Institute of Bioorganic Chemistry
Polish Academy of Sciences

Program
Abstracts
List of participants

2nd EUROPEAN NITROGEN FIXATION CONFERENCE
and
NATO ADVANCED RESEARCH WORKSHOP

Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture

September 8-13, 1996, Poznań, Poland

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Program

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and
NATO ADVANCED RESEARCH WORKSHOP:
Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture

Sunday, September 8

17.00	**Opening session**	
	Welcome addresses	**ANDRZEJ B. LEGOCKI**, Poznań **ALFRED PÜHLER**, Bielefeld
18.00	Keynote lecture	**DENIS DEAN**, Blacksburg *Nitrogenase: two decades of biochemical genetics*
18.45	Keynote lecture	**JEAN DENARIÉ**, Toulouse *Nod factors in perspectives*
19.30	Get-together party	

Monday, September 9

9.00 **CELL CYCLE AND PHYTOHORMONE GENES IN NODULATION**

Chair: Adam Kondorosi, Gif sur Yvette

9.05 **HERIBERT HIRT**, Vienna
In and out of the cell cycle: On the way to understand the molecular mechanisms how plant cell proliferation is regulated

9.30 **ANN HIRSCH**, Los Angeles
Legume nodulation: a developmental pathway that shares elements with other plant developmental pathways

9.55 **MARTIN CRESPI,** Gif sur Yvette
The encoded peptide and the 3'UTR of Msenod40 induce Msenod12A expression in alfalfa roots

10.20 **MARCELLE HOLSTERS**, Gent
Plant gene expression during stem nodule development on Sesbania rostrata

10.55 **HORST RÖHRIG**, Cologne
Lipo-chitooligosaccharide signaling in Tobacco protoplasts

11.10 Coffee break

11.30 **SIGNAL PERCEPTION AND TRANSDUCTION IN NODULATION**

Chair: Ben J. Lugtenberg, Leiden

11.35 **GARY STACEY**, Knoxville
Specificity determinants in the Bradyrhizobium japonicum – soybean symbiosis

12.00 **HERMAN SPAINK**, Leiden
The molecular basis of host specificity of the Rhizobium-plant interaction

12.25 **WILLIAM BROUGHTON**, Geneve
Early events in Nod-factor recognition

12.50 **CHRISTIAN STAEHELIN**, Gif sur Yvette
Perception and inactivation of nodulation signals

13.15 Lunch

15.00 **PLANT GENES INVOLVED IN NODULATION**

Chair: Kjeld Marcker, Aarhus

15.05 **TON BISSELING**, Wageningen
Modification of phytohormone response by a peptide encoded by ENOD40

15.30 **JAN KIJNE**, Leiden
Induction of root cortical cell divisions by heterologous nodulation factors

15.55 **JULIE CULLIMORE**, Toulouse
Studies on early stages of the Rhizobium meliloti – Medicago symbiosis

16.20 **ERIK JENSEN**, Aarhus
Identification of trans-acting factors regulating nodulin gene expression

16.45 Tea time

17.15 **BACTERIUM – PLANT SURFACE INTERACTION**

Chair: Ursula Priefer, Aachen

17.20 **RUSSEL W. CARLSON**, Athens
Rhizobial capsular and lipopolysaccharides: evidence for there importance in Rhizobium-legume symbiosis

17.45 **K. DALE NOEL**, Milwaukee
Changes in Rhizobium lipopolysaccharide structure induced by host compounds

18.10 **KARSTEN NIEHAUS**, Bielefeld
Symbiotic suppression of the Medicago sativa plant defence system by Rhizobium meliloti oligosaccharides – a molecular analysis of signal molecules and signal transduction

18.35 **JOS VANDERLEYDEN**, Leuven
Azospirillum genes involved in chemotaxis and adhesion to plant roots

19.15 - 21.00 **ROUND TABLE DISCUSSION AND POSTER SESSION**

CONTROL OF PLANT AND BACTERIAL GENES DURING SYMBIOSIS

moderator: **EVA KONDOROSI**, Gif sur Yvette

NATO Advanced Research Workshop

BIOLOGICAL FIXATION OF NITROGEN FOR ECOLOGY AND SUSTAINABLE AGRICULTURE

Tuesday, September 10

9.00 **Opening and introductory remarks**

HERMANN BOTHE, Cologne
ANDRZEJ B. LEGOCKI, Poznań

9.15 **MOLECULAR MICROBIAL ECOLOGY**

Chair: John Beringer, Bristol

9.20 **FRANS DE BRUIJN**, East Lansing
Molecular approaches in microbial ecology to assess diversity and monitor stress-induced gene expression in rhizosphere bacteria

9.45 **DONALD PHILLIPS**, Davis
Plant regulation of Rhizobium meliloti root colonization

10.10 **FERGAL O'GARA**, Cork
Enhancing the potential of microbial inoculants through molecular microbial ecology

10.35 **MICHAEL SADOWSKY**, St. Paul
Host-controlled restriction of nodulation by Bradyrhizobium japonicum strain USDA 110 and characterization of the genes regulating nodulation

11.00 Coffee break

11.30 **NITROGEN FIXING SYSTEMS: PART I**
Chair: Claudine Elmerich, Paris

11.35 **MARK O'BRIAN**, Buffalo
Regulation of heme synthesis in the Bradyrhizobium japonicum-soybean symbiosis

12.00 **STEFAN NORDLUND**, Stockholm
Metabolic regulation of nitrogenase in Rhodospirillum rubrum

12.25 **TERESA THIEL**, St. Louis
Genetic analysis of two systems for alternative nitrogenases in the cyanobacterium Anabaena variabilis

12.50 **IVES JOUANNEAU**, Grenoble
Molecular studies of the electron transport pathway to nitrogenase in Rhodobacter capsulatus

13.15 Lunch

15.00 **NITROGEN FIXING SYSTEMS: PART II**
Chair: Alfred Pühler, Bielefeld

15.05 **JAMES COOPER**, Belfast
Biochemical and molecular analyses of rhizobial responses to legume flavonoids

15.30 **JAIME MORA**, Cuernavaca
Aerobic and fermentative metabolism in Rhizobium and its relation with the symbiotic process

15.55 **ROBERT HASELKORN**, Chicago
Heterocyst differentiation and nitrogen fixation in cyanobacteria

16.20 **BRIGITTA BERGMAN**, Stockholm
Trichodesmium has cells specialized for nitrogen fixation but lacks heterocysts

16.45 Tea time

17.10 **NITROGEN FIXATION IN SUSTAINABLE AGRICULTURE**

Chair: Dietrich Werner, Marburg

17.15 **CARROLL VANCE**, St. Paul
Use of nitrogen fixing crop species for sustainable agriculture

17.40 **ESPERANZA MARTINEZ-ROMERO**, Cuernavaca
Nitrogen fixers: population diversity and effects of fertilizers

18.05 **IGOR TIKHONOVICH**, St. Petersburg
A plant genetic potential for improving the beneficial interactions with the soil microflora

18.30 **HERMANN BOTHE**, Cologne
Distribution of N_2 - fixing, denitrifying and nitrifying bacteria in soils monitored by DNA-probing

19.15 **ROUND TABLE DISCUSSIONS AND POSTER SESSION**

SAFE APPLICATION OF GENETICALLY MODIFIED MICROORGANISMS IN THE ENVIRONMENT
moderator: FERGAL O'GARA, Cork

LEGUME TRANSFORMATION AND REGENERATION
moderator: CRAIG ATKINS, Nedlands

Wednesday, September 11

9.00 Excursion day – visit to Gniezno, the first capital of Poland

18.00 **POSTER SESSION AND INFORMAL DISCUSSIONS**

Thursday, September 12

9.00 **CARBON-NITROGEN METABOLISM IN SYMBIOTIC SYSTEMS: INTEGRATION AND OVERALL REGULATION**

Chair: Nicolas Brewin, Norwich

9.05 **DESH PAL VERMA**, Columbus
Assimilation of reduced nitrogen in tropical legume nodules: regulation of de novo purine biosynthesis and peroxisome proliferation

9.30 **DIETRICH WERNER**, Marburg
Robinia pseudoacacia nodulation and nodule functions

9.55 **JEAN JACQUES DREVON**, Montpellier
Increased respiration for symbiotic nitrogen fixation under phosphorus deficiency

10.20 **VASSILY ROMANOV**, Moscow
Carbon and nitrogen metabolism in the plant-derived ineffective nodules of pea (Pisum sativum L.)

10.45 **JOHN STREETER**, Wooster
Unsolved mysteries in C metabolism in legume nodules

11.10 **RICHARD PARSONS**, Dundee
Contrasting evolutionary solutions to C supply and N assimilation and transport in symbiotic plants

11.35 Coffee break

12.00 **OXYGEN REGULATION IN NITROGEN FIXATION**

Chair: Pierre Boistard, Toulouse

12.05 **HAUKE HENNECKE**, Zürich,
The role of oxygen in regulation and bioenergetics of symbiotic nitrogen fixation

12.30 **JOHN WITTY**, Aberystwyth
Dynamics of oxygen regulation and nitrogen fixation In legume root nodules

12.55 **UELI HARTWIG**, Zürich
Is the variable oxygen permeability in nodules a physical or a physiological phenomenon?

13.20 **RAY DIXON**, Norwich
Regulation of nitrogen fixation genes by the NIFA and NIFL regulatory proteins

13.45 Lunch

15.15 **MODEL PLANTS FOR NITROGEN FIXATION**
Chair: Barry Rolfe, Canberra

15.20 **JENS STOUGAARD**, Aarhus
Recent advances in the molecular genetics of the model legume Lotus japonicus

15.45 **THIERRY HUGUET**, Toulouse
Molecular genetics of a model-plant: Medicago trunculata

16.10 **ANDRZEJ B. LEGOCKI**, Poznań
Advances in molecular characterization of the yellow lupin – B. lupini symbiotic model

16.35 **KATHARINA PAWLOWSKI**, Wageningen
Gene expression in actinorhizal nodules

17.00 Tea time

17.30 **LEGUME TOOLS FOR LEGUME BREEDING**
Chair: Noel Ellis, Norwich

17.35 **PETER GRESSHOFF**, Knoxville
Molecular insights into shoot control of nodulation

18.00 **GERARD DUC**, Dijon
Effect of nitrogen nutrition pathways on the quality of nitrogen storage compounds in legumes

18.25 **GYORGY KISS**, Szeged
Genetic mapping in alfalfa: regular and anomalous phenomena during linkage analysis

20.30 - 23.00 Concert at the National Museum and Conference Reception

Friday, September 13

9.00 **CO-EVOLUTION OF SYMBIOTIC SYSTEMS**

Chair: Desh Pal Verma, Columbus

9.05 **JEFF DOYLE**, Ithaca
Molecular phylogenetic perspectives on the origins and evolution of nodulation in the legume family

9.30 **JANET SPRENT**, Dundee
Co-evolution of legume-rhizobial symbiosis: is it essential for either partner?

9.55 **BARBARA REINHOLD-HUREK**, Marburg
Interactions between diazotrophs and grasses

10.20 **VIVIAN GIANINAZZI-PEARSON**, Dijon
Have common plant systems co-evolved in fungal and bacterial symbiosis?

10.45 **PAWEŁ STRÓŻYCKI**, Poznań
"Nitrogen fixing" systems and evolution of plant hemoglobins

11.10 Coffee break

11.30 **NITROGEN FIXATION IN BIOLOGY AND AGRICULTURAL SCIENCES**

Chair: A. Van Kammen, Wageningen

11.35 **WILLIAM NEWTON**, Blacksburg
Molybdenum-nitrogenase structure and function

12.20 Concluding remarks, final announcement and closing of the meeting

13.45 Lunch

LECTURES

Friday, September 13

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Have common plant [illegible] and bacterial symbioses?

10.45 **PAWEL STROZYCKI**, Poznań
"Nitrogen [illegible] globins

11.10 Co[illegible]

11.30 NITROGEN FIXATION [illegible] AND AGRICULTURAL SCIENCES

Chair: A. Van Kammen, Wageningen

11.35 **WILLIAM NEWTON**, *Blacksburg*
Molybdenum-nitrogenase structure and function

12.20 Concluding remarks, final announcement and closing of the meeting

13.45 Lunch

NITROGENASE: TWO DECADES OF BIOCHEMICAL GENETICS

Johan Spee[1], Jeverson Frazzon[2], Limin Zheng[2] and Dennis R. Dean[2]

[1]Department of Biochemistry, Agricultural University, Wageningen, The Netherlands; [2]Center for Biotechnology, Virginia Tech, Blacksburg, Virginia USA.

In the mid 1970's serious biochemical-genetic strategies aimed at elucidating the biochemical mechanism for nitrogenase assembly and catalysis were initiated both at the University of Wisconsin's Center for Nitrogen Fixation Research and at the Sussex AFRC Unit for Nitrogen Fixation. Since then, many investigators, scattered throughout the World, have contributed to our knowledge of nitrogenase assembly, catalysis, and regulation. In this lecture we will briefly review the bases for the initial biochemical-genetic strategies. We will then discuss our current knowledge of the biochemical events that lead to formation of an active nitrogenase system. We will also discuss how site-directed mutagenesis strategies and gene-replacement techniques developed in this laboratory are being used by us and by others to probe the mechanism for nitrogenase catalysis. This discussion will be placed in the context of the nitrogenase crystallographic structural models proposed by Doug Rees and colleagues from the California Institute of Technology and the kinetic model for nitrogenase catalysis proposed by David Lowe and Roger Thorneley, now located at the John Innes Centre.

L-A-2

MOLYBDENUM-NITROGENASE STRUCTURE AND FUNCTION

William E. Newton

Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0308, USA.

Mo-nitrogenase is the primary biological catalyst for N_2 fixation. It exists as a complex of two proteins; the smaller Fe protein (a homodimer of 63kDa encoded by *nifH*), which functions as a MgATP-binding, specific electron donor to the larger MoFe protein (a heterotetramer of 230kDa encoded by *nifDK*), which contains the substrate-binding site. Recent x-ray diffraction studies have been used to solve the three-dimensional structures of both proteins and to gain insight into both their mode of association in the complex and the interaction with MgATP. The conformation adopted by the Fe protein and the redox potential of its single [4Fe-4S] cluster are dependent on whether or not MgATP is bound and the switching between conformations, driven by MgATP binding and hydrolysis, has been interpreted as a nucleotide-dependent switch that ensures the unidirectional flow of electrons to the MoFe protein. The MoFe protein contains two types of prosthetic group: two P-clusters, each of which consists of Fe_8S_{7-8} in two[Fe-S] clusters bridged by the γ-S of two cysteinyls (αCys-88 and βCys-95); and two FeMo-cofactors (FeMoco or M centers), each of which has the composition, $MoFe_7S_9$(homocitrate). Each MoFe-protein α/β subunit pair contains one of each type of prosthetic group. The P-clusters are believed to act as electron acceptors from the Fe protein, whereas the FeMo-cofactors bind and reduce substrates.

Substrate (N_2, C_2H_2, H^+, etc.) reduction results from the sequential association and dissociation of the two component proteins and involves the hydrolysis of two molecules of MgATP for each electron transferred from the Fe protein to the MoFe protein. The MoFe protein must accumulate as many as 6-8 electrons for N_2 reduction, even though electrons are delivered singly. How and where these electrons are accommodated is unknown. The apparently contradictory patterns of mutual inhibition among nitrogenase substrates can be best rationalized if both multiple, possibly overlapping, binding sites exist along with the involvement of several redox states. This model of nitrogenase catalysis has been tested by site-directed mutagenesis through which ligating amino-acid residues of either prosthetic group are substituted individually and the catalytic and spectroscopic consequences monitored. Correlated changes in EPR spectra, which arises in FeMo-cofactor, and catalytic properties occur on substitution at the FeMo-cofactor, indicating its intimate involvement with substrate reduction. Some atypical phenotypes are C_2H_6 from C_2H_2, CO-sensitive H_2 evolution and, most surprisingly, N_2-sensitive H_2 evolution in non-N_2-fixing strains. Substitution at some P-cluster ligands results in full EPR activity but decreased catalytic activity with, in some cases, the P-clusters exhibiting unusual redox states.

L-B-1

IN AND OUT OF THE PLANT CELL CYCLE: ON THE WAY TO UNDERSTAND THE MOLECULAR MECHANISMS HOW PLANT CELL PROLIFERATION IS REGULATED

I. Meskiene, W.-C. Yang*, C. de Blank*, L. Bögre, K. Zwerger, M. Brandstötter, M. Mattauch, E. Heberle-Bors, T. Bisseling*, and H. Hirt

Inst. of Microbiology and Genetics, Vienna Biocenter, Dr. Bohrgasse 9, 1030 Vienna, Austria
*Dept. of Molecular Biology, Wageningen Agricultural University, Dreijenlaan 3, 6703HA Wageningen, The Netherlands

In all eukaryotes, cell proliferation is regulated at different checkpoints, allowing cells to enter, transit or exit the cell cycle. Checkpoints monitor internal variables such as the growth rate of the cell or chromosomal replication as well as external signals like nutrient availability and growth factors. Transition through a checkpoint is mediated by the activation of protein kinase complexes composed of catalytic cdk (cyclin-dependet kinases) and regulatory cyclin subunits. Protein kinase activity of the complexes is controlled through the transient synthesis of different cyclins, the actions of specific kinases and phosphatases, and association with other regulatory proteins. Over the past years, we have identified various cdk and cyclin genes from alfalfa. Whereas the expression of the cdk genes appears to be more or less constitutive, cyclin expression is highly regulated and correlates with the transition through different checkpoints, making these genes perfect cell cycle markers to investigate the cell cycle state of any one cell. In alfalfa, we have studied recently of differentiated cells into the cell cycle in two systems. When mature, nondividing leaf cells that are arrested in the G_0 state were activated by addition of plant hormones, the temporal induction of various cell cycle marker genes allowed us to distinguish immediate early, early and late events occur during recently into the cell cycle. This pettern also occurs in root cells after mitogen stimulation by rhizobia or Nod factor suggesting that differentiated cells in all parts of the plant follow a predetermined pathway during reentry into the cell cycle. Exit from the cell cycle was observed in outer root cortical cells that formed the infection threads during nodulation. Like inner cortical cells, these cells entered into the G_1 phase of the cell cycle and proceeded through S phase. Unlike the inner cortical cells which started to proliferate subsequently, these outer cortical cells never entered into mitosis, suggesting that they became arrested in G_2. However, this situation seems to be a special case and exit from the cell cycle mostly occurs in the G_1 phase and appears to be prerequisite for differentiation. Typically G_0 cells do not synthesize cyclin genes, but cdk expression may still occur and may be necessary for the ability to reenter the cell cycle at a later time point.

L-B-2

LEGUME NODULATION: A DEVELOPMENTAL PATHWAY THAT SHARES ELEMENTS WITH OTHER PLANT DEVELOPMENTAL PATHWAYS

Ann M. Hirsch, Larry M. Brill, Yiwen Fang, Ying Li, Michelle Lum, Rob Satterthwaite, Wei-gang Yang and Pieternel van Rhijn

Department of Molecular, Cell, and Developmental Biology, 405 Hilgard Ave., University of California, Los Angeles, CA 90095-1606 USA.

Alfalfa (*Medicago sativa* L.) nodules, like other cylindrical nodules, develop from the *de novo* initiation of cell divisions in the already differentiated cells of the inner root cortex. In contrast, spherical nodules, like *Lotus*, soybean (*Glycine max* L.) or bean (*Phaseolus vulgaris* L.) originate from cell divisions initiated within the outer root cortex followed by mitoses in the inner cortex and pericycle. In both indeterminate and determinate nodules, cell divisions are triggered soon after inoculation with *(Brady)Rhizobium* or after treatment with Nod factor, a substituted lipooligosaccharide produced by *nod* gene products. In alfalfa, we study the expression of several early nodulin genes, one of which, *Msenod40,* is expressed rapidly in response to Nod factor and cytokinin application. *Msenod40* is expressed in various other regions of the plant besides the nodule, however, including incipient lateral roots, leaf marginal meristems, and floral tissue. We have also been studying plant proteins, such as lectins, which are not specifically expressed upon *Rhizobium* inoculation, but are developmentally regulated and although found in a number of plant organs, are also detected in nodules. Alfalfa probably has three lectin genes, two of which have been cloned and completely sequenced and antisense plants have been generated. These loss-of-function plants show a number of significant defects not only in their nodulation responses, but also in floral development. Transfer of a soybean seed lectin gene to *Lotus* enables the gain-of-function transgenic plants to form small nodule-like swellings in response to inoculation with *Bradyrhizobium japonicum*, the normal symbiont for soybean. In contrast, transfer of soybean seed lectin to alfalfa does not result in nodule formation with *B. japonicum.* Because of the difficulty with working with alfalfa, an out-crossing tetraploid, we have initiated studies on white sweetclover (*Melilotus alba* Desr.), a dwarf, small-seeded, diploid legume that nodulates with *R. meliloti.* We are in the process of screening a genomic library for several of the early nodulin genes and also for lectin genes.

L-B-3

THE ENCODED PEPTIDE AND THE 3'UTR OF *MSENOD40* INDUCE *MSENOD12A* EXPRESSION IN ALFALFA ROOTS

Crespi M.[1], C. Johansson[1], Fillod C.[1], Poirier S.[1] and Kondorosi A.[1,2]

[1]Institut des Sciences Vegetales-CNRS, 91198 Gif sur Yvette, France
[2]Biological Research Center, H-6701 Szeged, Hungary.

Rhizobium meliloti is able to induce the formation of nitrogen fixing nodules on the roots of *Medicago* spp. under nitrogen limitation. These organs start by divisions in the root inner cortex which show amyloplast deposition. We had identified genes expressed in the nodule primordium: *Msenod12A, Msca1* and *Msenod40. Msenod12A* and *Msca1* code for a proline-rich protein and a carbonic anhydrase, respectively. Sequencing of several *enod40* genes showed that only a small ORF (12 or 13 aminoacids) is common between them (1) despite a strong conservation of the nucleotide sequence. *Msenod12A* expression was detected in the dividing cortical cells using transgenic alfalfa plants carrying the promoter of this early nodulin fused to the reporter *uidA* gene (Bauer *et al.*, in press). *Msenod40* is expressed very early during nodule organogenesis even before the initial cortical cells divide suggesting that it may act upstream of *Msenod12A* in the transduction pathway leading to nodule development. We have shown that the *enod40* genes code for structured RNAs and that these genes might act as "riboregulators" a novel class of 3'UTR involved in certain differentiation processes (2). In addition, it has recently been shown that both the *enod40* gene and the small encoded peptide render tobacco protoplasts tolerant to auxin (3). We have developed a transient assay in alfalfa roots using the particle gun. By assaying our *Msenod12A* transgenic plants, we were able to show that bombardment of *Msenod40* induces cortical cell division and *Msenod12A* expression in *Medicago* roots. Both the region spanning the small peptide as well as the 3'UTR of *enod40* could elicit these responses. These results were correlated with a phenotype found in *M. truncatula* plants overexpressing the *enod40* gene. Therefore, *enod40* might have a role in the elicitation of phytohormonal imbalances in the inner cortical cells, and might be one of the elements involved in nodule initiation.

1. Vijn et al., (1995) *Plant Mol. Biol.*, **28**, 1111-1119. **2.** Crespi et al., (1994) *EMBO J.*, **21**, 5099-5112. **3.** Van de Sande et al. (Science, in press).

L-B-4

PLANT GENE EXPRESSION DURING STEM NODULE DEVELOPMENT ON SESBANIA ROSTRATA

Marcelle Holsters, Sofie Goormachtig, Sam Lievens, Viviana Corich, Marcio Alves-Ferreira, and Marc Van Montagu

Laboratorium voor Genetica, Department of Genetics, Flanders Interuniversity Institute for Biotechnology (VIB), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

In the *Rhizobium*-legume symbiosis, N_2-fixing nodules typically arise on the roots of the host plant. In a few cases stem-located nodules are formed. A well studied example is *Sesbania rostrata*, a tropical legume that, as an adaptation to growth in waterlogged conditions, carries vertical rows of dormant root primordia all along its stem. These primordia provide predetermined nodulation sites. Upon infection with the microsymbiont *Azorhizobium caulinodans*, they develop into aerially determinate nodules. Simultaneous inoculation of many primordia can be performed and leads to synchronous development. This provides a good model system to study gene expression during initial stages of the development. We have studied stem nodule ontogeny using several molecular markers for *in situ* hybridizations. Development starts with dedifferentiation of cortical cells opposite infection pockets that are filled with multiplying bacteria (crack entry). Next, intercellular infection threads grow towards the nodule primordia and bacteria start to invade plant cells. Simultaneously with the formation of a zone of infected cells, a nodule meristem is delimited. To acquire more insight into the spatio-temporal organization of the meristem, we studied the expression of cell cycle-related genes. A *S. rostrata cdc2*, mitotic cyclin and histone H4 homologue were characterized. Also the expression pattern of the early nodulin genes *Srenod2* and *Srenod40* was studied. Finally, using a mRNA display approach, we isolated several new clones with nodule-enhanced expression. Amongst these are genes for a hydroxyproline-rich protein, a chalcone reductase, and two chitinases. The latter show totally different expression patterns. Approaches to determine the substrates of the chitinases will be discussed. A general conclusion from our observations is that although the mature stem nodules are determinate, their development shows more characteristics of indeterminate nodules.

L-B-5

LIPO-CHITOOLIGOSACCHARIDE SIGNALING IN TOBACCO PROTOPLASTS

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There is accumulating evidence that lipo-chitooligosaccharides (LCOs) play a general role as plant growth regulators. Therefore, a simplified procedure for the synthesis of these compounds was developed. Using tobacco protoplasts we tested the effects of different synthetic LCOs on nonleguminous plant cells. We found that glycolipid signals carrying fatty acids with double bonds in the *trans* configuration were most efficient to alleviate the requirement for auxin and cytokinin to sustain growth of cultured tobacco protoplasts as well as in activating the expression of *AXI1*, a gene implicated in auxin signaling. The effect of the carbohydrate backbone of synthetic LCOs on the activation of the *AXI* promoter was determined.

L-C-1

SPECIFICITY DETERMINANTS IN THE *BRADYRHIZOBIUM JAPONICUM* - SOYBEAN SYMBIOSIS

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Our laboratory has been investigating the basis for host specificity between *B. japonicum* and various host plants (e.g., soybean, cowpea, sirato, mungbean, rice bean, etc.). Research has found that host specificity can be determined at the level of *nod* gene expression and also by the plant response to the lipo-chitin nodulation factors. Our recent results indicate that the mechanisms by which host specificity is determined are more complex than previous envisioned. For example, the *nodVW* genes of *B. japonicum*, although not required for nodulation of soybean, are required for nodulation of other hosts. We have now shown by in vitro biochemistry that NodV encodes a protein kinase that phosphorylates NodW in response to the addition of isoflavone inducers of *nod* gene expression. Moreover, NodW phosphorylation is required for the induction for *nod* gene transcription. These data suggest that NodVW is an independent system for *nod* gene regulation and likely responds to isoflavones produced by the alternative hosts of *B. japonicum*. NolA is another gene product that plays an important role in host specificity and is a regulator of gene (i.e., *nolA* and *nodD2*) expression in *B. japonicum*. We have found that the *nolA* gene encodes two proteins, $NolA_L$ and $NolA_S$. Only $NolA_L$ possesses a helix-turn-helix motif and likely acts as a transcriptional regulator. Expression of $NolA_L$ or $NolA_S$ appears to be controlled transcriptionally by the production of two mRNA. It is generally accepted that the specific chemical structures of the lipo-chitin oligosaccharides (LCO) nodulation signals produced by a given rhizobial strain controls to large degree the host range of that strain. Therefore, we are interested in understanding the mechanism by which host plants recognize these LCO nodulation signals and respond. Our recent work suggests that nod signal recognition involves at least two unique recognition systems. For example, we have found that the early nodulin ENOD40 can be induced by both the LCO signals of *B. japonicum* and simple chitin oligomers. However induction by the later compounds is transient. Likewise, the induction of the early nodulin ENOD2 requires the cooperative action of two or more nod signals, one of which can be a non-acylated chitin oligomer. These data suggest that two systems exist for nod signal recognition that differ significantly in their chemical specificity. A *nodZ* mutant of *B. ajponicum* is unable to produce the normal fucosylated LCO signals. Fucosylation is required for biological activity when the purified pentameric LCO molecules are tested for their ability to induce cortical cell division on *Glycine soja* roots. However, this is a paradox since the *nodZ* mutant nodulates soybean and other hosts (e.g., rice bean). One explanation is that this mutant does produce a non-fucosylated tetrameric LCO which can induce nodules on *G. soja*. However, this tetramer is not active on rice bean. Recently, we have found that the addition of two or more of the non-fucosylated nod signals to rice bean will induce nodule structures. Therefore, we hypothesize that it is this response which allows the *nodZ* mutant to nodulate this host plant. Taken together, these results suggest a surprising complexity redundancy in the nod signal recognition system. This recognition likely involves multiple receptors with differing chemical specificity. In addition, in the absence of specific chemical substituents, the recognition system can still respond to a mixture of nod signals produced by a given rhizobial strain. These results suggest that it will be a formidable experimental challenge to elucidate the nod signal reception pathways in legumes.

L-C-2

THE MOLECULAR BASIS OF HOST SPECIFICITY OF THE RHIZOBIUM-PLANT INTERACTION

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Signal molecules which determine the host specificity of rhizobia for particular leguminous plants include flavonoids secreted by the host plant roots and the bacterial lipo-chitin oligosaccharides (LCOs). The role of these factors in root infection and nodule formation will be illustrated with special emphasis on the role of the regulatory *nodD* gene and structural elements of the LCOs. For instance, a highly unsaturated fattyl moiety plays a major role in the specificity of rhizobia which associate with indeterminate-nodulating plants. The genes *nodF* (accyl carrier protein), *nodE* (β-ketoacylsynthase) and *nodA* (acyl transferase) are involved in the production of LCOs containing such a special fatty acids. For determinate nodulating plants a fucosyl residue (located at the reducing *N*-acetylglucosamine residue of LCOs) plays a major role in determining specificity. The *nodZ* gene which is present in various rhizobia such as *Bradyrhizobium japonicum*, *Rhizobium loti* and *R.etli*, is a fucosyltransferase involved in the addition of this fucose. The role of other determinants of host specificity, such as *nodS*, *nodU*, *nodX* and *nolL* will also be discussed.

L-C-3

Early Events In Nod-factor Recognition

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Legumes respond to Nod-factors through deformation of the root-hairs and the development of nodulation foci in the root cortex. Cellular events in nearly mature, susceptible root-hairs, were studied in root-segments loaded with fluorescent calcium indicators. Application of 10^{-9} M Nod-factors of the broad host-range *Rhizobium* sp. NGR234 to the homologous legume *Vigna unguiculata* resulted, within seconds, in plateau-like increases in intracellular free calcium {$[Ca^{2+}]_i$} in root-hairs and root epidermal cells. Nod-factors of *R. meliloti* at 10^{-9} M also caused rapid increases in $[Ca^{2+}]_i$ in the root-hairs and epidermal cells of the conditional-host, *V. unguiculata,* and also induced root-hair deformation. The chitin tetramer, *N-N'-N"-N"'*-tetracetylchitotetraose, which represents the backbone of Nod-factors, induced neither root-hair deformation nor changes in $[Ca^{2+}]_i$ in *V. unguiculata.* Root-hairs and epidermal cells of the non-legume non-host, *Arabidopsis thaliana*, showed neither $[Ca^{2+}]_i$ increases nor root-hair deformation in response to both factors. NodNGR-factors were biotinylated and the cellular location of the streptavidin-biotinylated Nod-factor complex studied by confocal laser scanning microscopy. Nod-factors preferentially bound to the tips of nodulation competent root-hairs in an asymmetrical manner.

L-C-4

PERCEPTION AND INACTIVATION OF NODULATION SIGNALS

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The lipo-chitooligosaccharide signals (Nod factors) of *Rhizobium* have a chitooligosaccharide backbone which is substituted by an *N*-linked fatty acid moiety. Additional modifications are present at the reducing and non-reducing end which are essential for cell proliferation and nodule organogenesis. Perception of purified Nod factors can be investigated by measuring rapid plant responses such as depolarization of the plasma membrane and intracellular alkalinization of the root hairs. A repeated treatment with the sulfated Nod factors of *R. meliloti* induced much weaker an intracellular alkalinization of *Medicago* root hairs. However, the cells retained their ability to respond to non-sulfated Nod factors indicating a different perception system for sulfated and non-sulfated molecules. Desensitization of the perception system of mitogenic Nod factors might be an important mechanism to avoid uncontrolled cell proliferation and the induction of defense reactions. An additional regulation can be envisioned at the level of Nod factor inactivation by plant hydrolases. Using a set of tetrameric and pentameric Nod factors as substrate, six *Medicago sativa* hydrolases differing in their substrate specificity were distinguished on the basis of their cleavage sites. One of them showed an up to 6-fold stimulated activity after pretreatment of *M. sativa* roots with *R. meliloti* Nod factors indicating a rapid feedback inactivation of the Nod factors after their perception. This enzyme is a novel glycosyl hydrolase that lacks chitinase activity, but effectively releases lipodisaccharides from all Nod factors of *R. meliloti.* The Nod factor hydrolase was purified from Nod factor-treated young *M. sativa* roots. The enzyme is a concanavalin A-binding glycoprotein with a molecular weight of about 65 kDa, as determined by SDS-PAGE. The hydrolase was found in root exudates, roots and nodules, but not in the aerial part of *M. sativa.* The enzyme was detected in roots of several *Medicago* species which are host plants of *R. meliloti.* Up to now, however, this hydrolase was neither identified in roots of other legumes nor in non-leguminous species. We suggest that the dimer-forming Nod factor hydrolase is an important determinant in the interaction of *R. meliloti* with its host plants.

MODIFICATION OF PHYTOHORMONE RESPONSE BY A PEPTIDE ENCODED BY ENOD40

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Rhizobial Nod factors probably induce cell divisions in the cortex of legume roots by inducing a local change of the auxin/cytokinin ratio. The expression of a few nodulin genes is induced by Nod factors. One of these genes, ENOD40, is first expressed in the root pericycle opposite to the future nodule primordium. The expression precedes the induction of cortical cell division which suggests that ENOD40 changes the response to phytohormones. To test this we used tobacco protoplasts as a model system. We isolated an ENOD40 homolog of tobacco, which as does ENOD40 from legumes, encodes an oligopeptide of about 10 amino acids. In tobacco protoplasts these peptides change the response to auxin at concentrations as low as 10-12 - 10-16M.

L-D-2

INDUCTION OF ROOT CORTICAL CELL DIVISIONS BY HETEROLOGOUS NODULATION FACTORS

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Formation of nitrogen-fixing nodules on roots of leguminous plants results from specific signalling between plant and rhizobia. Essential rhizobial signals are lipochitin-oligosaccharides (Nod factors), which induce a variety of nodulation-related phenomena in host plant roots. Structure-function studies of Nod factors require a bioassay in which recognition effectuates a relevant response in the host root. An essential response of host plant roots to Nod factors is cell division activity in the root cortex. Usually this response is host plant-specific, for example, Nod factors from *Rhizobium leguminosarum* biovar viciae induce cortical cell divisions in roots of vetch but not in roots of clover. Host plant-specificity is in part determined by the decorations of the chitin oligosaccharide.

We found that externally applied Nod factors from *R. leguminosarum* biovar viciae, *R. meliloti* (symbiont of alfalfa) and *R. loti* (symbiont of lotus) each induce mitogenic activity in hairy roots of red clover transformed with the pea lectin gene. Cell divisions were observed in the inner root cortex. This mitogenic response was not observed in hairy roots of control plants. In a second approach, mitogenic activity could be induced in *Vicia* (vetch) roots by direct ballistic introduction of certain chitin oligomers together with uridine. Uridine appeared to act as an auxin enhancer, and possibly plays a role in root cell division activity rather than in signal recognition.

These results demonstrate that under certain circumstances induction of cell divisions in the inner cortex of clover and vetch roots not necessarily requires host plant-specific decoration of Nod factors. Possible roles of pea lectin and uridine will be discussed.

L-D-3

STUDIES ON THE EARLY STAGES OF THE *RHIZOBIUM MELILOTI-MEDICAGO* SYMBIOSIS

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Two approaches are being used to study the plant genes and proteins involved in the establishment of the symbiosis between *R. meliloti* and its plant hosts of the genus *Medicago*. Firstly a biochemical approach is being used to study the interaction of the rhizobial Nod factors with plant extracts. Using radiolabelled ligands, two binding sites have been characterised for Nod factors. The first (termed Nod Factor Binding Site 1 or NFBS1) was initially characterised in a particulate (3000g) fraction of *M. truncatula* roots and exhibits a Kd for the principal Nod factor of *R. meliloti* (NodRm-IV (Ac,S,C16:2)) of 86nM. Sites with approximately similar characteristics have also been identified in *M. varia* cell cultures and also in a non-legume, tomato. The second site, NFBS2, exhibits a higher affinity for the Nod factor ligand (approximately 2nM) and was characterised in the microsomal fraction of the cell cultures. Its presence in roots is being investigated. Whether these sites represent receptors involved in the transduction of the Nod factor signal in the symbiotic interaction will be discussed.
Secondly, we have used a subtraction library approach to isolate new genes induced in the early stages of the symbiosis. A cDNA library of 4-day old *M. truncatula* nodules was screened with a subtractive probe and eventually clones for 29 new genes which are induced by 4-days after inoculation were isolated. Only a few of the clones, in the parts sequenced, showed similarities with sequences in the data bases. Selected clones are being used for more detailed analyses to define the steps involved in the early stages of nodulation.

L-D-4

IDENTIFICATION OF *TRANS*-ACTING FACTORS REGULATING NODULIN GENE EXPRESSION

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Functional studies of nodulin gene promoters in transgenic legumes have identified a number of *cis*-regulatory elements important for nodule specific expression. To identify transcription factors interacting with these elements, soybean and *Vicia sativa* nodule-specific expression libraries were screened using oligonucleotides derived from the soybean *lbc3* and the pea early nodulin gene *PsENOD12* promoters, respectively, as probes.

We identified 58 soybean cDNA clones encoding ten different proteins binding to the *lbc3* promoter. Three of the clones were similar to Histone H1, two clones showed homology to High Mobility Group proteins and one clone was homologous to a myb-like factor previously isolated from parsley.
Two clones, however, encoded novel DNA binding proteins. The DNA binding domains of these proteins were delimited by studying binding properties of truncated bacterially expressed peptides. The binding domain of the protein denoted Nodox was homologous to a homeodomain. In Noz, two cysteine-rich domains constituted the binding domain.

The screening of a *Vicia sativa* cDNA library using *PsENOD12* promoter sequences as probes identified 4 different cDNAs. One of cDNA's represented a gene encoding a protein of 1641 amino acids, ENBP and it was shown to interact with a positive element in the ENOD12 promoter.

The structure, expression and biological significance of the Nodox, Noz and ENBP1 proteins will be discussed.

L-E-1

RHIZOBIAL CAPSULAR AND LIPOPOLYSACCHARIDES: EVIDENCE FOR THERE IMPORTANCE IN *RHIZOBIUM*-LEGUME SYMBIOSIS.

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Rhizobial lipopolysaccharides (LPSs) are important for the development of infection threads in determinant nodule-forming hosts, or release of bacteria into the root cortical cells in indeterminate nodule-forming hosts. Mutants which lack the O-chain polysaccharide portion of this molecule are symbiotically defective. In addition, it has been shown using monoclonal antibodies that subtle structural changes occur in the LPS during symbiosis. The rhizobial LPS that has been most studied is from *R. leguminosarum*, and from *R. etli* CE3 (formerly known as *R. leguminosarum* bv. phaseoli CE3). The LPSs from strains of *R. leguminosarum* contain a variety of O-chain polysaccharides, and a core oligosaccharide that is common to all strains;

Kdo2→6Gal1→6[GalA1→4]Man1→5[GalA1→4(GalA1→5)Kdo2→4]Kdo.

Its lipid A is unusual in that it is totally devoid of phosphate, and consists of an acylated GalA1→4GlcN1→6GlcN-onate trisaccharide. The structure, biosynthesis, and epitope changes of this LPS during symbiosis will be discussed.

Rhizobia have also been found to produce a class of capsular polysaccharide (CPSs) analogous to the Group II capsules of *E. coli*. A common feature of these CPSs is that they are all rich in Kdo. The CPSs from *R. fredii* vary from strain to strain but their repeat oligosaccharide unit has a common Kdo→Hex disaccharide motif. These Kdo-containing CPSs have been found in all species of rhizobia examined, as well as in some plant pathogenic bacteria. In the case of *R. meliloti* strains which carry the *rkpZ* gene, these CPSs have been shown to play an important role in symbiosis, and can functionally substitute for EPS. At least three CPS-specific gene regions have been identified in *R. meliloti* by Kondorosi and coworkers. The effect of mutations in these regions on symbiosis and on CPS structure will be described. Supported by grants from NIH (GM89583), DOE(DE-FG05-93ER20097, and NSF (IBN-9305022).

L-E-2

CHANGES IN RHIZOBIUM LIPOPOLYSACCHARIDE STRUCTURE INDUCED BY HOST COMPOUNDS

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Studies with several rhizobial species have shown that rhizobial mutants deficient in lipopolysaccharide (LPS) O-antigen are defective in infection of host roots. Previous work in various laboratories has also established that in the nodule the LPS structure differs from that of the bacteria grown in normal laboratory media. In Rhizobium etli CE3 the differences in bacteroid LPS are subtle and most easily detected by monoclonal antibodies that have been generated by N. J. Brewin. We have discovered that exudate from host Phaseolus vulgaris seeds and roots induce similar changes in the LPS. The major inducers from the germinating seed are anthocyanins, with delphinidin glucosides being the most abundant. Anthocyanins also induce R.etli nod genes, but the nod inducers naringenin and genistein do not induce the LPS modifications. Moreover, the LPS changes occur in mutant strains lacking the Sym plasmid. Therefore, this effect seems to depend on a pathway for responding to host flavanoids that is independent of nodD. Currently, we are isolating and analyzing mutants of R. etli CE3 that produce LPS carrying O-antigen but do not alter its structure during growth in exudate compounds. The goal is to determine whether these LPS changes are important in the symbiosis and to dissect the pathway of this response to host metabolites.

L-E-3

Symbiotic suppression of the *Medicago sativa* plant defence system by *Rhizobium meliloti* oligosaccharides - a molecular analysis of signal molecules and signal transduction

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Mutants of the symbiotic soil bacterium *R. meliloti* that fail to synthesize the exopolysaccharide EPS I were unable to establish effective root nodules and induce a plant defence response. From these observations we propose that the LMW EPS I acts as a suppressor of the plant defence system, enabling the symbiont *R. meliloti* to infect the host plant alfalfa. In order to test this hypothesis we established elicitor responsive cell cultures of the host plant alfalfa, and as a control of the non host plants tobacco and tomato. Beside other defence related reactions, the cell cultures reacted to the addition of small amounts of the non specific yeast elicitor with a strong transient alkalinization of their culture media, a bioassay for elicitor perception. Because of their chitin-backbone we tested the *R. meliloti* nodulation factors for a possible elicitor activity in the cell culture alkalinization assay. N-acetyl chitin oligomers provoked a transient alkalinization in alfalfa, tomato and tobacco cell cultures, while the nodulation factors were active only on the nonhost plants tomato and tobacco. Using this assay system purified homologous and heterologous EPS and LPS were analysed for possible suppressor functions in the three plant suspension cultures. Only in alfalfa cell cultures the elicitor induced alkalinization could be suppressed by the simultaneous application of LMW EPS I or LPS. These data provide a strong evidence for a specific recognition of the *R. meliloti* LMW EPS I and LPS by the host plant as a suppressor of the plant defence system enabling the symbiont to infect the plant. In addition these observations suggest that the host specific *R. meliloti* nodulation factors, elicit a fast plant defence response on the non host plants, whereas the the host plant alfalfa does not perceive them as elicitors.

L-E-4

AZOSPIRILLUM GENES INVOLVED IN CHEMOTAXIS AND ADHESION TO PLANT ROOTS

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Azospirillum brasilense colonizes the roots of plants. Best documented is the colonization of wheat roots by *A. brasilense* strains Sp245 and Sp7 (Vande Broek *et al.*, 1993; Arsène *et al.*, 1995).

A. brasilense mutants blocked in chemotaxis and adhesion are strongly impaired in plant root colonization (Vande Broek *et al.*, 1996). Chemotaxis of *A. brasilense* strains Sp7 and Sp245 towards D-galactose, L-arabinose and D-fucose, depends on the activity of a periplasmic sugar binding protein, encoded by *sbpA* (Van Bastelaere *et al.*, 1996). Expression of *sbpA* depends on the presence of these sugars in the growth medium, and is regulated by a LysR-type regulator, GbpR. The SbpA protein shows high similarity with the *Agrobacterium tumefaciens* ChvE protein, involved in chemotaxis towards and induction of *vir* genes by D-galactose, L-arabinose and D-fucose. However, the *A. brasilense sbpA* gene cannot complement an *A. tumefaciens chvE* mutant for *vir* gene induction.

Chemotaxis and motility in general is dependent on the presence of flagella. *A. brasilense* has a mixed type of flagella: a single polar flagellum, present under all growth conditions tested, and several lateral flagella, expressed when cells are grown in high viscosity medium. In *A. brasilense*, the polar flagellum not only plays a role in motility but also in adhesion of bacterial cells to plant roots (Croes *et al.*, 1993). Although it cannot be excluded that an unknown small adhesin, associated with the polar flagellum, is responsible for adhesion to plant roots, it is more likely that the polar flagellin, which is a glycoprotein, is responsible for adhesion to plant roots (Moens *et al.*, 1995). Interestingly, hindrance of the rotation of the polar flagellum, either by growing cells in high viscosity medium, or by adding an anti polar flagellin specific antibody to liquid grown cells, induces expression of the *lafI* gene, the structural gene of the lateral flagella.

L-F-1

MOLECULAR APPROACHES IN MICROBIAL ECOLOGY TO ASSESS DIVERSITY AND MONITOR STRESS-INDUCED GENE EXPRESSION IN RHIZOSPHERE BACTERIA

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The recent development of nucleic acid-based analytical methods and novel marker/reporter genes has significantly contributed to the birth of a new subspecialty in the field of the ecology of microbes: Molecular Microbial Ecology. Here, I will present two novel methodologies and their applications. First, I will discuss the development of a PCR based technique (rep-PCR) to fingerprint bacterial genomes and its use in assessing microbial diversity and determining phylogenetic relationships between closely related bacteria. I will primarily focus on the use of rep-PCR fingerprinting to identify and classify plant-associated (rhizosphere) bacteria, and discuss the utility of computer-based phylogenetic pattern analysis programs, including backpropagation neural network approaches. Secondly, I will briefly summarize a project designed to study environmental control of gene expression in *Rhizobium meliloti*, using the luciferase (*luxAB*) genes of *Vibrio harveyi* as a reporter of transcription. I will discuss the isolation and characterization of a large collection of nutritional ($C/N/O_2$) limitation-induced rhizobial genes and the determination of their role in microbial persistence and competition in the rhizosphere. I will also briefly discuss the use of another reporter/marker gene (green fluorescent protein; *gfp*) for microbial ecology studies.

PLANT REGULATION OF *Rhizobium meliloti* ROOT COLONIZATION

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Rhizobium meliloti colonization of alfalfa roots (*Medicago sativa* L.) involves bacterial growth. Our group studies root factors that affect intrinsic growth of the bacterial population. We currently are defining how biotin and root-derived CO_2 affect colonization. In our experimental system a few cells (50-100) of *R. meliloti* 1021 (Rm1021) placed on a germinating seed on day 0 double every 7 h between day 3 and day 6 until they reach a density of 10^5 cells/root. We feel this approach is more realistic than experiments that study an inoculum of 10^6-10^8 bacteria which survive but probably do not grow in the rhizosphere. Our results show that biotin derived both from internal bacterial synthesis and from root exudates is a major factor affecting root colonization. We hypothesize that the biotin operates as both a cofactor and regulator for acetyl-CoA carboxylase, which catalyzes the first committed step in fatty acid synthesis required for membrane formation. We focus on acetyl-CoA carboxylase because rhizobia require CO_2 for growth and because increasing the cell surface/volume ratio may confer an advantage on rhizosphere bacteria competing for carbon substrates in root exudates. Rm1021 may obtain CO_2 to implement this strategy by promoting root respiration. To explore these ideas we are studying three aspects of the alfalfa-*Rhizobium* interaction: 1) biotin synthesis and uptake in Rm1021; 2) Rm1021 factors that promote root respiration; and 3) CO_2 utilization in Rm1021.

Our initial results show that 1) Rm1021 contains biotin synthesis genes which can be complemented by the *E. coli bio* operon; 2) Rm1021 has several biotin uptake systems; 3) nodulation factors and other products from Rm1021 elicit increased CO_2 evolution in alfalfa roots; and 4) an unexpectedly large number of biotin-regulated genes can be found when the transposable promoter probe Tn*5*-B22(*lacZ*) is used to generate mutants in a biotin auxotroph of Rm1021. We are using these biotin-regulated mutants to identify biotin transporters and to search for additional bacterial products that promote root respiration.

L-F-3

ENHANCING THE POTENTIAL OF MICROBIAL INOCULANTS THROUGH MOLECULAR MICROBIAL ECOLOGY

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The application of innovative biotechnology is continuing to make significant impact in the agricultural sector. In the context of food production, there is a strong demand from consumers for food products that are free from residues and produced in an environmentally friendly way. Against this background, the desirability of promoting sustainable and integrated systems for food production is a high priority, and this has focused attention on the effective exploitation of microbial inoculants in modern agricultural biotechnology.

The promotion of sustainable agricultural systems essentially requires the integrated use of microbial inoculants as biofertilizers, biopesticides and phytostimulators. Modern biotechnology provides the strategies and options to modify and improve the performance of individual inoculant strains under laboratory conditions. However, unless the potential of these strains can be harnessed in practical field conditions, then the true benefit for the promotion of sustainable agriculture will not be achieved. In this regard, the development and application of molecular microbial ecology is greatly assisting this process.

In the framework of a multidisciplinary EU project called IMPACT, the biotechnology and ecology of microbial inoculants is under investigation. Key traits involved in N_2-fixation, biopesticide and phytostimulation actions have been modified by genetic engineering, and modified microbial inoculants have been developed. These genetically modified inoculants are being investigated and tested using molecular microbial ecology procedures. The impact associated with the deliberate release of biocontrol strains on other key microbial inoculants, such as *Rhizobium* and mycorrhizal fungi, is being investigated in microcosm and pilot-scale field experiments. The results of these experiments and approaches, together with the development and testing of a containment system for rhizobial inoculants, will be discussed.

HOST-CONTROLLED RESTRICTION OF NODULATION BY *BRADYRHIZOBIUM JAPONICUM* STRAIN USDA 110 AND CHARACTERIZATION OF THE GENES REGULATING NODULATION

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Bradyrhizobium japonicum strain USDA 110 is restricted for nodulation by soybean genotype PI 417566. Nodulation and genetic diversity studies indicated that not all serogroup 110 strains are restricted for nodulation by this PI genotype. We previously reported the identification of a USDA 110 *Tn*5 mutant, D4.2-5, which had the ability to overcome nodulation restriction conditioned by PI 417566. In this study, we report the cloning and characterization of a negatively-acting gene from USDA 110 which is involved in the genotype-specific nodulation of soybean. The *Tn*5 integration site in mutant strain D4-2.5 was localized to a 5.2-kilobase *Eco*RI fragment from genomic DNA of wild-type USDA 110. Saturation *Tn*5 mutagenesis and homogenitization studies indicated that a 0.9-kilobase DNA region was involved in the genotype-specific nodulation of PI 417566. Sequence analysis and *Tn*5 saturation mutagenesis established that a single open reading frame (ORF) of 474 nucleotides constituted the negatively-acting genotype-specific nodulation gene, *noe*D. No similarity was detected between the *noe*D DNA sequence or the predicted amino acid sequence of any known genes or their products in the nucleotide and protein databases. However, a region upstream of *noe*D had high similarity (71%) to the N-terminal region of the *R. leguminosarum* bv. viceae *nod*M gene. Southern hybridization analysis indicated that *noe*D is not closely linked to the main or auxiliary nodulation gene clusters in *B. japonicum* and that both nodulation-restricted and -nonrestricted *B. japonicum* serogroup 110 strains contain a noeD homolog. Transcriptional analyses done using *nod*C::*lac*Z gene fusions indicates that *noe*D positively regulates the expression of the common nodulation genes. High performance liquid chromatography and FAB-mass spectroscopy analyses indicated that the *noe*D mutant produces a greater amount of acetylated nodulation factors than does USDA 110. Our results suggest that specific LCO signals molecules may be one of the major factors influencing nodulation specificity in this symbiotic system.

