained from the pots. Aerobic bacteria and fungi were enumerated by the soil dilution plate method. Free living N_2-fixing bacteria were estimated by the MPN technique. Specific activity of dehydrogenase was reported as µg triphenylformazan released per 1 g dry soil.
Nitrogenase activity was similar for all treatments, except for *Lolium* 80 $t.ha^{-1}$ treatment where the ARA was significantly higher. Similar result was obtained for MPN of free living N_2-fixing bacteria. Values of dehydrogenase activity and heterotrophic bacteria decreased in amended soils sown with *Trifolium subterraneum*. In contrast in pots sown with *Lolium multiflorum* microbial populations evaluated increased as a function of sewage application. Largest numbers occurred after application of 50 $t.ha^{-1}$. In general, dehydrogenase activity reflected the results of the microbial populations.
The results emphazise the potential "value" of sewage sludge as biofertilizer and the importance of vegetation to pattern of recovery of amended soils.

S8-P35

A GROUP OF *METHYLOBACTERIUM* NODULATES AND FIXES NITROGEN IN SYMBIOSIS WITH LEGUMINOUS PLANTS

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Rhizobia described so far belong to three distinct phylogenetic branches within the α-2 subclass of the Proteobacteria. Here we report the discovery of a fourth rhizobial branch constituted by bacteria isolated from the African legume *Crotalaria*. On the basis of 16S rDNA analysis, these bacteria were shown to belong to the *Methylobacterium* genus and assigned to a new species, *M. nodulans*. We demonstrated that these rhizobia grow on methanol, which is a characteristic of *Methylobacterium* spp., but a unique feature among rhizobia. Genes encoding two key enzymes of methylotrophy and nodulation, the *mxaF* gene encoding the α subunit of the methanol dehydrogenase and the *nodA* gene encoding an acyl-transferase involved in Nod factor biosynthesis, were sequenced for the type strain ORS2060. Plant tests and *nodA* amplification assays showed that *M. nodulans* is the only nodulating *Methylobacterium* identified so far. Phylogenetic sequence analysis showed that *M. nodulans* NodA is close to *Bradyrhizobium* NodA suggesting that this gene has been acquired by horizontal gene transfer.

S8-P36

CHARACTERIZATION OF MUTANTS OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE* STRAIN Z25 DEFECTIVE IN BACTERIOCIN PRODUCTION.

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Rhizobium leguminosarum bv. *viciae* Z25 (CECT 4585) was isolated in 1995 from nodules of faba bean (*Vicia faba*), one of the most important legume crops in Mediterranean areas. Strain Z25 shows high infectivity and effectivity for the nodulation of legumes in laboratory tests, as well as production of a bacteriocin able to inhibit growth of closely related strains classified within the same biovar (1). The purpose of this work is the isolation and characterization of gene(s) involved in the production of bacteriocin by strain Z25, in order to understand if this activity is a determinant of competition for nodulation and an advantage for its use as inoculant, as well as the possible construction of improved strains for legume inoculation.
Random Tn5-mutagenesis of an spontaneous streptomycin resistant derivative of wild-type strain Z25 has led to isolation of 4 mutants defective in bacteriocin production. All mutants retain immunity to the bacteriocin produced by the wild type strain and are not impaired in growth. Eckhardt gels of strain Z25 and its mutants show the presence of 3 megaplasmids with molecular weights of 380, 320 and 150 MDa, calculated using previously sized megaplasmids from *R. leguminosarum* bv. *viciae* strain 8401 pRL1JI, and *Azospirillum brasilense* Sp7, as standards (2). The *HindIII* 3.5 Kb band from Tn5 has been used as a probe to locate the insertions by Southern blot, indicating that the mutants are placed inside the 320 MDa plasmid in all mutants. Hybridization analysis of total DNA of the mutants digested with *EcoRI* and *EcoRI/BamHI* using the same Tn5 probe shows that the insertions are located in all mutants in a *EcoRI* band of ca. 12-13 Kb. Cloning and further characterization of the DNA region are in process.

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S8-P37

SOYBEAN RESPONSE TO N RATES IN NO-TILL AND CONVENTIONAL TILLAGE SYSTEMS IN THE BRAZILIAN CERRADOS.

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In Brazil, *Bradyrhizobium* inoculation has replaced successfully the use of N fertilizer on soybean crops. However, with the expansion of no-tillage cropping systems in the Cerrados region, it has become widespread the idea that it is necessary to use small N rates at the sowing to overcome problems related with N immobilization, mainly when the soybean is cultivated after a non-legume crop. In this study we examined the soybean response to small rates of N fertilizer under no-till (NT) and conventional tillage (CT) systems. The experiment (a completely randomized block with five replicates) was carried out in a red yellow oxisol, with an established *Bradyrihzobium* population (4.0 x 10^4 cells. g^{-1} soil), during two years (1998/1999 and 1999/2000). The experiment design was a factorial with two levels for management systems (no-till and conventional-tillage) and four levels of urea (0, 10, 20 and 30 kg N /ha). All treatments were inoculated with *Bradyrhizobium japonicum* strain SEMIA 5080 and *B. elkanii* strain SEMIA 5079, in the proportion of 1kg of a peat inoculant (10^8cells g^{-1}) per 50 kg of seeds. The N fertilizer was applied at the sowing. The parameters evaluated were number and dry weight of nodules at flowering and soybean yield. In both experiments the soybean was cultivated after corn. The interaction between management systems and N rates was not significant in any of the experiments. In both years there was a significant effect of management systems on the number and dry weight of nodules and also on the soybean yield. In 1998/1999, there were significant reductions on the number and dry weight of nodules and of the soybean yield in the conventional-tillage system (37%, 35% and 14%, respectively). The opposite was observed in the experiment carried out in 1999/2000, where the reductions in number and dry weight of nodules and also on the soybean yield were observed in the no-till system (25, 16 and 8% respectively). In the two experiments, there was no effect of N rates on the number and dry weight of nodules and on the soybean yield, demonstrating that it is not necessary to apply small rates of N fertilizer on the soybean even when it is cultivated under no-tillage systems.

S8-P40

DIVERSITY OF RHIZOBIA IN SENEGAL

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Senegal is a small but ecologically constrasted tropical country in West Africa, with 250 mm rainfall in the North and 1300 mm rainfall in the South. Legumes play important ecological and agronomical roles: protection and enrichment of soils, fodder, cash crops, etc. During the past ten years we intensively investigated the diversity of local symbiotic systems, trees (*Faidherbia albida, Acacia* and *Pterocarpus* species), waterlodged legumes (*Sesbania, Aeschynomene* and *Neptunia* species), a number of small annual and perennial legumes growing in different places throughout the country, and some cultivated legumes like *Phaseolus vulgaris*. Associated rhizobia were found very diverse : some were identified as already described species (*R. tropici, R. etli, B. japonicum, B. elkanii*), but the majority of them were different and represented new species of the genera *Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium, Bradyrhizobium*, but were also surprisingly more closely related to *Methylobacterium*. This addresses the question of the biological significance of this large rhizobial phylogenetic diversity. The particular metabolic properties found at least in some of these rhizobia, i.e. photosynthesis, free-living nitrogen fixation, methylothrophy could account for their adaptation to the variety of legume plant species and ecological habitats in Senegal.

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S8-P41

FIELD RESPONSES OF GRAIN LEGUMES IN MEXICO TO INOCULATION WITH SELECT STRAINS OF *RHIZOBIUM* AND FERTILIZER APPLICATIONS

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Grain legumes are of great interest in Mexico. Many million people use these species as a basis of their foodstuff. In the context of Sustainable Agriculture, Biological Nitrogen Fixation optimization is one of the best target technologies to be used.
Some comercial (NitraginR and RhizobiolR) and experimental (Fertimex) elite strains of *Rhizobium spp* were assayed in experimental field plots in Oaxaca, Mexico. Grain legumes used were common bean (*Phaseolus vulgaris*), peanut (*Arachis hypogea*) and chickpea (*Cicer arietinum*). Fertilizer Nitrogen and Phosphorus were included, with lower dosis than the recomended ones. Nodulation was consistently depressed with Nitrogen application, whereas Phosphorus act as nodulation activator.
Grain yield was considered the best indice of strain efficiency. In common bean, comercial inoculant NitraginR and all experimental strains, except FM-114, reach the same yield that the Nitrogen dose 60 kg ha-1 produce. Inoculant RhizobiolR result ineffective with this species. In peanut, nor inoculation or fertilization have some positive effect in grain yield. All the treatments have the same yield, satistically. In chickpea, the results wee more or less as in peanut, with no effects of inoculant nor fertlization. For both species, these results indicated that the native strains are present and have high efficiency level for Biological Nitrogen Fixation.

S8-P42

NITROGEN-FIXING PEA PLANTS ARE MORE TOLERANT TO MODERATE WATER STRESS THAN NITRATE-FED PLANTS.

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The effect of the source of nitrogen nutrition (nitrogen fixation or nitrate assimilation) on the response of pea plants to a gradual and moderate water stress was studied. Growth declined under water deficit but nodulated plants were less sensitive to drought than nitrate-fed plants. Also, stomatal conductance and internal CO_2 concentration decreased, but both were higher in nitrogen-fixing plants throughout the drought period, leading to better maintenance of carbon assimilation rates under water deficit. Glycolate oxidase, a key enzyme in the photorespiratory cycle, declined by 50% in nitrogen fixing plants under water deficit, whilst it was not affected in nitrate-fed plants. Nitrogen assimilation declined during the drought period and was independent of nitrogen sources. Water stress led to carbohydrate accumulation in pea plants grown with both nitrogen sources, but it was higher in nitrogen-fixing plants. Free amino acid content declined in leaves of plants grown under both nutritions, reflecting the decrease in nitrogen assimilation. It is concluded that the nitrogen source is a major factor affecting pea responses to water stress, although this different sensitivity seems not to be related to the nitrogen assimilation process but to complex interactions with photorespiratory flux and stomatal conductance.

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S8-P43

THE CONSTRUCTION AND APPLICATION OF RECOMBINANT SOYBEAN RHIZOBIA STRAINS BY THE INTRODUCTION OF EXTRA COPY *DCTABD* AND/OR *NIFA* GENE

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At first, the 5.75kb DNA fragment containing C_4-dicarboxylic acid transport (*dctABD*) genes from *Sinorhizobium meliloti* was subcloned into the stable-broad-host plasmid pTR102 through pIJ2925, A inducible expression recombinant plasmid pHN202 was obtained. The reporter gene *luxAB* from plasmid pDB30 was also inserted into pHN202 and the recombinant plasmid pHN205 was constructed. And also a recombinant plasmid pHN207 containing *dctABD* , *parCBA/DE* genes from pTR102 and reporter genes *luxAB* was constructed by using pLAFR3 as the vector. A recombinant plasmid pHN306 containing *nifA* of *Klebsiella pneumoniae* and *luxAB* was also constructed by using pTR102 as the vector. And finally a recombinant plasmid pHN307 containing *dctABD*, *nifA* and *luxAB* genes was constructed.
The recombinant plasmids pHN205, pHN207, pHN306 and pHN307 were then introduced into *Sinorhizobium fredii* HN01, YC4 and GR3 and *Bradyrhizobium japonicum* TA11 and CB1809 by tri- or bi-parental mating. A series of recombinant engineering rhizobia strains were screened. Plant pot experiments were carried out for the recipient strains and their transconjugants to compare their nodulation ability and symbiotic nitrogen fixation efficiency. The results showed: 1) Recombinant strains HN01(pHN205) and GR3(pHN205) could significantly increase the shoot dry weight (biomass) and total nitrogen content of the plant with soybean varieties of Ningzhen N0.1 and Chuanzao No.1 respectively. Transconjugant YC4 (pHN205) could also significantly increase the shoot dry weight (biomass), total nitrogen content of the plant and root nodule fresh weight with soybean variety Heilong 33. 2) The introduction of *dctABD* genes could significantly improve the symbiotic nitrogen fixation efficiency of TA11 and CB1809 with soybean varieties of Heilong 33, Ningzhen No.1 and Yudou No.1. Compared with the control, the shoot dry weight (biomass) and total nitrogen content of the plants tested were significantly increased. 3) Recombinant strains HN01(pHN306) and GR3(pHN306) could significantly increase the root nodule number, nodule fresh weight, shoot dry weight (biomass) and total nitrogen content of the plant with soybean varieties of Yudou N0.1 and Heilong 33 respectively. Transconjugant YC4(pHN306) could also significantly increase the nodule number, nodule fresh weight and total nitrogen content of the plant with soybean variety Yudou No.1. 4) The introduction of pHN307 into the soybean rhizobia did not result in the expected further significant improvement effects on the symbiotic nitrogen fixation ability for all the recipient strains. The soybean yield inoculated with recombinant strains of *S. fredii* with extra copy of *dctABD* gene was significantly greater than that of inoculated with recipient strains or nitrate fertilizer.
The results of plant pot and field experiments indicated that: the extra copy introduced *dctABD* gene or *nifA* gene could significantly increase the nitrogen fixation efficiency and the nodulation ability of the recipient strains respectively, and the effects were relevant to many factors such as soybean varieties, soil fertility and recipient rhizobia.
By means of the luminescence detection, it was confirmed that all the recombinant plasmids constructed by pTR102 in this work were stable under free-living and symbiotic conditions, and discovered that *parCBA/DE* genes could increase the stability of pLAFR3 in the transconjugants.

