ASSOCIATION OF RICE AND WHEAT PLANTS WITH NITROGEN FIXING BACTERIA

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Introduction

Nitrogen is one of the main nutrient elements of cereals which substantially affects the quantity and quality of the crop yield. The intensive crop production technologies utilize huge amounts of artificial nitrogen fertilizers. Thus, the crop productivity is considerably affected by the expenses of nitrogen fertilization. Besides, our environment, the soil and ground water sources, the drinking water bases are greatly loaded and polluted by the accumulation of the artificial nitrogen fertilizers, giving rise to risks of human diseases and deteriorating the quality of life. The nitrogen supply of plants completely by environment-friendly way is still an open question. However, the replacement of artificial nitrogen fertilizers partially by biologically fixed nitrogen appears to be a promising perspective.

During the biological nitrogen fixation, diazotrophic bacteria fix nitrogen from the atmosphere and reduce it to ammonia, and, when living in association with plants, they provide this ammonia to the host plant.

Nowadays, remarkable research capacity is focused on diazotrophic, plant growth promoting rhizobacteria (Nif-PGPR) which live in associative symbiosis with roots of cereals and tropical grasses. The associative *Azospirillum* spp. mainly live on the surface of cereal and grass roots, while certain strains of them are also established within the root and shoot. Diazotrophic *Herbaspirillum seropedicae* is a natural endophyte in rice of tropical areas, it lives exclusively in inner tissues of its host plant.
Azospirillum brasilense and Azospirillum lipoferum, as inoculants for cereals, are already utilized in substantial amount mainly in mediterranean regions of Europe. These bacteria are also resident in Hungarian soils, although in very low amount. Their application as inoculum appears to be a promising way to reach them and bring them into action in cereal cultures. However, the effectiveness of such inocula has to be carefully estimated under Hungarian climatic, weather and soil conditions. Moreover, the capability and susceptibility of Hungarian cereal cultivators to live in associative symbiosis with these bacteria have to be also extensively tested.

In my dissertation the associations of wheat and rice plants with Azospirillum and Herbaspirillum species are studied.

To establish effective nitrogen fixing association between the wheat and Azospirillum, it is obligatory to reveal the biological events on the root surface during the colonizat ion, the communication between the plant root and the colonizing bacterium. In the first part of my dissertation the interaction between the Azospirillum cell and the root lectin, as specific signal molecule of the root surface, is ascertained at molecular level.

In the second part of my work various Hungarian rice and wheat cultivars are characterized in nitrogen fixing associations with Azospirillum and Herbaspirillum bacteria.
Aims of the work

In the first part of the work the interaction between the wheat root and the *Azospirillum lipoferum* cell is studied during the root colonization:

– a recognition event between the bacterium cell surface and the root lectin, wheat germ agglutinin (WGA) is presented;
– an enhanced nitrogen fixation capacity of the bacterium is evidenced as a consequence of the WGA binding to the cell;
– a stimulus induced by WGA binding and a putative signal transduction pathway to the regulation of the nitrogen fixation is analyzed in the bacterium;
– a lectin binding receptor is identified on the bacterium cell surface.

In the second part of the work:

– associative symbioses are established:
  plant partners: three Hungarian rice cultivars, one Hungarian wheat cultivar
  bacterium partners: associative *Azospirillum brasilense*, endophytic *Herbaspirillum seropedicae*, both are nitrogen fixing, plant growth promoting rhizobacteria;
– the associations are characterized:
  – establishment of the bacterium within the plant, and the effect of the association on the growth of the plant are described;
  – capability of the cultivars to benefit from the association is reported;
– a preformed cellulose bead based inoculum formulation technique is presented and efficiency of the formulated inocula is characterized.
Materials and Methods

Methods of molecular biology experiments.

Bacterial strains, plasmids, culture media and growth conditions. *A. lipoferum* SpBr17R is rifampin-resistant mutant of the wild type parent. WGA-nonbinding (WGA\(^{-}\)) mutant strain carries transposon insertion (SpBr17R::Tn2706 (Rif\(^{r}\), Cm\(^{r}\))). pAB358 (*nifH-lacZ*), pAB576 (*nifA-lacZ*), pAB904 (*glnBA-lacZ*) and pAB912 (*glnA-lacZ*) plasmids carry *A. brasiliense* promoter-*lacZ* transcriptional fusions, pAB53 and pAB914 carry *A. brasiliense* *nifA* and *glnB* genes. *A. lipoferum* and *Escherichia coli* strains were grown in Luria-Bertani medium at 30\(^\circ\)C with continuous aeration. For nitrogenase enzyme activity assay *A. lipoferum* strains were grown in N-free minimal medium.