L-G-1

REGULATION OF NITROGEN FIXATION GENES BY THE NIFA AND NIFL REGULATORY PROTEINS

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Over the past ten years it has become apparent that mechanisms for regulating expression of nitrogen fixation (*nif*) genes are surprisingly novel in a number of different respects. Firstly transcription from most *nif* promoters requires a novel form of RNA polymerase holoenzyme containing a unique sigma factor, σ^N (σ^{54}), which is competent to bind its target promoters but is inactive in the absence of a specific transcriptional activator. Secondly, the activator binds to upstream enhancer-like sequences and contacts the downstream bound σ^N-containing RNA polymerase via DNA looping. The *nif* system thus provided one of the first examples of long distance transcriptional activation in prokaryotes. The principal role of the activator is to catalyse the isomerisation step in transcription initiation by interacting with the σ^N-holoenzyme to promote the transition of the closed promoter complex to the open complex. An unusual feature of this reaction with respect to prokaryotic transcription systems is that it requires the hydrolysis of a nucleoside triphosphate, principally ATP or GTP. While none of these features are in fact unique to *nif* gene regulation, studies of the *nif* system have considerably enhanced our knowledge of the mechanism of σ^N-dependent transcriptional activation in bacteria.

The enhancer binding protein, NIFA is ubiquitous among many diazotrophs as an activator of *nif* gene transcription but the activity of this protein as well as the expression of *nifA* is regulated in a variety of different ways in nitrogen fixing bacteria. Various mechanisms for regulating the expression and activity of NIFA in response to environmental oxygen and the level of fixed nitrogen will be reviewed. I will focus on our recent finding that the *Azobacter vinelandii nifL* gene product is a flavoprotein which directly modulates NIFA activity in response to a redox-sensitive switch. In addition to its ability to act as a redox sensor, NIFL is also responsive to adenosine nucleotides, particularly ADP. These observations suggest that energy charge, in addition to redox and nitrogen status, may be an important determinant of *nif* gene regulation *in vivo*.

L-G-2

METABOLIC REGULATION OF NITROGENASE IN *Rhodospirillum rubrum*

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Nitrogenase activity in the photosynthetic bacterium *Rhodospirillum rubrm* is regulated at the metabolic level through a cascade leading to reversible ADP-ribosylation of dinitrogenase reductase. This metabolic regulation is manifested *in vivo* as a decrease in nitrogenase activity upon addition of ammonium ions, glutamine or NAD^+, or by shifting the cells to darkness. A major question is how the activities of the two enzymes catalyzing modification/ demodification, i.e. DRAT/DRAG, are controlled. A number of possible metabolites have been studied in many laboratories, but so far no conclusive answer has been obtained.

We have hypothesized that both pmf and the concentration of oxidized NAD are involved in controlling the activity of DRAT/DRAG. While the activity of DRAT is suggested to be strongly dependent on the concentration of NAD^+, our models predicts that DRAG is inactive when associated to the chromatophore membranes. In this presentation further studies to verify our model of the processes at the molecular level of the reulatory cascade will be presented.

L-G-3

GENETIC ANALYSIS OF TWO SYSTEMS FOR ALTERNATIVE NITROGENASES IN THE CYANOBACTERIUM *ANABAENA VARIABILIS*

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Anabaena variabilis ATCC 29413 is a heterocystous cyanobacterium that is capable of photoautotrophic growth as well as photoheterotrophic and chemoheterotrophic growth using fructose as sole carbon source. This organism fixes dinitrogen using nitrogenases encoded by three different genetic systems. Two of the nitrogenases are Mo-nitrogenases, or type-1 nitrogenases, encoded by the *nif1* and *nif2* gene clusters. The third nitrogenase is a V-nitrogenase, or type-2 nitrogenase, encoded by the *vnf* gene cluster. There is no evidence in this strain for a non-Mo, non-V, type-3, nitrogenase.

The *nif1* system is encoded by a cluster of genes that includes *nifBSUHDKENXW*. Like the related strain, *Anabaena* sp. PCC 7120, the *nifD* gene is interrupted by an 11-kb excision element that is excised during heterocyst differentiation. A reporter gene, *lacZ*, under the control of the *nifH1* promoter was expressed only in heterocysts under either aerobic or anaerobic growth conditions; thus, the *nif1* system cannot function in vegetative cells.

The *nif2* system is also encoded by a large cluster of genes that includes *nifBSUHDKENXW* as well as ferredoxin genes and several ORFs similar to those found in the *nif1* cluster. A major difference between the two *nif* clusters is the absence of the *fdxN* gene, the absence of the 11-kb excision element, and the fusion of *nifE* and *nifN* into a single gene, *nifEN*, in the *nif2* cluster. The entire sequence of this cluster is available from the GenBank database. The *lacZ* reporter gene, under the control of the *nifH2* promoter, was expressed only under anaerobic conditions in vegetative cells and in heterocysts. Therefore, the *nif2* system appears to be under environmental control, regulated by oxygen and the availability of fixed nitrogen. Expression of the *nif2* nitrogenase, although sufficient to support growth, does not repress heterocyst differentiation and subsequent expression of the *nif1* genes. Preliminary data suggest that the *nif2* system is normally repressed in vegetative cells, but is activated by a protein synthesized after cells are shifted to anaerobic conditions.

The V-nitrogenase is encoded by a small cluster of genes that includes the fused gene *vnfDG*, as well as *vnfK*, *vnfE* and *vnfN*. The fusion of *vnfDG* suggests that the α and δ subunits encoded by these genes are physically, and perhaps functionally, linked. Unlike *Azotobacter vinelandii*, there is neither a *vnfH* gene, a ferredoxin gene, nor a *vnfX* gene near this cluster. However, we have preliminary evidence for a *vnfH* gene distant from the *vnfDGKEN* cluster. The V-nitrogenase depends on the NifB1 protein of the *nif1* system; however, the NifS1 and NifU1 proteins are not essential for either the heterocyst-specific Mo-nitrogenase, or for the V-nitrogenase. It appears likely that other gene products, perhaps normally used for synthesis of Fe-S clusters in photosynthetic proteins, function in the absence of NifS1 or NifU1 for nitrogenase synthesis. Preliminary data suggest that the *vnf* system is controlled by a repressor protein that is made in Mo-grown cells, but not in V-grown cells.

L-G-4

MOLECULAR STUDIES OF THE ELECTRON TRANSPORT PATHWAY TO NITROGENASE IN *RHODOBACTER CAPSULATUS*

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The nitrogenase-catalyzed reaction requires reductants which are provided by a small electron carrier operating at a low redox potential (<- 400 mV). In the photosynthetic bacterium *R.capsulatus*, the electron donor to nitrogenase was identified as a specific ferredoxin, called FdI. It is a ferredoxin containing two [4Fe-4S] clusters having an average mid-point redox potential of - 510 mV. The molecular interaction between FdI and dinitrogenase reductase (Rc2) was studied through the analysis of FdI variants obtained by site-directed mutagenesis. One particular aspartate residue (Asp36), presumably located at the surface of the molecule, was shown to participate in the interaction with Rc2.

Deletion of the *fdxN* gene encoding FdI greatly impaired nitrogen fixation. Although *R.capsulatus* contains five other ferredoxins, none of these alternate electron carriers could replace FdI in providing reductants to nitrogenase. The *fdxN* gene belongs to a *nif*-controlled operon that also includes *fdxC,* encoding a [2Fe-2S] ferredoxin and Orf14, coding for a putative flavoprotein. The latter gene was overexpressed in *E.coli* and its product purified as a yellow protein containing one FMN group per monomer. The flavoprotein did not react with pyridine nucleotides, but was readily reduced by dithionite and methyl viologen. It was also reduced by the ferredoxin encoded by *fdxC*, suggesting that the two proteins might belong to the same electron transport pathway. The flavoprotein displayed significant sequence similarity with a protein associated with a hydrogenase in *Methanobacterium thermoautotrophicum.* As a Orf14-deleted mutant did not show a distinctive phenotype, the role of the *R.capsulatus* flavoprotein is still unknown.

Besides the common *nif* genes found in all N_2-fixing bacteria, *R.capsulatus* has additional genes, called *rnf*, that may code for a *nif*-specific electron transport system. Based on sequence analysis of the deduced polypeptides, the *rnf* gene products may form a membrane complex that would function as a ubiquinol-ferredoxin oxidoreductase. As the *rnf* genes appeared to be expressed at a low level in *R.capsulatus*, we have attempted to overexpress six of them in *E.coli,* as a means to isolate the corresponding polypeptides. This strategy allowed to puitfy the *rnfB* product as a brown protein containing as yet unidentified Fe-S centers. Using antibodies raised against this recombinant protein, RnfB was shown to be mainly associated with the membrane of *R.capsulatus*, consistent with the expected localisation of the Rnf complex. The *rnfC* gene product, another putative Fe-S protein, was produced in truncated form, while the *rnf* gene products predicted to fold as transmembrane polypeptides failed to accumulate in significant amounts in *E.coli.* As the heterologous expression of some genes of interest was unsuccessful, another approach will be employed consisting of overexpression of the *rnf* genes in the natural host.

L-H-1

BIOCHEMICAL AND MOLECULAR ANALYSES OF RHIZOBIAL RESPONSES TO LEGUME FLAVONOIDS

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Legume flavonoids induce the expression of *nod* genes in rhizobia. They also elicit a number of other responses in this microsymbiont, including degradation of the flavonoid itself, expression of genes which share no homology with nodulation gene promoters and exudation of new proteins into the surrounding environment. We have used both biochemical and molecular approaches to investigate the mechanisms of flavonoid degradation exhibited in rhizobia, the fate of a *nod* gene inducer during Nod factor synthesis and the full extent of rhizobial gene expression during exposure to *nod* gene inducing flavonoids.

GC-MS analyses of extracted culture media showed that many rhizobia degrade individual flavonoids by mechanisms which originate in a cleavage in the (central) C-ring of the molecule. Subsequent fissions in the same ring release a variety of conserved A- and B-ring monocyclic aromatics which may include protocatechuate, a universal growth substrate for rhizobia. Label tracing experiments with ^{14}C naringenin at *nod* gene inducing concentrations confirmed that the compound was degraded and demonstrated that carbon atoms originating in this flavanone were incorporated into the lipid moiety of a *Rhizobium leguminosarum* bv. *viciae* Nod factor. A range of separation/detection techniques (TLC - UV, HPLC - UV, CE - UV and GC - MS) was used to identify naturally occurring flavonoids in seed and root exudates of *Lotus pedunculatus* and to demonstrate that new flavonoids and monocyclic aromatics could be detected when exudates were incubated with *R. loti*.

A combination of molecular techniques was used to identify flavonoid dependent non-*nod* gene expression in *Rhizobium* sp. NGR234: subtractive hybridisation of paired genomes, competitive RNA hybridisation to *Xho* I digests of an ordered cosmid library of the NGR234 symbiotic plasmid, differential RNA hybridisation to the same library and RNA arbitrarily primed PCR (RAP-PCR) of induced and uninduced NGR234 cultures. Examples of genes detected include a homologue of a *R. fredii* cultivar specificity gene, *nolU*, a symbolically active locus with strong homology to the leucine responsive regulatory protein of *E. coli* and a sequence with high homology to a hypothetical protein from *Haemophilus influenzae*.

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L-H-2

AEROBIC AND FERMENTATIVE METABOLISM IN *Rhizobium* AND ITS RELATION WITH THE SYMBIOTIC PROCESS

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During serial subcultivation in minimal medium (MM-succinate-ammonium) *Rhizobium etli* and other species of *Rhizobium* change their metabolism from aerobic to fermentative. As a part of this metabolic response the polymer poly-β-hydroxybutyrate (PHB) is accumulated (1). *R. etli* in symbiosis with *Phaseolus vulgaris* also accumulates PHB. For this reason, a mutant strain that has interrupted the phaC gene, responsible for the synthesis of PHB does not accumulate this polymer and excretes, instead, large amounts of organic acids to the medium (2). This mutant strain when in symbiosis with *P. vulgaris* while not synthesizing PHB fixes more N_2, something that results in an increase of the nitrogen content of the plants and seeds. In contrast, *R. meliloti*, although accumulates PHB during subcultivation in MM, lacks PHB when in symbiosis with alfalfa. However, when the operon with the genes for PHB synthesis of *Alcaligenes euthropus* was cloned into *R. meliloti* 1021 strain under the regulation of the nifH promotor of *R. etli* (3), PHB granules were present in this strain in symbiosis with alfalfa. The acquisition of the capacity to synthesize PHB during symbiosis resulted in a severe decrease in the size of the plants as well as, in the total nitrogen content (4). The physiological role of PHB in *Rhizobium* will be discussed in relation with the carbon distribution and utilization by this bacteria, and also as a sink necessary to drain the reductive power generated when *Rhizobium* is fermenting in low oxygen.

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L-H-3

HETEROCYST DIFFERENTIATION AND NITROGEN FIXATION IN CYANOBACTERIA

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The cyanobacterium *Anabaena* differentiates specialized cells for nitrogen fixation at regular intervals along its filaments of vegetative cells, under conditions of low fixed nitrogen availability. The conversion of a carbon-fixing, oxygen-evolving vegetative cell into a nitrogen-fixing, anaerobic heterocyst requires the orderly expression of many hundreds of genes in a cell-specific manner. In the strain *Anabaena* PCC 7120, heterocysts generally differentiate at the ends of filaments and at regular intervals, approximately every tenth cell, along each filament. These cells are readily distinguished by their double-layered envelope, consisting of novel polysaccharides and crystalline glycolipids, and prominent polar granules comprised of cyanophycin. Within the heterocyst, synthesis of proteins for carbon fixation stops while the enzymes for nitrogen fixation and generation of ATP and reductant for nitrogen fixation and assimilation increase. Several DNA elements interrupting *nif* and *hup* operons in vegetative cell DNA have to be excised. Systems for the orderly transport of fixed nitrogen out of the heterocyst and carbohydrate into it have to be established. To learn how these conversions are accomplished, we have isolated mutants blocked in the development of functional heterocysts and the genes complementing the mutations. These include the regulatory genes *hetR*, required for the early commitment of a cell to the differentiation path, *patA*, required for HetR function, and *patB*, required at a later stage. Relationships among these gene products are being elucidated by W. J. Buikema using fusions of their promoters to GFP. C. C. Bauer and K. Black identified a set of genes controlling heterocyst glycolipid synthesis and another required for proper secretion and assembly of the glycolipid envelope. Y. Guo has shown that sucrose synthase is an essential enzyme for nitrogen fixation. With these and other results it is possible to begin modelling the regulatory circuits controlling heterocyst differentiation and pattern formation.

L-H-4

TRICHODESMIUM HAS CELLS SPECIALIZED FOR NITROGEN FIXATION BUT LACKS HETEROCYSTS

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Trichodesmium is a filamentous cyanobacterium that unlike other non-heterocystous cyanobacteria has the nitrogen-fixing enzyme, nitrogenase, confined to a subset of cells within each colony (1, 2) or filament (3). In the two non-heterocystous cyanobacteria, *Oscillatoria limosa* and *Plectonema boryanum*, nitrogenase is found in all cells of the filaments (4, 5).

Cells with nitrogenase in *Trichodesmium* amounts to around 14% of the total cell population, i.e. only a small proportion of the cells synthesize the protein (2). This resembles the situation in heterocystous cyanobacteria which typically convert 5-10% of the photosynthetic vegetative cell population into nitrogenase harbouring non-photosynthetic cells, heterocysts.

Moreover, the nitrogenase containing cells of *Trichodesmium* never occur one by one along the filament but are always consecutively arranged. Such stretches of nitrogenase containing cells may show varying locations within the filaments but most frequently they are located in the center (3).

Recent data also suggest that cells with nitrogenase do not fix carbon dioxide as stretches of consecutively arranged cells, often located centrally in the *Trichodesmium* filaments, are devoid of ^{14}C-label (6). Not only the location but also the proportion of cells lacking ^{14}C label, constituting approx. 13% of the cell population, correlates extremely well with that containing nitrogenase. This is the case for both naturally growing and laboratory cultures of *Trichodesmium*. Moreover, "lighter" areas correlating in size and location to the cell stretches with nitrogenase are easily detected by light microscopy in certain natural *Trichodesmium* species.

Taken together we therefore suggest that, although *Trichodesmium* is classified as a non-heterocystous cyanobacterium, they differentiate certain photosynthetic cells into a specific non-^{14}C-fixing cell type in which nitrogen fixation takes place, i.e. they practice division in labour through cell differentiation.

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L-I-1

USE OF NITROGEN FIXING CROP SPECIES FOR SUSTAINABLE AGRICULTURE

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Individual protein consumption of the 5.3 billion people on earth averages about 70 g of protein/day, or 23 million tons of nitrogen (N)/annum. To maintain this level of intake in the face of a doubling of the earth's population over the next 40 years, will necessitate a doubling or tripling of crop production. This enhanced production will need to be achieved despite a significant deterioration of much prime agricultural land and will require the utilization of large areas now considered marginal for meaningful production. Judicious management of N in the environment will be essential to the development of more productive and sustainable agriculture. Nitrogen acquisition and assimilation is second only to photosynthesis in terms of importance for plant growth and development, yet N is perhaps the single most important factor currently limiting crop yields. The striking increase in cereal grain yields in developed countries between 1950 and 1990 was directly attributable to a more than 10-fold increase in N fertilizer use over the period. However, further increases in crop production are unlikely to be achieved by increased N fertilization. The increased use of N in developed countries has led to leaching of toxic NO_3^- into groundwater and volatilization of N oxides into the environment. By contrast, in developing countries the high cost of N fertilizer and the energy requirements for production will limit its use, especially for smaller landholders. Biological N_2 fixation (BNF) accounts for 65% of the N currently utilized in agriculture and will be increasingly important in future crop productivity especially for sustainable systems, small scale operations, and marginal land utilization. Plants capable of symbiotic N_2 fixation provide important sources of forage, food, and firewood. Clearly, it is imperative that we not only understand the importance of symbiotic N_2 fixation to agriculture but also that we enhance its presence in cropping systems. Moreover, it is critical that new uses for legumes be developed to stimulate the incorporation of these species into nontraditional management systems. For example, legumes may be used as sources of biomass for electrical energy generation, as bioremediation agents for toxic materials in soils and groundwater, and to improve the availability of other nutrients such as phosphorous. Such roles for legumes in cropping systems not only provide growers with the traditional commodities but also give extra added value to sustainable initiatives.

L-I-2

NITROGEN FIXERS: POPULATION DIVERSITY AND EFFECTS OF FERTILIZERS

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One of the reasons for the interest in the research on nitrogen fixation is its potential for application in agriculture. The contribution of nitrogen fixation in legume crops is well documented, and it can partially substitute for the chemical nitrogen fertilizer needed by the plants.

The recent discovery of nitrogen fixing organisms inside the stems and roots of diverse plants such as sugar cane opens the possibility of extending biological nitrogen fixation to other crops important for nutrition. In addition to *Acetobacter diazotrophicus*[1] we have isolated *Klebsiella* spp. and *Azospirillum* spp. from sugar cane. Our results show that populations inside the plant are different from the bacterial populations encountered in the rhizosphere, outside the plant. It is imperative to evaluate the contribution of the different nitrogen fixing species to the plant. Besides this, we are also interested in isolating nitrogen fixers associated with other plants such as coffee and maiz (*Zea maiz*). The latter originated and diversified in Mexico; therefore, native bacterial populations from non-fertilized areas traditionally cultivating this crop may be analyzed. We have recently isolated different nitrogen fixers from maiz. From coffee plants, we have isolated *Acetobacter diazotrophicus* strains identified by biochemical traits, by MLEE (multilocus enzyme electrophoresis) and by RFLP's of ribosomal and *nif* genes. Thus coffee constitutes a new host plant for this endophytic bacteria.

Nitrogen fertilization inhibits nodulation and nitrogen fixation in legumes. We have proposed that it may also have deleterious effects on the population of nitrogen-fixing micro-organisms[2,3]. To test this hypothesis we have undertaken different experiments with *Phaseolus vulgaris* beans and sugar cane to evaluate the effect of nitrogen fertilization on bacterial biodiversity.

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L-I-3

A PLANT GENETIC POTENTIAL FOR IMPROVING THE BENEFICIAL INTERACTIONS WITH THE SOIL MICROFLORA

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Using of the biological nitrogen in the sustainable agriculture is a part of a more general task of improving the plant abilities to interact with different beneficial microorganisms. Various groups of endosymbiotic, rhizospheric and epiphytic microbes can contribute sufficiently to fulfilling the basic plant functions - mineral (N, P) nutrition, growth regulation, defense from pathogens, etc. - thus permitting us to minimize the application of the ecologically dangerous and expensive agrochemicals. Many cultivars of both legume and non-legume crops which have been developed under the conditions of the "intensive agriculture" are characterized by a decreased ability for the microbial interactions which is well expressed in the wild-growing and low-cultured genotypes. In order to restore and even to improve the natural potential for the microbial interactions the new approaches in the plant breeding are required which up to now have been developed mainly for the legumes (analysis of the genetic polymorphism, developing the screening systems and breeding strategies, coordinated plant-microbe selection, etc.).

For the non-legumes this work is only in its beginning now. In our Institute several dozens of new preparations based on different rhizospheric microbes have been developed. An efficiency of these preparations depends greatly on the plant genotype which controls a quantity and biological activity of the root exudates in different plant species and cultivars. Being the sources of energy and of the growth signals for the soil microbes, these exudates might determine the plant-stimulatory effects of the rhizosperic microflora. A sufficient potential for breeding the non-legumes for enhancing these effects is evidenced by a high intra- and inter-cultivar variability for an intensity of rhizospheric N_2 fixation, for an ability to support the numerous populations of the beneficial microbes on the root surface, for the content and biological activity of the root exometabolites.

Several breeding programs have been developed for both legume and non-legume crops which will improve sufficiently the plant potential to develop the high, nutritionally valuable and ecologically safe yields by using interactions with various symbiotic and associative microorganisms.

Distribution of N_2-fixing, denitrifying and nitrifying bacteria in soils monitored by DNA-probing

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Using specific oligonucleotide primers and PCR, gene probes have been developed for N_2-fixation (*nifH* gene), for all steps of denitrification and for nitrification (*amo*A gene). These probes allow to assess the occurrence of genes with such traits in a wide range of microorganisms. Data of the DNA-DNA hybridizations with these probes generally match with known activity profiles. These probes have successfully been employed to monitor the distribution of these genes in *Hyphomicrobium* isolates which can hardly be grown under lab. culture conditions. The data showed that denitrifying *Hyphomicrobium* predominantly live in the sludge of a sewage disposal plant whereas N_2-fixing isolates of this genus preferentially occur in a N-poorer lake in the same geographic area (1). In four selected soils of the Cologne area, N_2-fixing and denitrifying bacteria predominantly occurred in the upper soil layer (about 5 cm depth), whereas the percentage of these bacteria significantly decreased in lower layers (e.g in around 25 cm depth of the soil). The absolute number of bacteria increased in the vicinity of plant roots, however, with no special enrichment of either N_2-fiixng or denitrifying bacteria. In an acid pine forest soil of the Black Forest in Southern Germany, the number of these bacteria showed large fluctuations during the vegetation period with peak levels in spring.

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ASSIMILATION OF REDUCED NITROGEN IN TROPICAL LEGUME NODULES: REGULATION OF *DE NOVO* PURINE BIOSYNTHESIS AND PEROXISOME PROLIFERATION

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The symbiotically reduced nitrogen is assimilated in the host cytoplasm with the help of cytosolic glutamine synthetase. We have shown that this enzyme is induced directly in response to ammonia. The glutamine formed in the cytoplasm is funneled to the plastids where it is converted to purines. The latter are synthesized via a *de novo* biosynthesis pathway. The purines are then oxidized into ureides inside the uninfected cells of root nodules. We have demonstrated that the *de novo* purine biosynthesis pathway is induced prior to the onset of nitrogen fixation, apparently due to the higher demand for purines for DNA endoreduplication in the infected cells. We have demonstrated that glutamine is capable of enhancing expression of the PRPP-AT gene, encoding the first enzyme of the purine biosynthesis pathway. This pathway is also regulated by the availability of carbon *via* PRPP synthetase. We have identified a protein which may act as a repressor of the *pur* genes in soybean, particularly PRPP-AT gene. This protein may interact to a procaryotic-type Pur box sequence in the promoter region of PRPP-AT gene. Because the access of purines is toxic to the cell, they are exported to the uninfected cells where they are oxidised. The purines apparently induce peroxisome proliferation where uric acid is converted to ureides. We have demonstrated that purines are able to induce peroxisome proliferation in yeast. Using urate as a source of nitrogen, we have been able to isolate several yeast mutants defective in peroxisome proliferation/assembly. We have complemented one of these mutants with a soybean gene encoding a "putative peroxisome proliferator" with similarity to G-box binding proteins. In addition, we have determined three pathways for the conversion of uric acid to ureides in root nodules and have isolated corresponding genes involved in these pathways.

L-J-2

ROBINIA PSEUDOACACIA NODULATION AND NODULE FUNCTIONS

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Hydrogen uptake is thought to increase the efficiency of nitrogen fixation by recycling H_2 produced by nitrogenase that would otherwise be lost by diffusion. We demonstrate the capacity of eight *Robinia* nodulating *Rhizobium* strains to take up molecular hydrogen. Uptake by nodule homogenates from *Robinia pseudoacacia* was measured amperometrically under nitrogenase repression. Markedly lower activities were found than in soybean nodules. In addition, hydrogenase activity was detected by the ability of bacteroids to reduce methylene blue in the presence of hydrogen. It was demonstrated that hydrogenase structural genes are present in the black locust symbiont, *Rhizobium* sp. strain R1, using hybridization with a plasmid which contained hydrogenase genes from *R. leguminosarum bv. viceae*.

Eight compounds exuded from young roots of black locust (*Robinia pseudoacacia*) were separated by two-dimensional HPTLC, HPLC and GC and identified by spectroscopic methods (ultraviolet/visible absorbance and mass spectrometry) as being 4',7-dihydroxyflavone, apigenin, naringenin, chrysoeriol and isoliquiritigenin. Comparison with authentic standards verified the chemicals structures of the aglycones and their capacity to induce ß-galactosidase activity in *Rhizobium* NGR234 containing a *nod* box:*lacZ* fusion on plasmid pA27. Every of the identified flavonoids respectively chalcone is a *nod* gene inducer. This is an indication of their important role in nodulation of this legume tree.

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L-J-3

INCREASED RESPIRATION FOR SYMBIOTIC NITROGEN FIXATION UNDER PHOSPHORUS DEFICIENCY

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Phosphorus deficiency is a major limiting factor of symbiotic nitrogen fixation in mediterranean and tropical zones. Being a partner of a multidisciplinary project to improve legume nitrogen fixation and yield in low-P soils, we investigated the effects of P deficiency on nodule respiratory (CO_2 evolution and O_2 uptake) and nitrogenase activities (C_2H_2 reduction).

Nodulated soybean (*Glycine max.*) and common bean (*Phaseolus vulgaris*) were grown in intensely aerated liquid nutrient solutions. Indeed, the hydroponic culture optimized (i) the P supply to the roots, (ii) the nodulation and growth depending upon nitrogen fixation, and (iii) the in situ measurement of the nodule gas-exchanges. During gas-exchange assays, the exposure of nodulated-root to C_2H_2 induced a decline of nitrogenase activity. This decline was less than 25% for control plants versus more than 50% for low-P plants.

By plotting CO_2 evolution as a function of C_2H_2 reduction during the decline, we found that nodules of low-P plants evolved significantly more CO_2 per C_2H_2 reduced than nodules of control plants. However, both types of nodules had similar nitrogenase activity, measured as pre-decline C_2H_2 reduction activity.

The nodule O_2 uptake, was measured as the variable component of the response of nodulated-root O_2 uptake to external pO_2 between 15 and 21 kPa O_2. The nodule O_2 uptake was also found to be increased by P deficiency.

The nodule permeability to O_2 diffusion, calculated with the nodule O_2 uptake and an estimate of total nodule area, was increased by P deficiency. However, the P deficienccy did not affect the nodule permeability of the common bean genotype G19839 for which the nodule nitrogenase activity was found to be relatively tolerant to P deficiency.

It is concluded that P deficiency increases the nodule CO_2 and O_2 consumption linked to nitrogenase activity, and that this effect may vary with sensitivity of legume nodule nitrogenase activity to P deficiency.

Further exploration of this phenomenon consists in analysing the effect of P deficiency on nodule cortex structure and nodule alternative oxidase activity.

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L-J-4

CARBON AND NITROGEN METABOLISM IN THE PLANT-DERIVED INEFFECTIVE NODULES OF PEA (*PISUM SATIVUM* L.)

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Organogenesis of legume root nodules and the construction of nitrogen fixation system require exchanges of molecular signals between *Rhizobia* and the host plant for activation of expression all necessary genes. Plant mutants with ineffective nodules are potentially useful for studies of the host-plant control and regulation of symbiotic nitrogen fixation. In this paper the nodules of three different pea mutants which were preliminarly characterized as Nod^+ Fix^- (Sym 13, Sym 31 and FN_1) as well as parent lines (Sparkle, Sprint 2 and Rondo), grown in the same cabinet, were compared for selected physiological and biochemical parameters. All mutants are monogenic (FN_1 - double), recessive and non-allelic.

What we are interested to know. (**1**) Nodule respiration and nitrogen fixation in relation to the appearance of nitrogenase proteins. (**2**) 0_2 diffusion - nodule structure and 0_2 concentration profiles in relation to leghemoglobin (Lb) and Lb apoprotein content. (**3**) Concen-tration of key metabolites of carbon metabolism and carbon reserved compounds. (**4**) Activity of key enzymes involved in C and N metabolism and immunodetection of enzyme protein.

It was shown: **1**. The plant-derived mutants of pea produced large (Sym 31) or small (Sym 13) white nodules which had no N_2-fixation activity, but show high rates of CO_2 evolution. FN_1 mutant was able to fix some nitrogen (about 15% of parent line), but carbon cost of the nitrogenase function was 2 times more than in the parent nodules. This data was confirmed by immunodetection of nitrogenase proteins. **2**. The structure of all mutant inner cortex suggests the existence of an 0_2 diffusion barrier and this was confirmed using O_2 microelectrode techniques. Oxygen concentrations in the central regions of mutant nodules were different, depending on Lb content. **3**. Starch contents in the ineffective nodules were similar or higher in comparison with parent lines. This suggests there was no block in carbon supply to the nodules from the shoots. **4**. Ononitol content was lower in ineffective (or partly effective) nodules, especially in Sym 31. This compound may play an important role in symbiosome formation. **5**. The lower activity of sucrose synthase (SS), phosphenolpyruvate carbxylase and alanine pyruvate aminotransferase in ineffective (or partly effective) nodules confirm that these enzymes play important roles in nitrogenase-linked metabolism of normal nodules. **6**. The high activity of alcohol dehydrogenase in ineffective (Sym 31 and Sym 13) nodules taken together with low 0_2 concentration, suggests that in these nodules part of the carbon coming from the shoots may be metabolized through anaerobic pathways. **7**. Glutamine synthetase (GS) activity was very low in Fix^- (Sym 31 and Sym 13) nodules, but not in FN_1 mutant which was able to fix even small amounts of nitrogen. **8**. The data from immunodetection of host nodule proteins confirm reduced levels of SS in all mutants and almost complete absence of GS in Sym 31 and Sym 13.

L-J-5

UNSOLVED MYSTERIES IN C METABOLISM IN LEGUME NODULES

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(Full references are supplied in the written report).

Poly-β-hydroxybutyric acid (PHB) is accumulated by many but not all bacteroids. In the fast-growing rhizobia (e.g., *Rhizobium meliloti*), mutants lacking the ability to synthesize PHB are capable of N_2 fixation rates similar to or greater than those in wild type bacteria. In the slow growing rhizobia (e.g., *Bradyrhizobium japonikum* and *B. elkani*), PHB is a major feature in electron micrographs of nodules. In spite of early reports, minimizing the importance of PHB, more recent reports, mainly from Bergersen *et al.*, have suggested that this carbon-rich polymer may supply bacteroids with reducing equivalents under circumstances of carbon limitation (e.g. dark). Even more intriguing is an 12-year-old report from Emerich indicating that *B. japonicum* mutants lacking the ability to utilize β-hydroxybutyrate are Fix $^-$. My recent efforts to confirm results of Emerich will be discussed.

α, α-trehalose (Tre) is a simple glucose-glucose disaccharide common among fungi but less common among bacteria. Interestingly, this sugar is produced by all nitrogen fixing organisms studied to date. Tre has many unusual chemical properties but the two most relevant to symbiotic systems are the ability to interfere with plant cell wall biosynthesis and to stabilize membranes subjected to heat or osmotic stress. Tre is synthesized and released by bacteroids in legume nodules. WHY? Previous efforts in my lab to obtain mutants of rhizobia unable to synthesize Tre were unsuccessful. These efforts were based on the assumption that the mechanism for Tre synthesis (and the relevant genes) were the same as described in Escherichia coli. However, Japanese workers have recently reported a totally new mechanism for the synthesis of Tre in bacteria; have been looking for the wrong genes? My recent efforts to establish the mechanism for Tre biosynthesis in rhizobia and to obtain Tre $^-$ mutants of *B. japonicum* will be discussed.

L-J-6

CONTRASTING EVOLUTIONARY SOLUTIONS TO C SUPPLY AND N ASSIMILATION AND TRANSPORT IN SYMBIOTIC PLANTS

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All N_2-fixing symbioses are characterised by the exchange of C and N at a specialist location within the plant where N_2-fixing bacteria are housed in an environment where C, N and O are rapidly and efficiently exchanged. A comparison of a range of important symbioses will be provided in a summary table prepared from the literature and, in some cases, work in our laboratory. Two important sets of conclusions can be drawn from this analysis:

(I) Similarities.
With the exception of the aquatic plant *Azolla*, all symbioses are located within the soil or at the soil surface. Each symbiosis involves one plant and one bacteria and, with the exception of the cyanobacterial symbioses, rbcL sequence analysis (Soltis *et al.*, 1996 *Proc. Natl. Acad. Sci. USA* **92**, 2647) groups all the other symbiotic nitrogen-fixing plants within a single broad clade within the dicotyledons. All symbiotic bacteria are eubacteria.

The transfer of fixed N seems to occurs in a similar fashion in each symbiosis. Ammonia, produced by nitrogenase within the bacterial cells, crosses the bacterial membrane as NH_3 or NH_4^+ and is taken up into the plant cytoplasm by an ammonium transporter (recently demonstrated for soybean by Tyerman *et al.*, 1995, *Nature* **378**, 629) and assimilated rapidly by cytoplasmic GS ensuring NH_3/NH_4^+ levels remain low and efficient transfer of N continues. The availability of substrates for the continued GS reaction is assured through operation of GOGAT and the combined action of these two enzymes provides glutamate and glutamine as the first forms of assimilated N in all symbioses examined. I am unaware of any strong evidence for N transfer as amino acids from the bacteria to the plant in any system.

(II) Differences.
Carbon to power nitrogenase activity is provided by the plant as malate and possibly succinate in the legumes, and perhaps also in *Parasponia* and the actinorhizal systems. Within the cyanobacterial systems, where the TCA cycle does not operate, sugars are transferred and metabolised via the pentose phosphate shunt.

The transfer of N from nodules to the rest of the plant occurs predominantly and initially in the xylem. However, different plants use a range of N rich compounds as transport molecules and this is achieved by the enhancement of different pathways of molecular synthesis within the root nodules of these different plants. Asparagine and glutamine are important compounds in a significant number of plants, but ureides and non-protein amino acids such as citrulline and 4-methylglutamate are also used. These differences are controlled by the genotype of the plant.

Regulation
In all systems the growth and activity of the symbiosis is closely regulated to match the demands of the plant for N. How this is achieved will vary in different symbioses because: (i) mechanisms to sense N status may be different with dissimilar N transport compounds, (ii) various mechanisms to supply C will have different regulatory steps and (iii) regulation of O_2 diffusion may be achieved by plant, or bacterial, or plant and bacterial, mechanisms.

L-K-1

THE ROLE OF OXYGEN IN REGULATION AND BIOENERGETICS OF SYMBIOTIC NITROGEN FIXATION

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A low free oxygen concentration in soybean root nodules (<25 nM) is the trigger for the induction of two sets of genes in the nitrogen-fixing endosymbiont *Bradyrhizobium japonicum*. One set of genes, organized in two operons (*fixNOQP*, *fixGHIS*), is controlled by a regulatory cascade composed of the two-component sensor/regulator pair FixL/FixJ and the Fnr-like regulatory protein $FixK_2$, in which the phosphorylated form of FixJ first activates the $fixK_2$ gene whose product in turn promotes transcription from the *fixN* and *fixG* promoters (1). The *fixNOQP* genes code for the subunits of a novel cbb_3-type cytochrome oxidase that supports microaerobic respiration by the endosymbiotic bacteroids, owing to a K_M for O_2 of 7 nM (2, 3). The products of the *fixGHIS* genes are important for the biogenesis of this oxidase (4). The second set of genes induced in response to microaerobiosis includes those that are concerned with the formation of an active nitrogenase enzyme complex. Apart from the nitrogenase structural genes (*nifH*, *nifDK*) and genes for metal cofactor synthesis (e.g. *nifEN*, *nifB*, *nifS*) this group also contains a special copy of the chaperonin genes, $groESL_3$ (1). All of these genes have in common that they are transcribed by the σ^{54}-RNA polymerase and activated by the oxygen-responsive NifA protein. Two overlapping promoters are responsible for expression of the *nifA* gene, and transcription from both is positively regulated in a complex way (5). There is a connection between the NifA cascade and the FixLJ-$FixK_2$ cascade in that the latter activates one of two σ^{54} genes ($rpoN_1$) needed in the former (1).

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L-K-2

DYNAMIC CONTROL OF OXYGEN DIFFUSION RESISTANCE IN THE ROOT NODULE

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Hydrogen production by undisturbed root nodules of soyabean (Clarke inoculated with either RCR 3442 or USDA 16) was monitored continuously in a flow through apparatus. A comparison of changes in H_2 concentration measured by micro-electrodes inside the nodule and rates of H_2 production when activity was altered by increases in pO_2, by Ar/O_2 mixtures and by detopping the plant show considerable changes in diffusion resistance. Both before and after these treatments were applied the H_2 concentration gradients across the infected zone were very shallow indication that the network of intercellular spaces in this region were interconnected. This provides further evidence that the site of the variable barrier to diffusion is outside this zone within the cortex.

Measurements of H_2 production also showed that the exposure of roots in air to relatively small increases in pO_2 (3-4%) resulted in large (40%) transient decreases in in both nitrogenase activity and associated respiration. Activity returned to its former rate over a 25 minute period in a series of characteristic oscillations. This observation cannot be ascribed to nitrogenase damage because further increases in pO_2 (from 40 to 60%) resulted in substantial increases in activity and the effect appears to be due to fluctuations in the resistance of the diffusion barrier. We were able to simulate these oscillations in a model of nodule dynamics only by incorporating a delayed feedback control for diffusion resistance.

L-K-3

IS THE VARIABLE OXYGEN PERMEABILITY IN NODULES A PHYSICAL OR A PHYSIOLOGICAL PHENOMENON?

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The regulation of nitrogenase activity in legume nodules is still poorly understood. Through a variable oxygen permeability it appears to be fine-tuned. Using highly structure-preserving cryo-techniques in combination with scanning electron microscopy and immuno-gold labeling, the morphology of lucerne and white clover nodules was examined with regard to the variable oxygen permeability. Decreasing the nodule oxygen permeability by either defoliating the plant or by increasing the rhizospheric oxygen partial pressure to 80 kPa had no effect on the nodule cortex structures. Likewise, the abundance of MAC236 (glycoprotein antibody) associated label in the intercellular spaces of the inner cortex did not alter due to defoliation or after the rhizospheric oxygen concentration has been increased. Unlike in other systems, the present results do not indicate a regulation of oxygen permeability through a physical occlusion of diffusion pathways in the inner cortex of lucerne nodules. In contrast, there is increasing evidence that the nitrogen metabolism is involved in the regulation of nitrogenase activity through a variable oxygen permeability. One new indication is the fact that the diurnal variation of nitrogenase activity appears to be dependent on whether the plants get fertilized with nitrate or not. This observation would be consistent with the possibility that physiological mechanisms are involved in the regulation of the variable oxygen permeability. However, the question how nodule nitrogen physiology might be linked to the phenomenon of a variable oxygen permeability remains to be answered.

L-K-4

REGULATION OF HEME SYNTHESIS IN THE *BRADYRHIZOBIUM JAPONICUM*-SOYBEAN SYMBIOSIS

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Sustaining Rhizobium-legume symbiosis is energy-intensive, and an increase in plant and bacterial heme protein synthesis is an essential feature of nodule ontogeny. Our focus is on the *Bradyrhizobium japonicum*-soybean symbiosis, and evidence shows that the heme biosynthetic pathway is regulated in both symbionts. The soybean *Gsa1* gene encoding glutamate 1-semialdehyde aminotransferase is strongly induced in young nodules and high levels of mRNA and protein expression are maintained throughout late development. *Gsa1*, *Alad* and *Copox* are all expressed in nodules at levels comparable to that observed in leaves for chlorophyll formation, indicating that soybean has the capacity for abundant tetrapyrrole synthesis in root nodules. On the bacterial side, *B. japonicum hemA* is not required for heme synthesis in nodules, and data infer that *hemB*, the gene encoding δ-aminolevulinic acid (ALA) dehydratase, is the first essential bacterial step for heme synthesis in the endosymbiont. Recent evidence shows that *hemB* is a highly regulated gene that is controlled by O_2 and iron. We found that *hemB* mRNA is rapidly induced over 100-fold in response to O_2 deprivation, and that this induction is not observed in a *fixJ* mutant. These observations suggest that O_2 control of *hemB* is mediated by a FixL/FixJ-dependent signal transduction pathway, and therefore under developmental control. We isolated a new gene called *irr* (iron response regulator) that encodes a protein that appears to mediate iron control of heme synthesis by repressing *hemB* transcription and by inducing iron uptake in response to iron deprivation. This control makes physiological sense because iron is part of the heme moiety, thus the pathway should be down regulated under iron limitation. Under those same conditions a high affinity iron uptake system is necessary to maximize the amount of iron acquired to maintain cellular functions.

L-L-1

RECENT ADVANCES IN THE MOLECULAR GENETICS OF THE MODEL LEGUME *LOTUS JAPONICUS*

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In an attempt to accelerate the progress in understanding the plant contribution to symbiotic nitrogen fixation, the model legume *Lotus japonicus* was suggested as experimental organism for a focused international venture (Handberg and Stougaard, 1992). At the Laboratory of Gene Expression the main emphasis has been concentrated on the molecular genetics of root nodule organogenesis, together with the signal transduction and the transcriptional gene regulation occurring during root nodule development. A progress report on the recent advances in developing the molecular genetic approaches will be given. The attempts to establish transposon tagging using the maize elements *Ac* and *Ds* will be presented together with the results obtained from a screening for symbiotic mutants in a tagging population. Results from a smaller programme employing T-DNA for insertion mutagenesis will also be reported. Phenotypes of the symbiotic plant mutants developing ineffective nodules or impaired in the nodulation process will be presented.
Gene inactivation after homologous recombination (gene targeting) would be a strong supplement to the random insertion mutagenesis (*Ac*,*Ds*,T-DNA) and a very valuable tool to establish the function of nodulin genes isolated by traditional molecular cloning techniques. Based on the negative selectable marker, cytosine deaminase established in *Lotus japonicus* (Stougaard 1993), a positive - negative selection scheme for identification of putative double recombinants has now been worked out. The molecular analysis of the putative targeting events, so far isolated with this procedure, will be summarised.
Finally the differential display approach initiated to generate a collection of expressed sequence tags (ESTs) corresponding to nodule expressed genes will be presented. The differential display PCR technique has been used on developing root nodules and compared to root and leaf ESTs. Several ESTs expressed after initiation of root nodule development have been isolated and characterised. A summary of the expression patterns and identities of the ESTs will be given.

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L-L-2

MOLECULAR GENETICS OF A MODEL-PLANT: *MEDICAGO TRUNCATULA*

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The mediterranean forage legume *Medicago truncatula* is used as a model-plant to study the nitrogen fixing *Rhizobium*-legume symbiosis because it is a diploid, autogamous plant of low DNA content (1C # 0.5pg) and with which transgenic plants can be routinely obtained. *M.truncatula* is nodulated by well-known *Rhizobium meliloti* strains and is of good agronomical value for mediterranean regions. Our objective is to make a genetic study of plant genes involved in symbiosis, based on symbiotic mutants and natural variants.

After irradiation with γ-rays, 18 symbiotic mutants were obtained at the M2 generation and were stable up to the M6 generation. They consist of 2 [Nod^-], 4 [$Nod^{+/-}$], 3 [Nod^{++}] and 9 [Nod^+Fix^-] mutants. Non-nodulating mutants [Nod^-] were also defective for mycorhizal symbiosis [Myc^-]. Supernodulating mutants [Nod^{++}] were nitrate-tolerant up to 15 mM (Nts). Genetic and molecular analysis of these mutants is under way.

Symbiotic variants were obtained by screening three natural *M.truncatula* populations and one cultivar (Jemalong) with wild-type *R.meliloti* strains, effective on alfalfa. From 123 genotype/strain combinations tested, 44 (36%) were deficient for nodulation or fixation.

The construction of a genetic map of *M.truncatula* is under progress, based on crosses between the above populations. We have been able to map 60 RAPD markers, 2 isoenzymes (PGM, PGD), 6 known genes (rDNA, ENOD12, MTGSa, MTGSb, MTLb1, MTLec2) and 1 morphological marker (pod coiling). Current research involves the study of genetic segregation and allelism tests of the identified symbiotic characters, their mapping and isolation by positional cloning. This genetic map will be also a powerful tool for the breeding of mediterranean forage legumes as well as for studying evolutionary processes within *Medicago* genus.

L-L-3

ADVANCES IN MOLECULAR CHARACTERIZATION OF THE YELLOW LUPIN (*LUPINUS LUTEUS*) - *BRADYRHIZOBIUM* SP. (*LUPINUS*) SYMBIOTIC MODEL

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The potential role of lupins in the farming systems developed on light, less productive and often polluted soils has already been well recognized. In Poland agronomic species of the genus *Lupinus* belong to the most popular grain legumes. Over the past several years, our research has been focused on the characterization of the symbiotic system of yellow lupin (*L.luteus*) and *Bradyrhizobium* sp. (*Lupinus*).

a) *Root nodule morphogenesis*. The successive stages of nodule development were characterized using cytological methods. The mitotic division of the root cortex parenchyma cells which initiated the formation of the nodule primordium were accompanied by structural changes in the root hairs and division in the root pericycle.

b) *Molecular analysis of lupin genes involved in symbiosis*. Among the identified sequences were ENOD-2 and ENOD-40, cell cycle genes as well as a pair of genes coding for pathogenesis-related PR10 proteins. Phylogenetic analysis of these genes indicates that *Lupinus* species are very old and might be somewhat distinct from other legumes

c) *Transformation and regeneration of yellow lupin* A reliable protocol for yellow lupin was elaborated which allows to induce stable transformed callus both from immature embryos and seedling mesocotyl using *A.tumefaciens* C58(p35SGUSINT). A transgenic character of callus sustained over one year in *in vitro* culture. The regeneration protocol *via* auxiliary buds multiplication was also developed for both mature and immature embryos.

d) *Characterization of nod cluster of Bradyrhizobium* sp. (*Lupinus*). The analysis of the physical maps of several overlapping cosmids and sequence analysis of the subcloned restriction fragments led us to the identification of the majority of the nodulation genes. The nodulation region carries genes *nodS*, *nodU*, *nodZ*, *nodL* in addition to 10kb fragment which includes *nodC*, *nodS*, *nodU*, *nodI*, *nodJ* and *nodJ* genes. Also, the presence of the *nod VW* genes whose products constitute an additional regulatory circuit resembles organization found in *B. japonicum*.

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L-L-4

GENE EXPRESSION IN ACTINORHIZAL NODULES

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Nitrogen-fixing actinomycetous bacteria of the genus *Frankia* can enter a root nodule-symbiosis with several dicotyledonous plant species from eight different familes. Nitrogen-fixing actinorhizal nodules are composed of multiple lobes, each of which represents a modified lateral roots without root caps, with a central vascular system and infected cells in the expanded cortex. Actinorhizal nodules formed on plants from different families show a considerable structural diversity, for instance in the oxygen diffusion pathways and in the organization of the infected cells in the cortex.
Nodule cDNA libraries were constructed of two different actinorhizal plants, *Alnus glutinosa* and *Datisca glomerata*. cDNA clones representing genes expressed at markedly enhanced levels in nodules compared to roots were isolated from these libraries via differential screening with nodule and root cDNA, respectively. The expression patterns of the corresponding genes in nodules were localized by *in situ* hybridization. Expression patterns of homologous genes in the different types of nodules were compared. The implications for the metabolic specialization of nodules and the differentiation of infected cells will be discussed.

L-M-1

LEGUME TOOLS FOR LEGUME BREEDING

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One of the main problems in the application of molecular tools for legume breeding is their intrinsic cost. Nevertheless many maps have been derived for legume species, often with an emphasis on the analysis of particular mutants. Several of these analyses will be discussed in this session, but a short discussion their application to breeding rather than genetics will be presented..

L-M-2

Molecular insights into shoot control of nodulation

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Mutational analyses in several legumes have confirmed the original observation by Carroll et al (1985) in soybean that the shoot, in particular the developing leaves, are controlling the number of successful nodulation events. Removal of leaves increases nodule number. Alteration of this control results in super- or hypernodulation. Interestingly, supernodulation mutants of all legumes studied so far exhibit increased nodulation in the presence of otherwise inhibitory levels of nitrate. Split root experiments suggest that supernodulation mutants are altered in their autoregulation of nodulation process. This process acts systemically, while nitrate acts locally. Approach graft experiments using different non-nodulation mutants of soybean indicate that autoregulation is induced by early cell division events in the root cortex. Induction of autoregulation is fast, requiring less than 24 hours in alfalfa, and showing 50 % effectiveness after 48 hours in soybean. *Bradyrhizobium* with functional *nod* genes is required. Genetic analysis of the soybean mutants indicates the following: 1) most mutant alleles are at the same locus on molecular linkage group H close to the pUTG132a RFLP marker, 2) most mutant alleles behave as recessives, 3) supernodulation is epistatically suppressed by non-nodulation genes, 4) the mutant phenotype is not observed naturally, and 5) reversions have not been observed. To determine biochemical changes in leaves following inoculation, molecular studies were conducted. *In vitro* translation of mRNA and 2-D gel electrophoresis failed to detect inoculation or mutation-dependent protein changes. Application of differential display-reverse transcriptase PCR (DD-RT-PCR) to soybean leaves revealed new proteins apparently responding to inoculation and the supernodulation status of the plant. Sequence analysis suggests possible functions of these.

L-M-3

Effect of nitrogen nutrition pathways on the quality of nitrogen storage compounds in legumes

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Legumes are well-known for their instability in the quantity and nutritional value of their nitrogen storage compounds.
A litterature survey will be given, showing that respective intensity of N2 fixation and assimilation pathways can explain some variations in these plant compounds. In general, these variations are greater in young plant tissues and in ureid-containing species.
An experiment using symbiotic pea mutants (non-nodulating and supernodulating plants) to create a range of nitrogen nutrition regimes will be described. The effects of nitrogen nutrition source (assimilation of nitrates or nitrogen fixation) and of nitrogen starvation have been assessed on the quantity of albumin, vicilin, legumin and non-proteic fractions in the seeds. We showed that most significant differences were observed after plant N starvation.
Consequences will be discussed in plant breeding and agronomy in relation with feed- and food- industries requirements.

L-M-4

GENETIC MAPPING IN ALFALFA: REGULAR AND ANOMALOUS PHENOMENA DURING LINKAGE ANALYSIS

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The highly saturated linkage map of alfalfa containing more than 1000 markers allows us to map mutations affecting different traits of *Medicago sativa* like sticky leaf and symbiotic nodulation and effectiveness. Closely linked molecular markers (within 0.5 centimorgan) can be used as starting points for chromosomal walking experiments towards the gene of interest. This map-based cloning strategy should enable us to identify and characterize genes required for leaf development, symbiotic nodule organogenesis function in alfalfa. During the linkage analysis, however, irregularities in inheritance (extreme distorted segregation) have been observed. This phenomenon forced us to apply different approache apart from mathematical calculation of linkage to establish linkage groups and gene orders of tightly linked markers.