S8-P44

ESSENTIALITY OF BORON FOR N_2-FIXATION AND FOR NODULATION CAPACITY OF *FRANKIA* BCU11501

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Frankia BCU11501 isolated from *Discaria trinervis* nodules (1) was unable to grow in the absence of boron (B) under N_2-fixing conditions. Using fluorescence microscopy after acridine orange staining of B-normal and B-deficient cultures, the majority of acridine orange-stained +B cells emitted green color, while most of cells from -B cultures, including hypha, sporangia, and vesicles, fluoresced orange. As orange fluorescence is typical of single-stranded nucleic acids (2), these results may indicate that DNA appeared denatured in -B *Frankia* cultures and hence, cellular death might occur under B deficiency. Because of these findings, the development of vesicles and N_2-fixation activity was investigated. The electron microscopic study of vesicles developed in B-deficient treaments demonstrated a very disorganized inner compartment. Moreover, the normal structure examined by scanning electron microscopy of vesicles control disappeared in B-starved cultures. Nitrogen fixation was studied both by gas chromatographic determination of ARA and by immunological detection of nitrogenase component I (CI) on western blots. While ARA activity of *Frankia* growing with B after 15 days was 94.4 ± 25.2 nmol ethylene·mg protein^{-1}·h^{-1}, activity of -B cultures was always under the detection limit of the chromatograph. These results can be confirmed by immunological identification of nitrogenase CI following SDS-PAGE and electroblotting to nitrocellulose sheets which demonstrated that there was not detectable enzyme in B-deficient treatments. To investigate whether B deficiency could also alter the symbiotic capacity of *Frankia*, *D. trinervis* plants were inoculated with B-sufficient or B-deficient bacteria. Nodulation was highly inhibited both in plants grown in the presence or in the absence of the micronutrient when B-deficient inocula were used.
Overall results suggest that B is required for the development of N_2-fixing *Frankia* vesicles and for early interactions between the symbiotic partners.

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S8-P45

MESORHIZOBIUM CHACOENSE SP. NOV. THAT NODULATES *PROSOPIS ALBA* IN THE CHACO ARIDO REGION (ARGENTINA).

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LMW RNA analysis was performed for the identification and classification of 20 Argentinian strains isolated from the root nodules of *Prosopis alba*. These strains were also studied by SDS-PAGE of total cellular proteins, determination of the DNA base composition, DNA-DNA reassociation experiments and physiological and biochemical tests, and the whole 16S rRNA gene was sequenced from one of the strains, *M. chacoense* LMG 19008^T. The results of the genotypic and phenotypic characterization showed that the strains isolated in this study belong to a group that clustered in the genus *Mesorhizobium*. The results of DNA-DNA hybridizations showed that this group is a new species of this genus. We propose the name *Mesorhizobium chacoense* sp. nov. for this species. *M. chacoense* is a Gram negative, aerobic, motile, non-spore forming rod. Their colonies on YMA are circular convex, white, opaque and usually 1 to 3 mm in diameter within 7 days at 28°C. The maximum temperature for growth is 37°C. The generation times are from 10 to 24 h in YM broth. The strains of this species produce acid from sucrose, galactose, lactose, L-arabinose, rhamnose, trehalose, maltose, adonitol and melibiose in media with ammonium nitrate as nitrogen source. These strains are resistent to erythromycin, ciprofloxacin and cloxacillin. All strains produce β-xilosidases, α-maltosidases and N-acetyl-glucosaminidases. This species show the same tRNA profile, and this profile is different to other species from *Mesorhizobium*. The plasmid present in the strains did not carry the *nifH* gene. The type strain LMG 19008^T has the characteristics described for this species. The G+C content of strains LMG 19008^T and LMG 19002, as determined by HPLC, was 61.7 and 61.5% respectively. The accession number of 16S rRNA sequence is AJ278249.

S8-P46

RADIONUCLIDES ACCUMULATION IN PLANTS: THE ROLE OF BACTERIUM *KLEBSIELLA OXYTOCA*

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There is a high public concern over health hazard of agrochemicals on the radioactively polluted territories where radiation enhances hazardous effect. As far the ecologically clean plant production is actual on polluted territories, application of inoculants may be a reasonable alternative to agrochemicals. Data, concerning the radionuclides accumulation in the plant tissue due to bacteria activity in the rhizosphere, encouraged us to examine a role of *Klebsiella oxytoca* VN 13 (planned in Ukraine for the use as a component of inoculants) in accumulation of radiocesium and radiostrontium in inoculated crops.
Radiocesium accumulation in biomass of agricultural plants maize (*Zea mays* L.) and buckwheat (*Fagopyrum esculentum* M.) grown on polluted soils in the Chornobyl area was investigated after the seeds inoculation by *K. oxytoca* VN 13 and klebsiella–based inoculants KLEPSâ and Duceol. The bacterium decreased ^{137}Cs accumulation in roots, straw and grain of plants. In addition, *K. oxytoca* lowered level of mentioned radioisotope in biomass of barley (*Hordeum vulgare* L.) when the latter was cross-inoculated with *Glomus intraradices* Sy 167. However, klebsiella in a couple with *G. intraradices* Gv promoted accumulation of ^{137}Cs in roots of alfalfa (*Medicago sativa* L.) for 3.1 times more than being applied alone. Nevertheless, the radionuclide did not migrate to stems. Synergistic action of both microorganisms resulted in enhancing accumulation of ^{90}Sr in the alfalfa stems: cross-inoculation of seeds led to 3.2-fold increase of radiostrontium content in stems, meantime, in the independent inoculations it was the same as in control stems.

S8-P47

POTENTIAL OF THE "DUAL", IMPROVED TECHNOLOGY OF INOCULANTS MANUFACTURE, FOR CROP PRODUCTION

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Bacillus mucilaginosus is known as a plant growth promoting rhizobacterium, producing phytohormones and improving the mineral nutrition of plants. In 60-s it was used in Ukraine for enhancing crops, however, intensive use of agrochemicals displaced the *B. mucilaginosus* spore inoculant from practice. Nowadays, interest in *B. mucilaginosus* is renewed because of it produces large amounts of exopolysaccharide (EPS). With the idea of using EPS as a carrier for inoculants development, practical formulation of inoculant was elaborated (Kozyrovska et al., 1996). The *B. mucilaginosus*-based technology of inoculant manufacture included co-cultivating the bacterium *B. mucilaginosus* and any bacterium of choice which determined the type of inoculant (biopesticide, biofertilizer). The first partner provided the living cells of the inoculant with a carrier, EPS, and caused a positive effect on the plant because of its beneficial traits. Second partner conferred beneficial activities and stimulated EPS production, so that *B. mucilaginosus* produced two times more EPS during 2-day co-cultivating with a partner. The inoculant, therefore, contained cells of both partners and large amount of EPS. And although two inoculants KLEPS® and PSEPS designed exhibited high efficiency in crop producing, potential lack of this technology could be in incompatibility of some PGRB with *B. mucilaginosus*.
The objective of this study was to improve the *B. mucilaginosus*-based technology, widening its potential for more species of bacteria. Next variant of technology, "Dual", is a one-stage as previous one, however, it separates cultivation of bacterial components (if incompatibility happens) in the time (i); zeolite plays role of a stimulator of the EPS production (ii). The inoculant of new generation contains cells of both partners and large amount of EPS as previous one, and in addition zeolite enriches the inoculant with microelements (up to 12).

S8-P48

PROSOPIS JULIFLORA NITROGEN FIXATION AND SYMBIOSIS WITH RHIZOBIUM AND ARBUSCULAR MYCORRHIZAL FUNGI

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The potential of *P. juliflora* to bear nodules, fix nitrogen and be associated with arbuscular mycorrhizal fungi was studied in Senegal in anursery and in the field.
The effect of *Rhizobium* inoculation was tested on seven provenances of Prosopis sp. and one provenance of *P. juliflora*. The experiment was conducted in a nursery in an unsterilized soil. The different responses to inoculation were estimated by comparing the inoculated with the uninoculated plants. After three months growth, inoculated plant height was increased by 31% to 147%, dry weight of shoots was increased by 15% to 213%
and dry weight of roots was increased by 12% to 231%. Although the growth of all the inoculated plants was increased, the results indicate that responses to inoculation vary markedly with provenance.
Plants of one provenance of *P. juliflora* were grown in polyethylene bags for 3 months and then transplanted into 1m3 microplots in a completely randomized design to estimate its symbiotic N fixation using 15N-isotope dilution method. The amount of N fixed was approximately 31g tree^{-1}. By extrapolation, fixation for a typical plantation would be about 20kg N ha^{-1}.yr^{-1} in the first year. The calculated pNdfa for *Prosopis* was relatively high, but the total amount of N derived was relatively low.
Roots were sampled for around *P. juliflora* and three other leguminous tree species (*Acacia tortilis, A. aneura and A. nilotica*) to determine their colonisation in the field by arbuscular mycorrhizal fungi. Mycorrhizal colonisation was highest on *P. juliflora* roots. Colonisation was not affected by distance from the tree and decreased with depth only in the *A. aneura* plot. Of the four tree species examined, root and mycorrhizal distributions of *P juliflora* and *A. nilotica* showed most promise of use in agroforestry systems.

S8-P49

CO-TRANSFER OF SYMBIOTIC EFFECTIVENESS, NODULATION COMPETITIVENESS AND PH TOLERANCE IN *R. LEGUMINOSARUM* BV. *VICEAE* BY CONJUGATION.

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We studied the plasmid-mediated transfer of symbiotic effectiveness, competitiveness and acid tolerance using conjugation between *R. leguminosarum* bv. *viceae* strains. The pSym of symbiotically effective *R. leguminosarum* bv.*viceae* strain 1-32 was identified by a conjugal transfer of Tn5-labelled replicons to the plasmidless of *A. tumefaciens* Gm1-9023. Tn5-*mob* labelled pSym1-32 was conjugated (with a help of pRP4-4) to *R. leguminosarum* bv.*viceae* symbiotically non-effective Y57 and low-effective Y14 strains. The transconjugants harbouring pSym1-32 induced Fix$^+$ nodules on *Vicia villosa* roots. They showed a significantly increased plant mass, N accumulation and acetylene-reduction as compared to recipient strains. Donor strain 1-32 and two recombinants of Y14 occupied 89%, 85% and 60% of nodules when co-inoculated (1:1) with recipient Y14. Strain 1-32 was able to grow in liquid 79 medium at pH=5.0, whereas Y14 was unable to grow at pH < 6.5. The Y14 (pSym1-32) transconjugants acquired the acid tolerance of donor strain. Therefore, genes required for symbiotic effectiveness, competitiveness and acid tolerance are located on pSym1-32.

S8-P50

GENETIC MODIFICATION OF *RHIZOBIUM* STRAINS AS A TOOL TO IMPROVE BIOLOGICAL NITROGEN FIXATION UNDER SEMI-ARID CONDITIONS.