Molecular biology techniques were done according to "Molecular cloning: a laboratory manual" (1989).

Assay of nitrogenase enzyme activity in the presence of lectins. Bacterial cultures were grown under nitrogenase enzyme derepressing conditions, in N-free minimal medium for 2 hours at 30\(^\circ\)C in 99,5:0,5 (vol/vol) N\(_2\)–O\(_2\) atmosphere, then 10 % (vol/vol) of acetylene and lectins were added anaerobically. Nitrogenase enzyme activity was determined by acetylene reduction assay.

Transfer of *A. brasiliense* *nifH-lacZ*, *nifA-lacZ*, *glnBA-lacZ* and *glnA-lacZ* fusions into *A. lipoferum* and \(\beta\)-galactosidase assay in the presence of lectins. pAB358, pAB576, pAB904 and pAB912 plasmids were conjugated into the wild type and WGA\(^{-}\) mutant strains.
Transconjugants were selected on minimal medium containing the appropriate antibiotics. Nitrogenase enzyme was derepressed as above, β-galactosidase activity was measured after 4 hours of the lectin addition.

**Differential lectin binding assay.** FITC-labeled lectins were used to test lectin binding to the surface of bacterium cells. Lectin binding was quantified by average epifluorescence of the cells measured at 525 nm ($E_{525}/10^7$ cells/ml). WGA–Neu and WGA–Glc were prepared by saturation of WGA with molar excess of $N$–acetyl–D–neuraminic acid and $N$–acetyl–D–glucosamine.

**Detection of capsular WGA-binding components.** Capsules of three-day-old cells, grown on solid minimal medium, were solubilized in HEPES-1%, Triton X–100 buffer at 4 °C-on for 18 hours. Protein components of the capsule preparations were separated by sodium-dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Protein patterns were transferred to nitrocellulose membranes and subsequently tested for WGA-binding with digoxigenin-conjugated WGA.

**Methods of plant inoculation experiments.**

**Bacterial strains, rice and wheat cultivars, culture media.** *A. brasilense* and *H. seropedicae* strains are rifampin-resistant mutants of the wild type parents. Karmina, Sandora and Ringola rice cultivars (Institute of Fish Breeding, Szarvas), and Mv–23 wheat cultivar (GATE Tangazdaság) were test plants. Bacteria were grown in minimal medium for plant inoculation, and in N-free minimal medium for measuring nitrogenase activity.
Production of liquid and cellulose bead based bacterium inocula.

For liquid inoculum, minimal medium was inoculated with bacterium culture of stationary phase. Cultures were grown for 16 hours at 30°C with continuous aeration, then washed in N-free minimal medium and used for plant inoculation. For production of the cellulose bead based inoculum, minimal medium containing preformed cellulose beads was inoculated as above. Cultures were incubated at 30°C for 7 days, with continuous aeration. The medium was renewed in every 48 hours. After 7 days of incubation, cellulose beads were resuspended in minimal medium for batch culture experiments, or in N-free minimal medium for plant inoculation experiments.

Assay of nitrogenase enzyme activity. Cultures were derepressed in N-free minimal medium for 2 hours at 30°C in 99.5:0.5 (vol/vol) N₂−O₂ atmosphere, then 10 % (vol/vol) of acetylene was added anaerobically. After subsequent 14 hours incubation nitrogenase enzyme activity was determined by acetylene reduction assay.

Plant inoculations in pots. Three-day-old plantlets were planted in washed and heat-sterilized sand (5 plants/pot), previously inoculated with 5 ml of liquid inoculum or 5, 10, 20 and 30 beads of cellulose bead based inoculum. Plants were grown for 28 days at 20–25 °C at 16 h/8 h light/dark cycle in greenhouse. Plants were watered as required. After 28 days, roots were cut, roots and shoots were air-dried and measured. Number of bacteria, established on the root surface, within the root and shoot and in the rhizosphere was determined and nitrogenase activity in the root and shoot was measured.
Results

The establishment and operation of nitrogen fixing association between wheat and *Azospirillum* requires lots of molecular interactions, including the recognition process between the root surface and the bacterium cell, especially the binding between the root lectin and the sugar moiety of the bacterium capsule.