L-N-1

MOLECULAR PHYLOGENETIC PERSPECTIVES ON THE ORIGINS AND EVOLUTION OF NODULATION IN THE LEGUME FAMILY

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Nodulation is not universal in Leguminosae, so the question arises as to when and how the syndrome evolved in the family. Addressing these questions requires a robust phylogenetic hypothesis for the family, which has not been available previously. Molecular phylogenetic results, in particular from the chloroplast gene *rbcL*, have provided hypotheses of evolutionary relationships of the three subfamilies and most of the tribes of the family. These results agree with traditional classifications and ongoing morphological phylogenetic analysis (J. Chappill, U. of Western Australia) in suggesting that nodulation may have originated three separate times in the family: 1) in the largest subfamily, Papilionoideae, which includes all of the major cultivated legumes; 2) in the lineage leading to Mimosoideae; and 3) in the caesalpinioid genus *Chamaecrista*. If this is the case, nodules and nodulation are not homologous in species of these three groups. This is the most parsimonious hypothesis, requiring fewer separate evolutionary events than, for example, a single origin followed by numerous independent losses of the syndrome. However, it is perhaps not the most likely, given the complexity of the nodulation syndrome, the apparent homologies of the basic nodule types found in all three groups, and the ease with which the ability to nodulate could potentially be lost. Tests of nodule homologies have been proposed that utilize multigene families among whose members are genes expressed in the nodule, including but not limited to "nodulin" genes in the classic sense. These tests involve determining the orthology relationships of genes expressed in nodules for two or more of the groups that are hypothesized to have originated nodulation independently. The basic idea is that independent origins of nodulation could involve non-homologous or paralogous genes, the latter produced either by independent recruitment or duplication events. Early results with glutamine synthetase suggest that the nodule-expressed GS gene of *Chamaecrista* is not orthologous with such genes in Papilionoideae, suggesting that nodulation in *Chamaecrista* and Papilionoideae may indeed be separate evolutionary events. Phylogenetic results also have implications for understanding the evolutionary polarity of differences in nodulation within Papilionoideae. For example, it appears that determinate types of nodules have arisen from ancestors with indeterminate types. It also seems likely that determinate types (e.g., the Desmodioid type of Corby) have arisen more than once in the subfamily.

CO-EVOLUTION OF LEGUME-RHIZOBIAL SYMBIOSES: IS IT ESSENTIAL FOR EITHER PARTNER?

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The paper will consider legumes and rhizobia in turn and then their interaction.

1. Legumes

There is now good evidence that potentially nodulated plants, both legume and actinorhizal form a district branch (clade) of dicotyledenous angiosperms. This raises several questions, including:-

(a) What was the selection pressure which led to the formation of the nodulating clade?

(b) Why are all genera in this clade not nodulated?

(c) Have non-nodulating members of the clade never acquired the ability, have they had it and lost it or are both alternatives possible?

2. Rhizobia

Accepting that rhizobia evolved long before legumes also raises several questions, including:-

(a) Was nitrogenase universal in primitive rhizobia?

(b) Were primitive rhizobia autotrophic (chemo- and/or photo-), heterotrophic or both?

(c) Are progeny of primitive rhizobia present in extant environments which have never been exposed to legumes?

3. Symbioses

Both nodulating legumes and their rhizobia can grow and reproduce in the absence of potential partners. Whilst the benefit to the plant of a nitrogen-fixing endosymbiont outweighs the high cost under certain conditions, the benefits to the rhizobia are much less clear. It will be argued that there is a wide spectrum of legume-rhizobial interactions. At one extreme both partners are better off living independently: at the other a high level of specificity, interdependence and co-evolution is advantageous.

L-N-3

INTERACTIONS BETWEEN DIAZOTROPHS AND GRASSES

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Nitrogen-fixing bacteria of the genus *Azoarcus* were found in high numbers in roots of Kallar grass in the Punjab of Pakistan. So far they have not been isolated from other grasses and cereals. Phylogenetic sequence analysis of structural genes of nitrogenase (*nifH*) coupled with analysis of *nifH*-specific PCR products from plant roots revealed, that *Azoarcus* spp. have a wider host range, being present in Asian rice roots, as well. Transcriptional reporter gene fusions of *nifH* with the ß-glucuronidase gene indicated that *Azoarcus* sp. expresses nitrogenase *in planta*, inside rice roots in gnotobiotic culture. Additionally, we are studying a unique feature of nitrogen fixation in *Azoarcus* sp., the formation if diazosomes. These intracytoplasmic membrane stacks are related to a special physiological state, when nitrogen-fixation rates and respiratory efficiency are increased. In order to understand functions of these structures and regulation of genes involved, we used several approaches. For analysis of differential gene expressio, we assessed global changes in protein composition by two-dimensional gel electrophoresis. We also characterized a mutant which is not capable of diazosome formation.

L-N-4

HAVE COMMON PLANT SYSTEMS CO-EVOLVED IN FUNGAL AND BACTERIAL ROOT SYMBIOSES?

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The ability of root systems to establish beneficial symbiotic relationships with soil microorganisms represents one of the most successful strategies that land plants have developed to cope with abiotic and biotic stresses. The two main categories of root symbioses found in extant plant taxa, arbuscular mycorrhiza and nodules, are both formed by legumes. Arbuscular mycorrhiza appeared very early in the history of land plants, well before legumes, and the fungal partners have successfully adapted to recent taxa during their evolution from primitive plants, so that the symbiosis is now widespread throughout the plant kingdom. At first sight the two root symbioses appear to share little in common apart from the cortical parenchyma being the same target tissue for mycorrhiza development and nodule organogenesis, and the formation of an extended interface between symbiont cells, but closer consideration of genetic and cellular events has revealed the existence of unexpectedly similar features. Studies of different legumes have shown that non-nodulating genotypes can often be altered in their mycorrhiza-forming abilities, and genetic analyses of isogenic pea mutants has provided evidence that some early infection stages are modulated by common plant genes in both symbioses. The most frequent mutant phenotype is characterised by complete resistance to the microsymbionts, and the three mutated loci identified so far as being responsible correspond to *sym* nodulation genes. Resistance-related reactions are strongly induced by arbuscular mycorrhizal fungi in these mutants suggesting that, as for *Rhizobium* interactions, suppression of plant defence responses is essential to symbiosis establishment.

Flavonoids which elicit *nod* genes can activate growth of arbuscular mycorrhizal fungi, and similar cellular events can accompany plant-microbe interactions in the two symbioses. A number of wall or membrane components linked to the nodulation process are associated with comparable plant-derived cell structures in host-fungal interfaces in mycorrhizal tissues, and some nodulin genes also appear to be activated in mycorrhizal roots. Whilst differences do obviously exist between the two symbiotic systems, more information about the extent to which they are regulated by common plant functions will greatly contribute to understanding how generalised the mechanisms involved may be. Moreover, seeing the ancestral nature of arbuscular mycorrhiza, we speculate that part of the plant processes leading to nodulation may have evolved from those already established for the fungal symbiosis.

L-N-5

"NITROGEN FIXING" SYSTEMS AND EVOLUTION OF PLANT HEMOGLOBINS

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The ability to reduce atmospheric nitrogen to biologically active forms is limited to a small group of prokaryotic organisms. For plants, the most effective ways of access to this source of nitrogen are symbioses with such bacteria like *Rhisobium* or *Frankia*.

Some plant species have evolved complicated communication systems with their bacterial symbionts. In the symbiotic systems, a part of the energy needed for the nitrogen fixation is supplied by the plant. In addition, a host plant protein - hemoglobin protects the bacterial oxygen sensitive elements.

Hemoglobin is known to be present in all nitrogen fixing systems analysed thus far. For a long time, the so called „symbiotic" type of hemoglobin was thought to be the only existing plant type of hemoglobin. A discovery of hemoglobin genes in nonnodulating plants have changed the understanding of the function and evolution of hemoglobins in the plant kingdom. Induction of the expression of these genes in response to anaerobic stress suggests that they play a more general function than that restricted only to the nitrogen fixation.

Because of the wide presence of hemoglobins in different types of nodules and in tissues of nonnodulating plants, the analysis of their amino acid sequences and structures of genes coding them allows to draw conclusions concerning the evolution of legume and nonlegune symbioses.

High sequence similarity of the „symbiotic" nonlegume hemoglobins to the „nonsymbiotic" hemoglobins may suggest that these hemoglobins emerged during the evolution after the duplication of hemoglobin genes had occurred. Symbioses of nonlegume plants may represent a „secondary" evolution of hemoglobin genes - a relatively recent event which occurred after the division of legume and nonlegume families. Lupin is probably the oldest genus which appeared as a first legume „branch" after this division. The evolution of the symbiotic type of hemoglobins seems to be accompanied by a substantial loss of amino acids in the polypeptide.

POSTER CONTRIBUTIONS

P-A-1

MUTATIONS IN TWO NITROGEN REGULATORY GENES AFFECT SYMBIOTIC SIGNAL PRODUCTION BY *RHIZOBIUM MELILOTI*

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The development of the *Rhizobium*-legume symbiosis is dependent on genes encoded by both organisms, and is governed by specific signal exchanges. The production of the first bacterial signal, the Nod factor, and the succinoglycan (EPSI), involved in nodule development, is controlled by the level of combined nitrogen (ammonia).

Two genes, *glnB* and *ntrR* have been studied for their role in the nitrogen regulation of signal production. The *glnB* gene is a member of the central nitrogen regulatory (*ntr*) system, which was shown to transmit the signal of nitrogen status to the regulation of *nod* genes, responsible for Nod factor synthesis. The *glnB* gene of *Rhizobium meliloti* has been identified and sequenced. A putative strong NtrC binding site and an NtrA binding site were found in the promoter region. The effect of *glnB* mutation on nitrogen assimilation (GSII), on signal production (Nod factor, EPSI), and on the efficiency of symbiosis with alfalfa was studied. The *ntrR* gene was shown to be involved in the repression of *nod* genes in nitrogen-excess conditions. The *ntrR* gene of *Rhizobium meliloti* was isolated and sequenced. An *ntrR-lacZ* fusion was generated to follow the expression of *ntrR* in free-living and in nitrogen fixing conditions. In a middle-scale plant test the symbiotic efficiency of the wild type and the *ntrR* mutant strains was compared. In the presence of combined nitrogen the inoculation by the mutant strain resulted in a 19% increase in plant dry weight, and 24% increase in nitrogen content of the shoot when compared to wild type inoculated plants.

P-A-2

IS *NOLR* A GENERAL REPRESSOR OF NODULATION GENES IN RHIZOBIA?

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Interaction of host plant flavonoids with the activator NodD proteins induces expression of nodulation genes in rhizobia. In many species nod gene expression is finely controlled and most likely involves additional regulatory proteins. Negative regulation of nodulation genes has been demonstrated in *Rhizobium meliloti* where the NolR repressor downregulating the common but not the host specific nodulation genes contributes to optimal nodulation of the host plant *Medicago* (Kondorosi et al, 1989, 1991; Cren et al, 1995). Negative regulation of nodulation genes has been proposed also for other *Rhizobium* species (Banfalvi et al, 1988; Schlaman et al, 1990; Firmin et al, 1993). One of the most evident cases for negative regulation appears to be in *R. leguminosarum bv viciae* strain TOM. This strain differs from other *R. leguminosarum bv viciae* strains by the presence of the host specific *nodX* gene and lower level of nodulation gene expression (Firmin et al, 1993). We investigated whether in strain TOM downregulation of nodulation genes is evoked by a NolR homolog repressor. Our studies demonstrate the presence, high conservation as well as the repressor function of the *nolR* gene in strain TOM. Southern hybridization of genomic DNA from different Rhizobium species to the internal *nolR* probe indicates the existance of *nolR* homolog gene in several species. These data suggest that NolR might act as a general repressor and involved in fine tuning of nodulation gene expression at least in certain classes of rhizobia.

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P-A-3

ROLE OF CHITINASES IN *SESBANIA ROSTRATA* STEM NODULE DEVELOPMENT

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For long it has been shown that chitinases are important components of the plant defense machinery against microbial attack. Two class III chitinase gene homologues, *didi-13* and *didi-24*, were isolated from mRNA of infected dormant root primordia and were shown to be specifically induced after treatment with *Azorhizobium caulinodans*. Do plants defend themselves against symbiosis? Are the defense mechanism tools converted to work for nodule development so that chitinases have a more direct function? Nod factors, the trigger for nodule organogenesis, are chitooligomers beautified with some decorating groups and can be a substrate for chitinases. Moreover some class III chitinases are known to have lysozyme activity and can, therefore, kill rhizobia. These observations let us to select the *didi-13* and *didi-24* clones for further characterization amongst several other clones which were all isolated by differential display and shown to be induced after infection.

Using Northern analysis, it was shown that the observed induction of the genes was a direct effect of the presence of the nodulating bacteria. A *nodA*⁻ mutant could not induce the genes whereas a lipopoly-saccharide mutant could, indicating that maybe Nod factors trigger the induction.

Furthermore, using *in situ* hybridization, it was shown that both genes have a completely different expression pattern. *Didi-24* transcripts were detected in one of the outermost cell layers of the root primordia whereas *didi-13* was expressed mainly in cells surrounding the nodule primordia and vascular bundles. During central tissue development *didi-13* transcripts were also detected in non infected cells. In mature nodules both genes are switched off. These expression patterns led us believe that both chitinases do have an active role during nodulation.

To investigate if both chitinases can degrade Nod factors, both proteins will be overproduced in *Escherichia coli* and using radiolabeled Nod factors as substrate, their activity will be analyzed. Preliminary experiments showed that in less than 30 minutes, radiolabeled Nod factors are degraded by crude protein extracts of induced primordia whereas even after overnight incubation non infected primordia almost do not have an effect.

P-A-4

Nod-factors and tri-Iodobenzoic acid Synergistically Stimulate Mycorrhizal Colonization, Chitinase and Trehalase Activities in Roots of *Lablab purpureus* (L.) Sweet

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In preliminary studies, the effect of the auxin transport inhibitor *tri*-iodobenzoic acid (TIBA) on root morphogenesis was tested on eight legume species. Subsequently, *Glycine max*, a legume only weakly affected by TIBA, and *Lablab purpureus* which reacted strongly, were treated with TIBA, or with Nod-factors purified from *Rhizobium* sp. NGR 234. Acetylated Nod-factors were only able to induce pseudo-nodules in the presence of TIBA on soybean. Either alone or in combination, both Nod-factors and TIBA elicited pseudo-nodules on *Lablab*, an effect that was also mimicked by kinetin application. Colonisation by the mycorrhizal fungus *Glomus mosseae* was increased when roots were treated with these growth regulators. Activities of chitinase and trehalase were enhanced in all pseudo-nodules. Both enzyme activities were slightly enhanced in sterile and mycorrhizal roots by the treatment with Nod-factors or TIBA. Morover, chitinase and trehalase activites in mycorrhizal roots were strongly enhanced when Nod-factors and TIBA were added in combination suggesting synergism between these growth regulators. Specific chitinase isoforms were strongly induced in pseudo-nodules and mycorrhizal roots. Activity gels of sterile roots treated with Nod-factors and TIBA showed a cluster of bands which were not detected following treatment with either substance separately. Yet, kinetin in combination with TIBA showed the same inducing effect as Nod-factors/ TIBA indicating that Nod-factors can replace kinetin under certain circumstances. Trehalase activity was enhanced in pseudo-nodules of both *Glycine* and *Lablab*.

P-A-5

The *O*-acetyl group at the non reducing end protects rhizobial nodulation signals against degradation by root hydrolases

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Nod factors (lipochitooligosaccharides) act as potent morphogens, triggering root nodule development at nanomolar concentrations. Structural variations at the reducing and non-reducing end determine the host-specific activity of Nod factors (1). For instance, the sulfate group at the reducing end of *R. meliloti* Nod factors is required for the activity on *Medicago* (2). Other structural parameters, as the length of the oligosaccharide backbone, the structure of the acyl chain, and the presence of an *O*-acetyl group at the non-reducing end, also influence the activity of Nod factors. The *O*-acetyl group enhances the nodulation on *Medicago* (3), but it has no effect on a rapid response (as plasma membrane depolarisation of root hairs) (4). This could be explained by the existence of different receptors in root hairs and cortical cells. Alternatively, nodule induction may require a prolonged presence of Nod factors in the rhizosphere. Since Nod factors are rapidly degraded by root hydrolases to inactive molecules (5,6), the different substituents of Nod factors may function by enhancing the stability. It has been previously shown that the sulfate group can protect Nod factors against degradation by class I chitinases (5), whereas it does not affect degradation by several other enzymes. To test whether *O*-acetylation at the non-reducing end affects Nod signal degradation, we have compared the stability of different Nod factors (NodRm-IV(Ac,C16:2,S), NodRm-IV(C16:2,S), NodRm-V(C16:2,S) and NodRm-V(Ac,C16:2,S)). We have shown that the *O*-acetyl group protects the Nod factors against hydrolysis by class I and III chitinases, and by a Nod factor specific hydrolase of *Medicago* (6). Furthermore, we have inoculated *Medicago* roots with *R. meliloti*, and followed the fate of the Nod factors, produced by the bacteria, in the rhizosphere. They are rapidly degraded to acylated dimers and trimers. After 48 h, only one Nod factor (NodRm-IV(Ac,C16:2,S)) is detectable in the rhizosphere. This Nod factor is the most active one in "long term" assays (leading to root nodule formation). These results suggest that Nod signal activity could be controlled by hydrolytic enzymes.

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P-A-6

EXTENT OF GENE EXPRESSION IN RHIZOBIA FOLLOWING EXPOSURE TO FLAVONOIDS AND OTHER AROMATIC COMPOUNDS

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Three techniques were employed to identify genes, other than *nod* genes, in *Rhizobium* sp. NGR234 which were either expressed in the presence of flavonoids or involved in the metabolism of aromatic compounds: RNA arbitrarily primed PCR (RAP - PCR); differential RNA hybridisation of *Xho*1 digests of an ordered cosmid library of the NGR234 symbiotic plasmid[1]; PCR amplification of NGR234 DNA using degenerate primers to target genes required for aromatic metabolism prior to sequencing and expression analyses.

RAP - PCR, applied to uninduced and daidzein - induced cultures of NGR234, identified a number of cDNAs with significantly increased expression in the presence of the isoflavonoid, including a 254 base pair fragment which had 93% homology to a flavonoid inducible cultivar specificity gene in *R. fredii* : *nolU*.[2] Hybridizations of Southern blots of a cosmid library digest of the NGR234 symbiotic plasmid with labelled RNA from uninduced or daidzein - induced NGR234 cultures also confirmed that the expression of many genes, in addition to *nod* genes, was induced by this isoflavone. Degenerate primer PCR was used to target NGR234 genes involved in catabolism of the monocyclic aromatic, protocatechuate: protocatechuate 3,4 dioxygenase genes (*pcaHG*), responsible for conversion of protocatechuate to beta - carboxy - *cis*, *cis* - muconate, and a regulatory gene, *pcaQ*, which has recently been shown to control the expression of five structural genes in the protocatechuate branch of the beta - ketoadipate pathway (including *pcaHG*) in *Agrobacterium tumefaciens*.[3]

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P-A-7

NOD FACTORS OF *AZORHIZOBIUM CAULINODANS* : STRUCTURE AND SECRETION

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Structural analysis of lipo-chitooligosaccharide (LCO) Nod factors synthesized by *Azorhizobium caulinodans* ORS571 revealed a complex population of LCOs. The majority is pentameric, a small fraction is tetrameric. The acyl chain is either a palmitoyl, a vaccenoyl or a stearoyl group. The non-reducing end is N-methylated and 6-O-carbamoylated whereas the reducing end can be substituted with a fucose and/or an arabinose residue. Data will be presented showing that the fucose and the arabinose residue are detected at the C6- and the C3-position, respectively. Azorhizobial Nod factors are synthesized upon expression of the regulator gene *nodD*, the *nodABCSUIJZnoeCD* operon and the *nolK* gene.

NodU is necessary and sufficient for carbamoylation because introduction of *nodU* in a *nodU* mutant restored the carbamoylation and because an *Escherichia coli* strain expressing the *nodABCSU* genes synthesized carbamoylated LCOs. An *in vitro* assay for carbamoyltransferase activity is presently worked out.

Based on the structure of LCOs produced by a *nodZ* and a *noeC* mutant and on an *in vitro* fucosylation assay, we propose that NodZ is a fucosyltransferase that uses GDP-fucose as a donor and that *noeC* and/or downstream genes are involved in arabinosylation. Fucosylation in a *nolK* mutant is completely abolished and NolK shows sequence similarity with *E. coli* proteins, supposed to be involved in the synthesis of GDP-fucose and GDP-colitose, suggesting that NolK is probably involved in the synthesis of GDP-fucose.

Nod factors are secreted by a NodIJ transport system as we demonstrated that in *E. coli* NodI as well as NodJ are necessary to secrete LCOs and that introduction of *nodJ* in a *nodJ* mutant restores secretion to wild-type levels. Using *E. coli* as a test system, the specificity of the NodIJ transporter will be determined.

P-A-8

PRODUCTION OF NODULATION FACTORS BY *RHIZOBIUM MELILOTI*: FERMENTATION, PURIFICATION AND CHARACTERISATION OF GLYCOLIPIDS

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Abstract

Lipooligosaccharides, synthesised by soil bacteria of the genera *Rhizobium*, are known to have multifunctional effects on a wide variety of plants as signal substances in symbiosis initiation, cell response elicitation and growth regulation. These so called nodulation (Nod-) factors represent interesting biotechnological products with respect to fundamental studies of symbiotic interactions as well as for potential applications. Therefore, a batch fermentation process on a scale of 30 l has been developed by means of the *Rhizobium meliloti* strain R.m.1021(pEK327) strongly overexpressing the genes for the synthesis of Nod factors. Induction by the flavone luteolin led to growth associated production of the lipooligosaccharides. Ultrafiltration was used for separating the biomass from the filtrate containing the extracellular Nod factors. Simultaneously, ultrafiltration reduced the amount of lipophilic substances, which would otherwise interfere with processes downstream. The second separation step consisted in adsorption on XAD-2, a nonspecific hydrophobic adsorptive resin. Adsorption of Nod factors was carried out by batch operation of a stirred tank. Desorption was performed by elution with methanol in a fixed bed column. A semi-preparative reversed phase HPLC (Polygoprep 100-30 C18) was chosen as the final purification step. The Nod factors were obtained after evaporation and lyophilisation. Thus, about 600 mg of Nod factors were produced from 20 l of fermentation broth. The Nod factors produced by *Rhizobium meliloti* R.m.1021(pEK327) were identified by liquid secondary ion mass spectrometry and by reversed-phase HPLC as fluorescent derivatives of 2-amino-benzamide. The biological activity of the products was demonstrated by means of the root hair deformation (HAD-) assay.

P-A-9

CALCOFLUOR, AN INHIBITOR OF CELLULOSE CRYSTALLISATION, ACTS AS A FUNCTIONAL ANALOGUE OF *RHIZOBIUM*-DERIVED LIPOCHITIN OLIGOSACCHARIDES

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We propose that the primary action of *Rhizobium*-derived lipochitin oligosaccharides (LCOs) is to disrupt some aspect of cellulose crystallisation (or biosynthesis) by direct action at the plasma membrane-cell wall interface. As a structural analogue of the nascent cellulose chain, the β,1-4 linked N-acetyl glucosamine backbone of LCO is presumably of prime importance in achieving this effect. According to this model, the effects of LCOs that have been observed in treated root hairs (e.g. membrane depolarisation; oscillations in cytoplasmic calcium; reorganisation of the cytoskeleton) would be downstream consequences of a primary disruption of cellulose biosynthesis at the plasma membrane. Subsequent effects of LCOs that have been observed in the root cortex and pericycle (e.g. reactivation of the cell cycle; cortical cell divisions; induction of early nodulin genes) would be downstream consequences of the initial cellular stress or wound-type response resulting from a perceived disruption in cellulose biosynthesis by LCO. A testable prediction of our model is that Calcofluor, an inhibitor of cellulose crystallisation, might act as a functional analogue of LCOs. We therefore investigated the effect of a transient application of Calcofluor on root hair deformation, transcriptional activation of "early" nodule-specific genes, and cortical cell division. We incubated pea roots for two hours in a solution of Calcofluor. This treatment induced deformation and swelling in the growing tip of root hairs. The morphology and timing of swelling was similar to the effects previously shown to be caused by the application of *Rhizobium*-derived LCOs. We then investigated whether the application of Calcofluor to pea roots induced the transcription of nodule-specific genes (nodulins). Our preliminary data show that ENOD12A expression was induced in plants sampled 48h after a 5 min. treatment with Calcofluor. We also tested whether plants treated with Calcofluor for 5 min. could induce the early stages of root nodule formation. No true nodules were formed on these plants, but large cortical swelling were occasionally observed on the primary roots. This suggests that Calcofluor treatment may lead to cortical cell divisions. These swellings were more frequent when Calcofluor-treated roots were also inoculated with a non-nodulating strain of *Rhizobium leguminosarum* (8401) that was unable to synthesise LCOs. Thus, our preliminary evidence indicates that transient application to pea roots of Calcofluor induces several effects similar to those of lipochitin oligosaccharides. These observations are consistent with our proposal that the primary action of LCO may be to disrupt biogenesis of a β-linked cell wall polymer (probably cellulose).

P-A-10

REGULATION OF *nolXWBTUV*, A LOCUS THAT CONTROLS NODULATION OF SOYBEAN BY *RHIZOBIUM FREDII*

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nolXWBTUV is a unique sym plasmid locus that negatively governs the ability of *Rhizobium fredii* strain USDA257 to nodulate many soybean cultivars. *nolW* is expressed constitutively, but both *nolX* and the *nolBTUV* transcriptional unit are inducible by flavonoid signals, even though they are not associated with *nod* box promoters. Neither *nolX* nor *nolBTUV* is expressed in a *nodD1*-negative genetic background, and so we employed gel retardation assays to seek cellular proteins (NodD1?) that interact with their promoters. Cellular proteins from both flavonoid-induced and uninduced cells retarded migration of a DNA fragment containing the *nolX* promoter, but not one containing the *nolBTUV* promoter. Retardation of the *nolX* fragment was *nodD1*-dependent, and the target site was localized on a 114 bp *Sac*II/*Taq*I fragment that begins 188 bp upstream of the transcript start site. Deletion of this fragment elevates expression of the gene, with no effect of flavonoid inducibility. *nodD*-dependent regulation of *nolX* and *nolBTUV* thus differs fundamentally from that of conventional *nod* genes.

P-A-11

INFECTION OF *SESBANIA ROSTRATA* BY *AZORHIZOBIUM CAULINODANS* ORS571 WITH A *lacZ* REPORTER GENE

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Azorhizobium caulinodans ORS571 nodulates stems and roots of the tropical legume *Sesbania rostrata* after infection by crack entry. However, the early stages of infection require further clarification. These early stages of infection are being studied using an ORS571 derivative harbouring a *lacZ* reporter gene. Germinating seeds of *Sesbania* growing in N-free medium in tubes were inoculated with ORS571 pXLGD4. The blue histochemical localisation of ß-galactosidase activity following *lacZ* gene expression in bacteria was revealed by incubating infected tissue, from 1-4 week old seedlings, with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal).

In transverse sections of primary roots of *Sesbania*, the blue-staining azorhizobia were seen within developing lateral roots, between the pericycle and the epidermis. Blue-staining azorhizobia were not observed within root hairs. This supports the suggestion[1] that crack entry is the mode of infection in roots of *S. rostrata*, and that the classic root hair curling pathway is not involved in root infection. Longitudinal stem sections stained with toluidine blue revealed what may be azorhizobia in the xylem; they appeared as darkly staining globular internal zones surrounded by paler, irregular curviform sheaths. Bacteria were not found in uninoculated material. Currently, the *lacZ* histochemical procedure is being used to confirm this observation, to investigate the possibility of infection and nodulation via the vascular system, and to increase our understanding of early stages of crack entry.

We have recently demonstrated that ORS571 is able to infect both wheat and *Arabidopsis thaliana* at the point of emergence of lateral roots[2]. Presently, ORS571 is being interacted with pot and tube-grown seedlings of wheat, oilseed rape and rice. Such infection by azorhizobia often modifies root morphology to give a high proportion of short lateral roots. A comparative assessment of infection in *Sesbania rostrata* and in these non-legume crops is being undertaken, using this *lacZ*-marked strain, to improve our knowledge of the early stages of the interaction of *A. caulinodans* with non-legumes.

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P-B-1

CYCLIN GENES IN YELLOW LUPINE

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The cell division in all *Eukaryota* is governed by a complex of $p34^{cdc2}$ protein kinase and cyclin, where the kinase composes a catalytic subunit and the cyclin has regulatory function.

We are interested in the role of different cyclins in regulation of lupine plant development and formation of root nodule.

Four clones, coding for putative cyclin proteins, were isolated (using soybean cyclin cDNA as a probe) from cDNA library of lupine roots infected with *Bradyrhizobium lupini*. The complete cDNA clone, *CycB1a-ll* is 1692 bp in length and encodes a putative 48.7 kDa protein consisting of 429 amino acids. The second full length 1796 bp clone, *CycB1d-ll* codes for 475 amino acid protein of predicted molecular weight 52.8 kDa. The two other clones: *CycB1b-ll* and *CycB1c-ll* comprise truncated cyclin cDNAs. Comparison of deduced amino acid sequences of four lupine cyclins showed the highest similarity of *CycB1c-ll* and *CycB1d-ll* (76% homology, 65% identity) and the lowest - *CycB1a-ll* and *CycB1b-ll* (64% and 46%, respectively).

The predicted proteins contain conserved *cyclin box* and other elements characteristic for plant mitotic cyclins, allowing to classify them as B1 type. Three clones *CycB1a-ll, CycB1c-ll* and *CycB1d-ll* comprise *destruction box* near amino terminus, whereas one of them, *CycB1c-ll*, posses additionally PEST-like sequence (both elements are responsible for rapid protein degradation).

The phylogenetic tree of plant mitotic cyclins was constructed by alignment of 37 adequate amino acid sequences.

Northern analysis of lupine cyclin expression during nodule formation showed higher transcript level in roots infected with *Bradyrhizobium* (in comparison with uninfected ones) from 4 to 16 days after inoculation. Since cyclins are expressed only in few cells during short periods of time, we plan to use more sensitive methods, RT-PCR and *in situ* hybridization, to determine the role of particular cyclins during plant developmental processes, including nodule formation.

We have also isolated four clones from lupine genomic DNA library, using one of cyclin cDNAs as a probe. We intend to analyze cyclin promoters, fused to GUS coding sequence, in transgenic plants. This experiment should help in further detailed characterization of cyclin expression in different types of dividing cells.

P-B-2

EXPRESSION OF GENES ENCODING ROOT-HAIR SPECIFIC EXTENSIN-LIKE PROTEINS IS MODULATED BY RHIZOBIA

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Three cDNAs (*ext*3, *ext*127 and *ext*26), isolated by differential screening of the *Vigna unguiculata* root-hair cDNA library, encode root-hair specific extensin-like cell wall proteins. Expression of the homologous genes is negatively regulated by inoculation with symbiosis competent *Rhizobium* sp. NGR234. Levels of transcripts which are abundant in untreated 4 day old root-hairs of *V. unguiculata* are drastically reduced. Modification of mRNA levels occurs as early as 1 day after inoculation with rhizobia and coincides with root-hair deformation. Interestingly, a mutant deficient in Nod-factor production (NGRΔ*nodABC*) also reduces transcript levels. Differences in transcript levels after inoculation with wild-type NGR234 and the mutant suggest that a part in the reduction of transcript levels can be attributed to the activity of Nod-factors. Another part is under control of a yet unidentified secondary signal(s). In contrast, inoculation with a rhizobial strain carryng a mutation in a gene encoding a transcriptional activator for *nod*-genes (NGRΔ*nodD*1) increased mRNA levels of *ext*3 and *ext*26, indicating that this strain is not recognized by plants as a symbiotic bacterium. Application of purified NodNGR factors themselves did not mimic the effect of the bacteria. The genomic locus corresponding to *ext*26 (Ext26G) was isolated. It possesses several potential TATA boxes and CAP signals in the 5' flanking region. Part of the promoter region shares homology with the *Pisum sativum* seed lectin promoter and *Nicotiana tabacum* nitrate reductase promoter. Nonetheless, the function of the homologous regions in gene regulation is unknown.

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P-B-3

CHARACTERIZATION OF *CIS*-ELEMENTS OF *Msenod40* PROMOTERS IN TRANSGENIC ALFALFA PLANTS

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Msenod40 encodes a gene product that is involved in the interaction between alfalfa and *Rhizobium meliloti*. We have found that *Msenod40* gene expression can be induced in alfalfa roots not only by Nod factor, but also by exogenous cytokinin. We are interested in the promoter elements of *Msenod40* that are important for its induction by cytokinin or Nod factor. We have isolated genomic clones of two *Msenod40* genes from alfalfa genomic library, and fused their promoter regions to the reporter gene, *uidA* (GUS). Alfalfa plants (*Medicago sativa* cv. Regen) were transformed with these constructs via *Agrobacterium tumefaciens*. In addition, a series of large deletions of one of promoters was also constructed and transformed into alfalfa. We found that the GUS expression directed by the two different promoters is not identical under non-symbiotic conditions. However, the two genes are expressed similarly during nodule development. Moreover, promoter deletion analysis showed that a 5' distal 604 bp-region is absolutely required for the correct expression of *Msenod40* in nodules as well as for the maximum induction of *Msenod40* expression by cytokinin and Nod factor. The responses of other truncated promoters to cytokinin and Nod factor application will be discussed.

P-B-4

CHARACTERIZATION OF TWO *ENOD40* CLONES ISOLATED FROM *SESBANIA ROSTRATA*

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An *enod40* clone of soybean (Yang *et al.*, 1993) was used to screen a cDNA library of *Sesbania rostrata*, a tropical legume on which nodules are formed not only on the roots but also at predetermined sites along the stem. This resulted in the identification of two clones, *Srenod40A* and *Srenod40B*. The former is thought to represent the full-length transcript and contains a sequence that encodes the conserved peptide. Using reverse transcriptase polymerase chain reaction, induction of both genes can be detected already at 8 hours after infection, both in the root primordia of the stems and in the roots, although the transcript is also present at a low level in non-induced material. The two *enod40* clones were used as probes for *in situ* hybridizations to study *enod40* expression during *Azorhizobium*-induced nodule formation. Non infected stem located root primordia, and developing stem nodules 1, 2, 3, 4, 6, and 20 days after infection were analyzed. The results show that the expression pattern of *Sesbania enod40* is comparable to that of the other legumes studied until now (Crespi *et al.*, 1994; Matvienko *et al.*, 1994; Vijn *et al.*, 1995). Moreover, a very localized signal has been observed also in non infected primordia, under the meristematic apical region. To investigate in more details this phenomenon, different meristematic regions from the plant (apical shoot and primary, lateral, and adventitious root tips) were prepared to check the eventual expression of the *Srenod40A* gene. Preliminary results show that very localized signals are present in all the regions tested.

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P-B-5

TWO *ENOD40* SEQUENCES FROM *LUPINUS LUTEUS*

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Enod40 homologous sequences from the evolutionary old legume plant *Lupinus luteus (1)* has been identified and evidences for the presence of two different *enod40* genes has been found. A first sequence *Llenod40A* was identified by RT-PCR amplification of cDNA from *Lupinus* nodules with specific primers for the *Medicago truncatula enod40* gene (2;*Mtenod40)* and a second one, *Llenod40B* was obtained by screening a *L. luteus* genomic library with the amplified probe. Sequence analysis of the identified clones indicated that the two sequences correspond rather to two different genes than to two alleles. Comparison of these sequences with the *enod40* genes from other legumes revealed the presence of the encoded small peptide of 12AA with high homology to other *enod40* peptides (3) in *Llenod40 B*. On the other hand, the nucleotide sequence of *Llenod40* A was highly homologous to *Mtenod40* whereas *Llenod40B* revealed lower conservation and certain deletions. Since morphogenesis of *Lupinus luteus* nodules is quite different from typical determinate and indeterminate nodule types, *in situ* hybrydization analysis of the expression patterns of these genes during nodulation is under investigation. These results may provide insight on the evolution of *enod40* sequences in legume plants.

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P-B-6

ISOLATION AND CHARACTERIZATION OF YELLOW LUPINE GENE CODING FOR NODULE-SPECIFIC PROLINE-RICH PROTEIN

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From the yellow lupine genomic library was isolated a clone containing gene coding for proline-rich protein. The core of this protein is made up of two groups of small pentapeptide units. The first is very conservative, but the second motif shows conservation only in the two first proline residues. In the predicted amino acid sequence was also identified a putative signal peptide. The sequence comparison of the whole protein with analogous sequences of known Enod2 proteins shows high level of homology and identity (around 60%).

In the promoter region was found several sequences which can regulate gene expression. Besides a conservative "TATA-box", as a result of comparing promoter regions of LlPRP2 and Enod2 genes was identified a 9 nt long A-T rich element. In the promoter were also found an element with high homology to a so-called "Organ-Specific Element", two long (150nt) DNA fragments forming a direct repeat sequence and a stretch of repeated (TA) motif. Also at 3' non-coding fragments were found some common sequences with other proline-rich protein encoding genes.

Analysis of expression of this gene was carried with the northern hybridization technique. In these experiments were used as probes: the fragment representing the 3'-end (almost half) of the gene and probes specific for the 5'- and 3'-ends (not containing these fragments of coding region which have repetitive properties). The results of hybridization experiments showed that the analyzed gene is expressed only in nodules. There is no expression in cotyledons, leaves, stems or flowers. The transcription begins 9 days after inoculation with *B. lupini*. To eliminate the possibility of non-specific hybridization in other experiments were used probes specific for noncoding ends. Obtained results led to three main conclusions: 1) there is only one transcript (about 3000 nt) containing poly(A) sequence, 2) this transcript is probably degraded from the 5'-end and, 3) in the RNA are present additionally at least two bands which do not contain a poly(A) sequence.

According to the results of hybridization with RNA, hybridization was performed with genomic DNA and DNA isolated from compartments in which the poliadenylation process does not occur. After hybridization were observed only lone signals always in a line containing nuclear DNA.

To examine whether the analyzed gene represents Enod2 gene family, an *in situ* hybridization experiment was performed. The results showed that this gene is expressed only in the infected, central tissue of the lupine nodule. For this reason it can be stated that the product of the gene from yellow lupine represents only a homologue of Enod2 nodulins.

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P-B-7

THE OVEREXPRESSION OF *ENOD40* INDUCES CORTICAL CELL DIVISIONS IN *MEDICAGO* PLANTS

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Rhizobium meliloti is able to induce the formation of nitrogen fixing nodules on the roots of *Medicago* spp. Certain *Medicago* plants can also form nodules spontaneously upon nitrogen starvation and we had characterized genes expressed in these nodules. One clone, *Msenod40*, homologous to the soybean early nodulin gene *Gmenod40* (1,2), was strongly expressed in differentiating cells of the nodule primordia and in spontaneous nodules, while minor levels were also detected in the root pericycle, stems and flowers. Several *enod40* genes have been sequenced and only a small ORF (12 or 13 aminoacids) (3) is common between them despite a strong conservation of the nucleotide sequence. We have shown that the *enod40* genes code presumably for structured RNAs and propose that these genes might act as "riboregulators"(4), a class of 3'UTR RNAs involved in differentiation. Recently, we succeeded in regenerating transgenic *Medicago truncatula* plants, a diploid autogamous species, overexpressing *enod40*. In normal conditions of growth, these plants did not show any particular phenotype. However, upon nitrogen starvation, root cortical cell divisions could be observed in the progeny of different independent transgenic plants overexpressing *enod40*. To gain further insight *enod40* action, we have also transformed a non-legume plant, *A. thaliana*, since the gene is not exclusively associated with nodule formation. The homozygotic transgenic plants showed a "short root" phenotype correlating to the level of transgene expression, but did not show cortical cell divisions.
In addition, we have developped a transient assay in alfalfa roots using the particule gun. The bombardment of *enod40* was shown to induce cortical cell divisions and *enod12* expression.
Based on all these results, we think that *enod40* might play a role in nodule initiation in legume plants and may affect certain cell responses to external elicitors in roots.

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P-B-8

STUDY OF THE DEVELOPMENT OF *SESBANIA ROSTRATA* STEM NODULES USING *IN SITU* HYBRIDIZATION

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A typical feature of the tropical legume *Sesbania rostrata* are the dormant root primordia which are lying in vertical rows along the stem of the plant. These root primordia develop into roots when immersed in water due to the activation of the apical located root meristem. Upon infection with the bacteria *Azorhizobium caulinodans*, ORS571 nodules appear due to the dedifferentiation of cortical cells at the base of the root primordium (Tsien *et al.* 1983; Duhoux, 1984). Stem nodulation is basically root nodulation but because of the special topology and abundance of nodulation sites, presents distinct advantages to study early stages of nodule development (Goormachtig *et al.*, 1995). To get more insight in the development of these stem nodules, *in situ* hybridizations were performed using genes as probes which have a well described expression pattern.
A cdc2 (Cdc2-1sr), mitotic cyclin (CycB1; Sr) and histone H4 (H4-1sr) clone were isolated from a *S. rostrata* developing stem nodule cDNA library and used as molecular markers to study the meristem organization during nodulation. Also the expression pattern of the *S. rostrata enod2* (Dehio and De Bruijn, 1991) gene was studied. Interpretation of these results revealed some typical §characteristics and it was demonstrated that although stem nodules are determinate, the development has more characteristics of indeterminate nodules.

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P-B-9

A PECULIAR cDNA CLONE FROM YELLOW LUPIN SELECTED WITH ALFALFA CHALCONE SYNTHASE cDNA SEQUENCE AS A PROBE

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Three distinctive cDNA clones (Ll CHS2-1, Ll CHS3-1, and Ll CHS4-1) have been selected from yellow lupin root and nodule cDNA library using alfalfa CHS encoding cDNA sequence as a probe (Ms CHS4-1 was obtained from Ann M. Hirsch). Two of the selected sequences (Ll CHS3-1 and Ll CHS4-1) reveal significant homology to each other, to their alfalfa counterpart, and to CHS encoding clones from soybean, pea and other legumes. Therefore, they can be considered yellow lupin chalcone synthase encoding cDNAs. The third clone (working designation: Ll CHS2-1), although hybridizing strongly with alfalfa CHS probe, shows only limited homology to its nucleotide sequence. It is also different from the remaining lupin clones revealing only about 40% of sequence homology with Ll CHS3-1 and Ll CHS4-1.
The genomic organization of Ll CHS2-1 is different from those of the two remaining clones, as it was shown by Southern blots probed with their inserts.
What is even more interesting, the expression patterns of three selected sequences also differ significantly. The "real" CHS clones (Ll CHS3-1 and Ll CHS4-1) reveal a typical for them expression in the uninfected roots including its strong, but transient enhancement shortly after inoculation, whereas Ll CHS2-1 can be detected by northern analysis only in the RNA preparation isolated from lupin roots harvested 6 hrs after inoculation. Its expression cannot be detected in uninfected roots.
It may be suggested that an interesting sequence has been selected showing limited homology to CHS, but expressed specifically at the early stages of the infection process.

P-B-10

THE CELL-SPECIFIC EXPRESSION PATTERN OF A CARBONIC ANHYDRASE GENE IS CONTROLLED BY THE PRESENCE OF *RHIZOBIUM* DURING NODULE DEVELOPMENT

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Under nitrogen starvation, *Rhizobium meliloti* is able to induce nitrogen-fixing nodules on the alfalfa roots. Certain alfalfa cultivars spontaneously develop pseudonodules in the absence of bacteria. We have identified a transcript, *Msca1*, expressed in spontaneous and *R. meliloti*-induced nodules, that codes for a carbonic anhydrase. *Msca1* was activated initially in all cells of the bacterially-induced nodule primordium and is also induced by cytokinin treatment of alfalfa roots. Thus, *Msca1* is a new early nodulin gene with a function that might be related to the increased carbon metabolism of the dividing cortical cells. During nodule development, *Msca1* transcripts were found mainly in the peripheral cells of the developing and mature nodules, as an envelope. This novel pattern of gene expression is presumably controlled by the presence of the bacterium inside the nodule. Sucrose synthase and phosphoenol pyruvate carboxylase, other genes of carbon metabolism, were expressed in the inner cortical cells but even stronger in the nitrogen-fixing region. These peripheral cells might be specialized for refixation of CO_2 leading to the establishment of the oxygen barrier required to nodule function. Thus, carbonic anhydrase may be involved in various carbon metabolism demands of different cell types in the root nodule.

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P-B-11

IDENTIFICATION OF *LOTUS JAPONICUS* NODULIN GENES BY THE DDRT-PCR METHOD

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We have set up a sensitive gene-identification system based on the differential display reverse transcription PCR method (DDRT-PCR) to isolate nodule expressed genes. We have chosen a methodology which could increase our chance of finding genes encoding low copy mRNAs and at the same time take an advantage of DDRT-PCR selectivity to discriminate between closely related mRNA species.

We have differentially displayed *Lotus japonicus* mRNAs isolated from different stages of nodule development. Two stages before the onset of nitrogen fixation were included since we are mainly interested in genes induced early in nodule development. Only a subset of genes showing induction of expression was chosen for further analysis after differential display. The genes were selected according to the observed expression pattern.

Sequence similarity between six differentially displayed genes and genes present in databases was already found as a result of analysis of PCR products. Similarities were pronounced both at the nucleotide and amino acid level.

The identified genes represent both known and unknown early and late nodulins. Some of unknown nodulin genes are homologous to genes whose biological function is already recognised.

In order to obtain uniformly and healthy developing seedlings we invented a method of growing hundreds of plants on a small surface. We have established this method especially for the purpose of this experiment in order to carefully control grow conditions. In the area of A4 format up to two thousands seedlings could grow in a stable and reproducible environmental conditions.

The efficiency of the system as well as a sequence analysis and expression pattern of the most interesting genes will be presented.

P-B-12

NEW cDNA CLONES CODING NODULINS FROM YELLOW LUPIN

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Using differential display reverse transcription PCR (DDRT-PCR) we have isolated many nodule specific expressed sequence tags (NSESTs) from twenty one day old yellow lupin nodule. Moreover, some sequences that are repressed in nodule in comparison to uninfected root tissues were also found. These sequences represent nodule repressed sequnence tags - NRSTs.

Specificity of several differentialy displayed probes were confirmed by northern hybridization. All probes, except dd4-A9, revealed on northern blot only one band. Hybridization pattern of dd4-A9 probe seems to be more coplex - two intense and three faint bands are visible.

Sequnce similarities between five cDNA clones identified by screening of cDNA library with differentialy displayed probes and sequences in data banks were analyzed. The identified clones represents the unknown genes. These genes have organ-specific properties and their characterization an the sequencing and transcriptional level is now in progress.

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P-B-13

IS THERE A NODULE SPECIFIC FERRITIN IN YELLOW LUPIN (*LUPINUS LUTEUS*) ?

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Iron-proteins (i.e. nitrogenase and leghemoglobin) are the main components of the nitrogen fixation chain, therefore nodules of the nitrogen fixing plants systems require high amounts of available iron. Ferritin is one of the key elements of the iron uptake and storage pathway, which is widely distributed among eukaryotes and prokaryotes. It is a protein with high capacity to sequester iron in a nontoxic form. Except for storage of iron for the synthesis of several proteins, ferritin also plays a detoxification role.

We investigated the expression of ferritin gene(s) (mRNA) in different lupin organs, especially in developing root nodules. Beside the forms of ferritin present in all analyzed tissues, hybridization data suggest the existence of another, nodule specific form. The concentration of ferritin mRNA seems to be coordinated with the development of infection and nitrogen fixation process. The highest level of transcription of ferritin gene(s) was observed in nodules around 24 days after infection with *Bradyrhizobium lupini*. Hybridization of the ferritin probe with the polyA$^+$ RNA confirms plant origin of both nodule specific and non-specific signals.

In two cDNA libraries, made from uninfected roots RNA and mixed root and root nodules RNA's, isolated 6, 14 and 21 days after the infection with *B. lupini* we have identified and sequenced thirty ferritin cDNA clones. These clones represented three different classes, with the highest sequence differences in the 3' and 5' nontranslated regions. The identity of the deduced amino acid sequences ranged between 78 - 82%. Only one of the classes, F43, was identified in the library from uninfected roots whereas all three were found in the "nodule" library.

PCR analysis using sets of synthetic primers specific to each ferritin clone is being performed to examine which (if any) of them is expressed in a nodule specific manner.

P-B-14

ISOLATION & CHARACTERIZATION OF SYMBIOTICALLY EXPRESSED SEQUENCE TAGS (SESTs) DURING THE EARLY STAGES OF INFECTION & NODULE DEVELOPMENT IN WHITE CLOVER

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Subtraction hybridization, differential display reverse transcription PCR (DDRT-PCR) and specific targeting of mRNAs using degenerate PCR primers, designed from conserved regions of known mRNAs from other genera or species, were used to isolate white clover root cDNAs which are uniquely or differentially expressed during the early stages of infection and nodule development. Subtraction hybridization of cDNAs from sterile and inoculated plant roots did not detect any uniquely induced plant genes (nodulins) in response to inoculation. Using DDRT-PCR, 90 differentially expressed cDNAs (dd-cDNAs) were detected and a selection of these was cloned and sequenced. Out of 40 of these dd-cDNAs which were sequenced, two were identified as *ENOD40* and phosphoglycerate mutase (PGM). Another dd-cDNA (dd23b) exhibited homology with *ENOD3* and *ENOD14*.

Using the gene targeting approach, clover homologues of *ENOD5*, cyclin and isoflavone reductase (IFR) were isolated and sequenced. Homologues of *ENOD12* or retinoblastoma (*Rb*) were not detected using this approach. Temporal RT-PCR expression analyses confirmed that *ENOD5*, *ENOD40*, cyclin, IFR, PGM, and four other dd-cDNAs were differentially expressed in clover roots following inoculation with *R. l.* bv. trifolii. One of the differentially displayed products, dd23b, was also found to be induced by treatment with *R. l.* bv trifolii Nod factor (CLOS).

In conclusion, a collection of white clover root symbiotically expressed sequence tags (SESTs), which are differentially expressed in the early stages of infection and nodule formation, has been isolated and characterised.

P-B-15

CLONING OF NODULE-SPECIFIC GENES OF *Galega orientalis* USING DIFFERENTIAL DISPLAY

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A cDNA-library representing mRNA of root nodules of *Galega orientalis* (goat's rue) consisting of 1.3 X 10^6 independent clones was constructed ino the Lambda ZAP® II vector. Differential display was applied in order to clone Genesexpressed exclusively or predominantly in nodule tissue compared to uninoculated root tissue. Seven fragments out of 45, which gave a nodule-specific signal in Northern hybridization, were cloned and subsequently sequenced. Two corresponding cDNAs were searched from the cDNA-library and sequenced. Three of the sequenced fragments were homologous to nodulin-gene sequences in databanks (EMBL, Genbank), among them *Glycine max* nodulin-20 and nodulin-75 and *Sesbania rostrata* ENOD2. The cDNA clone Ngo40 had a 556 bp region 63.3 % homologous to the *Medicago sativa* nodulin-25 gene (Nms25) and deduced repetitive amino acid sequences homologous with deduced amino acid sequences of Nms25 and the nodule-specific cDNA clone group 'VfNDS-L' of broadbean. Two of the fragments and the cDNA clone NgoM1 from the cDNA-library had no significant homology with sequences in the databanks.

P-B-16

ANALYSIS OF GENES EXPRESSED DURING ORGANOGENESIS AND FUNCTION OF BROAD BEAN (*Vicia faba* L.) ROOT NODULES

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A detailed screening for potential nodule-specific transcript sequences of a broad bean (*Vicia faba* L.) cDNA library constructed from root nodule polyA$^+$ RNA resulted in the isolation of 645 cDNA clones representing at least 44 different broad bean genes. Their expression was investigated in nodules, roots, leaves, seeds, epicotyls, stems, and flowers. Additionally, detailed sequence analysis were carried out for these clones. The transcripts exclusively detectable in nodules encode four different leghemoglobins (Lbs), the proline-rich proteins ENOD2, ENOD5, ENOD12, five different glycine-rich proteins (GRPs) not yet identified in other legumes, five different small cysteine-cluster proteins comparable to the *Pisum sativum* nodule specific proteins (nodulins) ENOD3/14, the modular nodulin NOD28/32 homologous to the *Medicago sativa* MsNOD25 and the ENOD18 showing no homology to known gene products. ENOD32, a gene encoding an $(\alpha/\beta)_8$-barrel nodulin with sequence similarities to class III chitinases is expressed in nodules and in flowers, whereas transcripts coding for ENOD40, a sucrose synthase, an extensin, and a lipoxygenase were detected in nodules and roots, but in a nodule-enhanced manner. By time-course experiments the ENOD12 gene was identified to be the first nodulin gene which is activated during broad bean nodule development. This gene is followed by ENOD2, the GRPs, ENOD32, ENOD5 and ENOD18 before the expression of the Lb genes and all other late nodulin genes started. Finally, CCP gene activity was detectable one day after Lb gene expression commenced. Furtheron, for most nodule-specific and nodule-enhanced transcripts the localization in the nodule tissues was established. The distribution of ENOD2, ENOD12, and sucrose synthase transcripts was essentially the same as in other legume nodules. The different GRP and CCP transcripts were exclusively located in the central nodule tissues, but differed significantly in their exact location. In addition, the expression of all 44 broad bean genes was analysed in roots devoid of rhizobial infections, which were infected by the mycorrhizal fungus *Glomus fasciculatum*. Interestingly, only one nodulin gene, the leghemoglobin gene Lb29, was expressed in mycorrhizal roots.