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Symbiotic nitrogen fixation suffers local environmental conditions that could cause reduction or inibithion of fixation or of nodulation. To globally face the problems found in Mediterranean semi-arid conditions we are running an INCO-DC project (IC18 CT98 0313, co-ordinator U.B. Priefer, Germany) in the aim to transfer the wide Rhizobium biodiversity collection catalogued in Marocco to investigate the failure of nodulation of French bean observed in Egypt. To this purpose we are progressing in three directions:

(v) we identified a new promoter sequence active at the onset of the stationary phase, induced by low pHs and by nutrient starvation, suitable to be used to express resistance genes both in the free-living and bacteroid state;

(vi) we constructed a gene bank from a *Rhizobium* ssp. nodulating Acacia and cloned it in a vector stable in *Rhizobium etli*. The first wild strain isolated in Marocco is resistant to many different unfavourable growth conditions and we are searching for new transferable resistance genes able to be expressed in a "sensitive" recipient strain;

(vii) to face the reduction of nodulation caused by heavy chemical nitrogen fertilisation found in Egyptian soils, we are testing genetically modified *Rhizobium etli* strains for their ability to improve plant biomass production in fertilised soils.

Our final goal is to produce improved *Rhizobium etli* inocula suitable to be used under semi-arid conditions.

S8-P51

SYMBIOTIC GENOTYPES DIVERSITY OF *SINORHIZOBIUM MELILOTI* ISOLATES SAMPLING FROM SOIL AND NODULES OF VARIOUS HOST PLANTS FROM THE CENTRAL ASIAN GENE CENTER OF ALFALFA.

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The 27 isolates of *Sinorhizobium meliloti* from both soil samples and nodules of *Medicago*, *Melilotus* and *Trigonella* were recovered from the Tadjikistan region of Central Asia. Nodule isolates predominantly carried a cryptic plasmid (average size of 200 kb) in addition to the megaplasmids and were in twice enriched in IS*Rm*2011-2 element copies per genome in comparison with soil isolates, among which two isolates free of IS*Rm*2011-2 were revealed. RFLP analysis of the 4 chromosomal, 4 megaplasmid1 loci and 2 megaplasmid2 gene clusters revealed the values of genetic heterogeneity (H) at chromosomal and megaplasmid2 loci were higher in soil isolates than in nodule isolates. The values of H at megaplasmid1 loci were similar in both soil and nodule isolates, but exceeded the values of H at chromosomal and megaplasmid2 loci. The combination of certain chromosomal and megaplasmid1 RFLP types was significantly non-random in both soil and nodule isolates, while the megaplasmid1-megaplasmid2 associations occurred to be specific in soil isolates.

S8-P54

EFFECT OF DOUBLE INOCULATION (*RHIZOBIUM* AND PGPR) ON BEAN (*PHASEOLUS VULGARIS*) AND SOYBEAN (*GLYCINE MAX* (L.) MERRILL)

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Plant growth promoting rhizobacteria (PGPRs) are beneficial bacteria inhabiting the soil ecosystem. They belong to several genera as *Bacillus, Pseudomonas, Streptomyces* and *Azospirillum* and can affect plant growth by direct or indirect mechanisms. Some of them produce hydrolitic enzymes and phytohormones (auxins, IAA), others produce phosphate solubilization or antibiotics, and others can improve root development.
The symbiotic association common bean/*Rhizobium* is described as non-effective when compared with other symbiotic associations. Several factors, both genetic and environmental, contribute to this poor performance.
We have tried to increase this association by double inoculation of *Rhizobium* and PGPRs. Bean plants have been coinoculated with different combinations of *Rhizobium*/PGPR under controlled conditions. Several symbiotic parameters as nodule mass and size, shoot and root dry weight and pods yield were analized. The best results were obtained with the double inocula CIAT 899 (*R. tropici*)/ATCC 33085 (*Phosphorobacillus latus*) on both cultivars tested (cv. Canellini and cv. Mutin). Increased nodulation on coinoculated treatment seems to be related to a larger root development, but not with phosphate solubilization. This bacterial combination was also evaluated under field conditions on cv. Mutin, which is commonly used by Andalusian farmers. Plants inoculated with this double inoculant nodulated more efficiently and an increase of 20% in pod production was observed with respect to the single *Rhizobium* treatment.
The strain ATCC 33085 was also evaluated on soybean plants when coinoculated with *B. japonicum* USDA 110. The results obtained were opposite to that on bean. The addition of *Phosphorobacillus latus* ATCC 33085 had a detrimental effect on symbiotic parameters under both, controlled and field conditions on the symbiosis *Bradyrhizobium*/soybean.

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S8-P55

INFLUENCE OF MICRONUTRIENTS IN BIOLOGICAL NITROGEN FIXATION ON BEAN (*PHASEOLUS VULGARIS*)

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The low efficiency for biological nitrogen fixation in the symbiosis *Rhizobium-Phaseolus vulgaris* under field conditions is well known (Graham, 1981; Piha and Munns, 1987). Since bean culture is considered of a great importance in many countries it would be interesting to increase bean yields when symbiotic nitrogen fixation is involved.
This work is an approach to the knowledge of some factors, such as microelements, concerned with this association. The effect of several microelements (Mo, Cu, Zn, Mn, B) has been studied using *Rhizobium* strains and commercial bean cultivars.
Phaseolus vulgaris cv. Canellini was inoculated with two *Rhizobium* strains (*R. tropici* CIAT899 and *R. etli* ISP23) and grown under controlled conditions in two greenhouse experiments. Different treatments involving the absence or not of each micronutrient in the nutritive solution were established. As in other legumes (Bolaños et al. 1999) boron seems to be the most necessary microelement for nodulation and efficient nitrogen fixation on bean. When the nutritive solution was deprived in boron, number of nodules did not differ significantly from controls, but nodules formed were white, inefective and smaller. Absence of boron affected more specifically the biological nitrogen fixation than the mineral nitrogen assimilation. Deprivation in other microelements affected negatively the nitrogen fixation in a lower proportion.
A field experiment with cv. Mutin (a fresh green bean commonly used by farmers in our region) was carried out in a loam soil ph 8.1. Inoculated treatments were supplemented with different microelements (applied on leafs) but no significant differences were observed, suggesting that other different factors, in addition to micronutrient content, must be involved in the low nodulation and nitrogen fixation observed in our field conditions.

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Graham, PH (1981) Field Crops Res. 4, 93-112
Piha MI, Munns DN (1987) Plant Soil. 98, 168-182

This work was supported by DGIFA, Consejeria De Agricultura y Pesca, Junta de Andalucía, Pir 95-26. Antonio Daza and María Camacho received postdoctoral felloships from INIA and DGIFA (Junta De Andalucía), respectively.

S8-P56

EFFECT OF SALT STRESS ON CARBON METABOLISM AND BACTEROID RESPIRATION IN CHICK PEA NODULES

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The present work investigates the relationships between nitrogen fixation, carbon metabolism and oxygen consumption by bacteroids, in order to establish whether some of the compounds which accumulate under salt stress may be used as respiratory substrates by bacteroids to fuel their own metabolism and nitrogenase activity, in nodules of chick-pea plants. Plants were cultured in a growth chamber, and salt stress was induced by adding NaCl (50 mM) to the nutrient solution at sowing. The data presented here show a rise in fermentative metabolism in nodules of chick-pea submitted to salinity, and suggest that proline, lactate or ethanol, may play an important role as energy-yielding substrates for bacteroids in this plant species. The bacteroids of *Mesorhizobium ciceri* could utilize glucose as a respiratory substrate in control and under saline conditions, while malate does not appear to be the preferred substrate under saline conditions.

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S8-P57

IDENTIFYING GENES SUITABLE FOR CONSTRUCTING PH AND SALT TOLERANT *RHIZOBIUM* INOCULANTS FOR IMPROVING FRENCH BEAN CULTIVATION UNDER SEMIARID CONDITIONS.

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The Phimed project is a collaboration of two European (RWTH Aachen, Germany and CNR-IIGB Naples, Italy) and two North African laboratories (University of Rabat, Maroc and GEBRI Alexandria, Egypt.). The project, aimed at improving french bean cultivation under semiarid conditions by constructing acid and salt tolerant rhizobial N_2-fixing inoculant strains, is financed by the EU under the INCO-DC programme (ERBIC 18CT 980313).

The role of our laboratory is to identify and clone genes involved in conferring resistance to acidic and osmotic stress in *Rhizobium* spp., to study the regulation of these genes and to understand the mechanisms of stress resistance. This knowledge may ultimately be used to construct *Phaseolus* inoculant strains with improved performance under semiarid conditions.
To date two gene clusters have been identified in *R. leguminosarum* and were studied in some detail. Homologues of the *S. meliloti act*RS two component regulatory system involved in the acid stress response, have been identified by hybridisation. A chromosomal fragment containing the *act*RS genes was cloned and mutants in these genes were constructed. The *R. leguminosarum act*RS mutants were impaired for growth under neutral pH conditions and failed to grow under acidic conditions on complex medium.
A second gene cluster was identified by Tn5-*lac*Z mutagenesis. A mutant was identified that showed increased *lac*Z activity under acidic conditions, when compared to neutral pH. The transposon was cloned with flanking DNA and was found to be located in a gene coding for a γ-amino butyrate (GABA) transaminase (*gab*T). The gene cluster also contains a gene coding for succinate semialdehyde dehydrogenase (*gab*D), which is the next enzyme in de GABA shunt pathway. Promoter-GUS fusion studies indicate that the *gab*T gene is induced by GABA and is highly expressed *in situ* during symbiosis. When introduced into a acid and salt tolerant *R. tropici* strain (RP163), the *gab*T promoter was found to be correctly regulated and additionally was induced at low pH on complex medium.

S8-P58

BIOLOGICAL NITROGEN FIXATION OF SUBCLOVER IN SOIL AMENDED WITH SEWAGE SLUDGE

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Sludge provides a source of organic matter and nutrients enhancing the growth of plants and soil micro-organisms. But besides beneficial plant nutrients it can contain materials potentially toxic to plants and soil micro-organisms, affecting their activity, including biological nitrogen fixation by the *Rhizobium*-legume symbiosis. To assess the effects of sewage sludge application on the biological nitrogen fixation of subterranean clover growing in a mixed pasture a four year field experiment was used.

An irradiated sewage sludge obtained from a treatment plant, collected on drying bed was used at the rates S1–5 t ha^{-1}, S2 – 10 t ha^{-1}, S3 – 20 t ha^{-1} and S4 – 50 t ha^{-1} was applied once at the beginning of the experiment. As control a standard mineral fertilization (PK) equivalent to 300kg of superphosphate (18%-P_2O_5) ha^{-1} and 60 kg of KCl (60%-K_2O) ha^{-1} was used. In the first year, a mixture of *Lolium multiflorum* cv. Prima (20 kg ha^{-1}) and *Trifolium subterraneum* cv. Clare (30 kg ha^{-1}) inoculated with a selected strain of *R. leguminosarum* bv. *trifolii* (10^6 bacteria $seed^{-1}$) were sown in each plot (1x1m). In the subsequent three years, the plots were resown without addition of sewage sludge or fertilizer; the legume seeds were non-inoculated and the grass was sown at the rate of 10 kg ha^{-1}. The plants were cut twice in the first and second years and once in each of the other years.

According to local experiments with pastures, 5% N atom excess ammonium nitrate (NH_4NO_3) was applied as a uniform spray at the rate of 2 kg N ha^{-1}, one month after sowing (clover plants with 3 leaves) or immediately after the cut. The ^{15}N isotopic technique was used to estimate the percentage of N derived from biological nitrogen fixation.

In an overall analysis (four year experiment), the % of nitrogen derived from fixation decreased when the rates of sludge increased. With mineral fertilization (PK) the % of nitrogen derived from fixation (80%) was significantly higher than S2 (72%), S3 (63%) and S4 (51%). These results for the lowest rates of sludge, S1 and S2, and for PK agree with values obtained for another authors. This sludge when applied at low rates can be used without harmful effects on the biological nitrogen fixation.