Lectins are proteins that recognize and reversibly bind to specific sugar chains of glycosylated macromolecules, polysaccharides, glycoproteins. Thus, lectins exposed on plant roots may contribute to the selective contact with bacteria in the rhizosphere, due to their ability to distinguish between sugar moieties on the cell wall of various bacteria.

In first part of the work the interaction between the wheat root lectin, wheat germ agglutinin (WGA) and *Azospirillum lipoferum* was studied: recognition and binding of the lectin by the bacterium and the subsequent response reactions in the bacterium cell.

WGA-nonbinding (WGA\(^{-}\)) transposon insertion mutant strain of *A. lipoferum* was isolated. The impaired DNA region of the mutant genome was identified by localization of the transposon insertion.

It was demonstrated that WGA is specifically able to stimulate the nitrogen fixing capacity of *A. lipoferum*, presumably after formation of a complex with the corresponding receptor on the bacterium cell surface. The stimulus, which leads to the upregulation of nitrogen fixation, is absent when the lectin–bacterium interaction is prevented, as in the WGA\(^{-}\) mutant strain. The stimulus is efficient with plant associated
diazotrophs, such as *A. lipoferum* and *A. brasilense*, but not with the free-living *Azotobacter vinelandii* and *Klebsiella pneumoniae*.

Terminal sugar residues on the cell surface of the wild type and WGA− cells were surveyed by a differential lectin binding assay. Impaired sugar moiety of the mutant cell surface was predicted. Next, it was identified whether which lectin-sugar contact is involved in the stimulation of the nitrogen fixation. Exclusively, the *N*-acetyl–D–glucosamine binding lectins were able to enhance the nitrogen fixation of the bacterium. The highest increase of nitrogenase activity was obtained by WGA.

Moreover, it was tested whether the lectin stimuli exert regulatory effect on the nitrogen fixation through modulation of the transcription of *nifH*, *nifA*, *glnB* and *glnA* genes. Expression of *nifHDK* operon (the structural genes of the nitrogenase enzyme) is positively controlled by NifA (encoded by *nifA*). NifA activity is modulated by the PII protein (encoded by *glnB*), the intracellular signal transmitter, in response to N-status of the cell. *glnA* is the structural gene of the glutamine synthetase, required for ammonia assimilation. Promoter fusions *nifH-lacZ*, *nifA-lacZ*, *glnBA-lacZ* and *glnA-lacZ* were introduced into the wild type and WGA− strains. β-galactosidase activities of the plasmid-born fusions were monitored upon stimulation with lectins. WGA and other *N*-acetyl–D–glucosamine specific lectins elicited increased expression of *nifH-lacZ*, *nifA-lacZ* and *glnBA-lacZ* fusions, and subsequently an enhanced nitrogen fixation.
Furthermore, a WGA–binding receptor was identified on the cell surface of the bacterium. The capsular material of the cells was solubilized and separated by SDS-PAGE, blotted to nitrocellulose membrane and developed with digoxigenin-conjugated WGA by enzyme–immunodetection assay. In the capsular preparation of the wild type cells single WGA-binding signal was detected as a 32 kDa polypeptide. Although a 32 kDa polypeptide was also present in the capsule of the WGA\(^-\) mutant cells, it was unable to bind WGA. It was also demonstrated that the 32 kDa capsular polypeptide of the wild type strain is glycosylated.

The results obtained by WGA–immunodetection, Schiff assay and WGA–affinity chromatography confirmed that the 32 kDa capsular protein can act as WGA–binding receptor (or a receptor component) on the cell surface of \textit{A. lipoferum}.

In the second part of the work associative symbioses of \textit{A. brasilense} and \textit{H. seropedicae} bacteria were established with Hungarian rice and wheat cultivars. The effect of the association on plant growth was assayed. The capability of the various rice cultivars were compared to accept and benefit from the nitrogen fixing associations. Established pattern and nitrogen fixation capacity of the bacteria within the plants were described.