P-B-17

LOCALIZATION OF BROAD BEAN (*Vicia faba* L.) TRANSCRIPTS IN NODULE TISSUES BY NON-RADIOACTIVE DETECTION METHODS

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A *Vicia faba* cDNA library containing root nodule transcript sequences was screened for nodule-specific and nodule-amplified sequences by differential hybridization. The localization of most of these transcripts was determined in the nodule tissue by tissue-print hybridizations. For this purpose longitudinal sections of mature root nodules were printed onto nylon membranes to transfer the RNA. Afterwards the membranes were hybridized to Dig-labeled antisense-riboprobes.
The transcripts of most of the genes examined were detectable in the central nodule tissue. Transcripts coding for five different small proteins with four conserved cystein residues each showed heterogeneous expression-patterns in the central nodule tissue. These cystein cluster proteins (CCP's) are comparable to the *Pisum sativum* nodulins ENOD3/14. The same is true for transcripts coding for five glycine-rich proteins (GRPs) with an markedly high glycine-content. This group of proteins contains early and late nodulins which are expressed in different zones of the root nodule. The asparagine-synthetase gene VfAS1 is expressed in the nitrogen-fixing zone III and weaker in the interzone II-III.
On the other hand, transcripts from some broad bean genes, e.g. VfENOD2 and VfLOX1, were detected outside the infected nodule tissue in the parenchyma. The distribution of these transcripts in broad bean root nodules was determined not only by tissue-print, but also by *in situ* hybridizations. While VfLOX1 hybridization signals form a closed ring around the central tissue, VfENOD2 hybridization signals were not detected above the meristem. In adition VfLOX1 transcripts were detected in the vascular bundles.

P-B-18

ALTERNATIVE EXON COMBINATIONS ACCOUNT FOR A SPECIFIC MODULAR STRUCTURE OF THE *Vicia faba* L. NODULINS Nvf-28/32

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The broad bean gene VfNOD28/32 encodes late nodulins termed Nvf-28/32. These proteins are composed of two types of repeating sequence modules flanked by N- and C-terminal unique modules. The N-terminal unique module contains a putative signal peptide [Küster *et al.*, Plant Mol Biol 24:143-157, 1994]. Although the repetitive modules are homologous between the Nvf-28/32 proteins and the Nms-25 nodulin [Kiss *et al.*, Plant Mol Biol 14:467-475, 1990] from alfalfa, their number and order is different. Moreover, specific repetitive modules are deleted from six isoforms of the Nvf-28/32 nodulins resulting in a characteristic modular structure of each isoform. We infer that the function of the Nvf-28/32 proteins is related to the individual modules rather than to their number or order. Southern blots indicate the presence of only one copy of the VfNOD28/32 gene. Sequence analysis of genomic PCR-fragments revealed that each module of the Nvf-28/32 proteins is encoded by one particular exon. Since all VfNOD28/32 exons encoding repetitive modules deleted in the six Nvf-28/32 isoforms are present on the genomic sequence, we propose a posttranscriptional generation of six VfNOD28/32 transcripts by the alternative splicing of primary transcripts. As a step towards the identification of functions for the Nvf-28/32 proteins in the symbiosis, VfNOD28/32 transcripts were localized in root nodules by tissue-print hybridizations. In that way, hybridizing transcripts were localized predominantly in the nitrogen-fixing zone III indicating that the late nodulins Nvf-28/32 play a part in the functional phase of broad bean root nodules. We propose that these proteins are exported to the peribacteroid space. At this interface of the macro- and the microsymbiont, the Nvf-28/32 proteins could act as binding proteins for solutes, e.g. ions, with the binding being carried out by the individual sequence modules.

P-B-19

THE *Vicia faba* LEGHEMOGLOBIN GENE *VfLb29* IS INDUCED IN ROOT NODULES AND IN ROOTS COLONIZED BY THE ARBUSCULAR MYCORRHIZAL FUNGUS *Glomus fasciculatum*

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The majority of legume species can establish symbiotic associations with two important groups of rhizosphere microorganisms, bacteria of the genus *Rhizobium* and arbuscular mycorrhizal fungi. The *Rhizobium*-legume interaction leads to the formation of a specialized plant organ, the root nodule. Following cross-hybridization experiments and sequence analysis, a broad bean (*Vicia faba* L.) nodule-specific cDNA library was subdivided into 44 clone groups representing different genes activated in the root nodule. In order to investigate whether these genes are also induced in arbuscular mycorrhiza, we carried out expression analysis in broad bean nodules, uninfected roots and roots colonized by the mycorrhizal fungus *Glomus fasciculatum*. Five of the genes active in all these tissues showed an increased expression in the mycorrhizal roots. These genes code for a sucrose synthase, two proline-rich proteins and two proteins not described until now. Additionally, we demonstrated the exclusive expression of 19 broad bean genes, including *VfENOD2*, *VfENOD5*, *VfENOD12* and three different leghemoglobin genes, in root nodules. In contrast, the leghemoglobin gene *VfLb29* was found to be induced not only in root nodules, but also in broad bean roots colonized by the mycorrhizal fungus. In uninfected roots, none of the twenty nodulin transcripts investigated was detectable. VfLb29 has an unusually low sequence homology with all other broad bean leghemoglobins as well as with leghemoglobins from other legumes. It can be regarded as a novel kind of leghemoglobin gene not described until now and the induction of which is common to symbiotic interactions of broad bean with both *Rhizobium* and a mycorrhizal fungus.

P-B-20

CELLULAR EXPRESSION OF NODULE-ENHANCED GENES INVOLVED IN NITROGEN ASSIMILATION IN THE EFFECTIVE AND PLANT- CONTROLLED INEFFECTIVE ROOT NODULES OF PEA (*PISUM SATIVUM* L.)

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The enzymes aspartate aminotransferase and phosphoenolpyruvate carboxylase play an important role in the assimilation of the symbiotically fixed nitrogen in root nodules of the legumes. Previously, the nodule-enhanced forms of these enzymes (AAT-2 and PEPC) have been described for pea (1,2). In this work, the expression of AAT-2 and PEPC genes was compared between two plant-controlled ineffective root nodules of pea. RNA from the effective root nodules of the SGE line was used for the construction of a pea nodule cDNA library. The library was screened for the clones encoding AAT-2 and PEPC. Sequencing of both 5' and 3' ends of the cDNA clones demonstrated a strong homology with the corresponding cDNA clones of alfalfa. RNA blot hybridization confirmed the nodule-enhanced character of the expression of AAT-2 and PEPC. Multifragment pattern of hybridization products for both AAT-2 and PEPC obtained in DNA blot analysis is discussed in relation to their gene structure. *In situ* hybridization experiments with antisense RNA probes demonstrated the expression of AAT-2 and PEPC in the interzone II-III and zone III of the effective pea nodules. Ineffective nodules of two non-allelic mutants of pea (SGEFix$^-$-1 and SGEFix$^-$-2) were examined for the expression of AAT-2 and PEPC. The decreased amounts of AAT-2 and PEPC enzymes in such nodules compared with those of the parental form SGE were the result of the decrease in the expression of AAT-2 and PEPC genes as demonstrated by RNA blot analysis and by *in situ* hybridization. However, differences in the expression of these enzymes were found also between SGEFix$^-$-1 and SGEFix$^-$-2 nodules. This phenomenon is discussed in connection to the nodule ultrastructure and the sequential functioning of the Fix$^-$-mutations.

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P-B-21

SYMBIOTICALLY REGULATED EXPRESSION OF YELLOW LUPIN GENES CODING FOR PROTEINS OF PR10 CLASS

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Intensive studies over the past few years leading to understanding the phenomenon of development and functioning of nitrogen-fixing nodules have been focused on identification and characterization of symbiotically regulated genes of both partners. However the majority of available data relating to the tissue specific expression of legume genes have not included the genes, whose expression is down-regulated because of the symbiosis development.

We have identified two homologous yellow lupin proteins representing a class of a small acidic polypeptides of M_r 17 000 which are constitutively expressed in roots of uninfected plant. The expression of corresponding genes shown by Northern blot analysis indicates, that their transcription is down-regulateds during development of functional symbiosis with *Bradyrhizobium lupini* and activated again in senescened nodule. The full-length cDNA copies coding for both analysed lupin proteins named LlPR10.1A and LlPR10.1B have been selected from cDNA library (Sikorski *et al.*, 1996). The deduced amino acid sequences showed that both genes code for polypeptides, each composed of 156 amino acid residues. These proteins exhibit 40-80% similarity to known pathogenesis-related and stress-induced proteins of other plants. The amino acid composition and physico-chemical properties of the identified yellow lupin proteins allowed us to classify them as intracellular pathogenesis-related proteins of PR10 class (van Loon *et al.*, 1994). These similarities established a class of conserved defence-related proteins what may suggest their common function in plant-microbe interaction. It has been recently suggested that nitrogen-fixing bacteria avoid induction of defence response. The question whether an active suppression of plant defence by invading microsymbiont is a general requirement for the establishment of functional symbiosis is still open. The data presented in this communication concern the first two genes coding for proteins of PR10 class which expression is presumably symbiotically-regulated.

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P-B-22

COMPARATIVE ANALYSIS OF POLYPEPTIDE COMPOSITION OF THE PERIBACTEROID MEMBRANE FROM EFFECTIVE LUPIN NODULES AND THE PLASMALEMMA FROM UNINFECTED LUPIN ROOTS

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The formation of a symbiosome, an organelle-like structure consisting of a bacteroid, peribacteroid space (PBS), and a peribacteroid membrane (PBM) within root nodule cells, comprises an essential stage of the interrelation between legume plants and Rhizobia. The appearance of PBM is one of the key events in the formation of the symbiosome as the metabolites exchange between both symbionts through the membrane occurs. However, PBM biogenesis and the nature of this newly formed subcellular compartment and the targeting mechanism of the PBM nodulins to this structure are poorly investigated. One of the hypotheses for PBM biogenesis suggests that PBM has the plant origin. In the present study we investigated the role of the plasmalemma in the formation of PBM structure by comparison of the protein composition of PBM from effective lupin root nodules with the plasmalemma from uninfected lupin roots.

It was shown that protein composition of PBM and plasmalemma reveals certain characteristic features. The majority of plasmalemma proteins are acidic with pI 5.1-4.0 with predominate proteins with pI about 5.1 and 4.3. We found in the case of PBM that the proteins with pI 8.8-8.9 and 5.8 are predominate. As analyzed by SDS-PAGE, the protein composition of PBM reveals several typical bands: polypeptide with mol. wt. 24, 37, 58, and 83 kDa, with the main band about 37 kDa. In the case of the plasmalemma the polypeptide bands that ranged in mol. wt. 20-30 kDa and protein band with mol. wt. about 37 kDa were not detected. The predominant protein is of mol. wt. about 60 kDa.

The PBM proteins are analyzed by Western blot hybridization using both nodule specific and root antibodies. In both cases the same protein pattern is observed. All proteins of PBM were visualized by these two types of antibodies.

Our data suggest that during the transformation of the plasmalemma to PBM in protein composition of the plasmalemma several changes takes place: some new proteins appear, some disappear, and some are enhanced. In the same time, not all the PBM proteins may be account for the plasmalemma proteins.

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P-B-23

PROTEIN HYDROLYSATE AS A POSSIBLE NUTRITIONAL SOURCE FOR *RHIZOBIUM* IN SYMBIOSOMES AND INFECTION THREADS

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The symbiotic legume root nodule is, in effect, a chemical engine converting carbon compounds derived from photosynthesis into nitrogenous compounds that are made available for plant growth. We have used biochemical and molecular genetic approaches to investigate the possibility that endophytic *Rhizobium* bacteria utilise plant-derived protein hydrolysate as a major nutritional source. Because there is strong evidence demonstrating that the peribacteroid membrane is impermeable to amino acids and sugars, it has been assumed that organic acids are the major carbon source for bacteroids. However, this hypothesis ignores the observation that glycoproteins (e.g Nodule lectin) are targetted by vesicle fusion into the symbiosome compartment where they might subsequently be degraded by proteases and glycosidases. (Similar arguments apply to the possible nutritional status of rhizobia in infection threads.) We present preliminary evidence supporting this model for protein turnover. (1) We have recently identified the major glycoprotein component of the pea nodule symbiosome compartment as a member of the legume lectin supergene family, termed *PsNlec1*. (2) Using the Promega Peptag assay, we have demonstrated protease activity in the peribacteroid fluid. (3) By RT-PCR, we have cloned cDNA sequences encoding plant thiolproteases from nodule mRNA and demonstrated by in situ hybridisation that one of these genes is strongly expressed in the invasion zone (where bacteria are contained in infection threads) and also in the central infected tissue (where bacteroids are contained within symbiosomes). (4) Using specific antiserum we have demonstrated that this protease is targetted to the lumen of the infection thread and is also probably associated with the symbiosome compartment. (5) Antiserum raised against the polypeptide component of Nlec1 (expressed in *E. coli*) demonstrates that in symbiotically defective (Fix^-) nodules the protein is virtually absent, even though *in situ* hybridization experiments reveal that the gene is actively transcribed in these nodules. (6) Antiserum against Nlec1 is also proving useful in determining protein vesicle targetting pathways to the symbiosome compartment. There are some indications that vesicle targetting is aberrant in the symbiotically defective pea line Sprint2 Fix^- (*sym31*). In our proposed model for the control of nutrient exchange in symbiosomes, the rate of protein synthesis, and hence of protein vesicle targetting to the symbiosome compartment, might be controlled by the nitrogen status of the host plant cell. The testable prediction of this model is that proteins targetted to the symbiosome compartment turn over very quickly and will disappear rapidly when biological nitrogen fixation is interrupted.

P-C-1

ANALYSIS OF THE *RHIZOBIUM LEGUMINOSARUM* BV. *PHASEOLI RPON* GENE CLUSTER

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Eubacterial sigma factors confer promoter specificity to the RNA polymerase holoenzyme. Promoters of the genes recognised by the alternative sigma factor σ^{54}, encoded by the *rpoN* gene, are characterised minimally by the presence of the dinucleotides GG/GC located at -24 and -12 nucleotides from the transcriptional start site.

Bacteria of the genus *Rhizobium* form nitrogen-fixing root nodules in a symbiotic interaction with their host. σ^{54} controls several symbiotic functions at different stages of the interaction. It is involved in competition for nodulation, symbiotic nitrogen fixation, dicarboxylate uptake and nodule organogenesis.

We cloned and sequenced the *Rhizobium leguminosarum* bv. *phaseoli rpoN* gene cluster. Three orf's, ORF265, Orf191 and *ptsN*, were found downstream from *rpoN*. ORF191 is homologous to *Escherichia coli* ORFII. The *rpoN* gene and the three orf's located downstream were mutagenised and their phenotype was tested. We determined melanin production, expression of a p*nifH-gusA* fusion and growth in minimal medium containing nitrate as nitrogen source. The results of these experiments are presented.

P-C-2

CLONING AND SEQUENCING OF THE NODULATION FUNCTIONS FROM LUPINE *BRADYRHIZOBIUM*

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The majority of lupine bradyrhizobia isolated in Poland form effective nodules on seradella and largely ineffective on *Lotus corniculatus*. Nodule formation on *L.corniculatus* suggests that the LCOs active in the infection process of lupines might be similar to those produced by *Rhizobium loti*. This observation was supported by the nodulation studies of lupines inoculated with different rhizobia, which LCOs had been already characterized. Among strains used were rhizobia harbouring a plasmid with the *nodD* gene, modified in such a way that enables induction of the *nod* functions independently on flavonoids (flavonoid independent transcription activation, or FITA). It appeared that lupines were additionally infected by *Rhizobium etli, Rhizobium tropici*, and *Rhizobium* sp.GRH2, but not by *Rhizobium fredii* nor *Rhizobium* sp.NGR234 (all strains except NGR234 beared the FITA plasmid). As it has been recently established, the main components of the LCOs of *R.etli*, *R.tropici* and GRH2 are identical. The most apparent difference between the Nod factors of lupine nodulating and non-nodulating rhizobia is presence (or lack) of N-methyl and carbamoyl groups on the non-reducing end, as well as character of the reducing *N*-acetylglucosamine residue. The LCOs of all lupine nodulating rhizobia are *N*-methylated, carbamoylated, and carry 4-O-*acetyl*-fucose at C-6 of *N*-acetylglucosamine of the reducing end.
Isolation of the nodulation region of *Bradyrhizobium* sp.(*Lupinus*) WM9 brought insight into the genetics of lupine nodulation. The *nod* region was fished-out from genomic library of WM9 constructed in a broad host-range cosmid vector (pRI40). Physical maps of several overlapping cosmids were carried out, and sequencing enabled identification of the most *nod* genes which are known to control the LCO biosynthesis. The genetic content is in congruence with the expected composition of the Nod factor. All *nod* genes, which products control either N-methylation (*nodS*), carbamoylation (*nodU*), fucosylation (*nodZ*) or 4-O-acetylation of fucose (*nolL*) have been identified. The presence of additional regulatory circuit encoded by the products of *nodVW* genes, as well as organization of *nodC-nodZ* resembles *B.japonicum*. However, comparison of the nucleotide or amino acid sequences indicates on their phylogenetic distinctness from the nodulation genes in other *Bradyrhizobium* strains. It is assumed that lupines as the whole tribe *Genisteae* are very ancient group of legumes. In this case, the observed dissimilarity of the *nod* functions might be a reflection of the coevolution of lupines and their microsymbiont.

P-C-3

THE *nol* L GENE OF *Rhizobium etli* IS INVOLVED IN 4-O-ACETYLATION ON THE FUCOSYL RESIDUE OF THE NODULATION FACTOR

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A complex exchange of chemical signals is necessary for the establishment of the *Rhizobium* -legume symbiosis. After induction with flavonoids exuded by the plant, *Rhizobium etli* CFN42 produces chitopentameric compounds of N-acetyl-D-glucosamine, which are N-methyl-N-acylated with cis-vaccenic acid or stearic acid on their non-reducing end and 4-O-acetyl-L-fucosylated at position 6 of the reducing glucosamine. A small fraction of these compounds is also carbamoylated at the C4 position of the non-reducing end (Poupot et al., 1995; Cárdenas et al., 1995). Unexpectedly, considering their phylogenetic dissimilarity and different host-ranges, Nod factors of *R.etli* and *R. loti* are identical (López-Lara et el., 1995)
The production of Nod factors in a strain carrying a deletion of 220 Kb on the pSym of *Rhizobium etli* CFN42 was studied. The strain (CFNX250) harboring this deleted pSym shows a different pattern of Nod factors as compared with the wild-type strain CFN42, i.e. analysis by direct-phase TLC revealed two bands corresponding to Nod factors in strain CFN42 but only one in strain CFNX250. Mass spectrometry analysis of the Nod factors produced by CFNX250 showed that they bear carbamoyl and fucosyl substituents at their non-reducing and reducing ends, respectively, but they are not acetyl-fucosylated.
This phenotype has been complemented with a defined sector of the pSym , leading us to the identification of a new pSym locus involved in the synthesis of Nod factors in *R. etli* CFN42. This locus is located 25 Kb away from the *nod* cluster region previously identified on the pSym. Sequence analysis of the new locus has shown that is has a significant similarity with the *nol* L gene from *Rhizobium loti* ,which is claimed to encode an acetyl-transferase. NolL is essential for *R.loti* to nodulate *Lotus pedunculatus* and *Leucaena leucocephala* (Scott et al. 1996). Acetylation of the fucose is not essential for *Rhizobium etli* to nodulate common bean (*Phaseolus vulgaris*), since strain CFNX250 shows a normal nodulation kinetics on this plant.
Thus, our results suggest that, (i) the *nol* L gene in *R. etli* is involved in 4-O-acetylation of the fucosyl residue in the nodulation factor; (ii) that acetylation is not necessary for the addition of the fucose to the reducing glucosamine and, (iii) the acetyl-fucose modification might be necessary for *R. etli* to nodulate some plants other than common bean.

Cárdenas et al. 1995. Plant Molecular Biology 29: 453-464.
López-Lara et al. 1995. Molecular Microbiology 15: 627-638.
Poupot et al. 1995. Journal of Biological Chemistry 270: 6050-6055.
Scott et al. 1996. Molecular Plant-Microbe Interactions 9: 187-197

P-C-4

ADDITIONAL ROUNDS OF REPLICATION ON THE pSym OF *Rhizobium etli* PROMOTE A DRAMATIC INCREASE IN REARRANGEMENT FORMATION

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We have shown that the symbiotic plasmid pSym of *Rhizobium etli* undergoes genomic rearrangements (deletions and amplifications) at a high frequency (3 x 10^{-4} and 4 x 10^{-3}, respectively). The formation of rearrangements involves homologous recombination among two reiterated operons for the nitrogenase[1,2]. These operons are 120 kb apart, and the region between them carries most of the genes needed to establish the symbiotic relationship. Data from other microorganisms suggest that the frequency of rearrangement formation may be altered by activation of cellular processes, such as replication[3,4]. This situation might be borne about by phenomena such as cointegrations among replicons, defects in plasmid partition or "runaway" copy number control.

In order to evaluate the effect of replication induction on rearrangement formation in the pSym, we inserted a Tn*5* -derivative harboring the replication origin from the broad-host range plasmid RK2 (OriV-RK2) at different locations on this plasmid. This origin employs an unidirectional mechanism. To induce replication from OriV-RK2, we constructed a plasmid carrying the *trf* A gene under the control of an inducible promoter. TrfA is the only protein needed to activate OriV-RK2.

Our results show that activation of OriV-RK2 induces a 2000-fold increase (6 x 10^{-1}) in deletion formation on the pSym. All the deletions have as recombination endpoints the nitrogenase operons. We have tested four different Tn*5* OriV-RK2 insertions, two located into the deletable zone and two outside and far from it (>100 kb away). In every case, high-frequency deletion of the 120 kb region was found upon activation of the origin. Thus, the increase in deletion formation is independent of the position of the OriV-RK2 in the pSym. In *rec* A strains there is no increase in deletion formation under induced replication. This RecA dependency, coupled with the use of long repeats, suggests that replication is enhancing a mechanism of rearrangement formation that involves homologous recombination.

Only the molecule harboring the active OriV-RK2 becomes highly recombinogenic. Cointegrates in other plasmid of *R. etli* are stable even when replication is started from the OriV-RK2 located in the pSym.

[1] Romero et al., J. Bacteriol., 173: 2435-2441, 1991
[2] Romero et al., J. Bacteriol., 177: 973-980, 1995
[3] Noirot et al., J. Mol. Biol., 136: 39-48, 1987.
[4] Morel-Deville and Ehrlich, Mol. Microbiol., 19: 587-598, 1996.

P-C-5

MULTIPLE RECOMBINATION EVENTS MAINTAIN SEQUENCE IDENTITY AMONG REITERATED *nif* HDK OPERONS IN *Rhizobium etli*

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A distinctive characteristic of the *Rhizobium* genome is the frequent finding of repeated sequences, which often constitute multigene families. Interestingly, members of these families usually maintain a high degree of nucleotide sequence similarity or even identity, as seen for the *nif* HDK family in both *R. etli* and *Rhizobium* spp. NGR234. It is commonly assumed that recombinational interactions (such as single- and double crossovers or apparent gene conversions) between repeated elements might lead to concerted variation among its members. However, the operation of these mechanisms has not been demonstrated yet for any member of the *Rhizobiaceae* family.

The nitrogenase multigene family in *R. etli* is composed of two identical direct repeats of about 5 kb, which are *nif* HDK operons; these operons are 120 kb apart on the pSym. The third element of this family is located in the middle of this region, consisting of a *nif* H- *nif* D* reiteration (1.5 kb) in an inverted orientation *vis a vis* the *nif* HDK operons. Homologous recombination between the *nif* HDK operons leads to genomic rearrangements (deletions and amplifications) at a high frequency [1]. This particular arrangement allows us to study the recombinational dynamics of a plasmidic multigene family.

To that end, we inserted a promoterless Km^r cassette into one of the *nif* HDK operons; this cassette is under the control of the *nif* H promoter. Expression of the Km^r gene was blocked by a polar insertion of 28 bp, leading to a Km^s phenotype. Selection for Km^r derivatives give us a positive system to identify events that lead to the loss or relocation of the 28 bp insertion, conceivably *via* recombination with the other members of this multigene family.

In a wild-type background, Km^r derivatives were obtained at a high frequency (4×10^{-4}). This frequency was reduced 100-fold (to 4×10^{-6}) in a *rec* A mutant background, thus confirming that homologous recombination is involved. A total of 71 recombinant products obtained in a wild-type background were further characterized by PCR, plasmid profiles and Southern blot hybridization. Fifty percent of the products were apparently generated by a single crossover, thus leading to plasmid rearrangements. Most of these provoke a tandem amplification of the 120 kb region; only one example of an inversion was found, indicating that this region is barely permissive for inversions. Among the events leading to the loss of the 28 bp insertion, only ten percent of them were generated by apparent gene conversion. The remaining forty percent were generated by multiple crossovers. The high frequency of multiple crossovers observed suggests that plasmid molecules are engaging repeatedly in recombination events, in a situation akin to phage recombination or recombination among small, high-copy number plasmids[2]. Our results indicate that a variety of events participate to maintain sequence identity among members of this multigene family.

[1] Romero et al., J. Bacteriol., 173:2435-2441, 1991; [2] Yamamoto et al., Mol. Gen. Genet., 212: 393-404, 1988.

P-C-6

CHARACTERIZATION OF THE PLASMID-BORNE *lpsβ* LOCUS OF *Rhizobium etli*

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The molecular characterization of the *Rhizobium* genome has been focused mainly on the symbiotic plasmid (pSym), where most of the genes required for symbiotic nitrogen fixation are located. Although plasmids other than the pSym may also influence the symbiotic process (1), very few sequences from these plasmids have been isolated and characterized at the molecular level. In an earlier study on *R. etli* CFN42 plasmids (2), we showed that plasmid b (pb), in addition to the pSym, was indispensable for symbiosis on common bean roots. This is due to the presence on pb of *lpsβ*, a genetic region involved in LPS biosynthesis. In addition to this *lps* locus, pb also contains sequences involved in the competitive capacity of this strain (2).
To initiate a molecular analysis of non-Sym plasmids we chose the *lpsβ* region of pb, due to its relevance in symbiosis.
The sequence analysis of a 2415 bp fragment from the *lpsβ* region revealed an ORF (*lpsβ* ORF1) essential for LPS biosynthesis and symbiosis. This ORF encodes a putative 683 amino acid polypeptide, which coud be involved in the synthesis of 6-deoxy or dideoxy sugars. This key sequence for LPS biosynthesis is highly conserved on plasmids of *R. etli* and *R. leguminosarum*, and absent in the genome of other *Rhizobium, Bradyrhizobium, Azorhizobium* and *Agrobacterium* species.

Using *lpsβ*$^-$ derivatives of *R. etli* CFN42, harboring the pTi plasmid from *A. tumefaciens*, we demonstrated that a deficiency in LPS synthesis also affects the crown-gall tumor induction.

1. García-de los Santos, A., S. Brom and D. Romero. 1996. *Rhizobium* plasmids in bacteria-legume interactions. World J. Microbiol. Biotechnol. in press.

2. Brom, S., A. García-de los Santos, T. Stepkowski, M. Flores, G. Dávila, D. Romero, and R. Palacios. 1992. Different plasmids of *Rhizobium leguminosarum* bv. phaseoli are required for optimal symbiotic performance. J. Bacteriol. 174: 5183-5189.

P-C-7

THE EFFECT OF A CONTROLLED EXPRESSION OF THE GLUTAMATE DEHYDROGENASE (GDH) DURING SYMBIOSIS OF *Rhizobium etli*

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As part of our ongoing studies between the ammonium assimilation (GS-GOGAT) and nitrogen fixation relationship in *Rhizobium etli*, we have previously communicated that the constitutive expression of GDH, a second ammonium assimilation enzyme, prevents nodulation of *Phasolus vulgaris* roots (Mendoza et al (1995) MPMI 8:584).

Here, we report the symbiotic effect of timing the expression of GDH in a *Nif* A dependent manner. To achieve this, the *E. coli gdh* A gene was placed downstream from the strong *nif* Hc gene promoter (Valderrama et al (1996) J. Bact. 178:3119) and cloned in the stably inherited vector, pTR101 (Weinstein, M. et al. 1992. J.Bacteriol. 174: 7486).

Expression of *Nif* A-dependent promoters during *P. vulgaris* infection by *R. etli* is triggered in late stages and nitrogenase genes transcription begins 12-14 days after inoculation. Timed expression of *gdh* A under a *Nif* A-dependent promoter allowed a normal infection to occur. Preliminary results indicate that the nitrogen fixation ability of the *R. etli* wild type strain harbouring the p*nif* Hc-*gdh* A construction is diminished by 20% (Some changes in nodule formation are under study).

The glutamate content of bacteroides isolated from nodules infected with the p*nif* Hc-*gdh* A construction is two times higher than in wild type bacteroides. The amino-nitrogen/total-nitrogen ratio is four times higher in the strain carrying the construction. In contrast, the ureides concentration of xylem sap obtained from plants inoculated with strains harbouring the construction is 20% lower than in the control plants. In conclusion, constitutive expression of gdhA hampers nodule formation in *P. vulgaris*. Timing its expression in a *Nif* A-dependent manner allowed nodule formation, but acquisition of the GDH activity not only impairs nitrogen assimilation inside the bacteroid, changing the nitrogen content balance, but also outside, changing the xylem sap ureides content, that is the common output from nodules to nourish the plant. This imbalance is correlated to a 20% reduction of nitrogen fixation.

P-C-8

THE TRANSCRIPTIONAL ACTIVITY OF THE SYMBIOTIC PLASMID OF *Rhizobium etli* IS AFFECTED BY DIFFERENT ENVIRONMENTAL CONDITIONS

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Global patterns of transcriptional activity in the symbiotic plasmid of *Rhizobium etli* were studied under a variety of environmental conditions, including some relevant to the symbiotic process. ^{32}P-sscDNA's synthesized from total RNA were used as hybridization probes against an ordered collection of cosmid clones that cover the whole pSym. Our results show that, under aerobic conditions, discrete regions in the pSym are differentially transcribed depending on the quality of the carbon and nitrogen sources employed. In general, poor carbon or nitrogen sources allow a better expression than rich ones. Time-course experiments with the *nod* gene inducer genistein lead us to the identification of new regions responsive to this flavonoid. Widespread transcription was observed in microaerobiosis, but not during aerobic conditions, indicating that oxygen concentration is a major effector of transcriptional activity in the pSym. This response is reduced, but not suppressed, in a *nifA* mutant, indicating the location of regions whose transcription may depend on other oxygen-sensitive regulators. During symbiosis, almost the entire pSym was actively transcribed. The transcription pattern observed in this condition was qualitatively similar to that observed during microaerobiosis. Our experimental approach allows the identification and localization of specific regions in the pSym whose expression depends on defined environmental stimuli.

P-C-9

ANALYSIS OF SYMBIOTIC AND FREE-LIVING FUNCTIONS OF *Rhizobium etli* PLASMIDS

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Rhizobium strains from diverse species share a similar genomic organization, where the DNA is distributed among the chromosome and plasmids which vary in number and size. In order to analyze the participation of these plasmids in diverse cellular functions, we have used *Rhizobium etli* strain CFN42 as a model. This strain contains six plasmids (pa to pf), in addition to the chromosome. Plasmid d corresponds to the symbiotic plasmid, as it carries most *nif* and *nod* genes. The strategy employed to initiate the study of plasmid functions, consisted in the isolation and characterization of derivatives cured (or deleted) of each plasmid. These derivatives were obtained using the Tn*5*-GDYN1 element (Flores et al., 1993). This element allows positive selection for its insertion, through antibiotic resistance determinants, and for its loss, through the selection of sucrose resistant derivatives. The analysis of the different derivatives obtained, showed that, in addition to pd, pb is also required for effective nodulation, due to the presence of LPS biosynthethic genes, and all plasmids except pa, are necessary for optimal competitive ability for nodulation. In regard to free-living functions, they were seen to affect the growth capacity, particularly pf, which is indispensable for growth on minimal medium (Brom et al., 1992). To proceed the analysis of plasmids, we constructed a set of derivatives containing different plasmid combinations. Analysis of these strains showed that some of these plasmids have cooperative effects for functions such as growth, nodulation competitiveness, nitrogen fixation and plasmid transfer. Presently, we are determining the role of plasmids in diverse metabolic functions, through their ability to utilize different carbon and nitrogen sources and the presence of enzymes by multilocus enzyme electrophoresis, as well as their requirement for optimal cellular viability. We will also discuss recent experiments regarding plasmid transfer, which suggest a possible correlation with certain plasmid rearrangements (amplifications). Finally, we have also initiated the identification and characterization of various *fix* genes which are localized on pf.

Brom, S., A. García de los Santos, T., Stepkowski, M., Flores, G., Dávila, D., Romero and R. Palacios. (1992). Different plasmids of *Rhizobium leguminosarum* bv. phaseoli are required for optimal symbiotic performance. J.Bacteriol. 174:5183-5189.

Flores, M., S. Brom, T. Stepkowski, M.L. Girard, G. Dávila, D. Romero and R. Palacios. (1993). Gene amplification in *Rhizobium*: Identification and in vivo cloning of discrete amplifiable DNA regions (amplicons) from *Rhizobium leguminosarum* bv. phaseoli. Proc.Natl.Acad.Sci. 90:4932-4936.

P-C-10

IDENTIFICATION OF IRR, A POSITIVE AND NEGATIVE EFFECTOR OF IRON METABOLISM IN BRADYRHIZOBIUM JAPONICUM

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Bradyrhizobium japonicum mutant strain LODTM5 accumulates the heme intermediate protoporphyrin, but is not deficient in any enzymatic step of the heme pathway. B. japonicum was shown to express a high affinity iron uptake activity that is induced under iron limitation, and that this activity is defective in mutant strain LODTM5. The gene mutated in strain LODTM5 encodes a protein named Irr (iron response regulator) that shows moderate (28%) identity to Fur, a bacterial transcriptional repressor of iron uptake systems that functions under iron replete conditions. The mutant phenotype suggests that Irr positively affects iron uptake under iron limitation, and therefore it has exactly the opposite activity of Fur. We also found that iron positively affects hemB, the gene encoding 5-aminolevulinic acid (ALA) dehydratase, at the message level. Unlike wild type cells, strain LODTM5 showed high levels of hemB expression in iron-deprived cells, suggesting that Irr normally represses hemB under those conditions. We suggest that the deficient iron uptake activity in strain LODTM5 results in a low cellular iron concentration that inhibits iron chelation into protoporphyrin for heme formation. In addition, porphyrin accumulation is exacerbated because the heme pathway is not effectively down regulated in response to iron stress. Finally, we propose thatIrr represses hemB and is a positive effector of iron transport, and that both activities are manifest under iron limitation. This regulation is physiologically reasonable because iron uptake is maximized and the heme pathway is repressed when synthesis of the end product is necessarily compromised.

P-C-11

MOLECULAR GENETIC AND SYMBIOTIC CHARACTERISTICS OF *Rhizobium leguminosarum bv.viciae* STRAIN A-1 AND ITS DERIVATIVES.

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The aim of this research is to classify all the genomic rearrangements observed in the derivatives of the. *R leg.bv.viciae* strain A-1 and to connect them with symbiotic characteristics. Strain A-1 is a field isolate and its general properties are the ability to infect Afghanistan peas, high effectiveness of symbiotic nitrogen fixation and high competitiveness in model experiments (1). 500 isolates of *R.leg bv. viciae* were selected from the soils with pea lines *sym2sym2* in greenhouse and field trials. Different types of soils were used: from several sites of field in Central Russia where A-1 was kept during 4 years and where A-1 was not introduced. It was shown that some of these isolates possess different genetic alterations in comparison with the original strain A-1(2). REP, ERIC PCR was used to detect and monitor the diversity of the A-1 soil derivatives. It was demonstrated that several isolates are not the A-1 derivatives but they content a *nodX-gene*. The conjugational transfer of *nod*-genes from strain A-1 to European strain CIAM 1026 was observed in the laboratory experiments. It is possible that this phenomenon reflects the real process in the soil. Besides two new strain were selected that are capable to inoculate Afghanistan peas in the control soil. Each group of similar bacterial PCR-patterns correlated with the specific LPS profiles. Five A-1 soil derivatives with different genomic rearrangements (revealed by RFLP with *nod*-genes as the probes, by RAPD and by plasmid content) were analyzed for the effectivity of nitrogen fixation and competitive ability in co-inoculation experiments on Afghanistan and European pea lines with the strains A-1 and CIAM 1026.

(1) Kravchenko L. et al., (1995) In: Nitrogen Fixation: Fundamentals and Applications, Tikhonovich I. et al. (eds.), p.313, Kluwer Academic Publishers, Dordrecht, the Netherlands.
(2) Kulikova O. et al., (1995) Ibid., p.700.

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P-C-12

GENETIC DIVERSITY OF *RHIZOBIUM MELILOTI* NATIVE ISOLATES FROM THE CENTRAL ASIA GENE CENTER OF ALFALFA

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The oldest gene center of alfalfa in Central Asia located in Tadjikistan was studied concerning genetic diversity of *R.(S.)meliloti*. More than 200 strains were isolated from different *Medicago, Mellilotus, Trigonella* and *Mellisitus* host plants. Different techniques were applied to study chromosomal and *sym*-plasmid structural polymorphism for 28 *R.meliloti* isolates effectively nodulating *Medicago sativa* in plant test. It was found that more than 50% of strains harboured one cryptic plasmid of 200 kb. Out of 20 different identified IS*Rm2011-2* fingerprint groups 17 were represented by a single strain harbouring between 0 and 19 copies per genome. Some of the strains isolated from the different plants and sites were similar in their IS*Rm2011-2* profile and were attributed to other three groups. IS-fingerprint grouping of the strains was supported by ERIC-PCR analysis. The analysed strains were found to be genetically closely related when RFLP-analysis was applied to study variation of chromosomal (*mucR* and *ala*) or megaplasmid 2 (*exo*-cluster and *eff*-798) gene regions. The highest RFLP was found for *nifHDK*, *nodH*, *nodABC* and *nodD1* genes from megaplasmid 1 responsible for the interaction with alfalfa. This finding may be considered as a characteristic feature for rhizobia from alfalfa gene center in Central Asia.

P-C-13

REGULATION OF THE BIOSYNTHESIS OF *RHIZOBIUM MELILOTI* EPS I AND EPS II WHICH ARE INVOLVED IN THE INVASION OF ALFALFA ROOT NODULES

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Rhizobium meliloti produces the acidic exopolysaccharide EPS I (succinoglycan). In addition, *R. meliloti* has the cryptic ability to synthesize the alternative and structurally different exopolysaccharide EPS II (galactoglucan). At least one of these exopolysaccharides is essential for the invasion of *Medicago sativa* root nodules by *R. meliloti*. Nineteen *exo* genes and two *exs* genes involved in the biosynthesis of EPS I are located on a 27 kb gene region of megaplasmid 2, whereas a 31 kb region comprising at least 21 *exp* genes involved in EPS II biosynthesis is located approximately 200 kb distant from the *exo* gene region. A mutation of the chromosomally located *muc*R gene resulted in the production of EPS II polymer and EPS I oligosaccharide precursors. The transcription of the *muc*R gene was negatively autoregulated. The MucR amino acid sequence contains a zinc finger motif of the C_2H_2 type indicating the ability to bind DNA. An electrophoretic mobility shift assay demonstrated that the MucR protein can specifically bind the *muc*R and *exo*Y promoter region. The transcription of the *exp* genes was positively influenced by a *muc*R mutation. In contrast, the transcription of most *exo* genes was not significantly influenced by a mutation in *muc*R, since only the transcription of an *exo*K-*lac*Z and an *exo*Y-*lac*Z fusion was reduced. *R. meliloti* produces a high molacular weight (HMW) and a low molecular weight (LMW) form of EPS I. Mutants characterized by ExoP* proteins lacking the C-terminal protein domain produced a reduced amount of EPS I. This reduction could be suppressed by a mutation in the regulatory gene *exo*R. The proportion of LMW EPS I to HMW EPS I was significantly increased in the *exo*P* mutant strains. The *exo*R/*exo*P* mutant produced large amounts of LMW EPS I, whereas no HMW EPS I could be detected. Nevertheless, the *exo*R/*exo*P* mutant was able to invade alfalfa nodules.

P-C-14

IDENTIFICATION OF THE GENES IN *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII* WHOSE PRODUCTS ARE HOMOLOGUES TO A FAMILY OF ATP BINDING PROTEINS

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In interaction between *Rhizobium* and its legume host some gene products should be exported to the milieu. In Procaryota there are two different export systems. One is dependent on the N-terminal signal sequence - so called *Sec*-dependent pathway. The other one is dependent on the presence specific proteins called ABC-transporters in the bacterial membranes. This system consist of an integral inner membrane protein with the ATP binding cytoplasmic domain and the accesory protein localized between inner and outer membranes. In some cases there is one more outer membrane integral protein. This system can export not only proteins but also non protein substrates such as polysaccharides. In the close linkage to the *exo::133* mutation *Rhizobium leguminosarum* bv. *trifolii* TA1 we have found two genes designated *resD* and *resE* (**rhizobial export system**).They are transcribed in one direction with an 15 bp intergenic region between them. There are potential RBS sequences upstream the *resD* and *resE* genes but no promoter sequence was found. These two genes share strong homology to the **ABC transporter** genes *prtDE* from *Erwinia chrysanthemi* and *aprDE* from *Pseudomonas aeruginosa* which control export of the proteases in these bacteria. ResD is a 570 aa protein with a deduced molecular weight 60.887 Da. It has respectively 44.4% identity (82.4% similarity) and 40.6% identity (76.8% similarity) to AprD and PrtD. ResD has five potential transmembrane hydrophobic regions (four in the N-terminal part) and large hydrophilic domain containing ATP/GTP binding sequence (A and B Walker sites). So, ResD can be the main inner membrane integral protein. ResE is a 433 aa protein with a predicted molecular weight 47.146 Da. It has 26.7% identity (72.1% similarity) with AprE of *P. aeruginosa* and 27.8% identity (71.4% similarity) with PrtE *E. chrysanthemi*. ResE has only one potential transmembrane hydrophobic domain in the N-terminal part and potential leucine zipper pattern in the central part. ResE protein can be an accesory factor in the transport system. We have inserted in vitro a kanamycin resistance cassette into the *resD* gene and introduced such construct into the genome of the wild strain *R. leg.* bv. *trifolii* TA1 by marker exchange technique. We have obtained *resD* mutant called TA1::34. This mutant form mucoid colonies resulting from the production of exopolysaccharide but it is unable to form effective nodules on clover. Recently we have found the third protein but the putative function of this protein remains to be resolved.

P-C-15

EXO GENES OF *RHIZOBIUM LEGUMINOSARUM* BV *VICIAE* VF39

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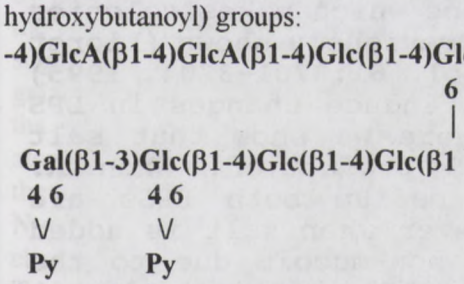
The exopolysaccharide from *Rhizobium leguminosarum* bv *viciae* VF39 seems to possess the most common structure for the polymers of this species. It is composed of octasaccharide repeating units substituted with piruvate, O-acetyl and O-(3-hydroxybutanoyl) groups:



The conclusion on the structure was drawn from the data on component analysis and ^{13}C NMR spectra of native, depyruvylated, deacylated and depyruvylated/deacylated polysaccharide.

Nucleotide sequence (5.5 kb) around Tn*5* insertions into chromosome of VF39 resulted in Eps$^-$Nod$^-$ phenotype was determined. Six ORFs, designated as *pss*CDEFGH, were localized. Tn*5* insertions were mapped within the *pss*G and *pss*H which are able to encode proteins of 19.3 and 17.4-kDa, respectively. The latter revealed local homology with glycosyltransferase from *Streptomyces* C5 and zeaxanthin glucosyltransferase from *Erwinia herbicola.* Amino acid sequences deduced from *pss*C, *pss*D, *pss*E and *pss*F showed similarity with ExoM, ExoO and ExoW from *R.meliloti,* and NodC from different *Rhizobium* species. Insertion of Km^R cassette into *pss*F led to reduction of exopolysaccharide synthesis.

P-C-16

EXOPOLYSACCHARIDES PRODUCTION IN *RHIZOBIUM MELILOTI* EFB1 IS MODULATED BY SALT AND BY THE DISPONIBILITY OF CARBON

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Rhizobium meliloti EFB1, an halotolerant strain isolated from nodules of plants growing on a salt marsh in Doñana National Park (South West Spain), produces several extracellular polysaccharides including two types of EPS: the typical calcofluor binding (EPSI) and a second one which makes colonies very mucoid (EPSII). We have previously shown (Lloret et al. Appl. Environ. Microbiol. 61:3701-3704. 1995) that salt and osmotic stress induce changes in LPS profiles and antigenicity. Here we show that salt also induces changes in EPS production. When *R. meliloti* EFB1 grows on TY medium both EPSs are simultaneously produced; however when salt is added to the medium, colonies are non-mucoid due to the lack of EPSII prodution, while EPSI production is not affected.

We have isolated a *Tn5lac* induced mutant that do not produce EPSII. This mutant has been complemented with a cosmid from a EFB1 gene-bank. Mutagenesis of the cosmid and complementation analysis have shown the presence of at least two complementation groups, spanning more than 15 kb implicated in EPSII production. A 2 kb fragment from this region has been sequenced revealing the presence of a long ORF which shows weak homology with several glucosyl transferases. All the mutants obtained so far are nod^+ and fix^+ and are non-mucoid unless grown on carbon-rich medium. These results indicate a role for extrcellular polysaccharides in adaptation to changes in the environment.

P-C-17

LIPOPOLYSACCHARIDES OF TRANSPOSON MUTANTS OF *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII*

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Random Tn5 mutagenesis of *Rhizobium leguminosarum* bv. *trifolii* strains 24.1 and TA1 was carried out for isolation of surface polysaccharide mutants. Slow growing or non- mucoid colonies were chosen for further studies. Two clones named 24.12 =Rt12 and 24.25 synthesized changed lipopolysaccharide (LPS) what was indicated by polyacrylamide gel electrophoresis (DOC-PAGE). LPS of 24.12 did not contain the main sugars composing O-side polysaccharide in parental strain 24.1. Other sugars that are known as core oligosaccharide components of *R. leguminosarum*, i.e.: galacturonic acid, galactose, mannose, Kdo, occurred at ratios different from that of the strain 24.1. The Tn5 Exo$^-$ mutant of TA1 strain, designated TA1::133 had been also changed in LPS composition. LPS from the mutant TA1::133 was deprived of the sugars indicative for the O-antigenic part i.e.: methyl- and 6-deoxyhexoses and 3-N-methyl-6-deoxyhexose. However, these differences were not reflected in the electrophoretic mobility patterns; the preparations from both strains (TA1 and TA1:133) contained O-side chains of similar molecular weight.

P-C-18

STRUCTURAL CHARACTERIZATION OF TWO LIPOPOLYSACCHARIDE SPECIES PRODUCED BY *RHIZOBIUM LOTI* STRAINS

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The lipopolysaccharides of the two strains of *Rhizobium loti* representing respectively the first group (HAMBI 1633) of rhizobia able to form effective nodules on a broad range of *Lotus* species and the second group (HAMBI 1129) inducing effective nodules only on *L. tenuis* and *L. corniculatus* var. *cree* have been studied. The application of the phenol-water procedure allowed to isolate two LPS species recovered from the aqueous phase (LPS-A) and the phenol layer (LPS-P) from both strains. The determination of their DOC-PAGE patterns revealed a set of bands in the high molecular weight range characteristic for S-type of the LPS-P and R-type character of the LPS-A. Chemical investigations of the polysaccharide portions obtained after mild acid hydrolysis of LPS-P showed the presence of large amount of 6-deoxytalose along with lower amounts of Rha, Glc and Hep but as the major components of polysaccharides from LPS-A of both strains occurred Glc, GlcN, Hep and Kdo. Lipid A's derived from hydrophilic and hydrophobic LPS preparations of the strain 1633 contained Glc, GalA, GlcN and 2,3-diaminoglucose, whereas in lipid A's from LPS-A and LPS-P of the strain 1129 2,3-diaminoglucose was the only sugar compound. LPS of both strains gave complex fatty acids profiles. As the main amide linked fatty acids; 3-hydroxyacids (with 12, 13, 18, 20 carbon atoms) and 4-oxo-eicosanoic acid were detected, whereas the ester linked acids comprised; palmitic, *iso*-heptadecanoic, stearic and 27-hydroxyoctacosanoic acid. Noteworthy, this long chain hydroxyacid characteristic for rhizobial lipid A's has been detected only in trace amount in the LPS of the strain HAMBI 1633.

P-C-19

THE CHARACTERISTIC FATTY ACID COMPOSITION OF *RHIZOBIUM HUAKUII* LIPOPOLYSACCHARIDES

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The phenol and water soluble fractions of the lipopolysaccharides of the *Rhizobium huakuii* strains carried a number of amide-linked 3-hydroxylated fatty acids including: 3-OH-12:0, 3-OH- *iso*-13:0, 3-OH-20:0, 3-OH-22:0 and unsaturated 3-OH-22:1, 3-OH-23:1. The first three above mentioned acids are the main amide-linked fatty acids in the LPS preparations.
The main ester-bound fatty acids comprise 16:0 i 17:0, 18:0 as well as 27-OH-28:0. Among minor constituents of lipid A's 25-OH-28:0 and 29-OH:30 together with some nonpolar fatty acids were found. Additionally, the presence of 4-oxo-20:0, 4-oxo-21:0 and 4-oxo-22:0 the amide-bound fatty acids as well as the 27-oxo-28:0 ester-linked fatty acid was proved. To our present knowledge oxo fatty acids are very rare constituents of lipopolysaccharides and **27-oxo-28:0** was found for the first time in the LPS preparations from members of *Rhizobiaceae*.

P-C-20

CYCLIC β-GLUCAN SYNTHESIS IN *BRADYRHIZOBIUM JAPONICUM*

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Bradyrhizobium japonicum synthesizes periplasmic cyclic β-(1,3);β-(1,6)-D-glucans during growth in hypoosmotic environments and in nodules and evidence is growing that these molecules may have a specific function during plant-microbe interactions in addition to osmoregulation. Site-directed Tn5 mutagenesis of the DNA region upstream of the *ndvB* locus resulted in identification of a new gene (*ndvC*) involved in β-(1,3);β-(1,6)-glucan synthesis and in nodule development. The predicted translation product was a membrane polypeptide (ca. 62 kDa). It contained a conserved sequence characteristic of a nucleoside-sugar binding motif and had 51 % similarity with B-glucanosyl transferase from *Candida albicans*.

B. japonicum carrying a Tn5 insertion in *ndvC* resulted in synthesis of altered cyclic β-glucans composed almost entirely of β-(1,3)-glycosyl linkages. The mutant strain was only slightly sensitive to hypoosmotic growth conditions as compared with the *ndvB* mutant but it was severely impaired in symbiotic interactions with soybean (*Glycine max*). Nodulation was delayed by several days and many small nodule-like structures were formed which were devoid of infection threads or viable bacteria. This new class of glucan molecules appears to functioned as osmolytes but not in supporting symbiotic effectiveness. Thus segregation of the pleiotropic phenotypes due to mutations in the glucan synthesis locus was observed for the first time. These results suggest that the structure of the B-glucan molecule is important for a successful symbiotic interaction, and that B-glucans may have a specific function in addition to their role in hypoosmotic adaptation. (Supported in part by USDA CSRS NRI Competitive Research Grant #93-37305-9233 to DLK and AAB)

P-C-21

RHIZOBIUM MELILOTI MUTANTS DEFICIENT IN PHOSPHOLIPID *N*-METHYLTRANSFERASE STILL CONTAIN PHOSPHATIDYLCHOLINE

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Phosphatidylcholine (PC) is the major membrane-forming phospholipid in eukaryotes. In addition to this structural function, PC is thought to play a major role in lipid turnover and signalling in eukaryotic systems. A definition of the precise role of PC in eukaryotes however, has been hampered by the fact that PC-deficient mutants could not be isolated so far and therefore PC seems to be an essential ingredient for the survival of eukaryotes.
In prokaryotes only some groups of bacteria, among them the *Rhizobiaceae*, contain PC. To understand the role of PC in bacteria, we studied *Rhizobium meliloti* 1021 which is able to form nitrogen-fixing nodules on its legume host plants and therefore shows a very complex phenotype.
R. meliloti was mutagenized with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and potential mutants were screened by a colony autoradiography procedure. Filters carrying lysed replica of mutagenized colonies were incubated with *S*-adenosyl-L-[methyl-^{14}C]methionine. The enzymatic transfer of methyl groups to phosphatidylethanolamine (PE) led to the formation of PC and therefore to the incorporation of radiolabel into lipid material. Screening of about 24000 colonies for reduced incorporation of radiolabel into lipids led to the identification of 7 mutants which show a strongly reduced specific activity of phospholipid *N*-methyltransferase. *In vivo* labeling of mutant lipids with [^{14}C]acetate showed that the methylated PC biosynthesis intermediates monomethylphosphatidyl-ethanolamine and dimethylphosphatidylethanolamine could not be detected anymore and this was combined with a corresponding increase of the potential methyl acceptor PE. These results indicate that PC biosynthesis via the methylation pathway is indeed blocked in the mutants isolated. However, the lipid analysis also showed that PC was still present when the mutants had been grown on complex medium and that it was present in the mutants in wild type amounts. These findings imply the existence of a second pathway for PC biosynthesis in *Rhizobium*.