S8-P59

DIVERSITY OF RHIZOBIA ASSOCIATED WITH *ACACIA* SPECIES NATIVE TO MORELOS, MEXICO

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Many tropical legumes are hosts for symbiotic nitrogen-fixing bacterial whitin the genera *Azorhizobium*, *Bradyrhizobium*, *Mezorhizobium* and *Rhizobium* (including *Allorhizobium* and *Sinorhizobium*). Characterization of isolates from legumes in tropical regions have led to descriptions of new taxa and unique phylogenetics branches, such as *Azorhizobium caulinodans*, *Allorhizobium undicola*, *Mezorhizobium plurifarum*, *R. etli*, *R. tropici*. *R hainanense*, *Sinorhizobium saheli*, and *S. teranga*.
Among these new taxa *R. etli* and *R. huautlense* were proposed for or recorded from the isolates from mexican soils. *Leucaena leucocephala* was reported to nodulated with *M. plurifarum* and *R. tropici* in South America and in Africa, but some new rhizobial group were found to nodulate this plant in mexican soils were *Leucaena* is native. So, extensive isolation and characterization of rhizobial from indigenous to Mexico could be very helpful to improve the taxonomy, to learn the origen, evolution and distribution of rhizobia.
Species in the genus *Acacia* have wide distribution in the tropical regions. These legume plants are trees or shrubs and they are important foliage for animal in the drought season. Some *Acacia* are native to Mexico such as *Acacia cochliacantha*. Diverse rhizobial populations associated to these plants in Mexican soils were obtained in this research and analyzed by MLEE, PCR-RFLP and sequence of 16S rRNA, plasmid patterns. From the results of 16S rDNA sequence analysis we identified some strains closely related to *R. mongolense*.
M. plurifarum LMG11892 (African isolate), did not form nodules with the mexican *Acacia* species tested and specificity was evaluated with the different native hosts and the groups of isolates.

S8-P60

ASSESING PGPR POTENTIAL OF RHIZOBACTERIA ON GERMINATION, GROWTH AND BIOLOGICAL NITROGEN FIXATION OF *L. ALBUS* VAR *MULTOLUPA*

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PGPRs can affect plant growth directly or indirectly. Direct promotion may be exerted by several mechanisms, such as biological nitrogen fixation (Chanway and Holl, 1991) or synthesis of plant hormones such as auxins (Gutierrez Mañero et al, 1996).
The aim of this study was to test the effect of bacterial culture media free of bacteria, selected in a previous study (Lucas García et al, 2000) on germination, growth and symbiotic nitrogen fixation of *L.albus* seeds or seedlings in order to select those that could behave as PGPRs. A total of 25 strains were selected: 11 *Aureobacterium* isolates (Aur 1 to Aur 11), 4 *Cellulomonas* strains (Cell 1 to Cell 4), 2 *Arthrobacter* (Arth 1 and 2), 2 *Pseudomonas* (Ps1 and 2) and 6 *Bacillus* (Bc1 to Bc6).
Culture media of each bacteria was assesed at 20 % on solid media for germination, evaluating radicle emergence every 24 h, for 6 days. Growth assays were carried out on 5 cm stem long seedlings, nodulated with *Bradyrhizobium*, assesing culture media at 20% with N-free crone solution. Plants were kept in a culture chamber for 10 days under controlled conditions. After harvesting, total nitrogen and dry weight were evaluated on dry plants; the fixed nitrogen was caltulated from the total plant nitrogen content minus the amount of nitrogen in the original seed (Zhang et al, 1996).
A principal component analysis (PCA) (Harman, 1967) was done with germination data. Bacterial strains separated into 4 groups, one of which included those strains that promoted germination, being the most outstanding Aur6, Aur9, Aur11 and Cell1. Activation of germination by culture media is indicative of the presence of active metabolites, probably hormones, such as gibberellins or cytoquinins, which have been shown to affect germination, although presence of other hormones of the auxin type cannot be ruled out.
According to ANOVA, results of biological nitrogen fixation revealed an outstanding activation of biological nitrogen fixation by Aur6 and Aur9 (5-fold and 2-fold, respectively); however, only the former also increased total weight, probably improving nutrient assimilation by the plant, due to an increase in root surface (data not shown).

S8-P61

EFFECT OF THE INOCULATION OF *LUPINUS ALBUS* WITH PGPRS ON NODULATION

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A lot of examples in bibliography showed a synergetic effect among fixation, nodule formation and the co-inoculation of leguminous with PGPRs (Plant Growth Promoting Rhizobacteria) (Chanway *et al.*, 1989; Dashti *et al.*, 1997). The reasons why this effect occurs are not well known, however many rhizobacteria are able to produce hormones which can change the infection (Dashti *et al.*,1998). Almost the whole of regulators of vegetable growth are involved in one or another way in the process of formation of the nodule, one of this, the ethylene, is showed to have a high effect in the process. Hunter *et al.* in 1992 had alredy demostrated that the ACC concentration increased till four times during nodule formation in the soya.
Different strains from *Lupinus* rhizosphere have been isolated. Of these was selected 3 *Aureobacterium* (Aur 2, Aur 11 and Aur 10), 1 *Bacillus* (BC6), and 1 *Pseudomona* (Ps2). All strains were able to degradate ACC in culture medium and used this metabolite as the only source of nitrogen (Glick, et al., 1995).
Our hypothesis suggest that these strains can use ACC, produced by plants, in the nodular primordium, and then it is released to the rizhospere. The degradation of ACC in the external medium allow the formation of a concentration gradient which will help ACC to be released from the nodule, therefore the internal level of ethylene will be clearly affected.
In this work 15 days old *Lupinus albus* plants have been inoculated with *B. japonicum*. One set was inoculated seven days before with each strains of PGPRs tested bacteria. Other, seven days after that, and finally the last one was inoculated at the same time of *B. japonicum*. In all cases 30 and 40 days after the inoculation with *B. japonicum* the visible nodules were counted (4 repetitions).
The results show powerful effect (an increase in the number of nodules) with the strain Aur 11 in case it was inoculated in combination with *B. japonicum* or seven days after the inoculation with this one. On the other hand, no strains present any effect in the nodulation if the inoculation is produced seven days before the inoculation with *B. japonicum*. Our results suggest a posible effect of the tested strains in accordance with the mentioned hypothesis, and could be used to consider other aspects related with the process of nodulation.

S8-P62

SELECTION OF POTENTIAL PGPRs FROM THE RHIZOSPHERE OF WILD LUPINS BASED ON GENETIC CRITERIA

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The isolation and identification of PGPRs is one of the most serious problems, since intraespecies variability may affect their PGPR condition and activity (Gutierrez Mañero et al., 1996). This study was conducted to isolate potential PGPRs from the rhizosphere of wild lupins assuming the selection that has taken place along the time (Sumner, 1990).
The 576 isolates from three populations of each lupin species at two sampling times were grouped into 11 genera, among which *Bacillus*, *Aureobacterium*, *Cellulomonas*, *Pseudomonas* and *Arthrobacter* were the most abundant. Each isolate was analysed by PCR-RAPDs with the three different random primers that gave the most reproducible results, number and quality of bands among the 20 primers from a random-primer kit (Operon Technologies, LA). This technique has prooved to be a reliable tool to determine genetic diversity amonog individuals in bacterial populations.
Isolates were grouped when similarity was 90% or over and 6 groups appeared in *Bacillus*, 11 in *Aureobacterium*, 4 in *Cellulomonas*, 2 in *Pseudomonas* and only 1 in *Arthrobacter*. Resulting from this analysis it can be assumed that each group involves strains with the same metabolic capacities, and therefore, with the same PGPR potential. It's noteworthy that strains with 100% similarity indexes were sampled from the rhizosphere of different plant species in *Aureobacterium* and *Cellulomonas*. This fact reveals i) the specificity between the plant and the bacteria, probably adaptative (Sumner, 1990) arising from the metabolic compatibility between them, and ii) the strong influence of the plants in the selection of rhizosphere bacteria through exudates (Bolton et al, 1992).
Once one bacterial genome from each group has been selected, the next step is assessing their PGPR potential on the plant physiology.

S8-P63

RHIZOBIUM STRAINS ISOLATED FROM BEAN NODULES IN SPAIN AND IN MEXICO SHARE SIMILAR PLASMID SETS.

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Rhizobium etli strains, which nodulate bean roots, were originated in Meso-america. We have found a series of characteristic plasmid-associated traits in these strains. Strains have been isolated from bean nodules in several soils of Spain. 16S rDNA analysis indicated that they belong to different rhizobial species, including: *etli, giardinii, gallicum, leguminosarum* and *fredii* (1,2).
We have compared these strains with mexican native strains. In our study we determined their plasmid pattern, and analyzed RFLP profiles of symbiotic as well as of non-symbiotic genes scattered among the different plasmids (*nifH, fixL, fixK, fixN, lpsβ, repABC*) and the chromosome (*leuA, katA, phaC*).
The results obtained indicate that *R. etli* strains isolated in Spain contain plasmid combinations similar to those found in native Mesoamerican strains, further supporting our view that extrachromosomal elements form an integral part of the genome of this species. Among the other rhizobial bean-nodulating species found in Spain, some show a typical *etli* symbiotic plasmid, indicating that these strains probably arose after acquisition of this plasmid by native Spanish non-symbiotic strains.

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S8-P64

UREIDE METABOLISM AND AMMONIUM ASSIMILATION IN ROOT NODULES OF THE COMMON BEAN UNDER SALT STRESS: EFFECT OF A TREATMENT WITH EXOGENOUS ABA

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The response of plant to salt stress depends on many factor, but phytohormones are thought to be among the most important endogenous substances involved in the mechanisms of tolerance or susceptibility of various plant species. This work explores the effect which the application of exogenous abscisic acid causes on growth, nitrogen fixation and nitrogen metabolism in the nodules of phaseolus vulgaris under saline conditions. For this experiment, a pretreatment was carried out with abscisic acid (1, 10 µm) two days before adding the sodium chloride. The variety of phaseolus was 'coco', inoculated with the ciat899 strain of rhizobium tropici.
The ABA promoted plant growth in the presence of 100 mM NaCl, while growth in control plants was inhibited. The root/shoot relationship increased with 100 mM of salt in the absence of ABA. The ABA in plants treated with salt stimulated the dry weight of the nodules. The specific nitrogenase activity was inhibited by ABA in control plants but appreciably stimulated in the saline treatment. In plants treated with NaCl up to 3 times, the ABA increased the ARAP, essentially by promoting nodulation. Glutamine synthetase was inhibited by salt in the absence of ABA. The ABA in the medium reversed the effect of salt stress. Also, glutamine synthetase presented a profile which was quite similar to that of GS. Meanwhile, xanthine dehydrogenase was inhibited by NaCl without the ABA. However, under saline conditions, the activity augmented with ABA. The uricase activity exhibited a response similar to that of XDH. In general, the ABA inhibited the enzymatic activities in control plants but stimulated them in the presence of NaCl. The content in proline, amino acids and ureides in the nodules diminished with ABA, but increased with NaCl when ABA was supplied.
Abscisic acid acts as a hormone that reduces growth of Phaseolus vulgaris plants, with an effect comparable to that of a high salt dosage. In addition, it inhibits the process of N fixation, affecting nodulation and nitrogenase activity. Nevertheless, the addition of ABA to the culture medium diminishes the harsh effects of the salt, and even improves the response of the salt-sensitive cultivar, both in growth as well as in N fixation and assimilation, in addition to reducing the accumulation of toxic ions.

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S8-P65

NITROGEN TRANSFER FROM LEGUMES TO NON-LEGUMES

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Several methods have been used to measure N transfer from legumes to non-legumes including the ^{15}N isotope dilution, $^{15}N_2$ labelling, split root ^{15}N labelling, leaf or stem ^{15}N feeding. Most of these methods have shown very little or no direct tranfer of N from legumes to a non-legumes when grown in a mixed cropping sysem. However, some studies have been able to quantify N rhizodeposition by legumes and the N transfer when the root system of a leguminous plant is decomposing, e.g. during or after cutting or stress.
The present study investigated the time course of N transfer from soybean and common bean to the associated wheat plants using the stem ^{15}N feeding technique under greenhouse conditions. The objective was to measure if any N transfer occured during the various growth stages of the leguminous crops.

S8-P66

THE IDENTITY AND DIVERSITY OF THE RHIZOBIA ASSOCIATED WITH *ACACIA MEARNSII*, *ACACIA DEALBATA* AND *ACACIA DECURRENS* IN SOUTH AFRICA AS DETERMINED BY SDS-PAGE OF WHOLE CELL PROTEINS.