Beneficial effect of \textit{A. brasilense} inoculation both in rice and wheat was expressed in increased root mass, more lateral roots, coupled occasionally with increased shoot mass production. In this early stage of
plant growth (28 days after the plant inoculation) the stimulation is mostly due to effect of the indol-3-acetic acid, produced by the bacterium and less by the biologically fixed nitrogen supplied to the plant.

It was emphasized that the beneficial effect is highly dependent on the host plant cultivar. Karmina and Sandora cultivars benefited from the inoculation with *A. brasilense* and produced considerably increased root mass. Distribution pattern of the bacterium within the plants revealed that the bacterium was able to establish even within the shoots, although its shoot colonizing capacity significantly differed in the two cultivars. In contrast, rhapsodic effect of the nitrogen fixing association between the rice cultivars and *H. seropedicae* was obtained. The Sandora cultivar responded to the *H. seropedicae* colonization with an unequivocal stimulation of shoot growth, while the establishment of the bacteria in the shoot was strictly limited. In Karmina plants, considerable increase in root mass coupled with moderate shoot growth promotion was documented when the colonization of the bacteria was restricted in the shoot. In contrast, an intensive shoot colonization harmfully affected the root growth. The association of both diazotrophic bacteria was unambiguously harmful with Ringola cultivar, resulting in considerable decrease in root mass.

*A. brasilense* and *H. seropedicae* intensively fixed nitrogen both in Karmina and Sandora plants.

Mv–23 wheat plants responded to the establishment of the two diazotrophic bacteria by completely different way. Inoculation of *A. brasilense* reliably and considerably increased the root mass. Bacteria
were mainly established in the root while less bacteria colonized the shoot. Bacteria within the root fixed nitrogen with extremely high capacity. In contrast, the association with *H. seropedicae* harmfully affected the growth of plants and resulted in significant root mass decrease. Colonization pattern of the bacterium within the plants was comparable to that of *A. brasilense*.

For the plant inoculation experiments two inoculum formula was applied: liquid culture and cellulose bead based immobilized inoculum. The immobilized formula can support the survival and competitiveness of the inoculum in the soil, providing a bacterium population of high cell number and constant quality around the germinating seeds and plantlets. Besides, it can ensure easy delivery and handling of the inoculum with standard machinery in the field.

The *A. brasilense* cells spreading out from the cellulose beads better survived and kept their nitrogen fixation of high capacity in the soil, and more intensively colonized both the rice and wheat plants.

**New results of the research**

– Nitrogen fixing associative symbioses of various Hungarian rice and wheat cultivars were established with associative diazotrophic *Azospirillum brasilense* and endophytic diazotroph *Herbaspirillum seropedicae*. 
The association with *A. brasilense* beneficially supported the plant growth, both in rice and wheat. The bacterium efficiently colonized both the root and shoot, and fixed nitrogen within the plants.

The association of the endophytic *H. seropedicae* with rice was balanced and beneficial when the host plant was able to restrict the radical spread of the bacterium within the shoot. The establishment of the bacterium was strictly rejected by wheat.

The plant genotype basically determined the efficiency of the association. Sandora rice cultivar benefited from the association with both diazotrophs. *A. brasilense* unambiguously supported the growth of Karmina plants while *H. seropedicae* triggered rhapsodic responses in this cultivar. Ringola cultivar rejected to live in nitrogen fixing association, with either bacteria.

*A. brasilense* and *H. seropedicae* in Karmina plants, and *A. brasilense* in Mv–23 plants produced nitrogen fixation of extreme high capacity.

A cellulose bead based, inoculum immobilization technique was developed. The application of the immobilized *Azospirillum* inoculum supported the survival of the inoculum population in the soil and helped to keep its nitrogen fixation capacity, both in rice and wheat rhizosphere.