P-C-22

CHARACTERIZATION OF A NOVEL ACYL CARRIER PROTEIN FROM *RHIZOBIUM MELILOTI* STRAIN 41

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Bacterial exopolysaccharides (EPS) and lipopolysaccharides (LPS) are known to play important roles in plant-bacterium interactions. The *fix-23* locus of *Rhizobium meliloti* strain 41 can compensate for exopolysaccharide-deficient (*exo*) mutations during symbiotic nodule development and is involved in the production of a novel polysaccharide, called K-like antigen, that is rich in 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) but different from classical LPS (Reuhs *et al.* 1993. J. Bacteriol. 175: 3570-3580). One of the four *fix-23* complementation units (unit I) contains 6 open reading frames (ORFs) coding for protein products that show a high degree of homology and similar organization to those of the rat fatty acid synthase multifunctional enzyme domains. The gene ORF6 was proposed to code for a novel Acyl Carrier Protein (ACP) named Fix23-6 (Petrovics *et al.* 1993. Mol. Microbiol. 8: 1083-1094).
We have now cloned the open reading frame (ORF6) and overexpressed the derived protein product (Fix23-6) in *Escherichia coli*. After purification, the Fix23-6 protein migrates as a single band with an apparent molecular weight of 5000 during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Fix23-6 protein can be effectively labeled *in vivo* by radioactive ß-alanine added to the growth medium. Also, if homogenous Fix23-6 protein is incubated with radiolabeled coenzyme A (CoA) in the presence of purified holo-ACP synthase from *E. coli* an *in vitro* transfer of 4'-phosphopantetheine to the Fix23-6 protein can be shown. The conversion from apo-Fix23-6 protein to holo-Fix23-6 protein seems to go along with a conformational change of the protein structure as the holo-Fix23-6 protein runs significantly faster in a native polyacrylamide gel electrophoresis than the apo-Fix23-6 protein. The quantitative conversion of the apo-Fix23-6 protein with holo-ACP synthase and CoA to holo-Fix23-6 protein can be followed easily by electrophoretic analysis under native conditions.
Our results show that in *R. meliloti*, in addition to the constitutive ACP and the flavonoid-inducible nodulation protein NodF, there is at least one more 4'-phosphopantetheine-carrying small, acidic protein (Fix23-6), presumably functioning as a novel ACP in the biosynthesis of an yet unknown ß-ketide.

P-C-23

A NEW INDUCIBLE RICH-RHAMNOSE CELL WALL COMPOUND FROM *RHIZOBIUM* NGR234 IS INVOLVED IN NITROGEN FIXATION IN *VIGNA UNGUICULATA*

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The symbiotic interaction between rhizobia and legumes results in the formation of nodules on the roots of leguminous plants in which bacteria reduce nitrogen to ammonia . Specific inducers secreted by plants activate the bacterial regulatory NodD proteins which lead to the transcription of *nod* genes. Activated *nod*-genes are responsible of the synthesis of lipooligosaccharide signals. The Nod-factor molecules are involved in the early phases of the symbiosis and nodule development. Here, we show that the *Rhizobium* NGR234 regulatory NodD1 activates other inducibles genes (*fix* genes) that control the processes converting rhizobia into nitrogen fixing bacteroids. At least two of these *fix* genes are involved in the synthesis of novel rhamnose-rich lipopolysaccharides cell wall components of NGR234. Mutations in the *fix* genes do not affect the biosynthesis of Nod-factors, but abolish the formation of this new component and the capacity of the matant to fix nitrogen.

P-C-24

THE FATTY ACID COMPOSITION OF PERIBACTEROID AND BACTEROID MEMBRANES OF SYMBIOSOMES FORMED BY DIFFERENT SYMBIOTIC PARES

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The obviously condition of effective symbiosome existing is the presence the normal peribacteroid membrane (PBM) and the bacteroid membrane (BM), that are determined by their protein and lipid composition. The latest determines the transport properties too, because the transport activation energy also increases with increasing bilayer lipid acyl chain unsaturation. We report here the finding of detailed analysis of the fatty acids (FA) composition of PBM and total BM formed by the following symbiotic paires: *Lupinus luterus* L., inoculated by the strains *Bradyrhizobium lupini* 359a (Fix+) and 400 (Fix-); *Vicia faba* L., inoculated by the strains *Rhizobium leguminosarum* 97 (Fix+) and 87 (Fix-) in comparison with that of the plasmalemma and free-living rhizobia.

The data presented showed that the character of the transformation the free-living rhizobia to the bacteroid form is the same for both fast and slow-growing bacteria and undependent on the effectiveness of symbiosis. On the contrary the FA compsition of PBM from the investigated symbiosomes are different and determined by the host-plant. In the PBM of uneffective nodules the level of the polyunsaturated FA was decreased. The ratio 18:2/18:3 was equal in the PBM isolated from uninfected nodules and did not depended on the genius of plant as the ratio 18:2/16:0. We can suppose that a dramatic increasing the level of 16:0 it appears be characteristic feature of the FA composition of PBM all uninfected nodules investigated. According to our data PBM from the effective nodules was more fluid, this determined the more PBM physiological activity. In the case of PBM from effective nodules there is a high level of unsaturated FA (ca 50%), and it being determined trienoic FA for the PBM from lupin nodules and by dienoic FA for the PBM from broad bean nodules.

It was also shown that the bacterial FA take part in the formation of PBM in all cases as the plant FA take part in the formation of BM.

So the data presented confirmed that the FA composition of PBM and BM have some common feature of the plasmalemma and the total bacterial membrane.

We suppose that in the legume plants the control of the lipid composition of PBM and BM can take place at the posttrancriptional level during the process of symbiosis formation.

P-C-25

STUDY OF THE FUNCTIONS OF *RHIZOBIUM* AGGLUTININS

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Prior work has shown that rhizobial agglutinins, like *Azospirillum* and *Bacillus* lectins, may have a role to play at early stages in the establishment of a plant-bacterial system and have the function of adhesins. In the formation of plant-microbe associations, apart from adhesive processes, a considerable role is known to be played by various enzymatic processes. *Rhizobium leguminosarum* 252 agglutinins were found in this study to affect the activities of certain hydrolytic enzymes in the bacterial cell itself. An inhibitory effect of rhizobial agglutinins on the pectinolytic, proteolytic and β-glucosidase activities, as well as on the activities of acid and alkaline phosphatases, was observed.

P-C-26

INTERACTIONS OF BACTERIA OF THE FAMILY *RHIZOBEACEAE* WITH WHEAT ROOTS AND THE ROOT HAIR SURFACE

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The number of attached rhizobial (*R.leguminosarum, R.meliloti*) and agrobacterial (*A.tumefaciens, A.rhizogenes, A.radiobacter*) cells to wheat roots and wheat root hairs was evaluated by radioisotopic and light microscopy methods. We documented the quantitative differences between quick, non-specific (seconds) and specific (minutes, hours) contact steps of agrobacterial cells to wheat roots. Attachment of agrobacterial cells to the wheat root surface was observed 10 seconds after incubation. The surface of young (short) hairs was colonized more actively than older (longer) wheat root hairs. We did not observe any big differences between agrobacterial and rhizobial attachment to wheat and rice cultivars. The lack of some agrobacterial and rhizobial surface polysaccharides and proteins led to changes in hydrophobicity and the ability to attact to the wheat root surface. Unidentified agrobacterial surface proteins lacking lectin activity mediated the attachment process. We observed a repressive effect of EDTA pretreatment on the agrobacterial attachment, especially in a calcium-free buffer. However, growth on a medium (or incubation in buffer) with 0-14 mM calcium had no effect on the agrobacterial and rhizobial attachment. These data were confirmed by both the methods. Thus, we conclude that agrobacterial attachment to wheat roots is no calcium-dependent and differs from agrobacterial attachment to dicotyledonous and some monocotyledonous plants (Swart, 1994). NodD mutation of *R.leguminosarum* did no affect on the attachment ability. Contact molecules were removed from the agrobacterial surface by centrifugation (at 4000g, 20 min), and can be restored within 2h after centrifugation. This, however, was not the case with cells treated by a respiration inhibitor just after centrifugation. Treatment of *A.radiobacter* 5D-1 cells with antibodies to the cell surface polysaccharides decreased the number of attached cells of the wild strain, but not decreased the attachment ability of mutant 227. Upon pretreatment of cells of mutant 227 with antibodies to the cell surface polysaccharides, the attachment ability increased up to that wild of the strain. On the other hand a neutral protein (albumin) did not affect the attachment ability of mutant 227 and the cells of the wild strain. Our hypothesis is that the surface polysaccharides of *A.radiobacter* 5D-1 are not directly involved in to wheat root attachment.

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P-D-1

BACTERIOCIN PRODUCTION AND RESISTANCE IN *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE*

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In 1993 twenty isolates of *Rhizobium leguminosarum* bv. *viciae* were obtained from nodules formed by plants inoculated with soil samples from a field in the East Anglia region of the UK. These were analysed by RAPD PCR and Eckhart plasmid gels and found to be repeat isolates of a single strain. In 1994 the soil was sampled again and over 100 isolates were characterised revealing at least 13 strains. None was the same as the strain in the 1993 samples. We have screened these strains for bacteriocin production and resistance. The single strain observed in 1993 was found to produce an extremely potent medium molecular weight bacteriocin to which most of our indicator strains were sensitive. However, all of the strains isolated in 1994 displayed either total or partial resistance to the bacteriocin produced by the 1993 isolates, but did not produce it themselves. We believe that the bacteriocin produced by the single strain isolated in 1993 was responsible for the dominance of that strain in that year, and that this in conjunction with the absence of any strains sensitive to this bacteriocin in 1994 indicates a selective advantage conferred by bacteriocin production. The appearance of a variety of strains in 1994 may be as a result of the transfer of a resistance determinant between indigenous sensitive strains. We think that the strain isolated in 1993 has been out competed for nodulation by the resistant strains in 1994, and that this poor competitive ability in the presence of resistant strains is further evidence for the selective advantage of bacteriocin production.

P-D-2

ROLE OF N-ACYL HOMOSERINE LACTONE MOLECULES IN NODULATION BY *RHIZOBIUM LEGUMINOSARUM*

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N-acyl homoserine lactone (aHSL) molecules have recently been detected in culture media of *Rhizobium leguminosarum* (1,2) and *Rhizobium meliloti* (1). Little is known about the role of aHSL molecules in *Rhizobium*. A role in stationary phase phenomena and in stress signalling can be predicted from the results obtained with other bacteria. Since (nitrogen) stress plays an important role in symbiotic nitrogen fixation, aHSL molecules might be important in early stages of nodulation.
The following results will be on display on our poster:

i) Nodulation studies with a *R. leguminosarum* mutant deficient in production of a major aHSL molecule "Small bacteriocin".
We found a difference in its nodulation behaviour in comparison to strains having a normal "Small bacteriocin" production. The mutant nodulates later and induces formation of less nodules.

ii) Evidence for a second type of aHSL molecule present in *R. leguminosarum* strains harbouring a self-transmissible plasmid.
This second type represents an alternative for the aHSL molecule "Small bacteriocin", which is not produced by these strains.

Literature:

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2) Schripsema, J., K.E.E. de Rudder, T.B. van Vliet, P.P. Lankhorst, E. de Vroom, J.W. Kijne, and A.A.N. van Brussel. 1996. Bacteriocin *Small* of *Rhizobium leguminosarum* belongs to the class of N-Acyl-L-homoserine lactone molecules, known as autoinducers, and as "quorum sensing" co-transcription factors. J. Bacteriol. **178:**366-371.

P-D-3

THE ROLE OF IRON AND SIDEROPHORES IN THE RHIZOBIUM-VETCH SYMBIOSIS

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Iron is an essential element required by both partners in legume-rhizobium symbiose. Root nodule bacteria use a range of strategies to obtain iron. Under iron-limitation, one is to use siderophore-based iron uptake systems. These involve the synthesis and excretion of an iron-chelating molecule (siderophore) and its uptake by specific membrane-located transporters. *Rhizobium leguminosarum* bv. *viciae* WSM710, nodulates field peas (*Pisum sativum* L.) and vetch (*Vicia sativa*) and, under iron limitation in laboratory culture, synthesises the siderophore, hydroxamate K (HK). HK is a cyclic trihydroxamate, composed of 3 residues each of N5-acetyl -N5-hydroxyornithine and hydroxybutyrate. Two Tn5-induced mutants of WSM710 impaired in siderophore synthesis have been isolated; MNF7101 produces a non-acetylated form of HK, resulting in a large, well defined halo on CAS agar while MNF7102 is a non-producing strain. These mutants are being used to investigate the role of siderophore production in nodulation and nitrogen fixation in vetch. Here we report the results from some initial studies.

Vetch were grown for 6 weeks at 22°C in nitrogen-free nutrient solution containing FeEDTA (0, 0.2 or 5.0 (M) and inoculated with WSM710, MNF7101 and MNF7102. Plants grown in 0 and 0.2 (M Fe developed symptoms of iron deficiency at day 14. Those grown with the 5 (M Fe did not show symptoms of nutrient deficiency during the experiment.

An effect of iron on nodulation appeared prior to any foliar symptoms of iron deficiency. Visible nodulation was delayed from day 6 in plants grown with 5.0 (M Fe to day 10 in plants grown in 0 and 0.2 (M Fe. All plants grown in 5.0 (M Fe developed many, large (>2 mm diameter), pink/red crown nodules, with few lateral nodules. In contrast, plants grown in 0 and 0.2 (M Fe developed few small (<2 mm diameter), white or pale pink crown nodules, with many lateral nodules. At all Fe concentrations, there was no difference in the nodulation pattern between the wild-type and the two mutants. At harvest nodules were picked, and 60 nodules per treatment were typed, using phenotypic characteristics of the wild-type and mutants on CAS agar. In each case >53 nodules were the inoculum strain.

Every 2 weeks nutrient solutions were extracted and assayed for HK and HK7101. Neither of these siderophores was detected. A second experiment investigated the persistence of HK in an acidic soil (pH 5.3). HK (2 mg) was added to 5 g of sterile and non-sterile soil. After 10 days 76% was recovered from the sterile soil and 50% from the non-sterile soil.

These preliminary results clearly indicate that iron deficiency affects the establishment of symbiosis in vetch. If the iron level is too low, nodulation is delayed and nodules that do form appear to be ineffective.

P-D-4

INCREASE OF SYMBIOTIC NITROGEN FIXATION IN SYMBIOSIS OF CLOVER WITH *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII* BY FLUORESCENT STRAIN *PSEUDOMONAS* SP. 267.

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Many species of the genus *Pseudomonas* are members of the PGPR (plant growth promoting bacteria). These bacteria promote plant growth by several mechanisms. *Pseudomonas* sp. strain 267 isolated from soil promoted growth of different plants (patato, rice, cabbage, wheat) and enhanced symbiotic nitrogen fixation in leguminous plants (clover, pea, veth, lupine). Strain 267 produced yellow-green, pyoverdine-like siderophore and secreted vitamins of the B group. Many different environmental factors (temperature, pH, cations, source of organic carbon) affected the syntesis of siderophore and vitamins. We determined optimal conditions for the production of these compounds. Maximal secretion vitamins of the B group was obtained on M1 minimal medium with 1% glucose or 1% glycerol as a sole carbon source, at pH 5.5-6.0, temperature 28°C or 37°C with 20μM concentration of divalent cations Co^{+2} or Mn^{+2}. The production of the pyoverdine was most abundant on M1 minimal medium supplemented with 0.2% succinic acid at pH 7.0 and 28°C. The biosinthesis of the siderophore was completely inhibited by adding Fe^{+3} or Co^{+2} to the culture medium. The role of fluorescent siderophore in the beneficial effect of strain 267 on nodulated clover plants was investigated. Several non-fluorescent (Pvd^-) Tn5 insertion mutants of *Pseudomonas* sp. strain 267 were isolated and characterized. The presence of Tn5 insertion was confirmed by Southern analysis of *Eco*RI digested genomic DNA of each derivative strain. The non- fluorescent mutants were compared to the parental strain with respect to their growth promotion of nodulated clover infected with *Rhizobium leguminosarum* bv. *trifolii* 24.1. We found that all isolated Pvd^- mutants stimulated growth of nodulated clover plants in a similar manner to the parental strain under gnotobiotic and non-gnotobiotic conditions.

P-D-5

OCCUPANCY OF *Rhizobium leguminosarum* STRAINS IN LEGUME ROOTS IN THE SOILS CONDITIONS

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Symbiotic nitrogen fixation has a significant part in sustainable agriculture, but this process is dependent on indigenous rhizobia qualities in the soils. Unfortunately, indigenous rhizobia met in soils of Latvia not always are suitable to the demands of the legumes for effective symbiotic actions in nodules. To secure effective activity of rhizobia in the nodules of legumes in order to fix symbiotic nitrogen in production sowings, it is an important, but at the same time the problem difficult to solve.

Biological and other environmental factors, that influence occupancy of inoculate rhizobia in the roots of the host-plant in the soils conditions were determined by different experiments. The pot and field trials were made in various soils with indigenous rhizobia. Different *Rhizobium leguminosarum* Frank. *cv. vicea* strains of streptomycin resistant forms and host-plants - peas (*Pisum arvense* L.), vetch (*Vicia sativa* L.) and field beans *(Faba bone* Medik.*)* ware used.

Results testify that biological ability of rhizobia strains to compete is one of the decisive factors to occupy themselves in soil, but to make effective nodules in the roots of legumes, the choice of host-plant has an important role. In order to secure maintenance with symbiotic nitrogen the host-plant gives preference to definite strains, which can form effective nodules. Each of the compared host-plants has different demands and there are few strains, which are equally suitable to all these crops, but usually they are corresponding only to one of these legume plants. Inoculant rhizobia occupancy in the roots of field beans is better as in those of the vetch and especially in the roots of peas in completely identical conditions.

In the conditions of soil, inoculant rhizobia, first of all, must get into the host-plant root spreading zone to take part in competition of nodule forming. Therefore, in the production sowings, the effectivity of inoculation is mainly dependent on condition of arable layer of soil, which secure the inoculant rhizobia spreading. It specially refers to annual legumes, for the period of nodule forming is comparatively short and for peas it is only 4 -7 days,

P-D-6

EFFICIENCY OF THE NODULATING *RHIZOBIUM LEGUMINOSARUM* POPULATION UNDER THE INFLUENCE OF SLURRY DEPOSITION AND SLOPE POSITION

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The leghemoglobin content of legume nodules is, at different stages of plant development, correlated with the activity of nitrogenase, measured by acetylene reduction assay. The total leghemoglobin concentration (Lb/mg nodule) was used as measure for nitrogen fixation activity of the nodulating *Rhizobium leguminosarum* bv. viciae population. A simple spectroscopic method was adapted to determine the content of leghemoglobin in the nodules of *Pisum sativum* cv. Grapis.
In a two year study (1994 and 1995) the effect of slurry and landscape position on the leghemoglobin content (Lb) was measured in a grasscovered arid region . Until 1990 this site has been used for the deposition of bovine slurry over a period of five years.
Pea plants were grown under controlled conditions in soil from the unpolluted or polluted sites or inoculated in sterile liquid culture with soil extracts from these sites .
In experiments with inoculated plants the Lb content/ mg nodule was significantly higher than in soil experiments and the treatment was more pronounced.
In 1994 and 1995 no effect of slurry on the leghemoglobin content of the nodulating population was found, whereas significant differences were detected between slope position and year of sampling. At the southern slope the Lb content/ mg was in both years significantly higher than at the northern slope and in 1995 the nodules from all sites had a significant higher leghemoglobin content compared to 1994. A reason for this increasing activity could be changes in population structure of the nodulating *Rhizobium leguminosarum* bv. viciae population. (LABES et al, 1996).
The results showed that landscape position had a significantly greater influence on the population than the deposition of slurry. The leghemoglobin content of pea nodules can be used for characterizing the efficiency of the nodulating *Rhizobium leguminosarum* population at a landscape scale.

Literature:

LABES, G., ULRICH, A., LENTZSCH, P. 1996. Influence of bovine slurry deposition on the structure of nodulating Rhizobium leguminosarum bv. viciae soil populations in a natural habitat. Applied and Environmental Microbiology 62:17717- 1722.

P-D-7

POPULATIONS OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE* IN EAST ANGLIAN SOILS

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Nearly 1500 rhizobia were isolated from 14 sites, including both arable and uncultivated soils in E. Anglia over three years. Isolates were sampled from nodules on resident legumes or legumes inoculated with soil dilutions and were characterised by PCR-RAPD analyses, confirmed by plasmid profiles. Correlations between RAPD and plasmid profiles were very good. Diversity at most sites was extremely high, e.g. 181 profiles from 401 isolates at one site. At only two sites, one arable and one uncultivated, were nodules dominated by one profile. One is discussed in more detail in another poster (Wilson *et al.*). Diversity was not an artifact of RAPD analysis as there was a similar variation in plasmid profiles. Very few profiles were found in consecutive years, indicating a rapid turnover with time, or wide differences over small areas because subsequent samplings were not from identical pockets of soil. We were unable to show that cultivation *per se* had a noticeable effect on diversity with both cultivated and uncultivated soils showing examples of high and low diversity, although there were wide differences in diversity between cultivated and uncultivated sites in close proximity. It would appear that factors other than cultivation are more important for the maintenance of diversity.

Strains were not randomly distributed between plants even when single dilution series of well mixed soil were used. Whilst it was common for one profile to be found in more than one nodule on a single plant it was uncommon to find one strain in nodules of different plants. This is the first time this has been reported for self pollinating host legumes.

As a result of the high diversity of rhizobia in most soils, it is thought unlikely that introduced strains could dominate in soils. It is difficult to assess persistence of strains due to the high diversity and the possibility that strains are localised in small niches. The isolation of rhizobia using host legumes as traps may give a biased estimate of population but, if many different plants are used, it could give a reasonable indication of diversity, particularly of those strains of agricultural significance.

P-D-8

SCREENING BIODIVERSITY OF RHIZOBIA AND *AGROBACTERIUM* LIKE STRAINS

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Fast growing bacteria isolated from the root nodules of *Acacia senegal* and *Prosopis chilensis* have been shown to be both fenotypically and genotypically diverse. We wanted to expand the taxonomic studies of tropical tree rhizobia at a genetic level and introduce new genetic screening methods. Fifty-seven rhizobial strains were used in nonradioactive DNA-DNA dot-blot hybridizations together with reference strains from four recognized *Rhizobium* and four *Sinorhizobium* species. Scores given to the intensities of dots detected in the hybridizations were used in principal component analysis (PCA), which clustered the strains in separate DNA-homology groups. The strains were further studied by restriction fragment length polymorphism (RFLP) analysis of 16S rRNA coding genes amplified in polymerase chain reaction (PCR). The PCR-RFLP analysis was applied to 150 strains including strains representing ten recognized *Rhizobium* species, two *Sinorhizobium* species, five *Agrobacterium* species and other reference strains belonging to the family *Rhizobiaceae*. Thirty-nine composite genotypes were obtained from the combined data of RFLP analysis with ten different restriction enzymes. The results correlated well with the dot-blot hybridization results and with previously published data based on RFLP and sequence analysis of the 16S rRNA coding gene. The PCR-RFLP also showed that the genotype of many root-nodule derieved strains was quite similar to that of agrobacteria. Five Sudanese *Agrobacterium* like strains and a mixed culture were tested with *Acacia senegal* seedlings for their ability to form nodules. Only the mixed culture was able to nodulate the legume and no tumors were formed. Both dot-blot hybridization and the PCR-RFLP analysis serve as good genomic screening methods for new rhizobial isolates with unknown taxonomic status, and used together they complement each other, allowing preliminary species and genus assignement, respectively.

P-D-9

BIODIVERSITY OF *RHIZOBIUM MELILOTI* STRAINS ISOLATED IN ACID SOILS FROM ARGENTINA AND URUGUAY

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Within the scope of the project "Improvement of the symbiosis between *Rhizobium meliloti* and Alfalfa in acidic soils from Argentina and Uruguay", that is financed by the European Community, a strain collection of wild-type *R. meliloti* strains was isolated from acid soils (pH5.0-6.5). 36 of these strains were characterized with several different methods. The screening of the strains with regard to the acid tolerance pointed out, that 12 strains had the ability to grow at pH 5.0 in laboratory conditions. The biodiversity of the 36 strains was characterized with ERIC and ARDRA profiling, IS-typing, LPS analysis in SDS-PAGE, fatty acid analysis and sequencing of the 16srDNA of selected strains. Furthermore the nodulation efficiency and the nitrogen fixation were determined in plant tests on plates with nitrogen-free agar.

The non-acid tolerant *R. meliloti* strains showed a high variability according to the tested parameters. In comparison the 12 acid tolerant strains are nearly similar to each other, although they were isolated from different areas with acid soils in Argentina.

P-D-10

CHARACTERISATION OF INEFFECTIVE SYMBIOSES IN *Vicia faba*

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Various types of the ineffective symbiosis caused by *Rhizobium leguminosarum bv. viciae* in *Vicia faba* L. var. Minor root nodules are described. The strains of this bacterium isolated from faba beans grown on Latvian soils had varying symbiotical effectiveness. For the *Vicia faba* L. cv. Lielplatones the inoculation with the effective 501 strain resulted in great increase of plant top and nodule weights and in the increase of their nitrogen contents when compared with the ineffective 106 strain-inoculated plants at flowering. The symbiosome membrane fluidity, (measured with 1.6 diphenyl 1.35-hexatriene (DPH) significantly increased in the root nodules formed with the ineffective 106 strain which may be the reason of unstability of symbiosomes and of the early loss of nitrogen fixing ability of bacteroids.

In experiments with cv Aushra faba bean plants grown in sand culture (16mg of nitrogen per kg of sand) the inoculation with ineffective *Rhizobium leguminosarum* bv viciae 87 strain (from the collection of All-Russian Institute of Agricultural Microbiology) resulted in acute nitrogen deficits and decrease of plant top and nodule weights. The carbon content also decreased in the leaves in plant flowering stage. The acetylene reduction activity was 13 times lower, catalase activity - 4 - 5 times lower and peroxidase activity two times lower in 87 strain-inoculated small white nodules than in normal 97 strain red nodules.

For *Vicia faba* L. var. Minor cv Maris Bead grown in a glasshouse on nitrogen-rich organic soil, spontaneous inoculation took place resulting in the sporadical appearance on the lateral roots of large white nodules without the leghemoglobin and succinate-dehydrogenase activity. An electron microscope investigation showed the presence of abnormally elongated, enlarged bacteroids containing no PHB (poly-ß-hydroxybutyrate) granulae. Extremely large amyloplasts with starch were found in these nodules with abnormally thick cortical cell walls. The significant peroxidase activities detected in these unusual nodules may have been caused by the possible presence of an active host-defence reaction.

P-D-11

EFFECT OF HEAVY METALS ON THE *Rhizobium meliloti* STRAINS

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Heavy metals can have inhibitory or stimulatory effect on the growth of rhizobia and on the nodulation of legume plants.

The objective of this investigation was to examine the effect of different concentrations of Mo^{6+} as $(NH_4)_6MO_7O_{24}$, Ni^{2+} as $NiCl_2$, Pb^{2+} as $PbCl_2$ and Cd^{2+} as CdCl (10_{-4}, 10^{-6}, 10^{-8} moles l^{-1} medium on the growth of two *Rhizobium meliloti* strains (L_1, 7M) and on the nodulation of alfalfa. The heavy metals were added in a YM medium after 0, 24, 48, 72 and 96 hours of growin in the medium at 28°C in a shaker (200 rpm) the number of rhizobia was determinated according a method of fertile drops. The nodulation was being determinated during 40 days. The five days old alfalfa plants were planted into flasks with a nutrient medium and heavy metals. The plants were inoculated with 1 ml cca 10^8 cels ml^{-1} of strains of rhizobia (L_1, 7M).

The growth of rhizobia (strain L_1) in medium with 10^{-4} mol l^{-1} Cd^{2+} and the growth of 7M rhizobia strain, with 10^{-4} mol l^{-1} Pb^{2+} and Ni^{2+} was inhibited. The concentrations of 10^{-6} and 10^{-8} mol l^{-1} of all four heavy metals in medium decreased or had no effect on the growth of any rhizobia strain.

The nodules formed 13 days after inoculation in control and variants with low concentrations of heavy metals.

The nodulation was best in variants with Mo^{6+} and Cd^{2+} in nutrient medium.

P-D-12

STUDY FOR THE DEGRADATION OF HERBICIDE ATRAZINE BY USING *RHIZOBIA MELILOTI* STRAINS ISOLATED FROM A GREEK PASTURE AREA

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The aim of the study is to investigate whether Biological Nitrogen Fixation may contribute to the protection of the Environment from the hazardous effects of the herbicide atrazine, by using effective *Rhizobia meliloti* strains isolated from the indigenous population of subsurface and aquifer samples collected from a pasture area in Greece .

Atrazine is the most widely used s-triazine worldwide and it has been classified as a very persistent herbicide. It can persist from 10 to 57 weeks and thus contributes significantly to Environmental pollution affecting health standards .

A hope to solve that problem is to discover bacteria able to degrade atrazine forming non-toxic products, and this presently attracts worldwide attention and effort. Concerning the present effort, the introduction in a contaminated pasture field of effective *Rhizobia meliloti* unaffected by the high concentration of atrazine, able to use it as a source of Carbon or Nitrogen will be extremely important . However, to the best of our knowledge, no Rhizobia had been isolated thus far capable to use atrazine as a source of Carbon or Nitrogen and therefore degrade it.

From a pasture therefore area we collected, under sterile conditions, soil and water samples, each a column 50 cm long. That sampling went all the way down to the water level, at a depth 7.0 m, by using a dry percussion coring technique . From these soil and water samples we isolated more than 100 strains of effective *Rhizobia meliloti*

Study of the isolated strains indicates that:

1. *Rhizobia meliloti* are present below the soil layer and even at a depth 7.0 m
2. Growth curves show that in more than 30 strains the presence of 5 to 60 ppm atrazine has no effect either on Generation time or on the Onset of the stationary phase.
3. Incubation for 1 year in sterile soil containing 5 to 60 ppm atrazine shows that in more than 20 strains atrazine has no effect on survivability or N-fixing activity.
4. Using selective media with atrazine as the only source of Nitrogen or Carbon, more than 15 strains use atrazine as Nitrogen source and more than 5 (until now) use atrazine as a Carbon source.

These results clearly indicate that the above strains will be able to survive and also Fix Nitrogen in a pasture heavily contaminated with atrazine . They may also contribute to the degradation of atrazine . The definite proof of the latter, nevertheless, clearly demands further investigation. Such a possibility will be of great benefit both to the Environment and to Sustainable Agriculture .

P-D-13

ROLE OF BORON IN SIMBIOTIC DINITROGEN FIXATION

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Boron (B) is an essential micronutrient for the development of nitrogen-fixing root nodules. We have studied nodule development in legumes with determinate (bean) and indeterminate (pea) nodules.

Bean nodules grown without B in the nutrient solution were smaller in size and weight than the control. B-deficient nodules (2 and 3 weeks after inoculation with *Rhizobium*) examined by light microscopy showed dramatic changes in cell structure, mainly observed at the cell wall level when compared to control nodules. The use of 'tissue-printing' and western blotting indicated that the extensin and pectins decreased in B-deficient nodules.

Moreover, the use of monoclonal antibodies that recognize specific glycoconjugate components implicated in pea nodule invasion and symbiosome development allowed us to establish a possible new role of boron in nodule development by stibilizing some glycoproteins and glycolipids and modify bacteria-plant cell surface interactions mediated by these glycoconjugates.

Overall these data suggest that B is an microelement required not only for the stability of the nodule structure but also for the correct recognizition between plant and *Rhizobium* leading to the establishment of the symbiosis.

P-D-14

CHARACTERIZATION OF A MEGAPLASMID 1 LOCATED GENE CMP-37 RESPONSIBLE FOR NODULATION COMPETITIVENESS IN RHIZOBIUM MELILOTI

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Nodulation competitiveness (NC) is an important property of the nodule bacteria which determines field performance of the commercial strains. Previously, we marked by Tn5 a gene involved in control of NC in a highly effective R.meliloti strain CXM1-105. The Tn5-insertion was located on the megaplasmid 1 (Sym-plasmid) and resulted in a strong decrease of NC (measured under aseptic conditions and when alfalfa was grown in non-sterile soil) and delayed nodulation (1). By means of transduction (phage ØM12) we localized the Tn5-insertion 57 kb apart from fdxN gene. There is no difference between the Tn5-mutant and parental strain in hybridization patterns obtained with probes containing one of the following symbiotic gene regions: nodABCD, nodH, nifA, nifHDK, fdxN. Preliminary sequence analysis of the competitiveness gene revealed homologies with genes encoding fatty acid synthase and glycero-3-phosphate dehydrogenase. The Tn5 insertion which lead to decreased NC does not affect utilization of different carbon sources including glycerol. The NC^- mutant does not show any differences from the wild type strain in its phenotype on yeast-mannitol plates with TTX or Congo Red as well as in its motility. The described gene was called cmp-37. Previously the R.meliloti genes controlling NC were identified on the chromosome ("behavioral" genes, lps- gene) and a cryptic plasmid (nfe-genes) (2-4). Since, no NC-controlling genes have been found on the megaplasmid 1, we concluded that cmp-37 represents a novel gene responsible for NC in R.meliloti.

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P-D-15

ECOLOGICAL CONSEQUENCES OF A GENETIC MODIFICATION OF THE *Bradyrhizobium japonicum*/ SOYBEAN SYMBIOSIS

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The fitness consequences of a genetic modification for the legume symbiont *Bradyrhizobium japonicum* and its plant partner, soybean *Glycine max* were examined under conditions when the resulting phenotype was not expressed. The mutant strain, KL1, had its *putA* gene disrupted by the insertion of a spectinomycin resistance cassette (Straub & al. 1996. Appl. Environ. Microbiol. 62, 221.). Inter-strain competition between the mutant and its parent wild type was studied by growing the mutant and wild type strains in liquid medium under non-limiting conditions, re-inoculating as necessary to be kept in the logarithmic growth phase. Three different initial conditions were mixtures of wild type vs. mutant strains at ratios of 1:1, 3:1, and 1:3. During an incubation period of 10 days, the mutant bacterial strain showed no tendency to be outcompeted by the original wild type. Soybean plants grew equally well when inoculated with either strain or their mixtures showing no statistically significant difference in biomass, biomass allocation between above- and below-ground plant parts, total or mean nodule mass or total nodule number. Nodule occupancy tests showed that the mutant strain was not an inferior competitor for nodulation sites in relation to the wild type parent strain. We conclude that this *putA* mutant confers no disadvantage for the *B. japonicum*/ soybean symbiosis under non-selective conditions. These experiments constitute the first steps to build an ecologically realistic model system incorporating several trophic levels to study the ecological effects of genetic modification of the *Rhizobium*-legume symbiosis.

P-D-16

SURVIVAL AND COMPETITION OF *BRADYRHIZOBIUM JAPONICUM*

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Most of the soils of the Argentine soybean area possess naturalized strains of *Bradyrhizobium japonicum* . These strains occupy most of the soybean nodules and it is difficult to identify a new strain introduced by inoculation. Because of this reasons the objectives of the work were: 1.- To study specific competition between strains for nodulation, 2.- To establish the persistence of strains of *Bradyrhizobium japonicum* in the soil with a specific legume host and non legume species. Three experiments in soybean areas were carried out in soil without bradyrhizobia: 9 de Julio (1987 to 1993); Castelar (1988 to 1993) and Tres Arroyos (1989 to 1992). Randomised blocks with five treatments and 4 replicates were used as indicated in the following table:

Treatments 1st agricultural cycle, AC	**Treatments subsequent AC**
1. Soybean seeds inoculated with E110.	Maize
2. Soybean seeds inoculated with E110 with E112 in the soil.	Maize
3. Soybean seeds inoculated with E110 with E112 in the soil.	Soybean without inoculation
4. Soybean seeds inoculated with E110 with E112 in the soil.	Soybean inoculated (E110)
5. Soybean without inoculation (control)	Soybean without inoculation

The strains used were: E110 (29 W MIRCEN Brazil) represented a commercial inoculant and E112 isolated from the 9 de Julio area and representing the naturalized population. The E110 strain was inoculated at the rate of 6 x 10^5 bradyrhizobia per seed. The E112 was introduced into the soil with irrigation and incorporated with a hand cultivator at the surface 10cm, until reaching 1 x 10^5 bradyrhizobia per gram of dry soil which is the normal concentration at soybean area. Plots were protected with a construction of wires covered with a polyethylene film (100µ thick), dug down 10 cm into soil delimiting 1 m^2 (1x1m). Each plot was seeded with two rows of soybean, 0,5 m apart. Soil analyses were made every year before sowing: Organic matter (%), carbon (%), nitrogen (%), C/N relation, P (ppm) and pH in paste. Nodule samples of soybean plants from each experiment were taken at reproductive stage 2. Strains from nodules (treatment 3 and 4) were identified by ELISA. The percentage of nodule occupancy was calculated. The most probable number of bradyrhizobia in the soil before sowing was determined using the dilution plant technique. The analyses of the soil indicated phosphorus deficiency and low pH, mean quantity of organic matter and total nitrogen. At 9 de Julio and Castelar, inoculation with E110 increased nodule occupancy by E110 in treatment 4 respect to treatment 3 after three years, but not at Tres Arroyos because only three experiment years was carried out. The survival of bradyrhizobia in the soil was low in all the treatments, 10^2 to 10^5, depending on the years for 9 de Julio and Castelar. Tres Arroyos had higher survival of bradyrhizobia (10^4 to 10^5) also depending on the year.

P-D-17

ALTERED COMPETITIVENESS OF *Bradyrhizobium japonicum* USDA 123 AND USDA 110 STRAINS IN THE FIELD CULTURES IN POLAND

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Studies on the competitiveness between *Bradyrhizobium japonicum* strains USDA 123 and USDA 110 have been conducted since 1994 as the field experiment. The selected field site was free of indigenous strains capable to nodulate soybeans. Prior to the first sowing (1994) the seeds (*Glycine max* (L.) Merr. cv. Nawiko) provided for separate plots were inoculated with USDA 123, USDA 110, and the mixture of equal cell concentrations of both strains. An uninoculated control plot was also included into the experiment. Each experimental plot was prepared in four replicates. No inoculations were performed during subsequent experimental seasons.

The mixed inoculation experiment revealed the increased competitiveness of USDA 110 over USDA 123. About 70% of nodules were occupied by USDA 110, as it was estimated by immuno-spot-blot method using cross-reacted strain-specific antibodies. The domination of USDA 110 was observed also during the following years. Due to the natural reasons the areas occupied by both strains have expanded leading to overlapping their initial ranges. Still USDA 110 dominates, although both strains show a good survival rate.

The experimental data suggest that in the contrary to situation observed in American soils, USDA 110 shows different pattern of competitiveness. The possible explanation is that the intra-strain relations are very much dependent on the presence or absence of other soil microorganisms, especially the indigenous bacteria nodulating the same host plant.

P-D-18

POPULATIONS OF *Rhizobium* AND *Bradyrhizobium* NODULATING LUPINS IN POLAND

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Most of the Polish soils contain indigenous populations of bacteria capable to form nodules on lupin roots. However, this group of strains has not been characterized with respect to their genetic diversity.
Over 200 isolates of bacteria were obtained from lupin root nodules collected from over 25 field sites in 15 places in Poland. All three major lupin crops (*Lupinus luteus* L., *L. angustifolius* L., and *L. albus* L.) were considered.
In order to determine their taxonomic position the isolates were characterized with respect to their type of metabolism and growth rate. Further analysis was performed using several standard techniques. The intrinsic antibiotic resistance, SDS-PAGE protein profiles, *nod* and *nif* hybridization profiles were analyzed as well as REP-PCR analysis of numerous isolates.
Apparently, most of bacteria isolated from root nodules of lupins grown in Polish soils can be included into the genus *Rhizobium* since about 80% of strains reveals acidic metabolism and are fast-growers. Only 20% of isolates can be designated *Bradyrhizobium* sp. (*Lupinus*) according to these criteria.
The diversity among isolates belonging to either group is relatively high. It may be particularly important to exploit this observation since little is known about rhizobia which inoculate hosts belonging to lupin cross-inoculation group. It is a good chance that it would provide us with an important data on the nodulation specificity

P-D-19

THE ROLE OF HUP-PHENOTYPE BRADYRHIZOBIUM.SP / LUPINUS / IN THE FORMING OF N_2-FIXING SYMBIOSIS.

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In nitrogen-fixing microorganisms the enzymatic system of nitrogenase catalyses the ATP-depended reduction of nitrogen to ammonia and of protons to hydrogen. The production of the last one by nitrogenase is obligatory and it dissipate 25-50 % of energy used for symbiotic N_2-fixing. Howewer, some rhizobial strains (Hup^+ strains) have H_2-uptake hydrogenase system which oxidises H_2 to water. The symbiotic expression of hydrogenase activity in nodules is strongly controlled by the legume host of rhizobia, although it is not clear , what plant factors are required for induction of the Hup-system and what sense it has for the symbiosis.We investigated the symbiotic properties of the Hup^+ strains Bradyrhizobium sp./ Lupinus / and the role of Hup-phenotype in forming of the high-productive symbiosis with Lupinus sp. We also studied the role of Hup-function in symbiotic nitrogen fixation by comparing the phenotypes of the strains Hup^+ and Hup^-Bradyrhizobium sp./ Lupinus /. We were succeeded in isolation of such strains from the soils of our region.The inoculation of lupine plants with Hup^+ strains results in the increase of nodule mass, leghaemoglobin and ATP levels, ATP-ase and hydrogenase activities. The uptake hydrogenase activity was positively correlated with green biomass of plants and grain yield. Also this activity was depended of lupine variety.

P-D-20

ENVIRONMENTALLY EXPRESSED GENES IN *RHIZOBIUM*

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The ability to study micro-organisms within complex mixtures such as occur n soil and water, has often been impaired by the difficulties encountered in culturing important organisms because they are present in low numbers or are overtaken by faster growing organisms. A new technique known as optical trapping has been developed which represents an opportunity to isolate these organisms as well as obtain spatial information about them within their environment (Ashkin *et al.* 1987a,b).

Optical trapping relies on the radiation pressure caused by the refraction of coherent light by particles being trapped (Block 1992). We have set up a continuous wave Nd:Yag 1064 nm infra-red laser whose light path is directed down an epi-fluorescence microscope. Using 10-30 mW of TEM_{00} radiation cells are trapped at the centre of the light beam where the Guassian distribution of light is at its peak. The bacteria can be moved by manipulating the microscope stage. In this way cells can be individually selected and placed in sterile medium in a capillary tube and subsequently cultured. Cells can be selected on the basis of morphology or because they express fluorescent marker proteins.

To facilitate the identification of genes that are specifically expressed in the environment but not in laboratory culture we have developed a new class of promoter probe vector that contain promoterless copies of the marker genes GFP and akaline phosphatase. Genomic libraries of *Rhizobium leguminosarum* have now been constructed in these vectors and cells bearing plasmids that do not lead to gene expression in the laboratory selected. We are currently introducing bacteria containing these libraries back into soil and into the rhizosphere to look for individual bacteria that now express the marker protein. These bacteria will be identified and separated from the bulk population by the use of the optical trapping fluorescence microscope. Individual cells will then be cultured and the promoter probe vector isolated. This should allow the identification of genes specifically expressed in the environment but not in the laboratory.

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P-D-21

ACQUISITION AND EVALUATION OF MICROBIAL GERMPLASM FOR LEGUMES OF THE TEMPERATE CLIMATIC ZONE WITH POTENTIAL AS NEW CROPS FOR AGRICULTURAL PRODUCTION

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Rhizobium strains isolated from root nodules of the temperate zone legumes such as *Astragalus* spp., *Oxytropis campanulata*, *Hedysarum alpinum*, *Ononis arvensis*, *Glycyrrhiza* spp., *Coronilla varia*, and *Onobrychis* spp. (90 bacterial isolates) were analysed. Their phenotypic properties were compared with those of other *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* strains by means of numerical taxonomy. The dendrograms show that the strains formed several clusters separated from both *Rhizobium, Agrobacterium* and *Bradyrhizobium* reference strains. The sensitivity of these strains to bacteriophages of different *Rhizobium* species was in agreement with data of numerical systematics. The diversity of these groups was confirmed by DNA:DNA hybridization experiments with reference DNAs from recognized *Rhizobium* species and by the sequencing of a 260 bp 16S rRNA gene fragment. About 60% of strains tested had the symbiotic genes located on high molecular weight plasmids. Genomic characterization included also an analysis of restriction fragment lengh polymorphisms for symbiotic and chromosomal genes. Among the strains, the bacteria suitable for inoculant production were selected. The results have practical implications for the improvement of rhizobial germplasm used in agricultural production and for expanding of new legumes in the plant industry (as medicinal preparations, oils or fiber, in ecology as bioremediators of damaged soils or erosion control, as new legumes for crop production etc.).

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P-D-22

MUTANTS OF NITROGEN-FIXING *PSEUDOMONAS* 418 DEFECTIVE IN COLONIZATION OF ROOTS

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Using the vector pSUP2021, Tn5-induced mutants of nitrogen-fixing *Pseudomonas* 418 unable to colonize barley roots competitively, were isolated by roots replication method. A Southern blot analysis indicated that eight mutants carried Tn5 in the single EcoR1 fragments of 8,0 to 22,0 kb. In two mutants Tn5 was found to insert twice. The phenotypes of the mutants were characterized. They can be divided into the following groups: mutants unable to attach to roots surface, non-motile mutants deficient in the production of polar flagella, mutants lacking chemotaxis, mutants affected in exopolysaccharides production. The effect of some Tn5 insertions is pleiotropic.

P-D-23

COLONIZATION OF BARLEY ROOTS BY *PSEUDOMONAS* SPP. DERIVATIVES ALTERED IN NITROGEN FIXATION AND SIDEROPHORE PRODUCTION

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Colonization of barley roots by *Pseudomonas* 418, its Tn5-induced Nif^- mutants, and derivative derepressed in nitrogen fixation (Nif^c), *Pseudomonas fluorescens* 49, its mutants non-producing fluorescent siderophore (Flu^-) and producing it constitutively (Flu^c) was assessed by the laboratory test and in the microplot field experiment. No significant differences were observed between genetically altered and non-altered strains when tested in the laboratory and in the density of seedling roots colonization in the field. During the plants growth the number of Flu^c bacteria on the roots decreased as compared with Flu^+ and Flu^-, and the number of Nif^c bacteria - as compared with Nif^+ and Nif^- ones. The instability of Flu^c and Nif^c strains was not the major reason for their population decline during vegetation. The ability to fix nitrogen was found to determine an increase in the introduced Nif^+ bacteria population at reproductive stages.

P-D-24

BEHAVIOUR OF APPLIED NON-PATHOGENIC AGROBACTERIA IN THE RHIZOSPHERE OF WHEAT

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Usually, it is necessary to control the bacterial activity (number of bacteria of a definite species) in soil and the rhizosphere of plants after inoculation of agricultural plants with bacterial preparations. The workers sometimes used bacterial strains displaying improved charachteristics in field conditions. The monitoring of genetically modified bacteria in the environment conditions is a very important problem, since we have no precise information on the transfer of genetic information in soil conditions. We developed out and applied a method for monitoring plant-associated soil bacteria in collaboration with the Laboratory of Physical Chemistry of Cell Structures, IBPPM RAS (Saratov). The method is based on the monitoring of genetically modified (twice antibiotic labeled) strains, which were identified after growth on antibiotic-containing media by antibodies to bacterial surface polysaccharides.

This approach was applied to *A.radiobacter* strain 5D-1 and its Tn5 attachment minus and overattaching mutants. *A.radiobacter* strain *5D-1* was isolated from wheat roots and can attach to wheat roots [1] and appears is a good model for studying bacteria-plant interactions. We conducted vegetative and field experiments for wheat germ and seedling inoculation with *A.radiobacter* 5D-1 strain. Attachment charachteristics of *A.radiobacter* 5D-1 and its mutants defined under laboratory conditions and colonization capacity in field conditions were directly correlated. No sigfnificant differences were found in wheat crop yield upon inoculation of wheat seedlings with the wild strain and overattachming mutants in the field . We reisolated *A.radiobacter* 5D-2 from field grown wheat roots using antibiotic-containing media and a reaction with *A.radiobacter* 5D-1 antibodies and studied morphological, genetical, attachment and immunological characteristics in compared with *A.radiobacter* 5D-1.

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P-D-25

ANALYSIS OF INTRODUCED *A.RADIOBACTER* 5D-1 IN SOIL AND THE RHIZOSPHERE OF WHEAT AND INFLUENCE OF BACTERIAL INOCULATION ON WHEAT

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A search for conditions for introduction of non-pathogenic agrobacteria to the wheat rhizosphere and soil was conducted in field and vegetative experiments. Analysis of the number of bacteria was conducted by counting a colony, growing on the glucose and Khotinger media supplemented with 100 mg/L rifampicin for *A.radiobacter* 5D-1 and 50 mg/L kanamycin for *A.radiobacter* 5D-1 Tn5 mutants. Next, the growing colony was identified with antibodies to the surface polysaccharides. A significant increase in dry weight of above- ground parts of wheat was recorded after a two-fold inoculation of wheat seedlings with *A.radiobacter* 5D-1 and after a two-fold inoculation with bacterial preparation with Emistime (inductor of mycorrhiza) . An increase (14% higher than in the controls) of plant biomass was observed after a two-fold inoculation of seedlings with *A.radiobacter* 5D-1 with Emistime F. In the case of applying *A.radiobacter* 5D-1 with Emistime F for seed inoculation, the increase in dry weight was 9% higher than in the control. A single inoculation procedure of wheat seeds had no effect on wheat yield. A significant increase in wheat dry weight (99% of significance) was regcorded after inoculation of seedlings with *A.radiobacter* 5D-1 (10% of control). We did not record more active colonization of wheat roots by overexopolysaccharide producing mutant 5005 in field conditions, but found that in field conditions non-motile and flagella-negative mutants appear on the surface of wheat root about 50-100 fold less than the wild strain.

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P-D-26

THE MAIN MECHANISMS ON THE INTERRELATIONS OF NITROGEN-FIXING BACTERIA WITH PLANTS

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Biopreparations on the base of associative nitrogen-fixing bacteria belonging to genera *Azospirillum* (*A.lipoferum*), *Agrobacterium* (*A.radiobacter*), *Arthrobacter* (*A.misorens*) and *Flavobacterium sp.* were created and tested in the complex experiments (with wheat, rape, barley, rice, corn, potato, tomato). All of the studied strains were able to colonize the root surface of the plants. *A.radiobacter* and *A.misorens* were most effective colonizers. Their number became stable at the high level (1-50 mln/g roots). The quantity of the strain *Flavobacterium* decreased evently during the first 20-30 days after the inoculation from 1 to 0,03-0,1 mln/g roots. The population size of *A.lipoferum* raduced rapidly and her number became stable at the level 0,01-0,5 mln/g roots. The differences in colonization ability of bacterial strains among the plants varieties were not significant. The results of the present study suggest, that population density of present strains are independent of the species of plants and determined by bacterial genotype.

Besides of the well known mechanisms of action (nitrogen fixation, plant growth promotions, supressing of phytopathogenesis) this bacteria can increase resistance of plants to ecology stresses and can decrease uptake of the heavy metals and radionuclides in the plants.

Yield of inoculated crops increased by 10-20% (for cereal crops) to 20-40% (for vegetables) as was established in the numerous experiments in various soil-climatice conditions.

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P-D-27

NITROGEN FIXING PHYLLOSPHERE MICROFLORA OF *TILLANDSIA* RESPONSE TO AIR POLLUTION

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Air pollution poses a seriuos threat to the environment worldwide. It affects all living things, including all kind of vegetation. More significant effects can be observed generally at regional or local levels when associated with roadways, urban or industrial areas. Leaf surface generally harbours a plentiful microbial population and microbes are highly exposed to air pollutants so their ability to withstand the environmental changes can influence strongly their activity. *Tillandsia* (*Bromeliaceae)* plants, which have developped one of the highest level of epiphytic life represents the suitable vegetal model for studing the air pollution effects on phyllospheric microflora since the microbial population living on their leaves, especially nitrogen fixing species, contributes to plant nutrition.

We have investigated about the possible influence of air pollution on the composition of the nitrogen fixing phyllospheric microflora of two species of *Tillandsia* collected in two different air pollution conditions with the future aim to examine the nutritional contribution of other microbial group being part of the community living on their leaves and to estabilish possible relationships between the welfare of the plant and its associated microflora. Our investigations have shown that both in *Tillandsia caput medusae* and *Tillandsia schiedeana* the number of nitrogen fixing microorganisms is slighty affected by pollutants so this source of nitrogen seemed not to be reduced in polluted plants.