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One of the major challenges facing world agriculture today, is to increase crop and pasture production rapidly enough to meet the nutritional demands of the growing human population without degrading natural resources. Apart from the exponential increase in the human population during the last century, it is also estimated that the current need for fixed nitrogen will double by the year 2020 to meet the food requirements of the human population. Although both symbiotic biological nitrogen fixation (BNF) in legumes and industrial nitrogen fertilizers may provide in this need, BNF has significant advantages. However, in order to utilize biological nitrogen fixation efficiently, more diverse and competitive rhizobial inoculants, which are better adapted to local climatic and soil conditions and provide higher productivity of leguminous plants, will have to be developed. In order to do so, the existing biodiversity in legumes and strains of rhizobia has to be examined locally. The focus of this study was to determine the identity and diversity of the indigenous rhizobial species associated with the exotic Australian wattle species, *Acacia mearnsii*, *Acacia dealbata* and *Acacia decurrens*, in South Africa. The specific aim was to determine whether the Australian wattle species are nodulated by a variety of indigenous rhizobia as has been suggested. If indeed so, these *Acacia* species could be used as trap plants for the isolation of indigenous South African rhizobia. In this study more than 200 putative rhizobial strains were isolated from wattle specimens from different geographical areas in South Africa in order to obtain rhizobial isolates associated with these trees over a wide soil pH range. These putative isolates were subsequently characterized with sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins using the GelCompar 4.0 computer program (Applied Maths, Kortrijk, Belgium). The isolates, which were representative of the three *Acacia* species investigated, were of both the slow- and fast-growing type. However, the SDS-PAGE analysis indicated that the bulk of the isolates were closely related. The soil pH from which the wattle specimens were obtained ranged from relatively low (5.0) to 7.0. However, no correlation between the soil pH, geographic origin or wattle species and the protein electrophoretic groups were found.

S8-P67

GENETIC DIVERSITY OF RHIZOBIA NODULATING LEGUMINOUS PLANTS OF SOUTH AFRICA.

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Rhizobia are root nodulating bacteria, able to biologically fix atmospheric nitrogen in leguminous plants. Fixed nitrogen is usually deficient in soils leading to increased application of industrially produced nitrogen fertilizer for adequate and sustainable food production. South Africa has approximately 1400 legume species growing under diverse geographical and climatological conditions. Establishing well-nodulated legumes on agriculturally poor soils is a means to meet the increasing demand for more effective food production Previously, effective nitrogen fixation has been achieved by inoculating legume seeds with appropriate rhizobial inoculants before planting. However, more effective and competitive rhizobial strains need to be selected for extensive application in South African agriculture. Understanding the diversity of the indigenous rhizobia is therefore essential for developing better inoculants and ultimate new additions to the current rhizobial taxonomy. Since previous investigations into the identity of the indigenous rhizobia were exclusively focused on phenotypic traits, this study aims to include comparative nucleic acid sequence data of the 16S rDNA and restriction fragment length polymorphism of the *nifH*-gene. Rhizobia were isolated from nodules of indigenous and naturalized foreign legumes. Genomic DNA was prepared from lysed cells and the complete 16S- and *nifH*-region amplified. Phylogenetic relationships were inferred from comparative analyses of the sequence data. Sequence information for known rhizobial genera were retrieved from GenBank and included in the analyses. For RFLP analyses, *nifH* PCR products were digested with four different endonucleases and banding patterns scored manually and analysed by UPGMA algorithm. Most of the indigenous rhizobia showed high correlation with reference strains, although most were closely related to *Bradyrhizobium*. Isolates from *Aspalathus* and *Lotononis* spp. were present in an apparent host species-specific cluster. The *Aspalathus* isolates were closely related to *Mesorhizobium*, while the *Lotononis* isolates were related to *Bradyrhizobium*. These results indicate the diversity of the indigenous strains, and further analyses could lead to the description of additional rhizobial genera.

S8-P68

DINITROGEN FIXATION IN ALFALFA GROWN IN MONOCULTURE AND ITS TRANSFER TO ORCHARDGRASS IN MIXED SWARD

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A pot experiment was conducted a) to determine the biological N_2-fixation (BNF) by alfalfa (*Medicago sativa* L.) grown in monoculture using three reference plants (*Dactylis glomerata* L., orchardgrass; *Lulium perenne* L.; *Eragrostis curvula* L.) and associated with orchardgrass, and b) to assess the potential transfer of fixed N from the legume to the companion orchardgrass. To calculate the proportion of N in the legume derived from the air (%Ndfa) the isotope dilution technique was used. Ammonium sulfate at 9.811 atom % excess ^{15}N in solution of destilled water was injected into a tube placed in the center of each pot after germination and after harvests 1, 2 and 3 (being equivalent to a total of 25 kg N ha^{-1}). The soil used was a Typic Haplustoll and two inoculations with an effective strain of *Rhizobium meliloti* suspension previous to sowing and reinoculations after each harvest were done. Plants (four per pot) were submited to 5 harvests. The experiment was conducted using a randomized complete design with five replicates. Aboveground biomass of the legume and grasses in monocuture and in mixture -and roots biomass in the last harvest- were determined and samples were analysed for total N and ^{15}N. The %Ndfa of the legume assessed with three reference grasses averaged 70% at the harvest 1. During the second and third harvest intervals alfalfa derived 80% of its N from fixation, and then declined in the fourth and fifth with both orchardgrass and *L perenne* as reference plant. With *E. curvula* alfalfa yielded lower estimates of %Ndfa coincidently with a lower and more uniform ^{15}N enrichment of this reference plant during the study period. At the last harvest, *L perenne and E. curvula* presented significantly different ^{15}N enrichment and similar %Ndfa between aboveground biomass and roots. In three harvests, the N yield of alfalfa and orchardgrass was higher when grown in association. A higher contribution from BNF was observed in the legume when mixed, ranging 84-94% from the second to the fourth harvest. In this period, both pure and mixed orchardgrass yielded statistically similar ^{15}N enrichments and they provided similar estimates of alfalfa %Ndfa. Consecuently, no transfer was detected in this period. At harvests 1 and 5, the difference in atom % ^{15} enrichment between pure and mixed grass were statistically significant but a direct transfer of N from alfalfa in harvest 1 is unlikely. For the last harvest a N transfer of 10.0 y 7.8% (without a statistical difference), calculated considering the aboveground biomass and the whole plant, respectivelly, was detected. This transfer may be a more complex multicomponent process than N being derived originally from the atmosphere.

S8-P69

BIODIVERSITY OF *RHIZOBIUM* IN A METAL POLLUTED ECOSYSTEM

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Pollution tends to lead a decrease in microbial diversity in terms of species or strains richness due to extinction of those ones which lack sufficient tolerance to the stress imposed, and can potentially lead to the enrichment of a particular species (or strains) which survive well in the face of the stress. N_2-fixation is considered one of the most important microbial processes that is sensitive to heavy metal pollution. An assessment of the genetic diversity of *Rhizobium leguminosarum* bv. *trifolii*, by analysis of plasmid profiles, and the relationship between plasmids and the tolerance to heavy metals could provide valuable information about rhizobial genotypes that are well adapted to the stress conditions existent in polluted soils. For this study a soil was selected from an area with known pollution problems where heavy metals have been emitted by industry for nearly 40 years. *Rhizobium* strains were isolated from nodules of subclover grown in this contaminated soil and also from nodules of subclover grown in an uncontaminated soil with identical texture and similar organic matter. The polluted soil had higher concentrations of heavy metals, mainly Cu, Zn and Hg, than the unpolluted soil.
Plasmid profiles of the 57 isolates of *R. leguminosarum* bv. *trifolii* tested showed the existence of 12 different plasmids with molecular weight ranging from 460 to 70 kb. Three to 6 bands were detected in each isolate. Dominant isolates with a characteristic plasmid profile, were verified according the soil from which they were isolated. Isolates from polluted soil had predominantly 4 plasmids and isolates from unpolluted soil mainly 3 plasmids. On the other hand it was verified that all the isolates had always 2 large plasmids, respectively 460 and 351 kb. The great variation shown by plasmid profiles was seen in the remaining plasmids of smaller molecular weight, some being present only in isolates from polluted soil. To check the tolerance to heavy metals two *Rhizobium* strains with different plasmids contents, were selected. One was from the contaminated soil and had 4 plasmids, respectively 460, 351, 219 and 154 kb. The other strain was from the uncontaminated soil and had only three plasmids, respectively 460, 351 and 161 kb. It was shown that the former isolate tolerated higher quantities of Cu, Zn and Hg than the isolate from the uncontaminated soil. These results suggest that isolates from contaminated soil are well adapted to high doses of heavy metals. Survival and tolerance of these *Rhizobium* strains could be related to the presence of some plasmids, such as the plasmid 219 kb, which is present in about 86% of the isolates from the contaminated soil.

S8-P70

OBTENTION OF *SINORHIZOBIUM MELILOTI* SUSPENSIONS WITH HIGH CONCENTRATION OF EXOPOLYSACCHARIDE IN AN AIR-LIFT FERMENTOR.

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In this work, the influence of the media composition and aeration on the biornass and exopolysaccharides formation produced by strains of *Sinorhizobium meliloti is* considered (Lorda *et al.* 1999). The experiments were performed in a rotary shaker and in an air-lift fermentor (Balatti *et al,* 1996). The objective is the optimization of legume inoculant production. Strains of *Sinorhizobium meliloti* isolated in our laboratories were used. The microorganism was kept on sterile peat at 5ºC and in a medium containing manitol, yeast extracts and salts. The inoculum was added to achieve an initial concentration of 1 x 10^8 cell/ml. The process medium contained high rates of saccharose, yeast extract and nitrogen sources. Bacterial growth and survival rates were determined by viable cell counts. Exopolysaccharide concentration was determined by precipitation with ethanol. The experiments were performed in a rotary shaker at 250 rprn and 2.5 cm stroke, and in an air-lift type fermentor at different aeration levels. Aeration was determined by oxygen absorption rate. It was observed that in erlenmeyer flasks, exopolysaccharide was in the order 10 g/l However, in the fermentor, at low aeration rates, high values of exopolysaccharide were reached, 15 g/l, using aeration rates 2 l/min. The suspensions adjusted to pH 7 and with the addition of saccharose 6M were kept for periods of 180 days at 5ºC The cellular suspension showed high survival values in the order of 1 x 10^9 cell/ml. The experiments performed with alfalfa plants showed that the strains kept their symbiotic properties. On the other hand, it was observed that the suspension with higher levels of exopolysaccharides influenced favourably the nodulation rates.

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S8-P71

SUSTAINABLE USE OF RHIZOBIA IN IMPROVED FALLOW SYSTEMS

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The primary objective of using nitrogen fixing trees and shrubs (NFTs) in tropical agroforestry systems is to improve N economy of the fixers and that of associated crop. Improved fallow is an agroforestry technology, and is a derivation from the traditional fallow where fast-growing species are deliberately planted during a crop-resting period for rapid replenishment of soil fertility. Improved fallow species are usually leguminous herbs, shrubs or trees managed to optimise biomass productivity in as less time as possible. The commonly used fallow species are: *Sesbania sesban*, *Crotalaria* spp., *Tephrosia* spp. and *Calliandra calothyrsus*. However, little attention has been given towards ensuring that biological nitrogen fixation (BNF) is enhanced, but rather relying on natural or spontaneous nodulation. Benefits of using NFTs as fallow species can be substantial. For example, a 2.5-fold increase of maize grain yield has been achieved following spontaneously nodulating *S. sesban* fallow (as opposed to a natural grass fallow) on smallholder farms in the western highlands of Kenya where soils are impoverished. The key question is can we improve BNF in these systems and with what *Rhizobium* technologies?

Evaluations of these farming systems and those reported by other workes indicate that native rhizobila populations and diversity are variable, and are influenced by host and site factors. Native populations of > 10^5 g^{-1} soil have been estimated in cultivated and virgin lands, with varying taxonomic and functional diversity. They differ in host spectrum and nitrogen fixation ability. These varied forms of biodiversity present equally vaied challenges to the application of rhizobila technology and adoption, especially in the smallholding systems. Therefore, maximisation of benefits from BNF in improved fallow systems will depend on how well the processes and interactions of above- and below-ground components are understood, managed and applied to improve delivery and sustainability of rhizobial inoculants and other microsymbiont technologies. Some of the strategies that are currently employed to improve BNF in these systems are presented and discussed.

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S8-P74

DIVERSITY OF RHIZOBIA ISOLATED FROM *ASTRAGALUS ADSURGENS* GROWING IN DIFFERENT GEOGRAPHICAL REGIONS OF CHINA

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Ninetyfive rhizobial strains isolated from *Astragalus adsurgens* growing in different geographical regions of China were characterized and compared with 26 reference strains of some recognized species of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium* by performing polyphasic taxonomy, including numerical taxonomy, rep-PCR fingerprinting with four different primers (REP, ERIC, BOX and GTG5), AFLP fingerprinting, 16S rDNA PCR-RFLP analysis, the partial sequencing of 16S rDNA, cross nodulating test, determination of DNA base composition and DNA/DNA hybridization. The results showed that there is great phenotypical, genotypical and phylogenetic diversity among these isolates. Based on results of initial screening methods most new rhizobial strains clustered into three large groups (1,2 and 3). The partial sequencing (the first 900 bp) of 16S rDNA showed that the representatives of group 1 and 3 belonged to *Mesorhizobium* phylogenetic branch. The SDW045 formed a distinct branch and it possessed a mosaic kind of 16S rDNA sequence, which contains the specific sequence of *Rhizobium*, *Agrobacterium* and *Mesorhizobium*. Another strain NM179 has a 72 base insertion at the beginning of its 16S rDNA sequence. The DNA homology between the representative strains SDW014 of group 1 or SDW018 of group 2 and the type strains of the known species of *Mesorhizobium* ranged from 16.8 to 52.9 % and 11.3 to 46.2 %, respectively. The strains of these two groups had moderate to slow growth rates and produced acid on YMA. Based on all of these research results two new species of *Mesorhizobium* might be proposed.

S8-P75

CHARACTERIZATION AND IDENTIFICATION OF SOME INDIGENOUS SOUTH AFRICAN RHIZOBIA USING 16S rDNA SEQUENCE ANALYSIS

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The use of different characteristics (the polyphasic approach) to describe bacterial taxa is a prerequisite for a stable classification. The taxonomy of root- and stem-nodulating rhizobia is in a state of transition. As more legumes are studied, new species and genera of rhizobia are described. It is important to study the indigenous South Africa rhizobia, as without them a complete rhizobial taxonomy is not possible. Futhermore, strains with superior nitrogen fixation abilities may be discovered. Indigenous strains better adapted to the harsh South African environment are possible candidates for commercial inoculants for cropped legumes. Only two locals studies have been done on the diversity of the indigenous rhizobia. These studies revealed the diversity of rhizobia existing in the South African context. As part of a polyphasic approach used to identify and determine the diversity of the indigenous rhizobia, 16S rDNA sequencing analysis was performed on some selected rhizobial and putative rhizobial isolates. The aim of study was to characterise and identify the indigenous isolates by 16S rDNA sequencing analysis and compare our data with those available in the GenBank database. Results showed that most of the indigenous isolates were slow-growers belonging to the genus *Bradyrhizobium*. Two isolates from supposedly non-nodulating legume genera (*Cassia and Senna*) were found to belong to the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. The identity of five isolates was not clear and further studies need to be performed to unequivocally determine their taxonomic position. Partial sequence analysis of 16S rDNA proved a valuable tool to characterise and identify the indigenous isolates. However, the method was unable to clearly distinguish between closely related species and strains.

S8-P76

INFLUENCE OF CARBENDASIM AND IMAZETHAPYR ON DINITROGEN FIXATION ACTIVITY, CROP AND CHEMICAL COMPOSITION OF SERRADELLA (*ORNITHOPUS SATIVUS* L.)

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Legumes are crucial to the balance of the nitrogen cycle. The nitrogen contributions of legumes can be vital for maintaining soil productivity over long periods. The effect of various peticides on the legume partner of *Rhizobium*-legume symbiosis are discussed.
Pesticides are either applied to control the pests of legume or enter directly into the environment where legumes crops are common, thus affecting their growth nodulation and yield.
The aim of this investigation was to determine the effect of fungicide seed dressing Funaben T (a.i. 3% carbendasim) and herbicide Pivot Encorne (a.i. imazethapyr) on the yield, total protein contents, nodulation and *Bradyrhizobium*-serradella nitrogenase activity.
The pot and the field experiments were conducted separately during 1997 and 1998. Serradella seeds were dressed with carbendasim and inoculated with effective strains of *Bradyrhizobium*, directly before sowing. Imazethapyr was applied by means of preemergence. Plots and pots without pesticides were used as the control group. At the beginning and the full blooming plant phases, the activity of N_2 – fixation was determined directly on the field, using acetylene reduction method.
During both years, in field trials the herbicide imazethapyr reduced yield (8%) dinitrogen fixation activity (44%), and nodulation. In contrast, combined application of imazethapyr with carbendasim stimulated yield (25%) and nitrogenase activity (14%).
In the pot experiment carbendasim and imazethapyr delayed serradella germination and subsequently inhibited serradella growth. Both active substrate applied caused a clear decrease in the dinitrogen fixation activity (75%) nodulation and the development of lateral roots.
None of the pesticides significantly affected total protein contents.

S8-P77

DIFFERENTIAL GROWTH OF LEGUMES IN THE PRESENCE OF NITROGEN FIXING BACTERIA

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Four greenhouse experiments were carried out in order to investigate the effect of nitrogen-fixing bacteria of the genus *Bradyrhizobium* on the growth of two shrubby legumes and its ability to nodulate non-natural hosts. The selected species were *Cytisus multiflorus*, and *Bossiaea aquifolium*, both natives of two areas of Mediterranean-type climate. The two species have a similar life form and life cycle. *Cytisus multiflorus* is endemic to the Iberian Peninsula, whereas *Bossiaea aquifolium* is restricted to Western Australia. *Bradyrhizobium* strains were isolated from root nodules of *Bossiaea aquifolium* growing in natural populations in Western Australia. Treatments included combinations of soil sterilization and soil inoculation: (a) non.-sterile, non-inoculated soil, (-/-); (b) non-sterile, inoculated soil (-/In); (c) sterile, non-inoculated soil (St/-); (d) sterile, inoculated soil (St/In). Plants of the two species were grown in soils from populations of *B.aquifolium*, collected in Western Australia. Nodulation efficiency was measured by comparing biomass production in both species after six months of growth. The shoot/root ratio, the number of root nodules produced by each plant and the nodule biomass was also measured. The results show that bacteria isolated from *B.aquifolium* are capable of inducing effective nodulation in seedlings of *C.multiflorus*. This does not support the hypothesis of complete specifity among nitrogen-fixing bacteria and their host legumes, as previously reported for cultivated legumes. The final values of biomass and shoot/root ratio for B.aquifolium growing in its own soil were always greater than those reached by *Cytisus multiflorus* under the same growing conditions. Significant differences were found for the biomass production of the two species growing under the treatment (St/-). This result is interpreted as the lack of bacteria in the soil. Differences were also detected in the growth of *B.aquifolium* in the experiment (-/In), where biomass production was greater than that of any of the remaining treatments. Root nodules production was much higher in *C.multiflorus* than in *B.aquifolium*. However, nodules produced by *C.multiflorus* were much smaller that those from *B.aquifolium*, a fact that suggest a higher efficiency in nitrogen fixation for B.aquifolium.This is in consonance with the final biomass production and shoot/root ratio that were higher for the plants with bigger nodules. Despite this, bacteria isolated from *B.aquifolium* were able to infect seedlings of *C.multiflorus*; but the efficiency of nodulation is lower than that in the natural host plants. This is reflected by a lesser growth of the plants of *C.multiflorus* as well as a lower production of nodules with presumably a lower capacity to fix nitrogen.

S8-P78

SINORHIZOBIA EFFICIENT WITH M *LACINIATA* CONSTITUTE A NOVEL BIOVAR (BV *LACINIATA*) OF *S. MELILOTI.*

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Among the family of Leguminosae, *Medicago* is a very extensive genus comprising more then 60 different species, two-thirds of which are annuals and one-third perennials. They are associated with nitrogen-fixing bacteria that were initially grouped into the species *Sinorhizobium meliloti*. Recently, *S. medicae* and *Rhizobium mongolense* have been proposed as additional species which form efficient symbioses with the species *M. polymorpha* and *M ruthenica*, respectively (1, 2). Other varying degrees of host plant affinitics have been reported among *Medicago* species. Some *Medicago* species are described as promiscous pant hosts (*M. sativa, M. truncata, M. minima*) which fix nitrogen with a broad range of *Medicago*-infective isolates. while other *Medicago* species (*M. polymorpha, M. laciniata, M. sauvagei, M. noeana*) appear to be more specific.
To further describe the diversity of sinorhizobia associated with *Medicago*, we analysed root-bacteria from *M. laciniata*, a low promiscous species that seems to exhibit one of the narrowest associations with highly specific strains. We isolated bacteria from soil samples collected in the Mediterranean basin where *M. lacinata* is native and we determined their genetic and phenotypics relationships with other *Medicago*-nodulating species.
Rhizobial isolates from root nodules of *Medicago laciniata* were identified as *Sinorhizobium meliloti* on the basis of the results of PCR-RFLP analyses of small-subunit rRNA (16S) and intergenic (IGS) sequences, and of the 16S rDNA sequence. Both efficient and non-efficient isolates were isolated from *M laciniata*. None efficient bacteria were similar to well-characterized strains of *S. meliloti* (represented by the strain 2011) whereas efficient ones showed new characters. Bacteria efficient on *M. laciniata*, constituted however, a restricted group of lineages with low genetic diversity based on *nifKD* and IGS typings. Phenotypic characters were not successful for distinguishing bacterial groups. A new biovar bv. *laciniata*, is thus proposed within *S. meliloti* to encompass sinorhizobia efficient on *M laciniata*.

1. Rome et al 1996. Int. J. Syst. Bacteriol 46 972-980
2. Van Berkum et al. 1998. Int. J. Syst. bacteriol. 48:13-22

S8-P79

IMPROVEMENT OF SYMBIOTIC NITROGEN FIXATION IN CHINESE SOYBEAN CROPPING AREAS

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The results presented here have been obtained during the development of an INCO-DC project entitled "Improvement of symbiotic nitrogen fixation in Chinese soybean cropping areas" (ERBIC18CT970191).

Soybean cropping is extremely important in China where soybean seeds represent one of the major protein sources. The soybean-microsymbionts *Sinorhizobium fredii* and *Bradyrhizobium japonicum* are present in the soil of soybean cropping areas of China, which is the geographical origin of soybeans. However, very little is known about the distribution and relative predominance in Chinese soils of the two bacterial populations, their symbiotic nitrogen-fixation capacity as soybean inoculants and their competitive ability to nodulate soybeans in soybean cropping areas.

Quantitative analyses of the *S. fredii* and *B. japonicum* populations in soils of four different provinces of China (Hubei, Shan Dong, He Nan and Xinjiang) have been carried out. For this purpose, soil samples were collected and used as inoculants of Asiatic and Western soybean cultivars. Bacterial isolates recovered from soybean nodules were divided into fast- (*Sinorhizobium*) and slow-growers (*Bradyrhizobium*) by their speed of growth on YMA. All soil samples contained fast- and slow-growers. The percentage of fast-growers was generally higher than that of slow-growers, suggesting that *Sinorhizobium* is more abundant than *Bradyrhizobium* in the investigated areas. The size of the indigenous populations of soybean-rhizobia (*Sinorhizobium* and *Bradyrhizobium*) were estimated by the "Most Probable Number" (MPN) technique. All soils contained about 10^4-10^5 bacteria/gram of soil.

Two hundred *Sinorhizobium* strains have been further characterised by assay of the following traits: generation time, final pH of the cultures, intrinsic antibiotic resistance, colony morphology, melanin production ability, plasmid profiles, lypopolisaccharide (LPS) profiles, Nod-factor (LCOs) profiles, utilisation of different compounds as the sole carbon or nitrogen source, ability to grow at acid and alkaline pH and symbiotic efficiency with soybean cultivars. A catalogue that compiles this information has been produced.

Competition experiments between *Bradyrhizobium* and *Sinorhizobium* strains have been carried out. Our results strongly suggest that pH and soybean cultivar are important in determining the final outcome of the competition between the two groups of bacteria. *Sinorhizobium* strains that are highly competitive against *Bradyrhizobium* have been identified.