P-E-1

REGULATION OF NITROGENASE ACTIVITY BY NH_4^+ IONS AND AMINO ACIDS IN *Azospirillum lipoferum*

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We have been studying the regulation of nitrogen fixation in ***Azospirillum lipoferum*** FS, isolated from rhizosphere of rice, for the purpose of developing a biological nitrogen fertilizer. ***A. lipoferum*** is a microaerobic nitrogen-fixing bacterium living in association with the roots of nonleguminous plants such as corn, wheat and rice. It has a posttranslational regulatory system for nitrogen fixation involving dinitrogenase reductase ADP-ribosyltransferase (DRAT) and dinitrogenase reductase-activating glycohydrolase (DRAG). In the presence of NH_4^+ ions or glutamine, DRAT catalyzes ADP-ribosylation of dinitrogenase reductase to inactivate it. When these nitrogen compounds are used up, DRAG removes the ADP-ribosyl group from dinitrogenase reductase to reactivate it.
We cloned a 6.7-kb ***Sal***I fragment containing ***draT*** and ***draG*** genes, which encode DRAT and DRAG respectively, from ***A. lipoferum*** FS. From analysis of the nucleotide sequence, it is assumed that ***draT*** and ***draG*** are transcribed as a single operon (named ***dra*** operon). To monitor expression of the ***dra*** operon, we constructed a broad-host-range plasmid containing a ***draT*****::*****lacZ*** fusion gene and introduced it into ***A. lipoferum*** FS. The β-galactosidase activity was detected under microaerobic conditions, regardless of NH_4^+ concentration, but not under aerobic conditions. This indicates that expression of the ***dra*** operon is regulated by oxygen but not by NH_4^+.
Next, we constructed a derivative of transposon Tn5 in which ***nifA*** gene from ***Klebsiella oxytoca*** NG13 was inserted downstream of kanamycin resistant gene, and the Tn5 derivative was transposed onto the genomic DNA of ***A. lipoferum*** FS. One of the obtained transposants, named ***A. lipoferum*** TA1, showed nitrogenase activity even when it was grown in the presence of NH_4^+ ions or several amino acids although the nitrogenase activity of the parent strain, ***A. lipoferum*** FS was completely repressed under the same conditions. The phenomenon observed in ***A. lipoferum*** TA1 is probably due to the function of NifA protein synthesized from ***K. oxytoca nifA*** gene. But, when induced for nitrogenase activity under NH_4^+-free conditions, both ***A. lipoferum*** FS and TA1 exhibited similar response to addition of NH_4^+ ions or several amino acids. These nitrogen compounds added to the nitrogen-fixing cultures led to a rapid loss of nitrogenase activity in both strains, suggesting that the nitrogenase activity is regulated posttranslationally in these organisms. In contrast, when ***A. lipoferum*** TA1 was grown under NH_4^+-rich conditions, nitrogenase activity expressed by NifA from ***K. oxytoca*** was not influenced at all by addition of the same nitrogen compounds. These results suggest that the posttranslational regulatory system of ***A. lipoferum*** is not capable of regulating the nitrogenase activity which had been expressed under NH_4^+-rich conditions.

P-E-2

FREE - LIVING HALOFILIC BACTERIA FIXING - N_2

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Introduction and methods

Bacteria of the genus *Azospirillum* commonly occur in soil and rhizosphere of plants of tropical as well as moderate climate (Baldani V.L. et al.1983; De Coninck K. et al. 1988; Dobereiner J. et al.1976; Kulińska 1983; Jaśkowska 1994). The samples were taken from: the rhizosphere of barley grown on different soils, the rhizosphere of maize, the rhizosphere of *Elymus arenarius*. In order to record the occurence of *Azospirillum* in rhizoplane, the roots were rinsed in tap water, sterilized on the surface with sodium dichloroisocyanurate and cut into a 5 mm pieces. Several root pieces of a given above plant variety were put into each of ten test tubes, containing 5 cm^3 semisoild nitrogen-free medium (Dobereiner J. et al.1976). The cultures were incubated for 72 hours at 30^0C. Presence of *Azospirillum* was macro- and microscopically tested. Strains of bacteria were studied on growth in presence of 3% and 5% NaCl. Most probable number (MPN-Hegazi et al 1979) of *Azospirillum* was counted with the use of Mc Crady's statistical tables (Girard, Rougieux 1967).

Result and conclusions

Halofilic bacteria of the genus *Azospirillum* occurred in about 22 % of all samples and no correlation between frequency of their occurrence and the sampling date was observed. In the rhizosphere of barley occurrence of halofilic strains of *Azospirillum sp.** was found in 84% and *Acinetobacter-like*** in 16% of all isolates. In the rhizosphere of maize the presence *A.sp.* and *A.-like* were found in 23 and 0%, respectively. The rhizosphere of *Elymus arenarius* taken from Sobieszewo contained the *A.sp.* and *A.-like* in amount of 12 and 6%, respectively. *A.sp.* (5%) and *A.-like* (48%) were found in the rhizosphere of gramineum taken from Świoujście. Abundance of *Azospirillum* in rhizosphere of the examined plants was low, bacteria were not found to occur in all replications, usually amounting to some cells in 1 g of rhizosphere soil dry matter and sporadically coming to several thousand.

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* - growth in presence of 3% NaCl
**- growth and nitrogen-fixing in presence of 3% NaCl and growth on 5% NaCl.

P-E-3

EFFECT OF PLANT GROWTH SUBSTANCES ON THE ACETYLENE REDUCTION (ARA) IN BARLEY AND MAIZE ROOTS INOCULATED WITH *AZOSPIRILLUM LIPOFERUM*

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Introduction and methods

Plant growth substances, such as auxine, cytokinin, giberillin and abciscin were tested for their effect on growth and nitrogenase activity from batch culture grown *A.brasilense* (Christiansen-Weniger C. 1988) Barley and maize seeds were surface sterilized, using 0,2% $HgCl_2$ for 3 min. and inoculated with 0,1 mL of a 24-h culture (10^6 CFU) of *A.lipoferum.* Barley and maize seedlings were treated with 0 mg kg^{-1}; 0,25 mg kg^{-1}; 0,5 mg kg^{-1}; 1,0 mg kg^{-1}: 4-(3-indole)-butyric acid (IAM), indole-3-acetic acid (IAA), 2,4-dichlorphenoxy-acetic acid (2,4-D), 6-benzyl-aminopurine (PAB) and gibberillic acid (GA). The influence of plant growth substances on growth of *A.lipoferum* were studied. The plants were grown in vitro in glass tubes (length 35 cm, diameter 3,5 cm, 1 plant per tube) containing 80 cm^3 sterilized perlite supplemented with 30 cm^3 of a sterile N-free nutrient solution (Fahreus 1957). The acetylene reduction assay (ARA) was used to test for the N_2-fixing, using 4-5 plants per vial (12 cm^3) containing 6 cm^3 of nitrogen-free Nfb (Baldani 1980). ARA were performed after 24-h incubation (atmosphere in the vials was 10% acetylene) on 14-days old seedlings.

Result and conclusions

Plant growth substances, such as auxine, cytokinin and giberillin were tested for their effect on growth and nitrogenase activity in barley and maize roots inoculeted with *A.lipoferum.*The present result indicated that indole-3-acetic acid (IAA) and tryptophan (T) are of a stimulating influence on the bacterial growth. Treatments cereal roots with plant growth substances in the presence of 0,25 and 0,50 mg kg^{-1} and 1,0 mg kg^{-1} (2,4-D) were about 5 times more active in the N_2-fixation, as compared to the untreated roots. Only treatments of maize roots with 4-(3-indole)-butyric acid (IAM) and gibberillic acid (GA) showed lowly increasing the nitrogenase activity. The nitrogen fixation of the rhizosphere bacteria *Azospirillum* could be influenced by plant produced growyth substances.

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P-E-4

TRANSFORMATION-REGENERATION SYSTEM IN A GREEN MANURE LEGUME, RENGE-SOU (*Astragalus sinicus* cv. Japan), WHICH FORMS ROOT NODULES WITH *Rhizobium huakuii* bv. renge

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Chinese milk vetch or renge-sou (*Astragalus sinicus* cv. Japan) in Japanese, is one of the most popular legumes used as a green manure legume in China, Korea and Japan. In China, this milk vetch is cultivated over an area of nearly 5 million ha. The renge-sou is also used as fodder for animals and a source of honey for bees. The plant has a symbiotic relationship with soil bacteria, namely *Rhizobium huakuii* or *R. huakuii* bv. renge, which forms nitrogen-fixing root nodules. We also found that a soil bacterium, *Enterobacter cloacae* A105 isolated from rice paddy stimulated the nodule formation on renge-sou.

R. huakuii bv. renge carries an indigenous large plasmid, pRhYM (420 kb). By heat treatment, Nod^- and Fix^- strains, which were deleted a par of the DNA fragment or cured the plasmid, were isolated. The hydrogenase genes from *Pseudomonas oxalaticus* were introduced into *R. huakuii* bv. renge by conjugation. The transconjugants stimulated the nitrogen fixation of renge-sou nodules. *Agrobacterium rhizogenes*-mediated transformation and regeneration system of renge-sou was also established. Transformation of regenerated plantlets was demonstrated by detection of mikimopine, histochemical GUS activity and by Southern blot analysis. We also isolated several nodule-specific cDNAs from renge-sou nodules and root cells. Thus, we have developed transformation and regeneration systems in both bacterium, *R. huakuii* and its host plant, *A. sinicus*. This system has potentials as a symbiotic model and also application of genetically engineered renge-sou in rice field.

P-E-5

CLONING AND EXPRESSION IN *E.COLI* OF THE *RNF* GENES INVOLVED IN ELECTRON TRANSPORT TO NITROGENASE IN *R.CAPSULATUS*

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In *Rhodobacter capsulatus*, a non-sulfur, photosynthetic bacterium, a set of new genes essential to nitrogen fixation was previously discovered.[1] These genes, called *rnf* (**R**hodobacter **N**irtogen **F**ixation) code for either iron-sulfur proteins (*rnfB* and *rnfC*), transmembrane proteins (*rnfA*, *D*, *E* and *F*) or a periplasmic protein (*rnfG*). Based on the analysis of the polypeptide sequences, it has been proposed that the products of *rnf* genes assemble to form a transmembrane complex which might provide reductants to nitrogenase. The ferredoxin I was previously shown to serve as the final electron donor to nitrogenase.[2] To study the putative Rnf complex and assess its role in the proposed electron transport pathway, the recombinant *rnf* gene products were expressed in *E.coli* and purified by immobilized metal affinity chromatography (IMAC). Expression vectors were constructed to generate recombinant polypeptide carrying a polyhistidine tag fused either at the N-terminus or at the C-terminus. The expressed gene products were analysed by SDS-PAGE and UV-VIS spectrophotometry. The expression of RnfA, a putative membrane protein, yielded no appreciable amount of protein with either type of terminal modifcation. RnfB, a putative Fe-S protein, was successfully expressed and purified. The RnfB with fused N-Histag was obtained in the form of inclusion bodies, whereas the RnfB with fused C-Histag was obtained in soluble form. The RnfB with fused N-Histag could be solubilized in urea and purified as a brown protein. Its absorbance spectrum indicated the presence of a bound [2Fe-2S] cluster. The protein was unstable and lost its absorbance, which precluded further characterization of its clusters. RnfB with fused C-Histag was isolated in the apoform. The expression of RnfC with fused N-histag yielded a truncated polypeptide which was around half the size of the expected product. The expression of RnfC with fused C-Histag is currently being analysed.

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P-E-6

STUDIES ON P_{II} IN THE PHOTOSYNTHETIC BACTERIUM *Rhodospirillum rubrum*

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P_{II}, a trimer of identical subunits [1], is an important regulatory protein in the regulation of glutamine synthetase actiity [2] and for the regulation of NTRC dependent transcription from σ^{54} dependent promoters [3,4]. The most extensive studies on the P_{II} protein have been made in enteric bacteria, in which it is modified by uridylylation when the intra-cellular glutamine/α-ketoglutarate ratio is low [5].

In *Rhodospirillum rubrum glnB* is cotranscribed with *glnA* from tandem promoters strictly regulated by the nitrogen status in the bacteria. A *glnA* mRNA which is suggested to be the product from specific RNAse processing on the *glnBA* transcript was also identified [6].
P_{II} in *R. rubrum* is extensively modified during nitrogen fixing condition, the modification is rapidly lost when nitrogenase and glutamine synthetase activities are "switched off" by addition of NH_4^+. Addition of glutamine or glutamate both affected the extent of P_{II} modification. We also report on rapid demodification of P_{II} after addition of NAD^+ which indicates that the redox state is affecting the regulation of nitrogen metabolism in this photosynthetic bacterium.

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P-E-7

EFFECT OF NUCLEOTIDE(S) ON DINITROGENASE REDUCTASE ACTIVATING GLYCOHYDROLASE IN *RHODOSPIRILLUM RUBRUM*

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Nitrogen fixation in *Rhodospirillum rubrum* is not only regulated at the genetic level but is also regulated posttranslationally by ADP-ribosylation. The enzymes involved in posttranslational regulation are dinitrogenase reductase ADP-ribosyl transferase (DRAT) and dinitrogenase reductase activating glycohydrolase (DRAG). When cells are submitted to "switch-off" effectors, such as ammonia or darkness, an ADP-ribose is covalently bound to one of the two subunits of dinitrogenase reductase and nitrogenase activity is reversibly lowered. We have shown that NAD^+ can cause a similar modification, carried out by DRAT, with a reversible decrease of activity. This change in concentration of NAD^+ we suggest as an internal signal for DRAT activity. The internal signal for DRAG has not yet been clearly established, although the nitrogen status and/or redox state of the cell has been suggested to be involved. The DRAG enzyme is associated with the chromatophores, during preparation, but easily washed off with 0.5M NaCl indicating a role for the membrane in the activity of DRAG. A chromatophore fraction was treated with different nucleotides (ATP,ADP,GTP,GDP) to investigate the possibility for DRAG to be bound to an unknown membrane protein using a nucleotide dependant "switch" analagous to other signal transducing systems. Addition of GDP released DRAG protein from the chromatophores, as shown by Western blotting, subsequently protein activity was located in the supernatant, as measured by activation of inactive nitrogenase.

P-E-8

ELECTRON TRANSPORT TO NITROGENASE IN *Rhodospirillum rubrum*

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The electron transport pathway to nitrogenase has been established in detail only in *Klebsiella pneumoniae*. In this organism a pyruvate:flavodoxin oxidoreductase (the *nifJ* product) has been shown to transfer electrons from pyruvate to nitrogenase via a nitrogen fixation specific flavodoxin (the *nifF* product).
In the photosynthetic bacterium *Rhodospirillum rubrum*, pyruvate dependent nitrogenase activity has been demonstrated and a pyruvate oxidoreductase isolated and characterized [Brostedt & Nordlund 1991]. We have also isolated and sequenced the gene (*nifJ*) encoding the pyruvate oxidoreductase. However, studies of a *nifJ* mutant under different growth conditions, indicate an alternate electron transport pathway [Lindblad et al. 1996].

Initial studies to identify the components of this alternate pathway include experiments to determine the involvement of the membrane potential in generating reductant for nitrogenase. We will report on studies in which ATP/ ADP and nitrogenase acitivity were measured after addition of uncouplers or at different light intesities. These investigations aim at determining if there is a direct relationship between the light energy conservation (pmf) and electron transport to nitrogenase in this photosynthetic bacterium.

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P-E-9

GLUTAMINE SYNTHETASE AND SUCROSE SYNTHASE EXPRESSION IN *GUNNERA MANICATA*

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Gunnera is the only angiosperm known to form an intracellular nitrogen-fixing symbiosis with a cyanobacterium (*Nostoc*) (1). The cyanobacteria enter the plant through stem glands. In later stages of infection the tissue that contains the cyanobacterium is spread inwards the stem. As the cyanobacterium remains pigmented, this tissue, also referred to as an internal stem nodule (2), is easily discerned from stem tissues without cyanobacteria.
We have by using degenerate primers and RT-PCR isolated two cDNA clones from *Gunnera manicata*; one for a cytosolic glutamine synthetase (GS) and one for a sucrose synthase (SS). Further on we have studied the expression of these genes in various plant tissues by northern blot. In order to be able to extract RNA from greenhouse plant organs we have also modified a nucleic acid extraction method.
GS expression was found in all tissues examined, leaves, roots, stem tissues and stem tissues with cyanobacteria. SS was expressed in roots, stem tissue and stem tissue with cyanobacteria. The ratio between GS and SS expression in roots, stem tissue and stem tissue with cyanobacteria did not vary significantly. The presence of cyanobacteria did not enhance expression of the two genes notably. It has earlier been shown that the *Nostoc-Gunnera* symbiosis is different from the *Rhizobium*-legume symbiosis in that the newly assimilated nitrogen is mainly transported to other plant organs through the phloem (1). Our results suggest that the gene expression in the "internal nodule" of *Gunnera* also differs from that of the legume nodule.

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P-E-10

EFFECT OF AMMONIUM AND CYCLOHEXIMIDE ON THE GLUTAMINE SYNTHETASE MOLECULAR FORMS COMPOSITION IN PHYTOBIONT OF THE *AZOLLA FILICULOIDES-ANABAENA* SYMBIOSIS

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The only obligate cyanophytic nitrogen fixing symbiosis is the *Azolla-Anabaena* symbiosis. Pathways of the primary ammonia assimilation in both the host-plant and the cyanobiont have been studied intensively (Peters, Meeks, 1989; Sof'in et al., 1991), but there are some open questions in the field.

The glutamine synthetase (GS, EC 6.3.1.2) molecular forms composition has been investigated in the phytobiont of *A.filiculoides-A.azollae* symbiosis grown at different nitrogen nutrition conditions (without any exogenous fixed nitrogen; at presence ether 5 mM nitrate or 1 mM ammonium in the cultivation medium). The vacuum infiltration of the ammonium (25 mM) and cycloheximide (50 mg/l; an inhibitor of the protein synthesis at cytoplasmic 80S ribosomes) was also used.

Two forms of glutamine synthetase were found in *A.filiculoides* grown at nitrogen fixation or in the presence of nitrate. One was eluted at 0.15 mM KCl (low activity) and the other one at 0.18-0.22 mM KCl (high activity) during the ion-exchange chromatography of cell-free extracts on a Servacel column equilibrated with 10 mM Tris-HCl buffer containing 2 mM $MgCl_2$, 3 mM EDTA and 20mM 2-mercaptoethanol. Both forms are suggested to be chloroplastic. The third form eluted at 0.08 mM KCl arouse after 2 days cultivation in the presence of ammonium. This form is probably cytoplasmic. The infiltration of cycloheximide 1 hour before the ammonium infiltration significantly blocked the light-dependent increasing of the GS activity eluted at 0.18-0.22 mM KCl, the GS eluted at 0.15 KCl disappeared, and the high GS activity eluted at 0.08 mM KCl appeared. The exact mechanism of the cycloheximide stimulation of the GS activity eluted at 0.08 mM KCl is yet unclear.

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Acknowledgments

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P-E-11

GENETIC CHARACTERISATION OF *TRICHODESMIUM* SPECIES BASED ON 16S rDNA SEQUENCES

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The filamentous colony-forming cyanobacterium *Trichodesmium* is probably the most abundant nitrogen-fixing cyanobacterium in the world, and its nitrogen-fixing activity is potentially of immense value for the oligotrophic tropical and subtropical oceans. Despite its ecological importance, the taxonomy has not been reconcealed. Morphological and ultrastructural studies (1, 2) of the genus *Trichodesmium* collected in the North Atlantic Ocean indicate that at least four species exists, while most of the recent and past studies only recognise one or two species. These four species and two morphotypes have now been compared genetically by PCR-amplificication of natural samples.

The nearly complete 16S rDNA sequence (1437 nucleotides) of six clones representing different, naturally occurring, morphotypes were determined. These types were identified by light microscopy according to Janson *et al.* (2), prior to DNA extraction. The six types comprised the following: a spherical colony (puff) of *T. thiebautii,* a colony with parallel trichomes of *T. thiebautii* (tuft), a colony of *T. hildebrandtii*, a colony of *T. contortum*, several colonies of *T. erythraeum* and a colony of *T. tenue*.

Four of these sequences together with most cyanobacterial 16S rDNA sequences available in databases were used to construct a phylogenetic tree. The tree topology, regarding the *Trichodesmium* sequences, is consistent with the one published by Wilmotte *et al.* (3), using the 16S rDNA sequence of the cultured strain *Trichodesmium* sp. NIBB 1067. This confirms the close genetic relationship between *Oscillatoria* sp. PCC 7515 (the type species of the genus *Oscillatoria*)and *Trichodesmium*.

A 454 nucleotides long sequence at the 5' end of the sequences was chosen for intraspecies comparison of five different morphotypes. Within the 454 nucleotides of the sequences, a region, four nucleotides in length, was highly variable, and the four species described earlier (1) are definable within these four nucleotides.

The main conclusion that can be drawn from these analyses are that four species, *T. thiebautii, T. contortum, T. tenue* and *T. erythraeum* are definable within the analysed part of the 16S rDNA sequence. While *T. hildebrandtii* and the spherical form of *T. thiebautii* appear indistinguishable from *T. thiebautii* with parallel trichomes in the colony.

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P-E-12

CELL SPECIALISATION IN *TRICHODESMIUM*

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The widespread, filamentous, non-heterocystous cyanobacterium, *Trichodesmium*, performs nitrogen fixation and photosynthesis simultaneously unlike other non-heterocystous cyanobacteria. By immunolocalisation of nitrogenase and $^{14}CO_2$ autoradiography we can now show that cell specialisation exists in *Trichodesmium* IMS 101. Nitrogen fixation is limited through confinement of nitrogenase to 13% of the cells. The labelling also shows that chains of nitrogenase-containing cells are present within the trichomes (filaments). Carbon fixation on the other hand proved active in 85% of the cells leaving one region of cells in the centre of the trichome inactive. Based on labelling frequency and pattern we conclude that carbon fixation occurs in the majority of cells whereas nitrogen fixation is restricted to another set of consecutive non-photosynthetic cells often located in the centre of mature trichomes of *Trichodesmium* IMS 101. This is a new example of cell specialisation coupled to nitrogen fixation in a multicellular cyanobacterium.

P-E-13

ANALYSIS OF GENES, INVOLVED IN NITROGEN FIXATION AND CELL DIFFERENTIATION, IN MARINE NON-HETEROCYSTOUS CYANOBACTERIA

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Nitrogen fixation by cyanobacteria in marine systems is performed, in large, by filamentous non-heterocystous cyanobacteria. In tropical pelagic systems *Trichodesmium* is the dominating type, estimated to contribute with 4% of the total annual nitrogen fixation. Filamentous non-heterocystous cyanobacteria also form a visual layer in benthic microbial mats, however, the diversity of genera is greater than in the open ocean. *Microcoleus chthonoplastes* PCC 8002 originates from such a mat. It has, like *Trichodesmium*, the capability to fix nitrogen in an aerobic environment without the formation of oxygen protective heterocysts. This strain is also relatively easy to grow as an axenic culture, unlike most of known *Trichodesmium* isolates, and is therefore used as a model.

There is increasing evidence that *Trichodesmium* forms specialized subsets of cells within the filaments capable of nitrogen fixation (see the abstract by Bergman et al., this conference). In order to determine whether some differentiation mechanisms, described for other cyanobacteria, participate in forming such cells, special attention was given to homologues of the *hetR* gene, responsible for heterocyst differentiation in *Anabaena* and *Nostoc*. Degenerate oligonucleotides for amplification of the *hetR* gene were deduced using known sequences from *Anabaena* sp. PCC 7120 and *Nostoc* sp. PCC 9229, a symbiotic isolate. Appr. 500-bp DNA fragments internal to the *hetR* gene were amplified and cloned from *Trichodesmium erythraeum*, *M. chtonoplastes* PCC 8002 and *Plectonema boryanum* PCC 73110, known to perform nitrogen fixation anaerobically. Sequence analysis of the cloned fragments revealed a high degree of identity to the *hetR* genes from heterocystous cyanobacteria, although no apparent phylogenetic affiliation of the strains tested was observed. In addition, a 350-bp internal part of the *nifH* was amplified and cloned from *M. chtonoplastes*. Its sequence, analyzed with the phylogenetic software package PHYLIP, confirmed that this strain is closely related to *Trichodesmium*, thus approving the choice of this strain as a model. Northern blots of total RNA, isolated from *M. chtonoplastes* grown in the presence of combined nitrogen revealed an unexpectedly high expression of the *hetR*-like gene presented by a single transcript appr. 1.4 kb in size. Removal of combined nitrogen was followed by a gradual decrease in intensity of *hetR* expression. The lowest (although quite distinct) abundance of the *hetR* transcript was observed in the culture constantly grown without nitrogen. These results are opposing those obtained for heterocystous *Anabaena* sp. PCC 7120, where removal of combined nitrogen elicits enhanced muiltiple transcript *hetR* expression. In contrast, *nifH* was only expressed in *M. chtonoplastes* after removal of nitrogen as three distinct transcripts 1.45, 2.9 and appr. 5 kb in size. These reached their highest intensity at 24 hr after nitrogen step-down. Acetylene reduction was first detectable at 16 hr and reached a constant level at 24 hr after the initiation of nitrogen step-down.

The data obtained indicate that (i) *hetR* is not confined solely to heterocystous cyanobacteria and that its function as well as its role in supporting nitrogen fixation is yet to be clarified; (ii) the *nifH* gene may not only be expressed as a part of the operon. Investigation of the spatial distribution of the HetR protein witin non-heterocystous filaments is underway.

P-E-14

PECULIARITIES OF AMINO ACID CONSUMPTION BY *AZOSPIRILLUM BRASILENSE* SP 245 DURING INDOLE-3-ACETIC ACID BIOSYNTHESIS

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We have made a qualitative analysis of 18 amino acids for tryptophan - dependent indole-3-acetic acid (IAA) synthesis in the culture fluid of *Azospirillum brasilense* Sp 245 using the Salkowski reagent. Results of thin-layer chromatography and identification using Ehrlich reagent indicated that IAA synthesis by *A.brasilense* Sp 245 grown on media supplemented with different amino acids proceeded differently. By character of the changes involved, two groups of amino acids were identified:

1. Presence of an amino acid induces the synthesis of IAA alone.
2. Presence of an amino acid induces the synthesis of IAA and attendant indoles.

IAA production reached a minimum level with methionine and glutamic acid. Using a multifactorial analysis of the cooperative effect of 20 to 180 mg/L tryptophan and 20 to 180 mg/L amino acid on IAA excretion, we obtained regression equations and spatial mathematical models for IAA synthesis:

$$IAA = 0{,}31[Trp] + 0{,}26[Met] - 0{,}001[Met]^2 - 0{,}0008[Trp][Met]$$
$$IAA = 0{,}31[Trp] + 0{,}42[Glu] - 0{,}002[Glu] - 0{,}0008[Trp][Glu]$$

There was a linear rise in IAA production with increasing tryptophan concentration in the medium. Increasing glutamic acid and methionine content in the medium inhibited IAA biosynthesis in either case.

P-E-15

THE EFFECT OF SELECTED FUNGICIDES ON THE NUMBER AND ACTIVITY OF N_2 FIXATION BY STRAINS OF *AZOSPIRILLUM BASILENSE* IN CULTURES

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Bacteria from the genus of *Azospirillum* belong to the group of numerous soil microorganisms capable of atmospheric nitrogen fixation. Although the activity of *Azospirillum* bacteria is particularly evident in tropical climates, attempts are being made to utilize them in cereal production in countries with the temperate climate. In order to obtain the best possible results, efforts are undertaken to inoculate soil or grains with isolated *Azospirillum* strains which characterized by high activity of N_2 fixation.

The aim of the research project was to establish the influence of widely used fungicidal seed dressings (Baytan Universal, Oxafun T, Vitavax) on the bacterias quantity and activity of dinitrogen fixation by selected strains of *Azospirillum brasilense* isolated from soils in Poland.

For this purpose, bacteria were cultured on semi-liquid media containing: malate, malate and fungicide as well as on the medium in which malate was replaced by a fungicide.

In majority of cases the applied fungicides inhibited the growth of bacteria. However, there were also cases when the applied fungicide was utilized by *Azospirillum* as an additional source of nutrients and stimulated the growth of these bacteria. This depended on the strain of bacteria and the applied fungicide. It was also found that, in the majority of cases, the applied fungicides led to a reduction in dinitrogen fixation in cultures which was probably caused rather by their influence on the number of bacteria in the culture than by decreasing bacteria capability for dinitrogen fixation.

P-E-16

R-S DISSOCIATION IN *A. BRASILENSE* Sp7: IMMUNOCHEMICAL ASPECTS

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A. brasilense type strain Sp7 exhibits spontaneous variability of the morphology of colonies (Matveev, 1987; Kennedy, 1995), all typical morphological distinctions being observed in aged cultures only. It is known from the classic literature that bacterial R-forms, in contrast to S-forms, contain only the R-form of lipopolysaccharide (LPS), the carbohydrate chains of which are represented by the core oligosaccharide. We suggested that the R-S variability of azospirilla is a non-typical example of dissociation.

This communication presents data to elucidate immunochemical aspects of the R-S dissociation in *A. brasilense* Sp7. We obtained monospecific antibodies against the LPS of the initial strain Sp7-R and its spontaneous mutant Sp7-S. For immunization, both the cultures were grown following two procedures: for 18 h on a liquid medium (young cultures) and for 72 h on a solid medium (aged cultures). Results of an immunochemical analysis indicate that:

1) during growth of the aged culture Sp7-R on a solid medium, the deciding contribution to the immune response begins to be made by one of O-specific polysaccharides (O-PS), giving the colonies of this strain their peculiar morphology;

2) a complete set of somatic (LPS) antigens specific to the initial strain may be extracted from both Sp7-R and Sp7-S independent of the strain and the culture age;

3) the highest immunogeneity of these structures is exhibited in the young Sp7-R and aged Sp7-S forms;

4) the greatest number of immunoprecipitation bands (3) are formed by antigens isolated from the aged form Sp7-S with antibodies obtained against the young culture of the wild strain.

These findings corroborate our assumption that the R-S variability of azospirilla, in particular of type strain Sp7, is a non-typical example, indicating that the morphological distinctions between the R- and S-forms of this strain depend on age topographic changes of lipopolysaccharide antigens being considered.

P-E-17

LECTINS OF *AZOSPIRILLUM BRASILENSE* SP7 AND ITS Tn-5 MUTANTS IN LECTIN ACTIVITY AS REGULATORS OF HETEROGENOUS β - GLUCOSIDASE

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The presence of a fucose-specific lectin on the surface of associative nitrogen-fixing soil bacterium *Azospirillum brasilense* Sp7 has previously been demonstrated (1). Using *A. brasilense* Sp7.2.3, a Tn5 mutant in lectin activity,we have shown that the lectin has a role in bacterial attachment to wheat roots at its early stages (2). This work provides evidence for lectins as regulators of the activities of enzymes, in particular of plant hydrolases. A comparative study of the effects of various concentrations of *A. brasilense* Sp7 and Sp7.2.3 lectins on the activity of plant β-glucosidase showed that 70 mkl/ml lectin treatments can induce a decrease in enzyme activity from 100% to 85% and 32%, respectively. The dependence of the inhibitory lectin effect on the incubation time, temperature and pH was also documented.

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P-E-18

PROMOTION OF FLAVONOID SIGNALS, NODULATION AND GROWTH IN *PHASEOLUS VULGARIS* INOCULATED WITH *AZOSPIRILLUM BRASILENSE*

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The common bean (*Phaseolus vulgaris*) is considered a poor nitrogen fixer. Several genetic and environmental factors contribute to this poor performance. The nodulation process in the common bean-*Rhizobium* symbiosis is relatively slow. Thus, in determinate, bushy types of bean with a short growth cycle, N_2-fixation does not contribute significantly to the nitrogen nutrition of the plant.

The free-living N_2-fixing rhizobacteria of the genus *Azospirillum* live in close association with plants, and may promote plant growth and yield under appropriate conditions. Positive effects of *Azospirillum* inoculation are mainly attributed to improved root development and to the subsequent increase in the rate of water and mineral uptake. The effect of coinoculation of legumes with *Rhizobium* and *Azospirillum* has received increasing attention in recent years and positive effects have been reported for several legumes. Early nodulation, increased number of nodules, higher N_2-fixation rates and a general improvement of root development have been observed.

In the present study, attempts were made to evaluate the effects of combined inoculation with *R. etli*/*R. tropici* and *A. brasilense* on nodulation and growth of the common bean under controlled conditions and in the field, and to investigate possible mechanisms involved in this interaction.

Combined inoculation of potted bean plants with *Rhizobium* and *A. brasilense* significantly increased both upper and total nodule number and N_2-fixation as compared to inoculation with *Rhizobium* alone. When the combined inoculation was performed using a relatively low *Azospirillum* concentration ($5x10^6$ cfu/mL), positive effects on plant growth were observed. Inoculation with *Azospirillum* also promoted root hair formation in seedling roots. In a field experiment we observed an increase in nodule number, shoot dry weight accumulation and yield by inoculation with *Rhizobium*, and a further increase in those parameters was observed following combined inoculation with *Rhizobium* and *Azospirillum*. In experiments carried out in a hydroponic system, *Azospirillum* caused an increase in the secretion of *nod*-gene inducing flavonoids, as was observed by *nod*-gene induction assays of root exudates fractionated by HPLC.

We propose that the observed increase on nodulation caused by *Azospirillum* could be explained, at least in part, by the promotive effects of the bacterium on root hair formation, and by an increase in the secretion of *nod*-gene inducer signals by roots. Combined inoculation with *Azospirillum* and *Rhizobium* should increase legume yield under limited water and nitrogen availability. It may also lead to a decrease in the need for N-fertilization, lowering costs of production and reducing the negative impact on the environment.

P-F-1

NOVEL METHOD FOR APPLYING NITROGEN FIXING BACTERIA: FOLIAR DRESSING OF PLANTS

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The traditional method for applying nitrogen fixing preparations by inoculating the seeds enhances the productivity of leguminous, grain and vegetable crops. However, the inoculation is a biologically inadequate method because the seeds in most cases are not an optimal medium for living nitrogen fixers. A method is proposed for applying liquid nitrogen fixing cultures to the leaves of young plants (spraying) using the selected strains Flavobacterium, Klebsiella, Artrobacter, Bacillus, Azotobacter, Pseudomonas. This considerably promotes (by 20-30%) accumulation of biomass and increases the yield of grains. It is supposed that the mentioned method holds more potential for cultivating the vegetable crops - lettuce, cabbage, carrot, beetroot etc. The mechanism of the positive effect is unclear: either it is an activity of the bacteria proper (nitrogen fixation) or their metabolites forming when the bacteria are grown in liquid nutrient media. The possibility of combining the bacterial cultures, surfactants, micro- and macroelements is considered.

P-F-2

THE WAYS OF POTENTIAL INCREASE FOR NITROGEN - FIXING ACTIVITY IN GRASSES ROOT ZONE

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The row of grasses species (*Lolium perenne, Zerna inermis, Phleum pratense, Phalaroides arundinaceae, Dactylis glomerata, Festuca pratensis*) has a high nitrogen-fixing potential in root zone. Study of diazotroph complex for grass root sphere shows that associative composition for nitrogen-fixing microorganisms is specifical for every species of plant. So for *Lolium perenne* the most typical microorganisms are *Azospirillum lipoferum* and *Agribacterium radiobacter*, for *Dactylis glomerata* are *Pseudomonas sp.*, for *Phleum pratense* are *Bacillus subtilis* and *Enterobacter aerogenes*, for *Festuca pratensis* are *Bacillus polymyxa*.

The inoculation with active diazotroph strains of corresponding plant species increases the nitrogen-fixing activity and crop capacity. However as a rule cross inoculation does not lead to positive effect. More over *Festuca pratensis* inoculation by bacteria genus *Azospirillum* leads to decrease both nitrogen-fixing activity and crop capacity.

Since grasses are grown in mixture, the inoculation application is difficult as a positive effect from inoculaton of one species may be smoothed by unresponsivness of other species.

In these conditions the perspective ways of associative nitrogen-fixation increase will be the next: applying the phisiologically optimal dozes of mineral nitrogen, plant treatment with molybdenum and cobalt salts solution and also plant growth promoting. According our data mentioned substances indirectles provide associative nitrogen fixator with optimal conditions for molecullar nitrogen binding byhaving influence on root system growth, photosynthesis intensity and nitrogen plant metabolism.

P-F-3

INTERRELATIONSHIP OF *Azotobacter* AND SUGAR BEET

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Azotobacter chroococcum is known to have advantages as a nitrogen fixer. Upon the determination of its widespread exitance in agroecological conditions in the Vojvodina Province, especially in the rhizosphere of sugar beet, we isolated strains from the rhizosphere of two sugar beet hybrids, NS-Hy-11 and Dana (developed at the Institute of Field and Vegetable Crops).

Associations of four Azotobacter chroococcum strains (2, 5, 8 and 14) with two sugar beet hybrids were investigated. Dry mass, nitrogen content, nitrogenase activity, glutamate dehydrogenase and proteins of sugar beet plants were used as indicators of N_2 - fixation. The results obtained showed a specificity of strain 5 to the hybrid Hy-11 and strain 8 to Dana. These two associations proved to be the most effective in N_2 - fixation two weeks after inoculation.

P-F-4

DIAZOTROPHS AND THEIR ACTIVITY IN SUGAR BEET

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The study investigated the effectiveness of 8 strains of *Azotobacter chroococcum* as well as of one strain of each of the species *Azospirillum lipoferum* and *Beijerinckia Derx*. The effectiveness of the diazotrophs was determined in two sugar beet varieties. The plants were grown in vegetation pots on a calcareous chernozem soil and watered with for about 180 days. The experiment included 4 control treatments: no nitrogen, 0,7 g, 1,4 g and 2,0 g of N per pot. The effectiveness of the diazotrophs was shown to be dependent on the species used and the variety of sugar beet as well as on the traits under investigation. The study has shown that biological N can substitute for a certain amount of mineral N. The diazotrophs under investigation broutht about an increase in the mass and N content of the root and the above-ground plat parts. The introduction of the diazotrophs into the soil by may ofinoculation of the seeds resulted in an increase in the total number of bacteria, the number of ammonifiers, azotobacters, oligonitrophilous bacteria, actinomycetes, as well as in an increase in dehydrogenase and protease activity. On the other hand, the introduction caused a decrease in the number of fungi.

P-F-5

DIAZOTROPHS AND THEIR ACTIVITY IN MAIZE

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The activity of nitrogen fixation of free nitrogen fixing bacteria (*Azotobacter, Azospirillum, Beijerinckia*) was investigated in two maize hybrids (PKB-635 and NSSC-640). Inoculation with nitrogen fixing bacteria strains was made in seven variants, one variant was without fertilization and without nitrogen fixing bacteria, but in three variants different amounts of nitrogen fertilizers (30, 60 and 90 kg nitrogen/ha) were applied.

Yield of maize, number of bacteria, amonifiers, actinomycetes, oligonitrofiles, azotobacter, fungi and dehydrogenase activity were determined.

Larger activity of diazotrophs was obtained in PKB-635 hybride but some poorly in NSSC-640. In variants where diazotrophs were applied increase of number of all microorganisms groups become evident except fungi, as well as the increase of dehydrogenase activity.

P-F-6

NITROGEN TRANSFORMATION IN RHIZOSPHERE OF NON-LEGUME PLANTS INOCULATED WITH NITROGEN-FIXING MICROBIAL CONSORTIA

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One of the most perspective way to intensify associative nitrogen fixation in non-legume plants rhizosphere is application of natural diazotrophic microbial consortia (mixed cultures) as far as these communities produce stronger and more stable effect on agricultural plants than pure cultures of nitrogen fixers. However, inoculation of plants with microorganisms does not always lead to the desirable effect, because many of nitrogen-fixing bacteria can carry out denitrification and heterotrophic nitrification processes. Therefore ultimate results of microbial inoculation will depend on correlation among the activities of nitrogen transformation processes in rhizosphere.

Connection between nitrogen fixation and denitrification in natural bacterial diazotrophic mixed cultures has been investigated. 150 nitrogen-fixing consortia has been isolated from chernozemic and soddy-podzolic rhizosphere soil. Overwhelming majority of the consortia carried out both nitrogen fixation and denitrification under laboratory cultivation. Primary activity one of these processes was observed on different stages of mixed cultures growth, meanwhile the activity of the other was reduced. Positive correlation between nitrogen fixation and denitrification activities of the consortia has been revealed under laboratory conditions: consortia showing the highest nitrogenase activity were the most active denitrifiers.

Inoculation of rape and barley with the consortia has shown that plants can stimulate both nitrogen fixation and denitrification, but to a different extent. According to these differences it turned out to be possible to divide our collection of mixed cultures into three groups:

1)consortia, showing high nitrogenase activity (HNA) and low denitrifying activity (about 10% from total number of cultures);

2)cultures with high denitrifying (HDA) and low nitrogen fixing activities (15%);

3)consortia with middle levels of nitrogen fixation and denitrification activities (MND) (75%).

It has been found that some cultures possessing high nitrogenase activity under laboratory conditions become active denitrifiers in associations with plants and so their influence on plants growth and yield changes. These bacteria being able to carry out both nitrogen fixation and denitrification in axenic conditions primary fulfil only one of these processes in plants rhizosphere. Therefore it is necessary to consider this fact when microbial cultures are selected for agricultural use.

P-F-7

ESTABLISHMENT OF ARTIFICIAL ASSOCIATIONS OF PLANTS WITH BACTERIA

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Two main principles of a stable plant-bacteria association generation have been analyzed on models *Triticum vulgare* embryos - *Klebsiella* species and the *Nicotiana tabacum* leaf culture - *K.oxytoca VN13*: 1) the bacterial partner of association is a nonpathogenic bacterium, possessing the mechanism of distribution in the plant interior; 2) establishment of an association occurs in the limited trophyc conditions when mutualistic interrelationships take place due to metabolites of both partners. The latter meet mutually available coexistance in the conditioned growth medium used for a plant regeneration. The plants can be bacterized during the plant tissue regeneration in such a manner that regenerants would possess enough bacterial cells to get a positive effect from them. This approach is belived to gain more profit from the plants propagated asexually.

Cocultivation of the *Triticum vulgare* embryos with some species of *Klebsiella* genera stimulated the beginning of both regeneration and organogenesis on 2 days earlier as compared to control, and increased the wheat shoot and root length in variants of inoculation. Location of *K.oxytoca VN13* and *K.terrigena* 80-07 inside the wheat regenerant roots has been proven by electron microscopy analysis. Stimulation of the plant regeneration from tobacco leaf disks and a shortened period of organogenesis were observed in the artificial association of *N.tabacum* - *K.oxytoca VN13*. The associative interactions of bacteria with the plant have been stored at least in two cycles leaf-culture - regenerant.

P-F-8

THE EFFECT OF *AZOTOBACTERIN* ON THE CROP YIELD AND BIOLOGICAL ACTIVITY OF THE SOIL

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Crop yield responds markedly to nitrogen application. Much of the applied N - 60 % - may be lost, and this loss represents a potential environmental risk.The major processes responsible for loss of inorganic N are: (1) leaching, the physical removal of excess nitrate in waters draining from the root zone into ground waters and rivers. From an environmental standpoint, leaching is probably of most concern; (2) denitrification, the conversion of nitrate to the gases N_2 and nitrous oxide by bacteria under anaerobic conditions.

High levels of fertilizer N increase the crop yield decreasing the quality of crop at the same time. Having been accumulated in plants, nitrate show a harmful effect on the health of people and livestock.

Up to now, production of *Azotobacterin* has been unprofitable and labour-consuming. A technology for the production of *Azotobacterin* by Azotobacter chroococcumin fermenters with counter-flow mixers ensuring perfect turbulence and optimum aeration conditions has been developed.

Field experiments are carried out in which the effect of *Azotobacterin* upon sugar beet, potato and leaf vegetables is studied. The experiments have shown that the introduction of *Azotobacterin* to the soil with neutral to slightly acid pH results in stable crop yield increase both in dry and wet seasons. As a result, plants are more resistant to diseases, the soils' biological activity increases. It was confirmed by the quantitative increase of the total bacteria count and amount of ferments in the soil. The introduction of *Azotobacterin* to the soil provides increase in the sugar beet yield by 4.0 - 6.5 t ha^{-1} or 17-24 % compared with control. It has been observed that with *Azotobacterin* a potato yield increase by 3.62 - 3.96 t ha^{-1} or 16.7-21.4 % was achieved compared with control. Experiments with bacterial fertilizer confirmed the decrease of the amount of nitrate in large crops of leaf plants.

Azotobacterin is a most economical preparation to use in agricultural production when introduced to the soil prior to sowing. Its use will reduce production costs and avoid environmental pollution.

P-F-9

NOVEL INOCULANTS FOR AN ENVIRONMENTALLY-FRIENDLY AGRICULTURE

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Increasing concern over health hazard of agrochemicals, coupled with economical problems, has promoted research in the field of agrobiotechnology in Ukraine. Plant Growth Promoting Rhizobacteria (PGPR) are considered as a valuable means to reduce the input of agrochemicals. Simple and inexpensive technology of inoculant manufacture has been designed on the base of PGPR Bacillus mucilaginosus. This bacterium possesses a set of beneficial for plants characters , on one hand, and produces a large amount of exopolysaccharide (EPS), mucilan, on the other hand. Cocultivation of B.mucilaginosus and other PGPR of choice (klebsiella, pseudomonas) stimulates the EPS production considerably. The resulted gel contains 2.5-7.OE+10 cfu/ml of either culture. Mucilan protects both cultures under storage and serves as a carrier. The plant growth promoting activity of EPS itself enhances a profit of preparations. Novel inoculants developed in accordence to such a formulation ("Kleps", "Pseps") increased the corn and buckweat yield to 30% on the nitrogen-deficient lands and improved a quality of grain.

P-F-10

THE EFFECT OF INOCULATING SOYBEAN PLANTS WITH STRAINS OF *B. japonicum* AND *Azotobacter chroococcum*

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The subject of the study were the effects of inoculating soybean plants with a mixture of five different *B. japonicum* strains, with a mixture of these five strains and three Azotobacter strains, and, finally, with a mixture of the *B. japonicum* strains combined with the amounts of gibberellic acid (GA_3) that had been determined to be present in the Azotobacter strains. Uninoculated plants were used as control treatments. The experiment was carried out in the greenhouse using pots with filled with soil and two soybean lines, NS-2016 and NS-300168, and one variety, Afrodita. Seeds were inoculated immediately following the sowing. The length of and dry matter mass in particular plant parts as well as in the whole plant were determined at flowering. Protein content and the activity of enzymes of nitrogen metabolism (NR, GS and GOGAT) were also determined. The highest dry matter mass and nitrogen (N) content were found in Afrodita, followed by NS-2016 and NS-300168. The greatest effect on N content and the mass of the whole plant was achieved in treatments with *B. japonicum* strains combined with GA_3, and then in those with strains of *B. japonicum* and *Azotobacter chroococcum*. The study has shown that the positive effect of the inoculation of soybean with *B. japonicum* strains can be further increased by the application of *Azotobacter chroococcum* strains as well as by combining *B. japonicum* and GA_3.

P-F-11

EFFECT OF GIBERELINE AND INOCULATION OF SOYBEAN PLANTS (*Glycine max* L.) WITH *B.japonicum* AND *A.chroococcum* ON PRIMARY ASSIMILATION OF N

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In plants, nitrogen (N), whether derived from the atmosphere by symbiotic N_2 fixation or from the soil as NO^-_3, is reduced to NH^+_4 to become available for amino acid and protein synthesis. The primary assimilation of NH^+_4 into amino acids occurs via the concerted action of four enzymes. The first two reactions, collectively referred to as the glutamine synthetase (GS; EC 6.3.1.2) glutamate synthase (GOGAT; EC 1.4.1.14) cycle, catalyze the synthesis of glutamine and glutamate, respectively. Subsequent incorporation of N into aspartate and asparagine occurs via the action of aspartate aminotransferase (ATT; EC 2.6.1.1) and asparagine synthetase (AS; EC 6.3.5.4). The carbon (C) skeletons required for initial assimilation of N are derived from the tricarboxylic acid cycle intermediates α-ketoglutarate and oxalacetic acid. Phosphoenol-piruvate carboxylase (PEPC; EC 4.1.1.31) provides a substantial amount of C to replenish the organic acid pool and for the synthesis of aspartate. Moreover in root nodules oxalacetate is reduced by malate dehydrogenase (MDH; EC 1.1.1.37) to malate which is a primary carbon source for N_2 fixing bacteroids. Thys, primary assimilation of N is closely coupled with C metabolism.

The aim of the study was to investigate changes in the primary assimilation of nitrogen in soybean plants inoculated with some nitrogen fixation strains (*B.japonicum* and *A. chroococcum*) after gibereline treatment.

Plants werw grown in the greenhouse under controled conditions on the sterile medium (control), nitragine medium, with *Azotobacter*, with nitragine and *Azotobacter* and nitragine and gibereline medium.

Changes in the primary assimilation of nitrogen werw studied according to the activities of nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT) and soluble protein content.

Inoculation showed positive effect on the values of examined parameters, especially on plants grown on nitragine and *Azotobacter* medium. Plants grown on nitragin and gibereline medium showed higher activities as well.

P-F-12

ACTIVITY CHANGES OF NITROGENASE AND GLUTAMINE SYNTHETASE IN SOYBEAN ROOT NODULES UNDER THE EFFECT OF V AND Ti

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Taking into consideration that V can participate in the formation of the alternative nitrogenase (NG) and the absence of the studies concerning the role of Ti in NG and glutamine synthetase (GS) catalysed reactions, the aim of the present study was to determine the possible regulatory role of V and Ti in the activity of soybean root nodule NG and GS. In order to elucidate the effects of seed treatment by various doses of V and Ti compounds we grew plants on soil cultures under monitored conditions. The activity of bacteroid NG and nodule cytosol GS was measured at the stage of mass blossoming and seed formation. The doses of V and Ti which induced NG and GS activities, were found. We established a different pattern of the V and Ti action on NG and GS activity by growing plants on sand cultures from the seeds inoculated and non-inoculated with Bradyrhizobium japonicum and with and without their treating with V and Ti. It was shown that V had a direct effect on soybean nodule NG, while the inducing action of Ti on the NG activity was indirect.

P-F-13

EFFECT OF NITROGEN AND POTASSIUM ON NODULE FORMATION AND NITROGENASE ACTIVITY IN FABA BEAN PLANTS

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There was ivestigated the effect of different rates of nitrogen and potassium on root nodule formation, and on nitrogenase activity in plants roots at successive stages of plant development in faba bean var. Nadwiślański . The experiment was performed in pots filled with sand; the seeds of faba bean were inoculated with an active strain of *Rhizobium leguminosarum*. Five plants were grown in each pot (7,8 kg of sand). The following rates of nitrogen and potassium were applied: 110, 550, 1100 mg N/pot; 250 and 1000 mg K/pot. The other mineral nutrient components were given in amounts appropriate to assure the normal growth of faba bean plants. Some of plants received the whole dose of N before sowing. Another plants got half of nitrogen dose in each combination before sowing and the remaining one to the leaves in the generative phase of their development. Nitrogenase activity in the root nodules was measured as the amount of ethylene formed, as a result of acetylene ruduction, at successive stages of plant development.

It was shown that mineral nitrogen supplied in both methods of N management depressed the nodule formation and nitrogenase activity. The inhibitory effect of N was smaller when nitrogen was supplied in part to the soil and in part to the leaves , than in the case when the whole dose of N was given to the soil. The inhibitory effect of mineral nitrogen became less visible at later development stages and in this stages even the new nodule formation was observed. In general the effect of potassium fertilization on root nodule number and mass as well as on the nitrogenase activity and seed yield in the all objects which were under investigation was positive.

P-F-14

A COMPARISON OF THE COPPER NUTRITION EFFECTS ON N_2 FIXATION IN YELLOW LUPIN AND SOYBEAN

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In some symbioses, nitrogen fixation and yields may be specifically limited by low availability of copper. Copper deficiency was found to have an indirect effect on nodule formation in subterranean clover but direct biochemical role of copper in nodule function and symbiotic N_2 fixation is not yet clear. In Department of Plant Physiology IUNG in Puławy was stated the differntial responses of some grain legume species to copper nutrition under the conditions of pot experiments. The aim of the experiment described in the present paper was to compare of copper nutrition effects on N_2 fixation in yellow lupin and soybean. Those included the determination of influence of copper supply on nitrogen accumulation in plants, dry matter nodules, its N, Cu, leghaemoglobin concentration and acetylene reduction activity. Yellow lupin and soybean plants inoculated with an active Bradyrhizobium or Rhizobium strain, were grown in pots on Cu deficient peat soil, mixed with sand, at different copper treatment. The accumulation of dry matter of vegetative parts, flowers and pods was significantly increased by copper supply at the beggining of pod setting stage in yellow lupin. These effects could be related to the effect of copper supply on symbiotic N_2 fixation. There was the increase of nodulation as an effect of copper treatment and, above all, the significantly higher concentration of nitrogen and leghaemoglobin in nodules and higher nitrogen accumulation in the whole plants. Cu application to the soil increased N_2 fixation as measured by the acetylene reduction assay. By contrast, the increase of accumulation of dry matter and nitrogen by copper supply was not significant at beginning of pod setting stage in soybean. The effect of copper nutrition on the dry matter of soybean nodules, its N and leghaemoglobin concentration and acetylene reduction activity was also not significant. However copper nutrition significantly increased the copper concentration in nodules of both yellow lupin and soybean. The presented results suggest that there are agronomical importance of Cu requirements by symbiotic N_2 fixation in nodules of yellow lupin in contrast with soybean. Further experiments are performed to investigate the physiological aspects of differential sensitivity of symbiotic N_2 fixation of some grain legume species to copper nutrition.