Nitrogen-fixation capacity of more than 200 *Sinorhizobium* strains has been evaluated by measuring soybean dry-weight. Highly effective *Sinorhizobium* strains with Asiatic and/or Western soybean cultivars have been identified.
Sinorhizobium strain 042B(s) was originally isolated from Xinjiang Region. Strain 042B(s) nodulated all soybean cultivars tested. The structures of the LCOs produced by this strain have been determined. The results showed that the LCOs isolated from 042B(s) are typical of those produced by *Sinorhizobium fredii*.

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AUTHOR INDEX

Abbadi JM 42
Abbdelli C 43
Abdel-Fattah M 243
Abourouh M 267
Abril A 307
Agostini D 285
Aguilar M 39, 177, 271, 279
Aguirre JF 45
Ahmad MS 243
Aigner M 327
Akao S 152
Akentieva N 32
Alberich-Jordá M 172
Albus U 217
Alexander I 276
Alho L 270
Alvarado X 209
Alves BJR 255
Amarger N 43
Ammannato S 129
Amrani F 272
Amrani S 272
Andronov EE 313, 314
Ané JM 59
Antolín MC 251
Antonyuk LP 150
Aparicio-Tejo PM 105, 304
Aquani EA 43
Arahou M 267
Arcondéguy T 112
Arias A 96
Arrese-Igor C 104, 105, 304
Ashford D 170
Asis CA 152
Auguy F 176
Aurag J 43, 241, 245
Austin S 124
Azcón R 250
Azcón-Aguilar C 250
Bâ A 141
Bâ S 302
Baginsky C 131
Baker D 189
Balatti A 282, 332
Baldani JI 149
Balleza D 225
Bally R 142
Baquerizo E 59
Barea JM 250
Barraquio WI 258
Barrios H 81
Battistoni F 96
Battistoni J 96
Batut J 22, 28
Baumgarth B 201
Bazhenova MA 51, 53
Bazhenova TA 51, 53
Bazzicalupo M 79
Becana M 55, 120, 121
Becker A 201, 214
Becker BU 183, 221
Becker JD 61, 217
Bedmar EJ 77, 103, 248, 273, 274, 275
Bekki A 263
Bellogín RA 181, 187, 188, 192
193, 197, 341
Bellot J 275
Bendaou N 267
Benelli EM 247
Beremand P 66
Bergman B 139
Beringer J 213
Berkiaê A 244
Berraho EB 43, 245, 246
Bhagwat A 229
Bian XL 288
Biondi E 79
Bisseling T 33, 67, 71
Blanco L 100
Blanco-Rivero A 148
Blasco R 54
Blesiadka J 259
Bloem JF 40
Boddey RM 255
Boesten B 72, 319
Bogusz D 176
Boiffin V 183
Boistard P 28
Boivin-Masson C 80, 297
Bóka K 155
Bolaños L 182, 306
Bonatto AC 247
Bonfante P 146
Bonilla I 159, 182, 215, 277, 306
Bono JJ 34
Boot KJM 36, 199, 205, 207
Borisov AY 65, 68, 72
Boscá L 218
Bossi R 56
Bothe G 22
Bothe H 308
Bouhmouch I 241
Bourcier F 295, 302
Bradic M 335
Branco C 270
Bravo JM 289
Brelles-Mariño G 248
Brewin NJ 35, 121, 203

Brhada F 43, 244, 245
Brito B 127
Brom S 76, 84, 125, 325
Broughton WJ 23, 75, 86
Brown S 223
Brunel B 340
Buck M 247
Buendía A 184, 193, 196, 341
Buhrmester J 83
Burillo S 117
Burygin G 135
Bustillos-Cristales R 162
Butelli E 35, 203
Caba JM 208
Caballero-Mellado J 38, 45, 162
Cabanes D 28
Cabrerizo PM 105
Cacic N 136
Camacho M 193, 284, 315, 316, 317, 341
Camas A 100
Camerini S 312
Campo RJ 242, 294
Campos R 101, 126, 140, 318
Cardinet G 59
Carmona E 233
Carreau V 20
Carreño-López R 27
Carrero JJ 126
Carson K 96, 107
Carter RA 107, 185
Carvalho H 102, 123
Castaño C 332
Castillo F 54
Castro IV 320, 331
Castro S 222
Caturla-Goñi M 60, 69
Cavalieri D 129
Caviedes MA 211, 230, 234
Cebolla A 12, 223,
Cervantes E 153, 307
Cevallos MA 81
Chaintreuil C 141, 302
Chamber M 269
Chanfon A 108
Charon C 12, 224
Chatagnier O 20
Chebotar VK 152
Chen H 71,
82, 154
Chen R 229
Chen W 336
Chichkova S 116
Chinchilla D 186
Chiurazzi M 175
Chubatsu LS 249
Chueire LM 242
Cioni L 129, 130
Clark TA 16
Cleyet-Maret JC 340
Colombo MV 128
Colón Flores JJ 157, 280, 322, 323, 324
Complainville A 224
Contreras A 117, 118
Contreras S 90
Cook D 67
Coopman R 295
Cordoba E 116
Corna G 56
Cortina J 275
Covarrubias AA 62
Crespeau H 20
Crespi M 12, 177, 186, 224
Cubo MT 181, 187, 188, 190
192, 193, 195, 341
Cullimore J 34, 102, 123
Cuny C 142
Curti B 56
D'Erfurth I 70
D'Haeze W 69, 202
Dahiya P 35
Dakora FD 145
Dammann-Kalinowski T 313
Danino V 236
Dary M 211, 220, 230, 234
Dávalos A 125
Davey ME 44
Davies A 228
Dávila G 86
Daza A 284, 315, 316, 317, 341
Dazzo F 189, 211, 220
De Bruijn FJ 44, 89
De Felipe MR 62, 191, 218
De Lajudie P 91, 141, 295, 297, 301, 302
De Luis I 252
De Mallorca MS 274
De Vos P 295
Deakin WJ 75
Debellé F 80
Defez R 312
Delgado MJ 103
Demba-Diallo M 301
Den Engelsman J 206
Dénarié J 59, 80
Desnoues N 27
Diagne O 310
Dilworth MJ 98, 107
Diouf A 302
Dirix G 95
Dixon R 15, 119

Djordjevic MA 24, 82, 154
Doerfel A 25, 171
Domergue O 340
Domínguez A 76
Donate-Correa J 268, 269
Dörr J 31
Downie JA 228, 231, 236
Drepper T 26
Drevon JJ 43
Dreyfus B 91, 141, 297, 302
Druzhinina T 200
Duhoux E 76
Durand P 70
Dusha I 113
Eady RR 16
Ebel J 229
Eckert B 149
El Khadir M 43
Ellis THN 63
Elmerich C 27
Elyachioui M 283
Emmerich R 108
Encarnación S 90, 226
Endre G 21, 64, 66
Epple G 169
Espuny MR 181, 188, 192, 193
195, 197, 341
Estévez J 190
Estrada de los Santos P 38, 162
Fabiano E 96
Fairhurst SA 16
Fancelli S 79
Fani R 129, 130, 146
Farias N 282
Favilli F 265
Fedonenko-Yu P 160
Fedorova E 223
Felle H 12
Femia P 79
Fenner BJ 98
Fernández D 131
Fernández MP 307
Fernández-López M 78
Fernandez-Pascual M 218
Fernández-Piñas F 1 148, 159
Fernández-Sierra ML 273
Ferreira EM 296, 320, 331
Fetiharrison R 91
Filali-Maltouf A 241, 244, 245, 246
Fischer HM 108, 111
Florencio FJ 158
Flores E 30, 110, 137
Flores M 86
Franche C 175, 176
Fransz P 67
Frechilla S 304
Frías JE 137
Fricke F 175
Frioni L 290
Frosh S C 217
Frugier F 12, 177, 186, 224
Frühling M 217
Fuli X 341
Gadkari D 18
Galan M 237
Galibert J 22
Gálvez L 104
Gamas P 20
Gao J 336
Gao M 69, 202
Gaouar A 263
García de los Santos A 325
Garcia N 297
García P 54
García-Chaves MA 103, 273
García-Rodríguez FM 78
García-Trigueros C 190, 194
Gardiol AE 144
Ge SC 288
Geiger O 169, 257
Geney C 176
Geurts R 67
Ghérardi M 59
Gherbi H 43
Gianinazi-Pearson V 20
Gillis M 141, 295, 297, 301, 302, 307
Gil-Serrano A 193, 194,
Girard L 115, 125, 325,
Giraud E 80, 91, 141, 297, 302
Glenn AR 98
Goffeau A 22
Goicoechea N 251
Golvano M P 218
Gómez F 225
Gonçalves LMB 278
Gonnet S 254
González EM 104, 105, 304
Gonzalez MA 258
González-Cu GR 45
González-López J 161, 298
González-Sama A 191
Goormachtig S 69
Goris J 295
Górska A 148
Göttfert M 25, 77, 271
Gottschalk G 23
Gouzy J 20
Grabbe R 109

Grachev A 200
Grasso DH 177
Gressent F 34
Gross S 26
Grzadka M 292
Gualtieri G 67
Guerreiro N 24
Guerrero G 90
Guescini M 286
Gueye M 302
Guillén G 209
Guldmann LL 35
Guo X 27
Gutierrez D 128
Gutierrez-Mañero FJ 157, 242, 280, 322, 323, 324
Gutiérrez R 341
Gutiérrez-Zamora ML 38
Györgyey J 223
Györgypál Z 113
Gyurján I 155
Haddadj A 272
Hai Yu Z 341
Halliday N 106
Hamaoui B 42
Handschuh L 259
Hardarson G 327
Harper A 247
Harteveld M 172, 341
Hartmann A 149
Härtner T 289
Hartsch T 23
Hastie A 185
Heinz E 174
Hennecke H 25, 108, 111
Hernández G 116
Hernández M 90
Hernández T 90
Herrera-Cervera JA 19
Herrero A 30, 110, 137
Higgins J 82
Hirsch AM 147
Hirt H 218
Hogg B 228
Hohnjec N 61, 217
Holsters M 60, 69, 202
Hozbor DF 173
Huesca M 275
Huguet T 59
Hungria MA 242, 279, 294, 299, 300
Hur JI 66
Hurek T 31
Ibijbijen J 253
Ignatov VV 150, 160
Igual JM 153, 307
Imperial J 127, 128, 131
Irigoyen JJ 252
Irisarri P 254
Ishchenko NV 167
Iturbe-Ormaetxe I 121
Ivashina T 200
Izaguirre-Mayoral ML 274
Izquierdo-Sánchez J 81
Jaftha JB 329
Jaillon O 20
James P 23
Janczarek M 168
Jarabo-Lorenzo A 269
Jean V 70
Jebara M 43
Jepson B 124
Jiangke Y 341
Jiménez-Zurdo JI 78, 189, 220
Jing Z 341
Johann A 23
Johnston AWB 106, 107, 185
Joubert C 328
Jourand P 297
Journet EP 20
Junchu Z 305, 341
Khadri M 43, 326
Kahn D 20
Kalinchuk N 200
Kaló P 21, 63, 64
Kamnev AA 150
Kan F 336
Kannenberg EL 289
Kapp D 214, 217
Keister DL 229
Keller M 313
Kennedy CK 144
Kereszt A 21, 59, 63, 64
Kevei Z 21, 63, 64
Khbaya B 245
Kijne JW 36, 199, 205, 206, 207
Kim D-J 67
Kirchhof G 149
Kiss E 113, 210
Kiss GB 21, 63, 64
Kiss P 63, 64
Klein A 185
Klipp W 26, 54
Kock MM 337
Kolaka A 258
Kolter R 226
Kondorosi A 12, 70, 71, 186, 210, 223, 224, 230, 233, 234
Kondorosi E 12, 70, 210, 223