ALTERATION IN POPULATION DENSITIES OF SOIL AND PHYTOPHAGOUS MITES WITH SOYBEAN NODULATION AND POSSIBLE CONTRIBUTION OF SOIL MITES TO CROP PERFORMANCE

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A positive correlation exists between density of phytophagous mites and nutrition status of soybean [4]. Little information are available on population density of soil mites when soybean nutrition schedule comprise nodulation with the symbiotic-micropartner *Bradyrhizobium japonicum* (Bj), combined-N or seed coating with micro-elements (SC). Soybean in the Nile Delta was nodulated with or without seed coating by a combination containing chelated Fe, Mn, Mo and Zn along with urea at 0, 96, 144 or 192 kg N/ha applied in two equal doses: 25 and 45 days after sowing.

Parameters	-	Bj	-	SC	0N	96N	144N	192N
nodules/plant (mg)	340b	532a	460a	413a	679a	394b	388b	284c
plant weight(g)	23.5b	29.5a	25.9b	27.1a	19.6d	25.1c	31.7a	29.6b
N/plant (mg)	427b	612a	497b	542a	361c	479b	629a	610a
seed yield (t/ha)	3.09b	3.67a	3.22b	3.54a	3.13d	3.56a	3.48b	3.35c
phytophagous mite/5 leaflets	50b	60a	52b	58a	51b	63a	53b	51b
soil mites/m^2	10028b	11922a	9908b	12041a	7974c	9136bc	9868b	16919a

Means have the same letter (s) are not significantly different at the confidence level of 95%.

Nodulation, plant growth, seed yield and N-content,100-seed weight and the agronomic fertilizer N-use efficiency (kg seed yield / kg fertilizer-N) were enhanced by nodulation and/or seed coating. Enhancement due to nodulation was obvious with 0 or 96 rather than 144 or 192 kg N/ha. Mean of population densities of the phytophagous mite *Tetranychus cucurbitacearum* on the lower surface of 5 leaflets throughout six weekly estimations started 42 days after sowing increased with each or the combinations of nodulation, N-fertilization and micro-elements. Population densities of soil mites belonging to the suborders : Acaridida, Actinedida, Gamasida and Oribatida to soil depth of 15 cm [1] at 8 days after sowing, 10 days after each N-fertilization and 2 weeks prior to harvest showed gradual buildup with lapse of time. The results suggest a potential role of soil mites in enhancing crop performance [2] may be through soil organic matter degradation and a sort of action as predators and/or parasites for some growth stages of inguries mites and insects[3].

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P-F-16

ENHANCEMENT OF RICE PERFORMANCE AND INCIDENCE OF NATURAL FUNGAL AND INSECT DAMAGE BY CYANOBACTERIZATION WITH CERTAIN AGRONOMIC FACTORS

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Asymbiotic dinitrogen-fixation in the rice ecosystem is not the only contribution of cyanobacteria. Suppression of growth of aquatic macrophytes, increasing P-availability, decreasing sulphide injury and aiding soil particle aggregation were also reported. In the northern Nile Delta, where certain fungal and insect pests severely damage rice crop, fields of the rice variety Giza-176 were inoculated with a soil-based inoculum containing the dinitrogen fixing cyanobacteria : *Anabaena cylindrica* , *Anabaena oryzae* , *Aulosira fertilissima* , *Nostoc muscorum* and *Tolypothrix tenuis* along with urea at 72 or 144 kg N/ha applied in two equal doses: 25 days after transplanting and at the midtillering stage. The macro plus micro-elements combination Vitafort (N,P,K,Mg,Fe,Mn,Zn,Cu,B,Mo,Co, Vitamin B, meta indole acetic acid, amino acids and growth promotion substances), the fungicide Sportake-45 or 50/50 of them were applied as soon as the detection of fungal infection symptoms during the late panicle initiation stage. A second set of field experiments comprised the rice variety IR-28 with the same cyanobacterization and combined-N treatments along with application of the stem borer (*Chilo agamemnon* Bles) insecticides : Cyfen, Dursban or Fipronil (28.8, 12 and 24 kg/ha, with active ingredients of 5, 5 and 0.2%, respectively). Performance of rice, as indexed by plant height, productive tillering, grain and straw yields, N-contents, the harvest index (% of grain yield / grain + straw yields), grain size and the agronomic fertilizer N-use efficiency (kg grain yield / kg fertilizer-N) were maximized by cyanobacterization with 72 kg N/ha and 50/50 of Vitafort plus Sportake-45. Natural infection with *Pyricularia oryzae* , *Helminthosporium oryzae* , *Alternaria* sp. and *Sclerotium oryzae* , the causative agents of the rice blast, brown spot, leaf spot and stem rot diseases, respectively, was less with inoculation and 72 rather than 144 kg N/ha. Inoculation with 72 kg N/ha and Cyfen at 28.8 kg/ha registered the maximum grain yield and N-content and the highest agronomic N-use efficiency. Natural infestation with the stem borer indexed by numbers of dead hearts and white heads/m^2 increased with increasing urea fertilizer and was significantly less with cyanobacterization. A mechanism involving a sort of regulation of available-N in the rice ecosystem by the dinitrogen-fixing cyanobacteria is suggested to diminish excessive or sub-minimal N consumption and, consequently, decrease plant susceptibility to fungal and insect pests.

P-F-17

ASSESSING BIOLOGICAL FIXATION OF NITROGEN AND CULTURAL PRACTICES ON THE BALANCE OF MINERAL-N IN THE SOIL-CROP SYSTEM

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Biologcal fixation nitrogen enriches directly the soil by means of root biomass (nodulation, root excudat and root system). The improved role of the legumes crops, consist, mainly, on the nitrogen fixation capacity and on transfer possibility of this nitrogen to non-legume crops. Thus, a study for obtaining the information about biological fixation of nitrogen and its transfer to non-legume crops was conducted by us from 1993 to 1995. The objective of this study was to evaluate the contribution of symbioticly fixed nitrogen by bean (*Vicia Faba L.*) to the ray-grass (*Lolium Italicum L.*) crop in different cultivating conditions.

Our study was carried out in two typical soils of Albania (*Calcaric Arenosol* and *Mollic Gleysol*, FAO, 1992). The main physico-chemical properties of these soils are as follows: a. Calcaric Arenosol- Clay=16,4%, Silt=24,2%, Sand=59,4%, C=8,5‰, N=0,87‰, ECC= 18,3 meq/100g soil and b. Mollic Gleysol- Clay=54,0%, Silt=35,2%, Sand=10,8%, C=17,5‰, N=1,88‰, ECC= 23,7 meq/100g soil.

At two experimental sites were cultivated bean and lolium in rotation for three years and with three variants (10, 20 and 30 kg N ha^{-1} for bean and 100 kg N ha^{-1} for lolium crop, at each experimental site and year). The fertilizer form was that of ammonitrate ($NH_4{}^{15}NO_3$) with an isotopic excess of 10 and 2%, respectively, which was applied in the germination phase. Calculations of fixed nitrogen by bean are performed according to *Fried & Middelboe* (1977) method, considering the lolium as reference crop.

Results show that the Ndff (Nitrogen derived from fertilizer) values from bean crop vary from 2,74 to 4,20 and 1,72 to 2,30%, respectively, in first and second soil and three cropping years in rotation. These differentated values can be explained by the fact that physico-chemical immobilization of applied nitrogen as fertilizer, in clay soil, is higher in relation to the first soil, charachterized by a high sand percentage. The Ndfa (Nitrogen derived from atmosphere-Nitrogen symbioticly fixed) values are in inverse sense to them of Ndff. This hierarchy is caused by a root system more developed (especially in initial phases). The above mentioned hypothesis is supported on the fact that is observed a higher amount of total nitrogen upteaken by crop in clay soil. Although soil supplying (Ndfs) is higher, this mechanizm can not balance the symbiotic nitrogen.

The values of fixed nitrogen show the variation from 51,3 to 81% for the ensemble of three variants at two experimental sites. The differences of variants value are higher in sand soil where, in general, is observed and a more exigent response according to the increase of applied nitrogen dose. The effect of bean crop on lolium one is evident, improving the yield and the CAU (Coeficient Apparent Utilization) values (10-20%) in comparison with the control.

At the conclusion we can underline that: **a.** the values of fixed nitrogen by bean, measured in our study, are dependent upon the soil type and applied nitrogen doses; **b.** symbioticly fixed nitrogen is easily boidisposable for lolium crop.

P-F-18

BIOLOGICAL FIXATION OF MOLECULAR NITROGEN BY LEGUMES AND CEREALS WHILE USING AGROCHEMICAL VIDAKS

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A new complex agrochemical preparation VIDAKS was elaborated and proposed for use in agriculture. It contributes to fixation of atmospheric molecular nitrogen by microorganisms, improves the feeding of plants by nitrogen, promotes to obtaining ecologically clean production, allows cultivating agricultural crops with less quantity of energy expenditures.
During vegetation experiments on turfy, podsol and slightly loamy soil it was established that using agrochemical VIDAKS contributes to efficient symbiosis of nodule bacteria and legumes (clover, alfalfa). The nitrogenous activity of nodules was raised and the quality of the crops improved; such characteristics as the crop capacity of the above ground mass as well as the content of nitrogen and albumen being increased.
For example, while experimenting with VIDAKS during alfalfa cultivation the nitrogenous activity of nodules was 5 times higher than in the control group. The quantity increase nitrogen in crops reached 10% - 16%.
In the field experiments with cereals VIDAKS contributed to substantial activization of atmospheric nitrogen fixation process. For example, nitrogenous activity in the root zone of perennial and other grasses was 9 times higher than in control groups.
The data allow to use both methods, i.e. to apply VIDAKS as well as to inoculate seeds with nitrogen fixing bacteria.

P-F-19

AGRO-ECOLOGICAL ASPECTS OF BIOLOGICAL NITROGEN APPLICATION IN THE AGRICULTURE OF UKRAINE

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During the last twenty years complexed studies of possibilities of using biological nitrogen fixation in soil and climatic condition were provided. The influence of antropological factors on functionality of nitrogen-fixing microflora, nitrogen balance and its transformation in soil were investigated. The system of activation of nitrogen-fixing microflora was worked out by the addition to soil the biological preparates of nitrogen-fixing microorganisms and by some agrooperations. It was established that the most perspective technologyfor supplying of biological nitrogen to plants , for obtaining the high yields and quality of agricultura plants is using the biological preparates on the base of high-effective strains of nitrogen-fixing microorganisms.Regional system was worked out to produce such type of preparates as mentioned above according to the various soil and climatic conditions of Ukraine.

P-F-20

THE RESERVES OF INCREASING SYMBIOTIC NITROGEN FIXATION IN UKRAINIAN AGRICULTURE

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The legumes occupy near 10 - 12% of area under agricultural crops in Ukraine, pea and alfalfa occupy about 70% of this area. The protein deficit in forage now is more than 25%. For ecological solution of plant protein deficit problem it is necessary to extend legumes area in 2 - 3 times, increase legumes variety raise its yield productivity.

The soil of Ukraine contain natural populations (100 -100 000 bact./g) *R. leguminosarum bv. viciae, bv. trifoli, R. meliloti, R. loti, R. lupini, R. galegae* are able to form efficient symbiosis with coresponding legumes varieties and fix to 40 - 200kg nitrogen per hectare. The selective Rhizobium strains can raise this level to 10 - 25%, but the nodule bacteria preparations apply now less than 15000 hectares.

Bradyrhizobium japonicum, R. leguminosarum bv. phaseoli, R. ciceri usually are absent in the soils of Ukraine. Selective strains of this crops raise grain yield to 20 - 50% and protein content to 2 - 10%. Chemical and biological plant growth stimulators (lentechnin, wheat extract, biohumus extract, rhizoagrin, agrofil, flavobacterin) can increase nitrogen fixing activity pea and soybean to 34 - 145% and enhance grain yield to 10 - 23% (0.18 - 0.43t/he). By means of adaptation selection of macro- and microsymbionts received some high competition and efficient *R.leguminosarum bv. viciae* strains which increased pea varieties Charkovsky 85 and Intensivny 90 grain yield to 20 - 32% (0.6 - 1.1 t/he).

To start selection soybean and alfalfa with increasing ability to fix nitrogen in symbiosis with commercial nitragin inoculants.

The new soybean varieties Charivnytsya stepu, Krepysh, Uspech, Palmira can form grain yield to 3.0 - 3.5 t/he without nitrogen fertilizer.

P-G-1

AMMONIUM ASSIMILATION IN THE *Lotus japonicus-Rhizobium loti* SYMBIOSIS: MODULATION OF THE PLANT GLUTAMINE SYNTHETASE GENE EXPRESSION

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Ammonium, produced from fixed nitrogen by bacteroids in legume nodules, is assimilated by the plant through the glutamine synthetase (GS) - glutamate synthase (GOGAT) cycle. The general goal of our research line is to study the molecular physiology of ammonium assimilation, a key process in the *Rhizobium* - legume symbiosis. We are using *Lotus japonicus* which has been proposed as a model legume system, it establishes symbiosis with *Rhizobium loti*. We have determined the kinetics of expression of both GS and GOGAT in root and nodules of *Lotus* plants inoculated with *R. loti*.

The reverse genetics in plants (overexpression or antisense inhibition) allow to modulate specific gene expression and therefore to vary quantitatively the flux control of a specific metabolic process. We are using this approach to study the molecular physiology of transgenic *Lotus* plants with nodule-specific modulation of either one of the ammonium assimilation enzyme activities (GS or GOGAT).

In this work we present the study of the overexpression or partial inhibition of GS during the symbiosis of transgenic *Lotus* plants with *R. loti*. We have genetically transformed *Lotus* with chimeric gene constructs of the GS cDNA clone from alfalfa fused, either in sense or in antisense orientation, to the nodule specific promoter from the soybean (*lbc*3) or sesbania (*Srglbc*3) leghemoglobin genes. Several transgenic plants containing stable integrations of each of the chimeric genes have been obtained. Our results indicate the achievement of nodule-specific modulation of GS. For example, some of our GS-sense *Lotus* plants inoculated with *R. loti*, form nodules that show an elevated GS specific activity, up to 5-fold, as compared to nodules of control plants, while their GS activity remains unaltered in other plant organs (roots or leaves). Further molecular characterization, as well as the determination of the symbiotic phenotype of the transgenic GS-sense and GS-antisense *Lotus* plants obtained is in progress.

P-G-2

MALATE AND GLUCOSE TRANSPORT BY BACTEROIDS ISOLATED FROM THE NODULES OF THE DIFFERENT PLANT-DETERMINED INEFFECTIVE PEA MUTANTS (*PISUM SATIVUM* L.)

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It was known that in free-living culture *Rhizobium* are able to use as carbon sources carbohydrates, C_4-dicarboxylates, amino acids etc, but for differentiated bacteria (N_2-fixing bacteroids) C_4-dicarboxylates are extremely important. At present study the ^{14}C-malate and ^{14}C-glucose transport by bacteroids isolated from the nodules of pea mutants sym31 (Sprint 2 Fix$^-$), sym13 (E135f), RBT, parent lines - Sprint 2 Fix$^+$ and Sparkle and by free-living *Rhizobium leguminosarum* 250 as well, were investigated. Sym31 was preliminary characterized by a block in bacteroid differentiation and symbiosomes formation at the time corresponding to bacterial endocytosis (Borisov et al., 1992). Sym13 was characterized by early senescence of symbiosomes and nodules as a whole (Kneen et. al., 1990). The RBT line was constricted and carrying mutations in two loci (genetic model of second level) (Borisov et al., 1995).

The glucose-grown *Rhizobium leguminosarum* showed glucose transport rate 2,61 and malate transport rate 1,89 nmol$\cdot$min$^{-1}\cdot$(mg protein)$^{-1}$. The malate-grown *Rhizobium leguminosarum* showed glucose transport rate 4,31 and 5,82 nmol$\cdot$min$^{-1}\cdot$(mg protein)$^{-1}$ for malate transport rate. The bacteroids isolated from all nodules investigated showed low transport rate for glucose - about 2-4 nmol$\cdot$min$^{-1}\cdot$(mg protein)$^{-1}$. Bacteroids from sym31 and RBT nodules showed high malate transport rate - 33,76 and 17,08 nmol$\cdot$min$^{-1}\cdot$(mg protein)$^{-1}$, respectively. But bacteroids isolated from sym13 nodules showed low malate transport rate - 2,71 nmol$\cdot$min$^{-1}\cdot$(mg protein)$^{-1}$. Unexpectedly, bacteroids isolated from both parent lines nodules also had low malate transport rate about 3-4 nmol$\cdot$min$^{-1}\cdot$(mg protein)$^{-1}$.

The data presented showed that in pea nodules the malate and glucose transport by bacteroids didn't directly connect with nitrogen fixing process and the malate transport is probably a marker for undifferentiated bacteria inside the nodules.

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P-G-3

SYNTHESIS AND TRANSLOCATION OF IMPORTANT AMINO COMPOUNDS IN *ALNUS GLUTINOSA*

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In *Alnus glutinosa* plants the assimilation of ammonia released by symbiotic *Frankia* was determined by $^{15}N_2$ labelling and subsequent analysis of the isotopic enrichment of nodule amino acids over time by single ion monitoring gas chromatograph mass spectrometry. Following 30 seconds exposure to $^{15}N_2$ we were able to detect significant labelling of glutamine with one ^{15}N (4.8 APE) while all other amino acids detected including glutamate did not incorporate significant amounts of label. Within 3 minutes of exposure to $^{15}N_2$ significant amounts of label accumulated in glutamate, aspartate, alanine GABA and ornithine. To determine label incorporation into citrulline, an important transport compound in alder, an additional novel derivitisation method was required. Using this method, label was shown to accumulate in citrulline (determined as ornithine) and 81 minutes after exposure to $^{15}N_2$ citrulline had an APE greater than 30. This is the first time that significant amounts of citrulline have been shown to be directly assimilated from N_2 within minutes of label being applied.

In another study, symbiotic plants fed nitrate had a much higher citrulline content in the xylem sap compared with N_2-fixing or ammonium fed plants (10.9 μmol cm^{-3} compared with 5.8 and 4.4 respectively). To determine if citrulline was synthesised from nitrate, ^{15}N labelled nitrate was added to pot saucers at day 0 and enrichment in plant parts and xylem amino acids followed over 7 days. During this time nodule activity fell steadily and by 7 days was only 21% of the initial value. Citrulline content of the xylem sap increased with each sequential harvest. Twelve hours after the label was added over 50% of N in glutamate and only 19% of N in citrulline was from $^{15}NO_3^-$. By 7 days 79% of glutamate and more than 80% of citrulline was from the labelled pool.

These results show that citrulline is a direct product of both N_2-fixation and nitrate assimilation. We believe that N_2-fixation is likely to be regulated to match the N demands of the plant and this may or may not involve sensing the levels of citrulline in the plant.

P-G-4

SHORT-TERM EFFECT OF NITRATE AND WATER STRESS ON NITRATE REDUCTASE AND MALATE DEHYDROGENASE ACTIVITIES IN YELLOW LUPINE NODULES

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The supply of nitrate and water stress treatment has a complex inhibitory effect on legume plants nodule function and nitrogen fixation. It is well established hypothesis that both treatments are involved in a mechanism which enhances the resistance of the nodule oxygen diffusion barrier. In condition of limited oxygen availability in nodule interior stimulation of fermentative pathways in plant fraction and activation of denitrification enzymes in bacteroids can be expected.

After supplying 5 and 20 mM nitrate a transient 20% increase of cytoplasmic malate dehydrogenase was observed. Changes of cytoplasmic aspartate aminotransferase activity proceeded in the opposite manner. In restricted water conditions cytoplasmic malate dehydrogenase and aspartate aminotransferase activities decreased significantly, relative to control. These findings are consistent with suggestion of other authors that water stress lead to limited malate transport into bacteroids. The presented data may be evidences for anaerobic conditions, being produced in yellow lupine nodules under nitrate supply and water stress.

In vivo measured activity of nodule nitrate reductase had about 25 times higher level than detected in other organs of field grown lupine plants (7 weeks old). At least 97% of this activity was concentrated within bacteroids.

Water stress resulted in increased bacteroid nitrate reductase and nitrite reductase activities (respectively 50% and 130% relative to control) in spite of nitrate absence in the nutrient solution. Nitrate supply caused significant increase of bacteroid nitrate and nitrite reductase activities in spite of lack of nitrate accumulation within infection zone. During these treatments cytoplasmic nitrate reductase activity followed a pattern different to bacteroid enzyme. It is postulated that bacteroid nitrate and nitrite reductases can act as dissimilatory enzymes. It can be one of adaptation mechanisms to nodule microaerobic conditions.

P-G-5

A PROPOSED STRUCTURAL MODEL AND MUTAGENESIS ANALYSIS OF THE CENTRAL DOMAIN OF NifA

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NifA, the central regulator of nitrogen fixation, activates the expression of the great majority of the *nif* and *fix* genes in response to the cellular oxygen and/or nitrogen status. The *nif* and *fix* genes have promoters recognized by the RNA polymerase associated with the alternative sigma factor σ^{54} ($E\sigma^{54}$). To activate transcription NifA binds to promoter-distal enhancer-like elements denominated UAS. NifA belongs to a large family of regulatory proteins that activate promoters recognized by $E\sigma^{54}$ denominated Enhancer-Binding Proteins (EBP).To date the amino acid sequences of more than 50 EBP are know. These proteins are composed of three structural, functional, and evolutionarily different domains. The COOH-terminal domain (70-120 amino acids) has a DNA-specific binding activity, while the Central domain (220 amino acids) has the so called "positive control" function. The function of the amino terminal part (8-150 amino acids) is less clear, although for some members it has been shown that they serve regulatory roles.

A remarkable characteristic of the activation of transcription by the EBP is the requirement of nucleotide triphosphates. The NTP is bound at the Central domain and the energy of its hydrolysis is coupled to the isomerization of a closed to an open $E\sigma^{54}$-promoter complex.

Whitin the Central domain of NifA, as in the rest of the EBP, seven conserved regions have been observed; C1 to C7. C1 has the typical sequence of the Walker A motif located in a large number of proteins that bind mononucleotides and mutations there abolish ATP hydrolysis and, consequently, transcription. Region C3 has been proposed for several criteria to be involved in interaction with the $E\sigma^{54}$-promoter complex. A putative function of the rest of the conserved regions has not been assigned.

In order to gain insight into the structure and function of this domain we generated a structural working model for the Central domain of the *Bradyrhizobium japonicum* NifA protein. We propose that this domain might adopt the "classical" mononucleotide-binding fold related to EFTu whose cristalographyc structure is known. This is based on the result of several secondary structure prediction and fold recognition methods, that suggest that it consist of an α/β architecture. The mapping of correlated mutations support the proposed model. Interestingly, almost all the know residue substitutions on several EBP that impair the ATPase activity are located in loops equivalent to those involved in nucleotide-binding in EFTu/p21ras proteins. Conversely, mutations that affect other activation functions, leaving intact ATPase map in sites equivalent to the effector region of the GTPase superfamily, or in protein-protein contacts.

Taking into account the fold model and the phenotype of some C3 region mutants, we generated a collection of 20 NifA derivatives with substitutions in or near this region. All the NifA derivatives were strongly impaired in transcriptional activation but not in DNA binding. Presumably, the ATPase function is not affected because, as the model predicts, C3 region does not interact directly with the nucleotide. We propose that C3 region is equivalent to the effector/contact region of the GTPase superfamily, then, the NifA mutants described here could be affected in protein-protein interactions or in coupling the ATP hydrolysis to open promoter complex formation.

P-G-6

GROEL CHAPERONINS ARE REQUIRED FOR SYMBIOTIC NITROGEN FIXATION IN *BRADYRHIZOBIUM JAPONICUM*

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Five highly conserved, but differently regulated *groESL* operons are present in *Bradyrhizobium japonicum* (1). Most strikingly, expression of *groESL3* is co-regulated with symbiotic nitrogen fixation genes via the alternative σ factor, σ54, and the trancriptional activator protein NifA (2). Knock-out mutation of individual *groEL* genes does not impair symbiotic nitrogen fixation activity of the respective mutant strains. By contrast, the *groEL3/4* double mutant strain D4, which is mutated in those *groEL* genes that contribute most to the GroEL pool under symbiotic conditions, exhibits less than 5 % Fix activity as compared with the wild type. Expression of *lacZ* fusions to six representative *nif* and *fix* genes is, if at all, only marginally reduced in mutant D4. On the basis of Western blot analyses, the level of NifH protein was found to be drastically reduced in extracts prepared from D4 bacteroids or from free-living cells grown anaerobically. Taken together these results indicated that efficient synthesis of NifH requires GroEL chaperonin at a post-transcriptional level. Transcriptional fusions of the *groESL3* promoter (P3) to all five *B. japonicum groESL* operons as well as to *groESL* from *E. coli* were integrated into the chromosome of mutant D4 and the resulting strains were tested for their ability to restore the symbiotic defect of D4. Partial complementation was observed in strains harboring P3 fused to *groESL1*, *groESL2*, *groESL5* or *Escherichia coli groESL* whereas the wild-type phenotype was restored in strains complemented with P3 fused to *groESL3* (control) or *groESL4*. Thus, the function of GroEL3 and GroEL4 in symbiosis can be substituted partially by forced expression of alternative *B. japonicum* or *E. coli groESL* operons. Conversely, the growth defect and the impaired ability to allow propagation of bacteriophage λ of an *E. coli groEL* mutant could be at least partially restored by individual *B. japonicum groESL* operons. In conclusion, both series of complementation analyses were not indicative for a strict specificity of any of the *B. japonicum groESL* gene products which is in good agreement with their high degree of sequence conservation.

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P-G-7

MULTIGENE *groEL*(chaperonin)-LIKE FAMILY IN THE GENUS *Rhizobium*

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Using a polymerase chain reaction and degenerative primers for highly conserved internal region of *groEL*-like genes, a 290 kb DNA fragment from *Rhizobium etli* CFN42 was amplified and used as a probe in Southern blot hybridizations against digested total DNAs isolated from 35 *Rhizobium* strains. In the strain CFN42, four bands hybridizing with the PCR product were revealed. One gene was localized in the non-symbiotic plasmid pf, and other three were in the chromosome. Each of the copy was cloned separetely in the *EcoRI* site of pSUP202 vector. The sequencing data showed about 70-75% level of similarity with $groEL_a$ gene of *Rhizobium meliloti.* Southern hybridization of the PCR product with *EcoRI* or *BamHI* digested total DNAs from other *Rhizobium* species (*R.tropici, R.fredii, R.galegae, R.leguminosarum, R.loti, R.huakuii* and *Rhizobium* sp.) revealed the presence of a multigene family. Only one strain, *R.trolici* CIAT899 had a single copy of the gene. Other strains contained up to 5-6 fragments hydridizing with the *groEL* PCR product. The restriction fragment length polymorphism hybridization profiles obtained by using this gene probe are useful for discrimination between related *Rhizobium* strains.

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P-G-8

REGULATION OF THE TCA CYCLE IN *RHIZOBIUM* BY OVERFLOW METABOLISM

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Oxidation of C_4-dicarboylic acids via the TCA cycle is the principal route for energy generation in the bacteroid. There is considerable evidence from labelling studies that the TCA cycle may be limited, possibly by redox inhibition, particularly at the level of α-ketoglutarate dehydrogenase. This inhibition may lead to amino acid biosynthesis via transamination of accumulated α-keto acids. Such an explanation is consistent with the synthesis and excretion of amino acids such as alanine and aspartate by bacteroids of *Bradyrhizobium japonicum* and *R. leguminosarum.* We have therefore been studying the regulation of the TCA cycle paying particular attention to the α-ketoglutarate dehydrogenase complex and how the TCA cycle is regulated by amino acid excretion. Mutation, complementation and sequence analysis have been used to demonstrate that *mdh-sucCDAB,* which code for malate dehydrogenase, succinyl-CoA synthetase and components of the α-ketoglutarate dehydrogenase pathway, are clustered together in *Rhizobium leguminosarum* b.v. *viciae* strain 3841. *SucB* appears to define the end of the operon with the promoter for *sucCDAB* upstream of either *sucC* or *mdh.* Mutation of downstream genes causes an elevation in activity of the enzymes coded for by upstream genes. Strains mutated in *suc* genes were originally selected on the basis of resistance to high concentrations of aspartate. Two other classes of such mutants were also identified on this basis, one of which is mutated in polyhydroxybutyrate synthase (*phbC*). *Suc* mutants, unlike the wildtype, excrete large quantities of glutamate and α-ketoglutarate. The presence of aspartate in the growth medium increases the rates of excretion, presumably because it acts both as an amino group and keto acid donor for the TCA cycle. Concomitant with mutation of the *mdh-suc* operon the intracellular concentration of glutamate but not α-ketoglutarate is highly elevated suggesting that α-ketoglutarate normally feeds into the glutamate pool. This may be coupled to the excretion of amino acids as a part of an overflow pathway for regulation of the TCA cycle. Overall the data suggest that the TCA cycle in *R. leguminosarum* is regulated by amino acid excretion and polyhydroxybutyrate biosynthesis.

P-G-9

SYMBIOTIC EXPRESSION OF THE *RHIZOBIUM MELILOTI* C_4-DICARBOXYLATE TRANSPORT GENE (*DCT*A)

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During symbiosis between *Rhizobium meliloti* and the alfalfa (*Medicago sativa*) host plant, the energy required to fuel symbiotic nitrogen fixation is ultimately derived from the plant photosynthate. C_4-dicarboxylates (dCA) are the major source of carbon channelled to the bacteroids. Expression of the *R.meliloti* C_4-dicarboxylate transport gene (*dct*A) is essential for an effective symbiosis. The *dct* operon consists of the structural gene *dct*A, coding for the permease and the regulatory genes *dct*BD, coding for a two-component regulatory system. Under free-living conditions, the regulatory *dct*BD genes are required for activation of the *dct*A promoter in the presence of inducer (dCA) in the environment. By contrast, during symbiosis the *dct*A gene is expressed, even in the absence of the regulatory genes. Accordingly nodules induced by *R.meliloti* strains mutated in the *dct*BD genes generally fix nitrogen although at an reduced rate. This demonstrates that in this specific environment regulatory molecules, other than DctBD, are involved in the symbiotic expression of the *dct*A promoter. This alternative system of symbiotic activation (ASA) has not been identified to date.

An effective nodule is organized into different zones (Vasse *et al*,1990,J.Bact.172,4295), which correspond to a spatial and temporal pattern of bacterial and plant gene expression. As a first step towards elucidating the symbiotic control of expression of *dct*A, we set out to evaluate the temporal and spatial expression of the *dct*A gene during symbiosis in wild-type and mutant strains of *R.meliloti*.

A *dct*A::GUS and a *dct*A::*lac*Z gene fusion have been constructed. The reporter genes have been fused to the second ATG codon at position 13 from the start of the DctA protein, in order to avoid possible effects of DctA transmembrane helices on the stability of the plasmids expressing the chimeric genes. Like the *dct*A::*lac*Z gene fusions, the *dct*A::GUS gene fusion can be highly induced by dCA in free living cultures of a wild-type *R.meliloti* strain. Both gene fusions were found to be expressed strongly during symbiosis. To date, the expression of the *dct*A gene has been monitored for up to 28 days after inoculation of 4 day old *Medicago sativa* seedlings with wild-type *R.meliloti* strains. When inoculated with a wild-type strain, *dct*A expression can be observed on the root surface, in infection threads and in young nodules,as soon as they become infected. Expression of *dct*A in mature nodules was found to be located in the proximal part of infection zone II, the interzone II/III and the nitrogen fixing zone III.

The identification of the ASA and the symbiotic regulation of *dct*A expression could shed additional light on the exchange of signals between *Rhizobium* and its host during symbiosis.

P-G-10

NITROGEN ASSIMILATION IN NITROGEN FIXING SYSTEMS OF PEA PLANTS WITH *RHIZOBIUM LEGUMINOSARUM* NITROGEN RESISTANT MUTANTS

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Durable application of mineral fertilizers has negative effects on soil microflora, particularly on *Rhizobia* saprophytely existing in the soil out of host-plant. Among them there occur competitive forms resistant to a high nitrogen level, however, less efficient in symbiosis. Therefore, to increase the level of symbiotical nitrogen fixation it is necessary to use *Rhizobia* strains, which are able to provide nitrogen fixation under above mentioned conditions. So, the search of spontaneous forms and obtaining the induced mutants which are able to create efficient symbiosis under conditions of high content of rezidual nitrogen in substrate, is constantly carrying out. Mutants of pea *Rhizobia* resistant to 6—9 mM nitrogen in the soil are obtained using the method of chemical mutagenesis with EMS. Mutants were selected in sterile micro vegetative experiments. Their symbiotic properties have been studied. The obtained mutants in comparison with the initial strain have a number of peculiarities in assimilation of both biological and mineral nitrogen. They form nodules in pea in the presence of nitrogen concentrations (7—8 mM NO_3^-) usually inhibiting nodulation. Among mutants there have been revealed those which can essentially increase pea productivity. Nitrate reductase activity of mutants both in pure culture and in symbiosis with plants exceeds itself in comparison with the initial strain. Our further task is to study the peculiarities in functioning enzymes of the sequent nitrogen assimilation. Obtaining the mutants with nesessary properties and comprehensive study of their essence permit to approach to the creation of highly efficient symbiotic systems, which are able to fix nitrogen under stress conditions.

P-G-11

WHAT TRIGGERS THE DECLINE IN NITROGENASE ACTIVITY AFTER DEFOLIATING WHITE CLOVER?

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It is well known that defoliation of white clover leads to a drastic decline in nitrogenase activity within one to two hours. One effect of defoliation is a strong reduction of the transpiration-driven xylem transport. This may limit the export of N_2-fixation products, i.e. amino-compounds from the root nodules. Amino acid analysis of root nodules showed a significant increase of asparagine within one hour after a complete defoliation. This is consistent with the concept that a backup of amino-compounds in the root nodules could trigger the decline in nitrogenase activity through acting on a system which changes the nodule oxygen permeability.

P-G-12

NITROGENASE PHOTODEPENDENT REACTIONS. Av2 INTERACTIONS WITH EOSIN DURING DARK AND PHOTODEPENDENT PROCESSES

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In the course of the investigation of nitrogenase reduction by eosin - NADH containing electron donor photosystem the pre-steady-state and steady-state kinetics of light-dependent enzyme reaction were studied. The inhibition of acetylene reduction and hydrogen evolution by eosin at the concentrations more than $7x10^{-5}$ M has been shown. On the contrary, the dye did not influence to the MgATP hydrolysis. It has been established that K_m for eosin in the photoreaction conditions is equal $6x10^{-6}$ M.

Method of free Av2 determination in solutions containing Av1 and eosin was advanced by using stopped-flow spectroscopy. It permits to follow the redox state of Av2 Fe_4S_4 cluster and thus to look after the complex formation between Av2 and Av1 during nitrogenase turnover in the presence of small ($6x10^{-4}$M <) dithionite concentrations.

Study of the Av2 reduction by the photochemical donor system with using impulse laser photolysis method had been carried on [1]. It was shown that the rate constants of this reaction in the case of either $Av2_{ox}$:Av1 (1:1) - complex or $Av2_{ox}$ alone are the same and equal ~500 s^{-1} . These results are in contrast to data obtained for the dithionite-dependent $Kp2_{ox}$ reduction.

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P-G-13

NEW APPROACHES FOR INVESTIGATION OF NITROGENASE MECHANISM ACTION

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Developed in 1993 [1] novel donor for nitrogenase, photochemical donor system (eosin with NADH in the light) makes feasible the new approaches for investigation of nitrogenase mechanism action. Eosin as a physical label offers to follow Fe protein (Av2) in solution in the process of nitrogenase turnover [2]. In combination with method of kinetic laser spectroscopy this donor system enables to register the photoreduction of Av2 in μs-range. Recently the reaction kinetics on photoreduction of the $Av2_{ox}$.MgADP in the free state and in the enzyme nitrogenase (within the complex with Mo-Fe protein, Av1) has been studied. Both reactions could be described by similar rate constants about $(11\text{-}12)\times10^6 \pm 1.6\times10^6$ $M^{-1}s^{-1}$ at 20°. Hence in contrary to other electron donor, dithionite, photochemical donor has reduced the Av2 within complex as effectively as in free state in solution and does not require previous nitrogenase dissociation. Under study of photoreduction of nitrogenase complex 1:1 ($Av2_{ox}$:Av1)MgATP both initial stages of nitrogenase turnover, photoreduction of Av2 and electron transfer from Av2 to Av1, can be registered simultaneously. The reaction rate of last process was equal about 100 s^{-1} at 20°. Hence the rate-limiting step of nitrogenase turnover is not concerned with photoreduction of Av2.

The other possible approach is direct photoreduction of Av1 with formation of its super-reduced (s.-p.) state. The implementation of this reaction either with Av1 alone or in complex with MgATP, MgADP; study of defect (without FeMoco) Av1 can answer for such unsolved questions: 1) succession and mechanism of electron transfer through iron-sulfur clusters of Av1; 2) redox potentials of Av1 s.-p. metal-cluster(s); 3) how the charge separation after electron transfer from Av2 to Av1 against potential gradient is realized.

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P-H-1

OXYGEN SENSITIVITY OF NifA PROTEIN OF *Azospirillum lipoferum* FS INDICATED BY GENE CLONING AND EXPRESSION IN *Escherichia coli*

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We cloned and sequenced a 2.8-kb ***Sal***I fragment of ***Azospirillum lipoferum*** FS as a homologue of ***Klebsiella oxytoca nifA*** gene. The amino acid sequence deduced from an open reading frame of 1,872 bases showed 91% identity to that of the ***A. brasilense*** NifA, and the central σ^{54} interaction domain and the C-terminal DNA-binding domain were conserved. A Cys-X_{11}-Cys-X_{19}-Cys-X_4-Cys motif resembling metal-binding sites found in oxygen sensitive NifA proteins was also conserved in ***A. lipoferum*** NifA.

To examine the ***nif*** promoter-activating ability of ***A. lipoferum*** NifA in response to oxygen conditions, we constructed a cascaded expression system in ***E. coli*** cells using ***nifA*** driver plasmids and P***nifH-lacZ*** reporter plasmids. The ***nifA*** genes of ***A. lipoferum*** and ***K. oxytoca*** were expressed under the control of ***lac*** promoter and the activities of produced NifA to induce the transcription from ***nifH*** promoters of ***A. brasilense*** or ***K. pneumoniae*** were evaluated as the β-galactosidase activity. The ***E. coli*** cells harboring ***K. oxytoca nifA*** driver plasmid and P***nifH-lacZ*** reporter plasmids showed high β-galactosidase activities under aerobic conditions as well as microaerobic conditions, indicating that the ***K. oxytoca*** NifA produced in ***E. coli*** effectively activates the ***nifH*** promoters regardless of aerobicity. On the other hand, the cells harboring ***A. lipoferum nifA*** driver plasmid and P***nifH-lacZ*** reporter plasmids showed only trace levels of β-galactosidase activity under aerobic conditions, whereas they showed higher (20 to 30-fold) activities under microaerobic conditions. These results indicate that the ***A. lipoferum*** NifA does activate the ***nifH*** promoters particularly under microaerobic conditions. We suppose that oxygen sensitivity is an intrinsic property of the ***A. lipoferum*** NifA protein.

P-H-2

AEROTAXIS AND ITS ROLE IN N_2-FIXATION BY *AZOSPIRILLUM*

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The microaerophilic nitrogen-fixing bacterium *Azospirillum brasilense* formed a sharply defined aerotactic band in a spatial gradient of oxygen. The bacteria were attracted to a specific low concentration (3 - 5 mM) of oxygen. Bacteria swimming out of the aerotactic band were repelled by the higher of lower concentration of oxygen that they encountered, and returned to the band. This behaviour was confirmed using temporal gradient of oxygen. The cellular energy level in *A. brasilense*, estimated is function of membrane potential, was maximal at 3 - 5 mM oxygen. The proton motive force was lower at oxygen concentrations that were higher or lower than the preferred oxygen concentration. Bacteria swimming toward the aerotactic band would experience an increase in the proton motive force and bacteria swimming away from the band would experience a decrease in the proton motive force. It is proposed that the change in the proton motive force is the signal that regulates positive and negative aerotaxis. The preferred oxygen concentration for aerotaxis was similar to the preferred oxygen concentration for nitrogen fixation. We developed a novel technique to isolate aerotaxis mutants, and isolated 9 putative aerotaxis mutants. After testing for motility, chemotaxis, respiration, the cytochrome content, and the proton motive force, one mutant (JL7) was assigned as a true aerotaxis mutant. The mutant was unable to locate the preferred oxygen concentration in spatial oxygen gradients and did not respond to oxygen in temporal gradients. The aerotaxis mutant was unable to grow in nitrogen-free semisoft medium under conditions where locating the low oxygen concentration was necessary. The results suggest that aerotaxis is an important adaptive behaviour response that can guide these free-living diazotrophs to the optimal niche for nitrogen fixation in the rhizosphere.

P-H-3

STUDY OF ENZYMES INVOLVED IN DEFENSE AGAINST OXIDATIVE STRESS IN THE NITROGEN-FIXING UNICELLULAR CYANOBACTERIUM *GLOEOTHECE* SP. 6909

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The unicellular non-heterocystous cyanobacterium *Gloeothece* sp. 6909 is capable to fix N_2 aerobically on the light and thus the processes of the nitrogen fixation, the oxygenic photosynthesis, and photorespiration take place in the same cell. During the photosynthesis is well known to be evolved activated forms of oxygen and free radicals that damage different cell structures and rapidly inactivate nitrogenase. Mechanisms to defense the *Gloeothece* cells and nitrogenase against oxidative stress are poorly understood.

One of the defensive mechanisms is the Halliwell-Asada pathway of scavenging of active oxygen species. To study a role of this mechanism a collection of *Gloeothece* sp. 6909 mutants resistant to agents inducing oxidative stress (paraquat, plumbagin, and menadion) has been obtained. The mutants was proved to show the cross resistance to all three agents. It indicates the expression of common mechanism of defense against oxidative stress in all mutants tested.

The total superoxide dismutase (SOD) activity in both parent strain and resistant mutants was practically equal in the cells grown in BG11 medium without agents inducing oxidative stress. In the same conditions of growth the activities of NADPH-dependent glutathione reductase and glutathione dehydrogenase (ascorbate) were lower in the mutants comparing with the parent strain. When the inhibitory agents (paraquat, plumbagin or menadion) were added in sublethal concentrations into the growth medium one day before the analysis some increase of the glutathione reductase, glutathione dehydrogenase, and SOD activities was found in the wild type cells. The same treatment of the mutants by the agents resulted in more higher level of induction of the glutathione reductase and glutathione dehydrogenase (but not SOD) activities in comparison with the parent stain. Cultivation in the presence of higher concentrations of the agents lead to strong decline of all three enzymatic activities in the wild type cells as well as in the mutants. The possible role of the Halliwell-Asada pathway enzymes in the protection of the *Gloeothece* cells against the oxidative stress is being discussed.

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P-H-4

LEGHEMOGLOBIN AND OXYGEN CONDITIONS IN NODULES

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Leghemoglobin (Lb) - oxygen-binding hemoprotein of legume plants which plaies role of source of bound oxygen for bacteroids. When discuss its functioning it is necessary to connect it with oxygen conditions in nodules. It seems now that amount of oxygen in outer zone of infected cell is much higher that it was predicted earlier (Thumfort et al., 1994; Kuzma et al., in press). So conditions for Lb functioning differ through infected cell. The scheme of Lb functioning including different processes and taking into account various oxygen conditions was proposed. Interaction of Lb reduction, oxidation, oxygenation and deoxygenation was studied as well as different ways of these processes. It was shown that enzymatic reduction of Lb is the main way for supporting it in physiologically active state. Calculation of ways of Lb deoxygenation (donating of oxygen for bacteroids and mitochondria, and so-called spontaneous deoxygenation) was also carried out. The described scheme was examined at different situations with gaseous diffusion barrier in nodule cortex for understanding of Lb function as part of the last step of oxygen regulation in infected cell.

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P-H-5

PROPERTIES AND ROLE OF LUPIN LEGHEMOGLOBIN COMPONENTS

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Leghemoglobin (Lb), hemoglobin of legume plants, is a heterogeneous protein with several components. Comparison of properties of lupin Lb components with their functioning as source of bound oxygen for bacteroids was studied. Experiments with model systems containing bacteroids, Lb and, if necessary, met-Lb-reductase (enzyme reducing Lb) were carried out. Both oxidazed (Lb^{3+}) and oxygenated (LbO_2) forms of Lb increased nitrogen-fixing activity of bacteroids, especially LbO_2. Addition of met-Lb-reductase made this activity even higher. Although both LbI and LbII components increased nitrogen-fixing activity of bacteroids, their influence was not the same. LbO_2(I) itself increased this activity more than LbO_2(II), but at the presence of met-Lb-reductase level of nitrogen-fixing activity was higher with LbO_2(II). We propose that the reason of this phenomenon is difference of Lb components in their oxidative-reductive properties. LbII has higher red-ox potential, it can be easer reduced and can better function as source of bound oxygen at the presence of met-Lb-reductase. It is necessary to mark that Lb components also differ in their affinity to oxygen. In general, heterogeneity of hemoglobins (not only Lb) helps them to play their roles in various conditions. Since cortical barrier of gaseous diffusion changes in nodules of different age, oxygen conditions in the centre of nodules can be also different. It was earlier shown that lupin Lb components differ in time of the beginning of their biosynthesis (Szybiak-Strozycka et al., 1987). Thus changing of content of Lb components can play important physiological role in nodules if oxygen conditions there also change.

RERERENCE:

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IRON UPTAKE BY *RHIZOBIUM LEGUMINOSARUM*; REQUIREMENT FOR A TWO-COMPONENT TRANSCRIPTIONAL REGULATOR PLUS THE *CYC* GENES THAT ARE INVOLVED IN CYTOCHROME *C* SYNTHESIS

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The bacterial genes *cycH,JK* and *L* comprise a single operon in strains of *Rhizobium leguminosarum*, *R.meliloti* and *Bradyrhizobium japonicum*. Mutations in these genes prevent the bacteria from making mature cytochrome c and as a consequence, such mutants fail to fix nitrogen on their cognate legume hosts.

We have found that mutations in this operon confer other, unexpected phenotypes. Such mutants fail to make siderophores and are thus defective in the uptake of Fe and they were also found to accumulate protoporphyrin IX, the immediate precursor of haem. A model to explain the complex pleiotropic phenotypes of the *cyc* mutants is presented.

Close to the *cyc* genes of *R.leguminosarum* are two genes, *feuP* and *Q* which, from sequence analysis, appear to be members of the large family of bacterial transcriptional regulators. In this example FeuQ appears to be the sensor protein and FeuP the response regulator.

Mutations in *feuQ* did not affect transcription of the *cycHJKL* operon. However, *feuQ* mutants shared one feature with *cyc* mutants in that they too were defective in the uptake of Fe. The reasons for this must be different in the two types of mutants since the *feuQ* mutations do not abolish siderophore biosynthesis. Further, *feuQ* mutant strains retain the ability to fix nitrogen in pea nodules.

P-H-7

CHARACTERIZATION AND REGULATION OF *c*-TYPE CYTOCHROMES EXPRESSION IN *Rhizobium leguminosarum*

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Rhizobium leguminosarum biovar *viciae* free-living cells possess two covalently-bound haem proteins of 31 kDa and 23 kDa in the membrane fraction and one soluble cytochrome *c* of 14 kDa. Strains of *R. leguminosarum* mutated in the *fbcH* gene encoding the cytochrome c_1 do not form the component of 31 kDa and are unable to fix nitrogen. Mutations of the *R. leguminosarum cycM* gene abolish the formation of the component of 23 kDa, which however is not essential for nitrogen fixation.
The *cycHJKL* operon of *R. leguminosarum* has been shown to be involved in cytochrome *c* biogenesis and symbiotic nitrogen fixation. We have studied the regulation of the *cycHJKL* operon, and of the synthesis of *c*-type cytocromes in *R. leguminosarum*. The presence of mannitol in the culture medium of cells containing a chromosomally integrated *cycHJKL-lacZ* fusion, produced a increase of the β-galactosidase activity compared to succinate-grown cells. The promoter activity of *cycHJKL* decreased when mannitol-grown cells were shifted to restricted aeration. Iron depleted culture medium had a negative effect on the transcription of the *cycHJKL* operon. The utilization of mannitol as carbon source, instead succinate, under aerobic conditions produced *in R. leguminosarum* a increase of the haem-stained membrane-bound cytochrome c_1. Increased levels of the cytochrome c_1 were correlated with a decrease of the periplasmic cytochrome *c* of 14 kDa. Iron depletion had a negative effect on the content of both soluble and membrane-bound *c*-type cytochromes.
Collectively, these data suggest a regulatory effect of the carbon source, oxygen and iron on the transcription of the *cycHJKL* operon, and consequently on the levels of the covalently-bound haem proteins of *R. leguminosarum*

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P-H-8

GENETIC ANALYSIS AND REGULATORY PROPERTIES OF A SECOND *FNR/FIXK*-LIKE GENE AND AN UNUSUAL *FIXL*-HOMOLOGUE IN *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE*

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Genes coding for the regulatory elements NifA, FixK, and FixL of *Rhizobium leguminosarum* bv. *viciae* VF39 were isolated, sequenced and genetically analysed. The *fixK-fixL* region is located on plasmid pRleVF39c that also contains genes for LPS biosynthesis. The deduced *fixL* gene product as compared to other FixL proteins possesses an additional C-terminal domain that shows homology to the receiver modules of response regulators of two-component regulatory systems. The *R. leguminosarum* bv. *viciae fixL* gene product most probably is a cytoplasmic protein because no hydrophobic segments able to form transmembrane helices could be found. A putative heme-binding domain and a transmitter module are also conserved.

Apart from the previously identified *fnrN* gene *R. leguminosarum* bv. *viciae* possesses a second *fnr*-like gene designated *fixK*, as the encoded gene product is very similar to *Rhizobium meliloti* and *Azorhizobium caulinodans* FixK, especially in that a N-terminal cysteine motif is missing in these proteins. *R. leguminosarum fixK* and *fixL* mutants displayed a Fix^+ phenotype. In contrast, a reduced nitrogen fixation activity was found for a *fnrN* deletion/insertion mutant (approximately 30 % of the wild type activity). Nodules induced by a *fixK/fnrN* double mutant showed a Fix^- phenotype. The *R. leguminosarum* bv. *viciae nifA* gene is expressed under aerobic conditions and only slightly induced under microaerobiosis. In the root nodule *nifA* is highly expressed in the symbiotic zone. NifA expression is not impaired in *fixK* and *fixL* mutants. Expression of *fixK* and *fixL* in the free-living state is induced under microaerobic conditions. FixK induction is no longer detectable in a *fixL* mutant which suggests that *fixK* expression is controlled by FixL.

Another *orf* was identified downstream of *fixK-fixL* and codes for a product with homology to pseudoazurins from different species. Mutation of the *azu* gene showed that it is dispensable for nitrogen fixation. However, analysis of an *azu-gusA* reporter gene fusion revealed that the *azu* gene is expressed in the root nodule in a *fnrN* dependent way. A sequence motif with homology to the Fnr-consensus binding site is located upstream of the *azu* gene.

R. leguminosarum bv. *viciae* possesses two copies of the *fixNOQP* operon. One is located on the nodulation plasmid pRleVF39d and the second on the plasmid pRleVF39c that also contains the *fixK-fixL-azu* region. Both *fixN* copies are expressed strongly in the symbiotic zone of the root nodule. Expression of $fixN_c$ and $fixN_d$ is highly reduced in a *fnrN* mutant background whereas FixK is only marginally involved in *fixN* regulation. Furthermore *in situ fixN-gusA* expression analyses indicate that $fixN_c$ is more strictly dependent on FnrN than $fixN_d$.

P-H-9

A *FIX* REGION OF *R. MELILOTI* IS REQUIRED FOR PH ADAPTATION AND ENCODES FOR PROTEINS HOMOLOGOUS TO SUBUNITS OF DIFFERENT PROTON-TRANSLOCATING SYSTEMS

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The *fix-2* mutation of *R. meliloti* was isolated and located on the chromosomal map earlier (1). Recently it was shown that the *fix-2* mutant was unable to invade the nodule tissue causing the early abortion of the infection threads and resulting in "empty" nodules. The chromosomal region carrying the wild type allele was isolated in genetic complementation experiment (2).