Konnova SA 160
Korányi P 155
Koroleva TA 65
Kosier B 241
Kovacev L 136
Kovanecz I 64
Kovtunovych G 151, 308
Kozyrovska N 151, 308, 309
Krämer M 319
Krause A 25, 171
Król J 198
Król MJ 143
Krusell L 19
Ksenzenko V 200
Kulikova O 67, 71
Kurchak ON 311
Küster H 61, 217
Ladha JK 258
Lagares A 173
Lamont B 339
Lamrabet Y 188, 242
Lapeyrie F 276
Lar O 151, 308, 309
Lara-Flores M 100
Laranjo M 270
Laus MC 36, 206
Law IJ 40
Lawson D 112
Lázzari A 330
Leganés F 148, 159
Legocki AB 204, 259, 293
Leidi EO 315
Lejman M 198
León-Barrios M 268, 269
LeRoux C 91
Lievens S 60, 69
Ligero F 208
Lihoung M 341
Lima L 102
Lin M 27
Lindström K 37, 336
Lió P 130
Lipka V 319
Lisova N 237
Little R 119
Lloret J 215, 277
Lluch C 43, 101, 126, 140, 318, 326
Löhmus K 266
Lopes-Cardoso MI 102, 123
López JC 77
López MV 39, 271, 279
Lopez O 115, 125
López R 209
López-Lara IM 257
Lorda G 282, 332
Lorite-Ortega MJ 19, 274
Lorquin J 141
Lotocka B 204, 293
Lourenço APMS 315
Lucas García JA 157, 322, 323, 324
Lucas MM 218
Ludwig RA 52
Luque C 208
Lutova LA 167
Lyra MCCP 181, 192, 193, 341
Ma L 27
Maatallah J 246
Madinabeitia N 181, 192, 193, 341
Madsen LH 19
Maldonado R 118
Malek W 204, 292, 293
Mander Ü 266
Mangin B 80
Manyani H 190, 194, 279
Marie C 75
Maritano S 16
Martin DE 99
Martín dos Santos VAP 273
Martin M 215, 216, 277
Martínez G 90
Martínez-Abarca F 78
Martínez-Argudo I 117
Martínez-Luque 54
Martínez-Molina E 153, 189, 211, 220 307, 334
Martínez-Romero E 38, 321
Martínez-Salazar J 88
Martínez-Toledo MV 161
Martín-Figueroa E 158
Martín-Nieto J 117
Masepohl B 26
Masson C 302
Matamoros MA 120, 121
Mateos C 279
Mateos P 153, 189, 211, 220, 307, 334
Mathesius U 154
Matiru V 145
Matora L 135
Mauré L 340
Mavingui P 86
Mazur A 198
McGrath SP 331
Medina C 187, 195, 341
Megías M 186, 190, 194, 242, 279, 280
Meletzus D 156, 163
Melo P 102, 123
Mences IC 299, 300
Merchan F 186

Merino S 251
Merrick M 112
Mesa S 77, 103
Metspalu M 266
Meyer F 83
Meyer O 18
Mezei S 136
Michiels JFA 95, 235
Mihacea S 64
Milcamps A 44
Milic V 136, 264
Miller DA 213
Miller RW 16
Minamizawa K 258
Minchin FR 55
Minerdi D 146
Mingzhou C 341
Miranda-Rios J 114, 115
Mithofer A 229
Mitsui H 258
Molina A 189
Molouba F 302
Mondy S 70
Money T 124
Monteiro RA 249
Montesinos ML 30, 137
Monza J 254
Moore J 66
Moore M 247
Mora J 90
Mora L 298
Mora Y 90
Moran JF 120, 121
Morandi P 56
Moreira LM 217
Moreno FJ 184, 196
Morera C 114, 115
Moris M 235
Morón B 190, 279, 280
Morzhina EV 227
Mouhsine B 241
Moulin L 80, 295
Moussaid A 184, 193, 196
Mrkovacki N 136, 264
Müller P 183, 211, 222
Münchbach JM 23
Munier-Jolain N 122
Munive A 91
Muñoz E 78, 307
Muro-Pastor AM 30, 110
Mutch LA 170
Natera SHA 24, 82
Navarro F 158
Navarro M 114
Negrutska V 308, 309
Ney B 122
Neyra M 245, 297, 301, 302
Niebel A 20, 34
Nienaber A 111
Niewiadomska A 338
Nogales J 180
Nordlund S 32, 138, 156
Norén A 32
Normand P 245, 307
Noureddine N-E 272
O'Gara F 47
Ocaña A 126, 318, 326
Odee DW 333
Okon Y 42
Oláh B 113
Olamendi T 209
Olivares J 209
Olivares J 76, 101, 178, 179, 180
Oliveira A 296
Oliveira CA 281
Oliveira S 270
Ollero FJ 181, 184, 187, 188, 192, 193, 196, 197, 341
Olsthoorn M 341
Ortega E 140
Ortega JL 100
Ortiz E 88
Oruezabal IR 215
Ostakhina NV 150
Pacios-Bras C 172
Palacios JM 127, 128, 131
Palacios R 11, 86
Palomares AJ 71, 211, 220, 230, 233, 234
Pampulha ME 296
Pastor m 282
Pauling DC 52
Pavlova ZB 167
Pawlowski A 26
Pawlowski K 97, 175, 176
Payre H 43
Pedrosa F 242, 247, 249
Pérez A 220
Pérez NO 147
Pérez-Fernández MA 339
Pérez-Galdona R 268, 269
Pérez-Hormaeche J 71, 233
Pérez-Oseguera A 81
Perhald A 64
Perlick AM 61, 217
Perlova O 156, 163
Perret X 23, 75, 86
Perrine FM 154
Perry S 119

Perzi M 289
Perzyushi A 143
Petersen M 189
Petrova GN 51, 53
Piana A 56
Pich-Otero AJL 173
Picossi S 30, 137
Pierleoni R 285, 286
Pilli E 79
Platero R 96
Plessl J 31
Polidori E 285
Poole PS 185
Portelle D 22
Possani L D 209
Potenza L 286
Pozo C 161
Preininger É 155
Prell J 72, 319
Priefer UB 72, 241, 319
Prin Y 141, 297
Probanza A 157, 242, 280, 322, 323, 324
Prospéri JM 59
Provorov NA 311
Pueyo JJ 62, 191
Pühler A 22, 61, 83, 201, 214, 217
Purcell LC 256
Purcino HA 281
Qiu Y 336
Quester I 201
Quinto C 225
Ramenskaia M 97
Ramírez-Romero MA 81
Ramos B 157, 322, 323, 324
Ranjeva R 34
Rashid A 42
Ratet P 70, 71, 230, 233, 234
Rathbum EA 35
Ravasio S 56
Recalde L 208
Reddy PM 258
Redondo-Nieto M 182, 306
Redzepovic S 335
Reinhold-Hurek B 31, 99
Reth A 163
Revillas JJ 161
Reyes-Ramírez F 119
Ribeiro A 175
Riedel KU 26
Rivas-González R 334
Rivilla R 215, 216, 277
Robles C 303
Rodelas B 161, 298
Rodríguez A 290
Rodríguez C 88
Rodríguez-Navarro DN 197, 269, 279, 284, 315, 316, 317, 341
Rodríguez-Barrueco C 153
Rodríguez-Echevarría S 339
Rodríguez-Llorente ID 211, 230, 234
Rohmer M 289
Rolfe BG 24, 82, 154
Rome S 340
Romero D 85, 88, 115, 125
Ronson CW 89
Rosas VD 81
Rosenthal PA 23
Röthlisberger S 25
Roudier F 12, 223
Roumiantseva ML 313, 314
Royuela M 304
Rüberg S 201
Rubio MC 55
Ruffini D 265
Ruiz-Argüeso 127, 128, 131
Ruíz-Sainz JE 181, 184, 187, 188, 190, 192, 193, 195, 196, 197, 284, 341
Sá NMH 281
Saadallah K 43
Sacconi C 285, 286
Sadiki M 43
Sáez LP 54
Saito M 258
Sajnaga E 204, 293
Salinas P 117
Salon C 122
Sameshima R 258
Sánchez F 209, 225
Sánchez-Contreras M 216, 277
Sánchez-Díaz M 251, 252
Sánchez-Raya J 275
Sandal N 19
Sanjuan J 76, 101, 178, 179, 180, 246, 325
Sanjuan-Pinilla JM 178, 325
Santamaria C 284, 315, 316, 317, 341
Santi C 175, 176
Santoni S 59
Sarig S 42
Sarjiya A 18
Sawicka A 338
Schlaman HRM 172, 341
Schlüter A 319
Schmitz R 109
Schmitz RA 23
Schmitz S 289
Schneider A 46
Schoeters E 235
Scholte M 70

Schultze M 70
Schump O 43
Scotti MRMML 278, 281
See LS 276
Segubre E 258
Sem´yanova M 200
Senatore B 312
Senchekova S 200
Seres A 63
Sergeeva E 139
Serraj R 256
Sharypova LA 214, 313
Shashkov A 200
Shchyogolev S 135
Shibaev V 200
Shilov AE 17, 51, 53
Sierra G 330
Sifi B 43
Sikora S 335
Sikorski MM 259
Silvente S 100
Simarov BV 227, 311, 313, 314
Sinclair TR 256
Skorupska A 168, 198
Soberon M 114, 115, 125
Soberón N 81
Soboleva EF 150
Soria-Díaz ME 193, 194
Sorochinsky B 308
Soto MJ 76, 179, 287
Sousa C 12, 186, 194, 224, 279, 280
Soussi M 43, 318
Souza EM 247, 249
Spaink HP 172, 341
Squartini A 189
Stacey G 232
Staehelin C 23
Stefaniak J 154
Stepkowski T 204, 293
Stevens JB 105
Steyn PI 328, 329, 337
Stingel D 221
Stocchi V 285, 286
Stoffels M 149
Stolzfus J 44
Stougaard J 19, 68
Streit W R 23, 174, 212
Stuurman N 172
Sullivan JT 89
Svistoonoff S 176
Sy A 297, 302
Taguett F 272
Taïmani S 283
Tak T 199, 205, 207
Tamburini E 129
Tarayre S 210
Tejera N 101, 126, 140, 318, 326
Téllez-Sosa J 81
Temprano F 188, 197, 269, 284, 315, 316, 317, 341
Tereefework Z 336
Thomas-Oates JE 171, 341
Thoquet P 59
Tikhonovich IA 65, 68, 72
Tirichine L 59
Tiwari RP 98
Todd J 106
Toledo-García I 321
Tõnno I 291
Toro N 78, 287, 307
Torrecilla I 159
Torres A 184, 196
Town CD 66
Trabelsi M 43
Trinh TH 70, 230
Tripathi AK 41
Trzebiatowski J 44, 89
Tsyganov VE 65, 68, 72
Tun-Garrido C 84
Turner SL 87
Ureta A 138, 156
Urquiaga S 253, 255
Ushakov KV 227
Vadez V 256
Valdés M 147
Valencia-Morales E 85, 88
Valladares A 30, 110, 137
Vallorani L 285, 286
Valverde A 153
Van Brussel AAN 36, 199, 205, 206, 207
Van de Velde W 69
Van der Gucht K 301
Van Dillewijn P 287
Van Spronsen PC 36, 205
Van Tuinen D 20
VandenBosch KA 66
Vanderleyden J 29, 95, 235
Vanoni MA 56
Vargas MAT 299, 300
Vargas MC 226
Vasi M 264
Vega-Hernández MC 268
Velasco L 103
Velázquez E 153, 189, 220, 307, 334
Venter SN 328
Ventre N 59
Verdoy D 62
Verma SC 41

Villacieros M 216, 277
Villadas PJ 287
Villar-Egea M 334
Villatoro MAA 255
Villegas MC 340
Vilotte L 59
Vinardeli J M 12, 188, 197, 210, 223, 341
Vincent JL 35
Vinuesa P 219, 274
Viprey V 23, 75, 231
Vloemans N 301
Voisin AS 122
Von Bieberstein UR 175, 176
Von Scheven-Cordero E 45
Voroshilova VA 65, 68, 72
Vriezen C 44
Wabnitz P 97
Walker SA 231
Wall LG 306
Walsh U 47
Wang ET 321
Wdowiak S 204, 292
Webb KJ 55
Weber BO 149
Weidner S 83
Weinman JJ 82, 154
Werner D 183, 219
Wexler M 106
Wiemann SU 61
Wijfjes AHM 172
Wilkinson A 236
Willems A 295, 297, 302, 307
Wilson K 228
Wisniewski-Dyé F 142
Worsley PS 107
Xi C 95, 235
Yang SS 288, 341
Yates MG 247, 249
Yeoman KH 106, 107, 185
Yegorenkova IV 160
Youguo L 305
Young JPW 87, 170
Yousafzai FK 16
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Zerhari K 245
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