In order to find out the function of the gene affected in the *fix-2* mutant different physiological tests were carried out and the DNA sequence of the region was determined. First the motility of the mutant was examined since some simbiotic mutants were reported to show a nonmotile phenotype (3). The *fix-2* mutant was found not to be able to multiply in the "swarm" medium due to the potassium content of the medium.

Potassium is known to be involved both in osmotic and in pH adaptation of the bacterial cells (4; 5). No difference in the osmotic tolerance was found between the mutant and the wild type. Similarly, both the mutant and the control cells could propagate in the same pH range with an optimum of about 7.5. In the presence of K^+, however, the pH optimum of the mutant cells decreased to about 6.5 and they were no longer able to grow at alkaline pH. The lethal effect of K^+ could be recovered by increasing the Na^+ concentration in culture medium but the infection defect of the *fix-2* mutant could not be rescued by keeping the pH acidic and increasing the Na^+ concentration in the medium of the plant test.

Using directed Tn*5* mutagenesis a 6 kb segment of the *fix-2* region was identified where mutations resulted in both Fix^- and K^+ sensitive phenotypes. In this region the presence of seven ORFs oriented in the same direction was revealed. Putative products of the first three ORFs exhibit homology to the subunits of a Na^+/H^+ antiporter described from the alkalophilic *Bacillus sp.* strain C-125. Products of ORF 1 and ORF 4 exhibit also similarity to ND5 and ND4 subunits of the NADH-ubiquinone oxidoreductase (complex I), respectively. Based on computer analysis all the seven predicted proteins are highly hydrophobic carrying several possible transmembrane domains. Some of these domains were confirmed by generating active alkaline phosphatase (*phoA*) translational fusions.

The K^+ sensitive phenotype of the mutants suggests that they are defective in a K^+ transport system. Results of the sequence comparisions indicate that the genes in the *fix-2* region encode for a cation/proton translocating system that can be a novel type of K^+/H^+ antiporter involved in pH adaptation and required for adaptation to an altered environment inside the plant.

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2. Putnoky et al., (1988) *J Cell Biol* **106,** 597-607 .
3. Dylan et al., (1986) *Proc Natl Acad Sci USA* **83,** 4403-4407.
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5. Csonka (1991) *Annu Rev Microbiol* **45,** 569-606.

Study of *Rhizobium meliloti* mutants with increased respiratory activity

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We obtained a collection of 29 Tn5-mob induced mutants in ***R.meliloti*** CXM1-188 with increased 2,3,5-tripheniltetrazolium bromide (TTB) reducing activity (Red^{++}). While 9 of them demonstrated some alterations in their cell surface components, Red phenotype of other 20 mutants seemingly was caused by alterations in their intermediate metabolism. All of these 20 mutants contained Tn5 in the megaplasmid 2 at least in 7 different loci. Subsequent analysis of this group of mutants revealed that upon inoculation of host plants, most of them caused significant increase in plant yield as concerning shoot mass and total nitrogen accumulation. In addition, they showed increased evolution of CO_2 under conditions of static (microerobic) culture, which may be caused by the enhanced operation of TCA-cycle. By transductional analysis it was proved that Red-phenotype on TTB-plates and increased CO_2 evolution in 3 mutants were caused by Tn5 insertions. It is noteworthy that this ability to increased respiration was expressed in the background of genetically unrelated strain:while recipient was inferior to the parental strain, all transductants were as good as Red^{++} donors. Four mutants were demonstrated to have increased respiratory rate under symbiotic conditions (Yurgel et al, 1996, in press). We propose that increased O_2 consumption may lead to a higher ATP production, and consequently enhance activity of nitrogen fixation complex. Alternatively, if increased O_2 consumption is uncoupled from generation of ATP, it might play a role in O_2 scavenging and cause earlier expression of nitrogenase in differentiating bacteroids.

P-I-1

SIMULATION OF NODULE FUNCTION IN MATHEMATICAL MODELS BASED ON DIFFUSION

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Models of the infected cells and of central zone tissue comprising a 1 : 1 mixture of infected and uninfected cells have led to a number of significant predictions.

1. Variable diffusion resistance occurs as an intrinsic property of infected cells.

2. The mechanism of diffusion resistance at this level relies on the relative depth of a layer adjacent to the cell : gas space interface in which the oxygenation of leghemoglobin is saturated.

3. Resistance at the infected cell does not preclude fixed and/or variable resistance in the nodule cortex.

4. A consequence of diffusive resistance in the infected cell is that pO_2 in adjacent uninfected cells may be 1000 fold greater than the average free O_2 in the infected cell. In this way the disparite O_2 requirements of the terminal oxidases (Km 10-50 nM O_2) of the infected cell and urate oxidase (Km 30 μM O_2) in the uninfected cell can be accommodated.

5. As much as 25% of O_2 entering the infected cell may pass through the infected cell : uninfected cell interfaces.

These findings will be discussed in relation to a novel hypothesis for nodule functioning and as a basis for new experimental approaches to determine the mechanisms of nodule homeostasis.

P-I-2

GENETIC AND MOLECULAR TOOLS FOR ANALYSING THE INFECTION AND INFESTATION OF CLOVERS

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Our research has investigated the interactions involved in development, defence and symbiosis of clovers. We have used a variety of techniques and transgenic plants to examine the levels of phytohormones and flavonoids as well as the roles of pathogenic - related (PR) protein genes in these biological processes. Spot inoculation assays were used to introduce auxin, cytokinins, flavonoids, NPA (N-(1-naphthyl) phthalamic acid), *Rhizobium* and *Rhizobium* Nod factors to transgenic white clovers at precise sites on the roots. The results suggest that the application of *Rhizobium*, NPA, flavonoids or Nod factors results in alterations of the auxin level, via blockage of polar auxin transport, in the roots within 24 h of their application. In addition, rapid and localised increases in chalcone synthase expression occur after the application of *Rhizobium* and Nod factors.

A tobacco basic-chitinase promoter-GUS gene construct was found to show the same developmental expression patterns in both tobacco and clovers: high expression in roots tips and very low expression in healthy aerial tissues. Wounding in all tissues tested induced expression to high levels. Experiments using transgenic clovers to study *Rhizobium* - clover interactions found non significant expression in nodules over a period of 1 day to 14 days post - infection. However, *Rhizobium* infection induces a temporal expression in the cortical cells in the zone of infection between 1 and 4 hours post-infection which is absent from there after. In contrast, expression was observed during the early stages of development and emergence of lateral root formation.

Proteome analysis has been used to monitor global changes in gene expression in R 1. bv. trifolii strain ANU843 in response to exposure to plant signals; infected and non-infected roots and roots under attack by clover pests. In strain ANU843 over 1000 well resolved spots were detected using 2-D gel electrophoresis (2-DE) with a number of proteins being induced and the expression levels of some others being up- or down-regulated. The identity of 88 constitutively expressed proteins was examined by using amino acid composition analysis. In addition, 2-DE was used to study the global changes of *Trifolium* gene expression in response to the attack of predatory mites. Redlegged Earth Mite (RLEM) resistant cultivars of subterranean clover were examined and up to 15 individual proteins were shown to be either elevated or strongly induced during predation by RLEM. Thus, we have shown that 2-DE can be used to analyse genomic structure and global changes of gene expression both in *Rhozobium* under different biological conditions and in plants also under various situations.

P-I-3

GENETIC LINKAGE MAPPING IN ALFALFA

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Our research group has started the construction of the genetic map of alfalfa several years ago to develop a genetic system for *Medicago sativa.* A detailed linkage map can be used for breeding purposes, genetic analysis and finally for map based cloning type of work. The 138 individuals of the segregation population were produced by the self mating of an F1 plant (F1/1) originating from a cross between a yellow flowered diploid *Medicago sativa* ssp. *quasifalcata* and the purple flowered *Medicago sativa* ssp. *coerulea* belonging to the *Medicago sativa* complex. Mapping was carried out on this segregation population by determining the genotype of more than 1000 genetic markers. The morphological, isozyme, seed protein, RFLP and RAPD markers have been arranged into eight linkage groups corresponding to the basic chromosome number of alfalfa (n=8). The average physical equivalent of 1 cM corresponds to 1400 kb. Among others we determined the map location of more than 20 nodulin genes like ENOD2, ENOD10, ENOD12, Nod-22, Nod-25, leghemoglobins, glutamine synthetases, etc., as well as the location of two cytologically localized genes (the rDNA and a β-tubulin locus). Some genes (CHS, CHI, COMT, DHFR, F3OH and VR) expressed in the stress-induced phenylpropanoid pathways have also been mapped. Out of 182 RFLP loci 124, 16 and 8 loci coded for single, double and triple genes, respectively. Using this linkage map we were able to map an ineffective mutation which located on linkage group 7 of alfalfa. Our long term goal is to isolate genes involved in the symbiotic nitrogen fixation by map-based cloning strategy.

P-I-4

FURTHER SATURATION OF GENETIC MAP OF DIPLOID ALFALFA

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Basic genetic map of diploid alfalfa (*Medicago sativa* L., F2 mapping population from *ssp. quasifalcata x ssp. coerulea* cross), (Kiss *et al.*, 1993, *Mol. Gen. Genet.*, 238, 129-137) was further saturated using DNA markers. Eight linkage groups were recognized which correspond to the haploid chromosome set of *Medicago*. To saturate further basic genetic map, 160 RAPD primers were tested for their power to produce DNA polymorphism. The best 20 primers generated altogether **200 new RAPD markers**. Beside these non specific markers covering all linkage groups the set of 14 specific DNA markers were mapped having following nature: **t-RNA-like, M13-like (minisatellite nature), ZIF-like (zinc-finger sequence motifs), and microsatellite-like ($[CAC]_n$ and $[GTG]_n$).**

Future prospects are aimed toward generation of 1) STS (sequence-tagged-sites), 2) EST (expressed-sequence-tags) and 3) cytological anchors (telomeric and centromeric sequences).

P-I-5

AN ALFALFA REGION HIGHLY SATURATED WITH MOLECULAR MARKERS

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Our main goal is to isolate *Medicago* gene(s) playing role in the formation of symbiotic nodules as well as in plant development by map-based cloning approach relying on the genetic map for diploid alfalfa constructed by our research group (Kiss et al. Mol.Gen.Genet. 238:129-137, 1993). The concept of the map-based cloning is to find molecular marker(s) linked to the gene of interest, and then to "walk" to the gene via overlapping clones. In the case of large genomes (like genomes of most plants) chromosome walking is hindered not only by the large amount of DNA to be passed, but also by the presence of repetitive DNA. To reduce the number of the walking steps, tightly linked molecular markers are required, that is "mapping rather than walking" is recommended.

We used the so-called Bulked Segregant Analysis technique (Michelmore et al., Proc. Natl. Acad. Sci. 88:9828-9832, 1991; Giovannoni et al., Nucl. Acid Res. 19:6553-6558, 1991) in order to saturate the region around the *stl* locus (sticky leaf, morphological mutation) of the *Medicago* genome with molecular markers. More than 600 ten-mer primers were tested in PCR reactions with the two homozygote bulks and almost 80 RAPD markers were identified in the vicinity of *stl* locus in both direction. More accurate linkage data of these markers were determined with the help of increased number of individuals segregating the trait. Before starting the chromosomal walking towards the *stl* gene, the correlation between the genetic and physical distance has to be determined in the region. This work is currently under way by using Pulsed Field Gelelectrophoresis.

P-I-6

GENETIC MAPPING OF SEED PROTEINS IN ALFALFA (*MEDICAGO SATIVA* L.) BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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We have examined the patterns of alfalfa seed proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to assess the level of polymorphism and to follow their inheritance. This analysis was undertaken in an F2 segregating population originated from a cross between a diploid *Medicago sativa* ssp. *quasifalcata* plant and a diploid *M. sativa* ssp. *coerulea* plant.

68 individuals of the segregating population were investigated, 12 seeds of each plant were bulked for 2D analysis and the resulted protein patterns were compared on a pair-wise manner. When comparing two-dimensional gels from different genotypes the variability appears either as a presence or absence of a protein spot (quality variation) or differences in spot intensities (quantity variation). Of the 800 spots investigated (ranging from pH 5 to 8 and Mw. of ca. 18- to 90 kDa) 500 were further analysed. We have found more than 250 spots that exhibited variation among the 68 F2 plant studied, but only those ones showing qualitative variation were genetically analysed. 136 protein spots segregated (27.2% of the 500 spots analysed) representing 49 DNA regions. 29 of them (59.2 %) showed codominant inheritance, 20 (40.8 %) showed dominant inheritance, that accounted of the pattern of the variation of 112 and 24 spots, respectively (82.3 % and 17.6 %). The map position of these markers were determined and localised to all of the 8 linkage group of alfalfa.

On the base of their different salt solubility, molecular weight and abundance the main storage proteins - the 7S storage protein called alfin and 12S storage protein called medicagin - were identified. We could detect one alfin and two medicagin loci by 2D electrophoresis of seed proteins. The localisation of medicagin was confirmed by hybridisation with pea legumine cDNA clones to genomic DNA.

P-I-7

REPRODUCTION AND NODULATION OF TRANSGENIC ALFALFA PLANTS EXPRESSING ANTISENSE-LECTIN CONSTRUCTS

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Alfalfa expressing antisense-*Mslec1* are designated γ-plants; those expressing antisense-*Mslec2* are designated β-plants. γ-plants have more, and β-plants fewer flowers than vector-only control (con)-plants. Petals of γ-plants are yellow or pale yellowish-purple; those of β- and con-plants are dark-purple. Selfed γ- and β-plants have less efficient pod and seed production than con-plants. γ-plants nodulate abundantly following inoculation with wild-type *Rhizobium meliloti* 1021, then show premature senescence of many of their roots and associated nodule primordia. This is most dramatic when a high *R. meliloti* inoculum is used, and also under aeroponic growth conditions. γ-plants have fewer healthy, mature nodules than β- and con-plants in the presence of high *R. meliloti* inoculum in hydroponics or potting soil. However, γ-plants produce the highest nodule number and dry weight/g root dry weight, and the lowest root dry weight whether nodulated or not. Under some conditions, γ-plants develop multi-lobed nodules; these are ca. 50- to 75-fold larger than normal and often senesce prematurely. γ-plants are smaller than β- and con-plants when either fully nodulated or N-starved. β-plants may nodulate differently than con-plants in subtle ways. Preliminary results suggest that *R. meliloti* culture growth phase may affect nodulation. Support was provided by USDA Competitive Grants and NRSA Biotechnology Training Programs.

P-I-8

MORPHO-FUNCTIONAL ANALYSIS AND CLASSIFICATION OF PEA (*Pisum sativum* L.) Nod+Fix- MUTANTS

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Ten pea (*Pisum sativum* L.) symbiotic Nod+Fix- mutants isolated after experimental mutagenesis of initial genotype Finale*, were examined using transmission and scanning electron microscopy. Comparative histological and cytological analysis of these mutants allowed to characterize them on the basis of following morphological characteristics: (1) type of histological differentiation of nodules, (2) structure and a mode of development of infection thread and peculiarities of bacterial endocytosis into host-cell cytoplasm, (3) symbiosome structure, (4) rate of bacteroid differentiation and (5) peculiarities of nodule tissue degradation. Our study revealed differences in fine structure of mutants. The certain stages of symbiosis development in which macrosymbiont mutations can block nodule morphogenesis, have been discovered. Classification of these mutants based on their ultrastructure and genetic analysis, is discussed.

* These mutants were kindly provided by Dr. K.J. Engvild (1987) Nodulation and nitrogen fixation mutants of pea (Pisum sativum), Theor. Appl. Genet. 74: 711-713.

P-I-9

A MOLECULAR MARKER BASED LINKAGE MAP FOR PEA

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An updated version of the pea linkage map will be presented. This linkage map is based on the integration of maps from three recombinant inbred populations, and is aligned with the classical linkage map.

P-I-10

USE OF BULKED SEGREGANT ANALYSIS FOR MAPPING *Sym-31* GENE OF PEA (*Pisum sativum* L.)

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Pea is one of the important grain-legume crops in Northern European agriculture including Russia. From the genetic point of view pea is a convenient model because of the intensive genetic study conducted since early Mendel's experiments. Nevertheless the map-based cloning strategy is not available yet for any gene of the pea including symbiotic ones. Therefore the most efficient strategy at this moment is saturation of the genetic map of pea by morphological, isozyme and molecular markers as well as alignment of the maps derived from different crosses.

Our project is aimed at identification of DNA markers linked with *sym-31* gene which results in formation of the ineffective root nodules. For this purpose the Bulked Segregant Analysis (BSA) was used. For increasing the amount of visible markers we performed PCR with mini-hairpin primers coupled with DAF silver-staining methodology. In our poster we will report about several markers with the *sym-31* locus. Some specific features of BSA joined with DAF will be discussed.

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P-I-11

SEARCH FOR MORPHOLOGICAL AND BIOCHEMICAL CHARACTERS WHICH MARK THE STAGES OF SYMBIOSIS DEVELOPMENT CONTROLLED BY CERTAIN PEA (*PISUM SATIVUM L.*) SYMBIOTIC GENES

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The Nod^+Fix^- mutants of pea can be used to close the gap of knowledge about the interactions between symbionts at late stages of symbiosis development. Isolation and study of such mutants, construction of lines carrying more than one Nod^+Fix^- mutations are necessary for identification of pea genes controlling symbiosis development at late stages as well as the types of symbiotic gene interactions. Characterization of these mutant lines using morphological and biochemical techniques is the way to found molecular mechanisms and signals which determine interactions between symbionts at those stages. A collection of such mutant lines (15 Nod^+Fix^- and 3 "double mutant" lines) have been created and is being analysed at RIAM (see also abstracts: Lebsky et al. and Morzhina et al., this volume). The most detailed comparative morphological and biochemical study was performed using five pea lines: initial lines Sparkle and Sprint-2, "single" mutant lines E135f* (in gene *sym-13*) and Sprint-2Fix^- (in gene *sym-31*), "double" mutant line RBT (in genes *sym-13* and *sym-31*). It was found that mutation in gene *Sym-31* determined the absence of morphologically pronounced differentiation of bacteroids after endocytosis into host-cell cytoplasm, the abnormal (for pea) symbiosome structure (several bacteroids per one symbiosome unit), the decreased level of ononitol contents (10 times lower than in the other lines studied) in nodules (ononitol is a sugar specific for pea nodules) and the increased level (3-4 times higher) of malate uptake by bacteroids. The mutation in gene *Sym-13* do not change these characters comparing with initial lines. Thus, the biochemical characters mark the certain stage of bacteroid and symbiosome differentiation which is controlled by gene *Sym-31* during the pea-*Rhizobium* symbiosis development.

*This mutant was kindly provided by Professor T.A. LaRue (Boyce Thompson Institute for Plant Research at Cornell University, Tower Road, Ithaca, New York 14853-1801, USA).

P-I-12

A MORPHOMETRIC STUDY OF BACTEROIDAL TISSUE DEVELOPMENT IN PEA ROOT NODULES

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Root nodule in Papilionaceae is a totally new organ formed as a result of interactions between the plant and bacterium genomes the function of which is nitrogen fixation. The functioning of nodules has its structural bases resulting from proper cortex structure, spatial organisation of bacteroidal tissue, structural specialisation of infected and uninfected cells. The different effectiveness of nodules can have a simple structural base. It has been proved that nodule effectiveness in Lotus and Glycine depends on the degree of bacteria (bacteroids) multiplication in infected cells (Wood et al. 1985, Lin et al. 1988). What is more, Wood et al. (1985) connected 1) smaller size of peribacteroidal space and 2) bigger part of the infected cell occupied by cytosol with more effective symbioses. To my knowledge there is no data describing the structural changes characteristic for the development of the effective and ineffective bacteroidal tissues. That is why the morphometric methods were applied which allowed to obtain the data on the changes of volume and surface area of particular cell structures as the basis for correlative investigations in which the relationships between the structure and the effectiveness of bacteroidal tissue of pea root nodules were analysed. Pea plants were inoculated by effective (wild type) R. leguminosarum 250a strain or ineffective 1064 strain. The main results are as follow:

a) The disturbances in the cell vacuolation in ineffective bacteroidal tissue are observed. The increase of the cell volume in ineffective bacteroidal tissue can be explained by the increase of the volume of symbiosomes and starch (infected cells) or cytosol and starch (uninfected cells). In case of effective bacteroidal tissue the cell volume increases because of the increase of the volume of vacuome, symbiosomes and cytosol (infected cells) or vacuome and cytosol (uninfected cells),

b) For infected cells of the effective bacteroidal tissue the volume of vacuome and peribacteroidal spaces change in the dependent way which stresses common traits of these two hydrolytic compartments,

c) A co-operation between mitochondria and plastids as well as mitochondria and symbiosomes is probably characteristic feature of effective infected cells because volumes and surface areas of these structures change in dependent way.

P-I-13

EARLY STAGES IN THE MORPHOGENESIS OF LUPIN AND CLOVER ROOT NODULES

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In lupin, in the first 24 hr after inoculation the nuclei of several root cortex cells lying immediately under the epiblem were shifted towards one another and grouped under the root hair cell being infected. An increase in the cytoplasmic density was noticeable. 48 hr after inoculation the root hair cell started building the infection thread, the next layers of the root cortex dedifferentiated centripetally and the earlier dedifferentiated cells divided anticlinally. Until 72 hr after inoculation the periclinal cell division occurred in the newly divided cells lying immediately under curled root hair. The infection thread penetrated into proximal derivatives and they became bacteroidal tissue initials (BTI). Simultaneously, the root cortex cells continued their dedifferentiation and produced the layers of the nodule cortex initials and the strand of the cambium initials. In clover, the most initial primordia collected 96 h after inoculation revealed only transverse divisions in the pericycle cells. The observations of lateral roots and nodules more advanced in their development, showed that in both cases the initial divisions took place in the root pericycle. 72 hr after inoculation cell divisions were observed in the pericycle and endodermis and in the innermost layer of the primary root cortex. The differentiation of the root endodermis cells in the region of the root nodule primordium formation was inhibited. It was found out that the cell divisions in the endodermis did not keep up with the divisions in the pericycle. Thus, the pericycle derivatives pushed their way through the layer of endodermis derivatives and came into direct contact with the dividing cells of the primary root cortex. Then a vascular junction between the nodule and the root stele was formed out of the pericycle derivatives.

P-I-14

HAIRY VETCH (*VICIA VILLOSA* ROTH) - THE LEGUME WITH A HIGH POTENTIAL FOR SYMBIOTIC NITROGEN FIXATION

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Vetch plantings are used as forage crop, hay, pasture, green manure, cover crops, sources of nitrogen for summer grasses and wildlife feed. We report here results of a analyzing the hairy vetch (*Vicia villosa* Roth) for symbiotic nitrogen fixation potential as compared to another vetch species. In aseptic test-tube experiment N content in *V. villosa*, inoculated with the effective *Rhizobium leguminosarum* bv. *viceae* strains, dry weight was higher (4,6%) as compared to inoculated plants of 5 wild vetch species: *V. hyrcanica, V. peregrina, V. narbonensis, V. angustifolia, V. michauxii* (from 2,2% to 4.0%). In pot experiments N content in *V. villosa* was significantly higher (4,0%) than in widely cultivated species: *V. sativa* (3,1%) and *V. pannonica* (2,1%), while N-content in uninoculated plants was 3,6%, 1,7% and 2,5%, respectively. Two-factor analysis of variance demonstrated that N-content in dry mass was controlled mainly by the additive actions of host genotypes. The responses of hairy and common vetches to inoculation with effective *R. leguminosarum* bv. *viceae* strains and to carbamide application have been examined in pot test. In hairy vetch, inoculation with rhizobia resulted in a greater increase in dry weight and total nitrogen in plants (about 85%) than when carbamide was applied (45 - 50%). When hairy vetch was grown in a mixture with oats, vetch dry weight increased from 48 to 70% in response to inoculation, while oat yield remained at the level of uninoculated treatment. Common vetch, on the contrary, responded to applied carbamide (a 49,6% gain in dry weight) more strongly than to inoculation with rhizobia (16,2%). Two vetch cultivars studied responded differently to the nitrogen sources: cv. Belotserkovskaya 222 was less responsive to inoculation with rhizobia than cv. Nemchinovskaya 72, but was superior with respect to utilizing carbamide. In hairy vetch the individual plant variation for shoot mass was higher than in common vetch (coefficients of variation were 46,7% and 36,1%, respectively). Hairy vetch morphological mutants, characterized by fascinated stem, transformation of leaves into tendrils or increased width of leaves were inferior than initial cv. Stavchanka in symbiotic efficiency. These data allows us to suggest that *V. villosa* possess a high symbiotic potential and may serve as the useful models for elucidation of the genetic and physiological mechanisms of the effective symbiosis formation.

P-I-15

SYMBIOTIC CHARACTERISTICS OF THE NEW NORTHERN VERIETIES OF SOYBEAN

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In agronomy and breeding programs a very important step is to compare promising new genotypes with those already used in commercial production. In order to select a soybean line which may be suitable for temperate growing conditions of the UK, a comparative study was made between five very early ripening Blarus soybean lines in terms of their nodulation capability. Soybean plants were grown in perlite and irrigated with deionised water and fed with nutrient solution without nitrogen. Commercially available *Bradyrhizobium japonicum* was used to inoculate the seeds and young seedlings. To check the effectiveness of symbiosis root nodule fresh weight, numbers of nodule per plant, the content of hemoprotein leghemoglobin, the activity of succinate dehydrogenase - tetrazolium reductase and total water - soluble iron in the root nodule were determined. Leghemoglobin (Lb) was determined in sucrose+tris-buffer extracts of root nodules using the coefficient A_{404}1%Lb solution = 104. Succinate dehydrogenase was detected with 2,3,5-triphenyltetrazolium chloride (TTC) and calculated as mg of formasan. The amount Lb does normally correlate positively with the N_2-fixation activity for soybean. Variety 4 had the largest nodule weight per plant, but the amount of Lb per g of nodule fresh weight was larger in varieties 1 and 5. The activity of succinate dehydrogenase (TTC - reductase) was highest in variety 1. Water soluble iron in 1g of nodule was greatest for variety 5. This experiment demonstrated a high correlation between formasan per plant nodule and Lb per plant nodule (r^2=0.783) and also between formasan, Lb and plant nodule weight (r^2=0.811 and 0.924 respectively).

P-I-16

Regeneration capacity of various pea (*Pisum sativum L.*) lines

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The aim of this research is to increase the regeneration ability of pea on the basis of genetic heterogeneity. Earlier we have found that while the shoots could be rooted with low frequency on rooting media the capacity of stem expants of the pea lines to form shoots in vitro were not sufficient for a regular regeneration and even decreased in subculture.

The intraspecific variability for shoot formation capacity was revealed and several genotypes holding promise were selected. We managed to increase the frequency of shoot formation using a modified procedure (Schroeder et al, 1993) and using the immature pea embryos as a source for explants. According that protocol we have received organogenic calli from an immature embryos of 8 pea lines. All of them formed shoots initially, as well as were capable to restore the new shoots after removal but with a different frequency. A short vegetative period lines have about 100% of shoot formation, whereas the frequency for the lines with a prolonged vegetation does not exceed 60%. Nod mutants were characterized by a high capacity to shoot formation. The lines with intensive type of regeneration usually display also a high transformation ability. The genetic forms of pea differing from the shoot formation differ by the profile of main endogenous cytokinins and auxins as it was shown by HPLC and ELISA analysis.

So, the application of the highly effective regeneration protocol to the selected lines allows to reach the intensive transformation process in pea.

P-I-17

AGROBACTERIUM TUMEFACIENS MEDIATED TRANSGENIC CALLUS INDUCTION IN YELLOW LUPIN *LUPINUS LUTEUS* L

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Among different lupin species the yellow lupin *(Lupinus luteus* L.) is that one pasture legume that breeds have longest tradition in Poland. Expanding breeders interest in yellow lupin origin not only from nitrogen fixation ability, common legume feature, but is evoked also because some other advantages like high protein and oil content and superior ecological value. Although the breeding progress of yellow lupin is meaningful, the breeders are restricted still to use a traditional attempts since the complete biotechnological techniques are still not available for any lupin species.
The transfer of foreign genes to genus *Lupinus* has not been reported yet in literature. We present here, the transformation attempts for *Lupinus luteus* cv. Ventus. The transgenic callus tissue has been derived from yellow lupin immature embryos and seedlings using *Agrobacterium tumefaciens*. Different *Agrobacterium* strains has been tested, all carrying p35SGUS introne gene. The reliable protocol has been elaborated which allow to induce a stable transformed callus both from immature embryos and seedlings mesocotyl using *A. tumefaciens* C58 (p35SGUSINT). β-glucuronidase transgen expression in callus tissue has been proved through histochemical assays, fluorometric enzyme activity and Southern blot hybridization. The trasgenic character of the callus sustained through 10 months *in vitro* culture period. The regeneration procedure from immature embryos derived transgenic callus of yellow lupin is underway.

Acknowledgments

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P-I-18

REGENERATION AND TRANSFORMATION OF THE LEGUME *GALEGA ORIENTALIS*

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Legumes are considered to be a difficult group whith reference to tissue culture and transformation. Until now most of the work has been done on legumes of little agricultural value for the Nordic countries. In our group we are now working on an efficient protocol for regeneration of *Galega orientalis*, which is a perennial legume suitable for cultivation in Northern conditions. Compared to red clover, which is a very popular forage legume, *G. orientalis* is more tolerant to plant diseases and is thus particularly used in ecological farming.

G. orientalis is also very interesting to study from another point of view in that in oder to be able to fix nitrogen it must have a specific symbiotic partner called *Rhizobium galegae*. This *Rhizobia* is until now only found to infect *Galega* species.

To be able to study *G. orientalis* further we must first develop an efficient plant regeneration and transformation protocol. *In vitro* plants and greenhouse plants were used for the regeneration experiments. Different parts of the plants were placed on media with different hormone concentrations and compositions to induce shoots. The best shoot induction occurred from the nodes. Different media were also tested to see which gave the best growth and survival rate. After shoot induction the shoots were placed on media with 1.0 mg/l NAA and roots were induced. The regenerated plants were placed out in the greenhouse and are currently growing well. To be able to induce more shoots from each node, a pulse treatment with very high hormone concentration were also tried. The results from the different regeneration protocol will be presented.

For transformation of *G. orientalis* different methods were used. Ordinary *Agrobacterium* transformation with the strain LBA 4404 containing the plasmid pBI 121 were tried. Transient expression could be detected by GUS-staining. Only the cut surfaces showed expression. To improve the transformation rates, different methods for wounding of the tissue were compared: particle bombardment, piercing etc. The results from those comparisons will be presented. Another possible way to improve transformation is to use another more infective strain of *Agrobacterium*. To select possible transformants, media containing 100 mg/l kanamycin is used., As kanamycin has been shown to have deleterious effects on the regeneration of other legumes, other kanamycin derivatives will be tested

P-J-1

CAN WE RECONSTRUCT THE N_2-FIXING SYMBIOSIS EVOLUTION USING THE LEGUME MUTANTS?

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The formation of N_2-fixing symbiosis between Pisum sativum L. (which represents an evolutionary "advanced" legume tribe Viciae, subfamily Papilionoideae) and Rhizobium leguminosarum bv. viceae is characterized by: a) release of the bacteria from the infection threads (ITH) into the host cytoplasm which is followed by encapsulation of bacteria in the plant-origin peribacteroid membranes (PBM) i.e. the endosymbiotic system is established; b) arrest of the bacteria proliferation inside PBM i.e. each symbiosome contains a single bacterial cell; c) differentiation of the rod-shaped bacteria into the Y-shaped bacteroids. However, in some evolutionary primitive legumes from sbf. Caesalpinioideae the rhizobia cells are not released into the host cytoplasm (N_2 fixation occurs inside ITH); no visible differentiation of bacteroids is detected inside ITH (**1**). Previously numerous plant mutants impaired in the endosymbiosis formation were described in the legumes representing different genera of sbf. Papilionoideae: Glycine, Medicago, Melilotus, Phaseolus, Pisum, Trifolium, Vicia, Vigna. By mean of EMS mutagenesis several classes of Nod^+Fix^- mutations were obtained in Pisum sativum which result in the symbiotic structure similar to those in the "primitive" legumes. 1) Bacteria proliferation inside and their releases from ITH are arrested (hard lignification of the ITH walls occurs); an N_2-fixing endosymbiotic system is not formed (SGEFix^--2). 2) Release of bacteria from ITH is hampered but their proliferation leads to ITH hypertrophy, collapse and to the symbiosome formation (SGEFix^--1). 3) Bacterial release and encapsulation by PBM are normal but then the proliferation of bacteria occurs instead of their differentiation; the symbiosomes containing 5-10 rod-shaped bacteria are formed. Some of these large symbiosomes can be formed by the fusion of the small ones (Sprint-2Fix^-). 4) Bacterial release and the symbiosome formation are normal but the symbiosomes are attacked by the host lytic systems and the infected zone of the nodule is rapidly degraded by the mechanism resembling the hypersensitive reaction (E135f, FN1). These data allow to suggest that the obtained mutations switch off those plant genes that were included into the symbiotic system at the recent stages of its evolution and that the symbiosis formation process is reversed to its ancestral forms. A more detailed comparison of the genes, morphogenetic events and molecular mechanisms involved in the formation of N_2-fixing nodules in different legume taxa is required to elucidate the crucial stages of the symbiosis evolution.

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P-J-2

DIVERSITY OF 16S rRNA AND SYMBIOTIC GENES IN RHIZOBIA THAT NODULATE AFRICAN ACACIA AND PROSOPIS

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We have studied the diversity of both chromosomal 16S rRNA genes and plasmid-borne, symbiotic *nifH* and *nodD* genes in a collection of rhizobial strains isolated from *Acacia senegal* and *Prosopis chilensis* trees growing in Sudan and Kenya. The strains for this work were selected based on information from previous studies using numerical taxonomy, pulsed-field gel electrophoresis and plasmid profile analysis. We sequenced a 230 bp fragment of 16S rRNA gene from thirty strains. Twelve different sequences were found: four were identical to those of previously described species, eight were novel. Sequence comparisons indicated that one strain belonged to the *"Mesorhizobium"* phylogenetic branch (includes *(Rhizobium) loti* and *(R.) huakuii* among others), while the rest were *Sinorhizobium*, as they were close to *S. (Rhizobium) meliloti*, *S. fredii*, *S. teranga* and *S. saheli*. The sequences indicate that there might have been recombination between 16S rRNA genes among our isolates. In addition, in one isolate we found two 16S rRNA sequences differing at six positions. The type strain of *S. saheli* was also shown to have similar microheterogeneity among its 16S rRNA genes. PCR-RFLP was used to screen 45 strains for their *nifH* structure and we found twelve different banding patterns. However, *nifH* types were not correlated with the 16S rRNA sequences. We chose nine Sudanese and, in addition, three *"Mesorhizobium"* and eight *Sinorhizobium* isolates from other trees and countries in Africa and Latin America, for *nifH* sequencing. Analysis of the DNA-sequences of a 561 bp fragment showed that most of the changes in the sequence were actually third base substitutions, which did not affect the amino acid sequences. In a phylogenetic tree of the NifH proteins the "mesorhizobia" always grouped together. Also the sequences from African *Sinorhizobium* isolates resembled each other, although four slightly different sequences were found (one or two variable amino acids between them). Otherwise, the *Sinorhizobium nifH* sequences were divided into two clusters according to the continent of isolation. Within the *Sinorhizobium* clusters from Africa and Latin America different chromosomal backgrounds (16S rRNA) could contain either similar or different *nifH* copies. In Latin America the *nifH* genes found in sinorhizobia resemble more the genes from *R. etli* and *R. tropici* than African *nifH* types, which might mean that the genes can be exhanged even between different genera. RFLP hybridisations to a number of our isolates using *nodD* as a probe showed that most of the strains probably have two rather similar *nodD* genes as well as, in some cases, one rather different *nodD*. We have now a complete *nodD* sequence for one strain, and it is different from the other known *nodD* sequences, showing only 77% similarity to *S. meliloti nodD*3 and 75% to *S. fredii*. The partial sequences of three other *nodD* genes from Sudanese isolates are rather similar to each other and to the one complete sequence regardless of the differences in bacterial chromosomal background or the tree of isolation. On the basis of these results, *nodD* and *nifH* gene sequence types do not seem to be correlated.

P-J-3

GENETIC RELATIONSHIPS OF NON-NODULATING *ACACIA* SPECIES TO OTHER NODULATING AND NON-NODULATING MIMOSOID LEGUMES: EVOLUTIONARY IMPLICATIONS

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The relationships of non-nodulating *Acacia* species to other non-nodulating species of mimosoid legumes is an important prerequisite for understanding the evolutionary development of nodulation, not only of the genus *Acacia*, but of the subfamily Mimosoideae as a whole. Our laboratory evaluated these relationships using RAPD analysis and established that there was no grouping of all the non-nodulating species: non-nodulation occurred sporadically through the subfamily. RAPD analysis demonstrated the relationships of the genus *Acacia* to three other tribes examined. The genus *Acacia* and the species comprising the tribe Ingeae may have arisen from the same branch of the Mimoseae.

The evolutionary development of the genus is complex and is not fully understood, RAPD analysis combined with additional evidence e.g. fossil data suggests that the non-nodulating African *Acacia* species may have given rise to the nodulating African *Acacia* species of subgenus Aculeiferum, section Aculeiferum. Subgenus Acacia, subsection Acacia probably arose from the new world species of section Monacanthea, armed with stipels.

P-J-4

A NEW SPECIES AMONG TROPICAL TREE RHIZOBIA

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Leguminous trees are abundant in savannah and arid regions of Africa, and play essential roles in promoting or sustaining soil fertility, retarding soil erosion, preventing desertification. We previously evidenced diversity amongst rhizobia from *Acacia* and *Prosopis* in Sudan (Zhang et al, 1991) and in Senegal (de Lajudie et al., 1994). Using the total protein SDS-PAGE technique, we found a separate gelelectrophoretic cluster (the so-called cluster U) comprising 38 strains from different geographical origins, and including the group of strains described as protein gelelectrophoretic cluster 2 by Moreira et al. (1993). Strains from cluster U were further characterized by a polyphasic approach. PCR-RFLP and sequencing of the 16S-RNA gene showed that all strains of cluster U form a separate homogeneous group phylogenetically localized on the *R. loti* rRNA branch in the vicinity of *R. huakuii*, *R. ciceri*, *R. mediterraneum*, *R.* sp.(*Cicer*), *R. tianshanense* but distinct species. PCR-RFLP of 16S-23S rDNA spacer, DNA:DNA hybridizations and PCR fingerprinting techniques performed with REP (REP1R- 1 and REP2-1), ERIC (ERIC1R and ERIC2), BOXA1R, GTG5 or RAPD primers and three randomly designed decamers (OPB-10, OPB-12, OPB-14, Operon Technologies Inc.) revealed more heterogeneity within cluster U enabling the distinction of 5 different genospecies. After a thorough evaluation of all data obtained in our polyphasic approach we propose a single new *Rhizobium* species containing isolates from *Acacia, Leucaena, Prosopis* and *Chamaecrista* from West Africa (Senegal and Mali), East Africa (Sudan) and South America (Brazil). As all other species of the *R. loti* rRNA lineage this new species will be transferred to the new genus *Mesorhizobium* as proposed by the International Subcommittee on *Rhizobium* Taxonomy (Lindström et al., 1995).

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P-J-5

PCR-RFLP ANALYSIS OF 16S rRNA AND 23S rRNA GENE REGIONS OF *RHIZOBIUM GALEGAE* AND OTHER RHIZOBIA AND AGROBACTERIA

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The phylogenetic position of *Rhizobium galegae* varies depending on the sequence analyzed. In dendrograms based on genetic distances obtained by using the variable region of the 16S rRNA gene, *R. galegae* is found next to *R. loti*. A similar relationship was reported for PCR-RFLP analysis of the 16S-IGS region. On the other hand, comparisons of full 16S gene sequences and PCR-RFLP of the same gene group *R. galegae* with agrobacteria, though the distance to agrobacteria and other rhizobia is considerable.

To increase the understanding of the evolution of ribosomal operons of rhizobia and agrobacteria, PCR primers were designed that amplified a 2.6 kb fragment of the 23S rRNA gene, and PCR-RFLP with nine restriction enzymes was applied to the 16S and 23S rRNA genes of 43 rhizobial and agrobacterial strains, resulting in 27 and 34 different restriction patterns for 16S and 23S respectively. The strains were selected based on the previous phylogenetic studies. The RFLP patterns were transformed to genetic distances and UPGMA dendrograms were constructed from the data. Overall, the shapes of the dendrograms correlated well, with only a few strains having varying positions on the two dendrograms. The 23S tree generally had deeper branching than the 16S tree, allowing better discrimination between species and even strains. The eight *R. galegae* strains formed a homogenous 16S cluster.

We conclude that PCR-RFLP analysis of 23S rRNA provides additional information about rhizobial and agrobacterial genomes, which can be used in taxonomic and evolutionary studies. We suggest that RFLP analysis of ribosomal genes should be applied to several strains representing a taxon before type strains are selected for sequencing of these genes. Sequencing ribosomal operons from different *R. galegae* strains should reveal the differences observed between them during RFLP analysis.

P-E-19

WHEAT LECTIN IN THE WHEAT- *AZOSPIRILLUM BRASILENSE* ASSOCIATION

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Wheat lectin (wheat germ agglutinin, WGA) is located in surface layers of the embryo and young parts of the root and is available to *Azospirillum brasilense* both during the germination of the grain and during the first month of plant growth. It is believed improbable that WGA and WGA-like lectins from graminae are involved in adsorption of *A.brasilense* to roots [1] or determine specificity in associative symbioses. However, it is well documented that the binding of WGA to the cell surface of *A. brasilense* stimulates the basic processes which determine the efficiency of the wheat - *A. brasilense* association, *viz.* indole-3-acetic acid production and N_2-fixation followed by ammonia excretion [2]. In addition, WGA causes expression of a number of genes in the bacterium [2].
Calcium ion is known to act as an effector of stimulus-response coupling in the regulation of diverse cellular functions not only in the eukaryotic cell but probably in the prokaryotic cell too [3]. Reasoning from this knowledge, we inspected lectin-activated (0.5 μg/ml WGA, 1-h incubation) and non-activated cells of *A.brasilense* for intracellular concentration Ca^{2+}. By flame atomic adsorption spectrometry,it was shown that WGA-activated bacteria had 11% less Ca^{2+} in their cells than non-activated bacteria. This finding is in line with previous reports on the correlation between the level in intracellular concentration Ca^{2+} and level of N_2-fixation in *Nostoc* [3]. Ca^{2+} is suggested to be involved in the response of *A.brasilense* to WGA.
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P-E-20

NITROGEN FIXATION BY BLUE-GREEN ALGAE IN RICE FIELDS OF UZBEKISTAN

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The importance of Cyanobacteria in the rice ecosystems is connected with their ability to assimilate free atmospheric nitrogen, accumulate organic substances, affect soil biota activity, participate in redistribution and accumulation of biogenous elements, first of all nitrogen. They can also affect the higher plants through the action of their metabolic products: vitamins, phytohormones. More than 20 species and forms of nitrogen-fixing unialgal cultures were isolated and identified from rice fields of Uzbekistan. Gloeotrichia natans f.bucharica, Nostoc muscorum, N.spongiaeforme, Anabaena variabilis, A.variabilis f.rotundospora are the predominating species. Under growing in the conditions of laboratory on nitrogen free media nitrogen accumulation fluctuated within the range from 3,5 to 13,6 mg/100 ml medium per month. Vegetative and field tests showed that blue-green algae accumulate nitrogen from 15 to 60 kg/ha per year. Improving soil fertility blue-green algae increase rice yield by 15-25%. Thus, the role of blue-green algae in the nitrogen balance of paddy fields is great, thanks to them it is possible to cultivate rice crop without chemical fertilizers solving the actual problem of ecological monitoring.

P-F-21

THE ESTIMATION OF RICE GENOTYPIC VARIATION IN N_2 FIXATION USING ^{15}N STABILIZED SOIL

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Rice genotypes with high N_2 fixation stimulating traits (NFS) are desirable because they add N to the soil-water-plant system without additional farm inputs and dependence on fertilizer N. A screenhouse experiment in pot was conducted during the 1993 dry season to assess differences in NFS among 70 genotypes of diverse origin and growth duration, and to validate the use of ^{15}N enrichment of mineralized soil N as a reference for N_2 fixation measurement. The soil used had a stable ^{15}N enrichment that was obtained by incubating and frequent mixing with $(^{15}NH_4)_2SO_4$ (6.3 kg N ha^{-1}) in submerged condition for 6 wk. The ^{15}N enrichment of soil NH_4^+-N dropped exponentially, showing an initial rapid decline to half of the original level within 2 wk, followed by a slower, nonsignificant rate of decline after 5 wk. Whole plant atom percent ^{15}N excess was inversely correlated with growth duration and ranged from 1.78 in Oking Seroni (late genotype) to 2.45 in PTB-18 (early genotype). It was, therefore, necessary to compare NFS among genotypes of the same growth duration. Enrichment was the lowest in the roots, suggesting this as the site of active N_2 fixation; it was highest in the shoot, and intermediate in grain. Nitrogen derived from air (Ndfa) estimated using ^{15}N enrichments of mineralized soil N and reference rice genotype correlated highly ($r = +0.998^{**}$). Percent Ndfa ranged from 1.5 to 21.0, with an average of 10.2. Yelsulbyeo, Pankaj, and Oking Seroni had the highest Ndfa among early, medium, and long growth duration genotypes, respectively. Genotypes with high Ndfa, grain yield, and N-uptake are Oking Seroni, IR29337-36-3, OR-142-99, and Pankaj.

P-K-1

INTERCELLULAR COLONIZATION OF NON-LEGUMES BY *AZORHIZOBIUM CAULINODANS* IS STIMULATED BY SPECIFIC FLAVONOIDS

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Azorhizobium caulinodans, which forms root and stem nodules on the tropical legume *Sesbania rostrata* by crack entry infection, is unusual in that it can tolerate up to 12 μM dissolved oxygen while fixing nitrogen in culture. We have studied interactions between *A. caulinodans* ORS571 and two non-legumes, the monocot wheat and the dicot *Arabidopsis thaliana*. Strain ORS571(pXLGD4) was used, with a constitutive *lac* Z reporter gene fusion to locate bacteria. Plants (wheat variety Canon; *A. thaliana* ecotype Columbia) were grown aseptically in tubes with agar Fåhraeus medium (without nitrate for wheat; 0.25 mM nitrate for *Arabidopsis*) and inoculated with ORS571 (pXLGD4). ß-galactosidase activity was visualised after 1 (*Arabidopsis*) and 2 weeks (wheat) by light microscopy of the dark blue precipitate resulting from X-gal degradation, with most of the roots of plants being colonized. Bacteria colonized the surface of root tips and the bases of lateral roots (lateral root cracks; LRCs), entering 9.2% of LRCs for wheat and 10.5% for *Arabidopsis*. The flavanone naringenin and the isoflavone daidzein (both at $5x10^{-5}$M) significantly stimulated colonization of *Arabidopsis* roots. Succinate at the same concentration had no significant effect on colonization, indicating that these flavonoids were not acting as simple carbon compounds. Naringenin (10^{-4}M) also significantly stimulated the colonization of LRCs of wheat by ORS571, approximately the same as for *Arabidopsis*. We assessed the possible role of Nod factors in the colonization of wheat and *Arabidopsis* by ORS571. No difference was found between the levels of colonization by the *nodC* mutant of ORS571 (which does not produce Nod factors) and the wild-type strain. An ORS571 *nodD* mutant was also tested, because *nodD* could activate genes other than *nod* genes in the presence of naringenin. This mutant colonized roots as well as the wild-type strain. Colonization by the *nodD* mutant was also stimulated by naringenin, indicating that the mechanism by which naringenin stimulates colonization of non-legumes is not mediated by NodD protein. This work demonstrates that *A. caulinodans* can colonize the LRCs of wheat and *Arabidopsis* and that this colonization is *nod* gene - independent, unlike infection by crack entry in *Sesbania rostrata*, and implies that it is likely to be a general phenomenon of non-leguminous plants. This is supported by studies with rice inoculated with ORS571, which also show large intercellular pockets of bacteria at emerging lateral roots. The implications of these results will be discussed for the ultimate aim of establishing stable endophytic nitrogen fixing interactions between diazotrophs and non-legumes.

P-K-2

BIOREMEDIATION OF SOIL BY THE *GALEGA* RHIZOSPHERE

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We are developing a bioremediation system for gasoline polluted sites in Finland based on plant - bacteria interactions in the rhizosphere, and a molecular identification system by which organisms and genes used in the process can be monitored.

To evaluate the potential of the system, *Galega orientalis* and *Rhizobium galegae* were tested for tolerance of the model compound *m*-toluate separately and in symbiosis. The rhizobia grew in the presence of 1000 - 2000 ppm *m*-toluate and the plant in 500 ppm. At 500 ppm root hairs were deformed but no nodulation occurred. Plants, the growth of which was arrested at higher concentrations of *m*-toluate, recommenced growth and also nodulated when transferred into cleaner medium. When the *m*-toluate degrading TOL plasmid pWW0 in *Pesudomonas putida* PaW85 was added to the symbiotic system plant growth, root-hair deformation and nodulation occurred even at 1000 ppm *m*-toluate. Thus, we believe that degradative removal of the pollutant by rhizosphere inhabiting, toluate metabolizing bacteria can help the plant to establish itself in polluted soil.

The transfer of the self-transmissible pWW0 to rhizobia or other rhizosphere bacteria was studied on laboratory media and in greenhouse experiments with inoculated plants growing in contaminated soil. Strain- and species-specific PCR primers for *R. galegae* in combination with pWW0 and *xylE* (the gene encoding cathecol-2,3-dioxygenase) specific primers are used to detect the inoculated rhizobia and TOL genes, and rep-PCR and 16S rRNA analysis to screen and identify any recipients of pWW0. Alternatively, indigenous, *m*-toluate degrading plasmids from the *Galega* rhizosphere will be isolated, characterized and used for bioremediation.

Our system is based on naturally occurring, self-transmissible plasmids with inducible, toluate degrading genes. Its dynamics is governed by the availability of substrate and bacteria harboring the degradative plasmids are maintained by the rhizosphere. In the future, construction and application of recombinant plasmids or plants will be attempted. Those will obviously be subjected to more rigorous control, but probably constitute no bigger risk than the naturally occurring organisms.

P-K-3

TRANSFORMATION & REGENERATION OF LEGUMES

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A range of legume species have been genetically transformed and transformants regenerated to provide novel genotypes. The methods used for transformation are dominated by those involving Agrobacterium tumefaciens. Generally these methods have low rates of transformation (0.1-2%) and alternatives are being sought. As an example of the procedures and their outcomes the details of transformation of narrow-leafed lupin (Lupinus angustifolius L.) will be presented. In this case both GUS and the bar gene (for resistance to phosphinothricin, the active ingredient of the herbicide Basta) have been used. Herbicide-tolerant (Basta) lines of lupin are currently at the controlled field trial stage. They offer considerable agronomic potential for cropping systems of the Western Australian wheat belt. Other transformations of lupin, including altered phytohormone synthesis, seed quality and nodule metabolism will be described.

P-K-4

TRANSFORMATION-REGENERATION SYSTEM IN A GREEN MANURE LEGUME, RENGE-SOU (*Astragalus sinicus* cv. Japan), WHICH FORMS ROOT NODULES WITH *Rhizobium huakuii* bv. renge

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Chinese milk vetch or renge-sou (*Astragalus sinicus* cv. Japan) in Japanese, is one of the most popular legumes used as a green manure legume in China, Korea and Japan. In China, this milk vetch is cultivated over an area of nearly 5 million ha. The renge-sou is also used as fodder for animals and a source of honey for bees. The plant has a symbiotic relationship with soil bacteria, namely *Rhizobium huakuii* or *R. huakuii* bv. renge, which forms nitrogen-fixing root nodules. We also found that a soil bacterium, *Enterobacter cloacae* A105 isolated from rice paddy stimulated the nodule formation on renge-sou.

R. huakuii bv. renge carries an indigenous large plasmid, pRhYM (420 kb). By heat treatment, Nod^- and Fix^- strains, which were deleted a par of the DNA fragment or cured the plasmid, were isolated. The hydrogenase genes from *Pseudomonas oxalaticus* were introduced into *R. huakuii* bv. renge by conjugation. The transconjugants stimulated the nitrogen fixation of renge-sou nodules. *Agrobacterium rhizogenes*-mediated transformation and regeneration system of renge-sou was also established. Transformation of regenerated plantlets was demonstrated by detection of mikimopine, histochemical GUS activity and by Southern blot analysis. We also isolated several nodule-specific cDNAs from renge-sou nodules and root cells. Thus, we have developed transformation and regeneration systems in both bacterium, *R. huakuii* and its host plant, *A. sinicus*. This system has potentials as a symbiotic model and also application of genetically engineered renge-sou in rice field.

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