In wheat–*Azospirillum* association it has been demonstrated that:

- binding of wheat root lectin to *A. lipoferum* cells specifically increases the nitrogen fixation of the bacterium;
− a 32 kDa glycosylated protein acts as lectin binding receptor (or receptor component) on the cell surface of the bacterium;
− stimulation of nitrogen fixation specifically occurs when the lectin binds to the N–acetyl–D–glucosamine sugar residues of the receptor protein;
− the stimulation rate is depended on the number of sugar links between the lectin and the receptor;
− the stimulation, induced by lectin binding, is transferred to the regulation system of the nitrogen fixation machinery and resulted in enhanced biosynthesis of the nitrogenase enzyme;
− the signal first targets the expression of the PII and NifA regulatory elements in the nitrogen fixation machinery, and subsequently transferred to the promoter region of the nifHDK operon, resulting finally in an increased expression of the nitrogenase structural genes.

**Propositions**

Creation of nitrogen fixing transgenic plants, which carry and operate bacterial nitrogen fixation genes in their genomic DNA, are still remained prey for the future research. However, the natural associative symbiosis between cereals and nitrogen fixing, plant growth promoting rhizobacteria represents a real perspective in the environment-friendly crop production. *Azospirillum* spp. as phytostimulators, in association with the host plant, stimulate the seed germination, promote the defense
reaction of the plantlets against pathogenic attacks, increase the drought
tolerance of the plant, and besides, supply the plant with biologically
fixed nitrogen.

However, before widescale utilization of such phytostimulators in
the intensive crop production, it is obligatory to test them in association
with Hungarian crop cultivars, under Hungarian climatic and soil
conditions.

Our work with Hungarian rice and wheat cultivars will be extended
to field size and wide range of cultivars will be included. Our results
clearly showed that Ringola rice cultivar is disable to live in associative
symbiosis with *Azospirillum* and *Herbaspirillum* bacteria. However, the
inoculation of Karmina and Sandora cultivars with *Azospirillum* is highly
recommended. Moreover, the mixed inoculation with *Azospirillum* and
*Herbaspirillum* should also be considered and the efficiency of the mixed
inoculum in both cultivars should be tested. Worthy to note, that bacteria
within the Karmina plants fix nitrogen with extremely high capacity.
Probably the genetic and physiological background of this cultivar is able
to support generously the operation of the association.

Moreover, we also propose to test the efficiency of the inoculation
in deepwater and wetland rice production technologies, with special
attention to the mixed *Azospirillum/Herbaspirillum* inoculants.

In wheat cultivars especially the association of *Azospirillum* spp.
appears to be highly advantageous while the endophytic symbiosis with
*Herbaspirillum* is still an open question.
Our inoculants are not considered to GMO (genetically modified organisms), they do not carry any foreign genetic information in their genomes, thus their application in field is not GMO-law-restricted.

Beside wheat and rice, we have already tested *Azospirillum/Herbaspirillum/Acetobacter* mixed inocula in potato, tomato, sugarcane and various vegetable cultures. Moreover, we have isolated diazotrophic bacteria of moderate nitrogen fixing capacity, too, from rhizosphere of various cereals and grasses in Hungarian soils.

For widescale application of *Azospirillum/Herbaspirillum* inoculants the cellulose bead based inoculum formulation is recommended. This formula may provide constant quality, better survival and effectiveness of the inoculum, besides easy of delivery and handling with standard machinery. However, the reinoculation in each vegetation period seems to be necessary in our climatic conditions.

A further substantial question is whether we can extend the range of host plants which are able to benefit from associative nitrogen fixing symbiosis. In order to answer this question, we have to reveal and understand the complete system of the interactions and their regulation between the two associative partners, even at molecular level. We have to know the biological events during the root surface colonization, and we have to understand the specific communication between the bacterium and plant partners.

We have demonstrated that the wheat root lectin binds to N–acetyl–D–glucosamine sugar residues on the cell surface of *Azospirillum* and as a consequence of this binding, the nitrogen fixation
of the bacterium is stimulated. Searching protein databases, it is worthy to note that root lectins of numerous cereal and grass species, of potato and tomato also recognize and bind N–acetyl–D–glucosamine sugar residues. *Azospirillum* spp. live in highly efficient association with tomato, too. Consequently, the establishment specific sugar links between the root lectins and the colonizing *Azospirillum* cell surface might be an essential factor to determine the compatible range of host plants. For this reason, the recognition events on the root surface and the lectin induced stimulation of nitrogen fixation will be further studied in other associative symbiotic systems.

An exciting question is to estimate the maximum capacity of the lectin stimulated nitrogen fixation in the bacterium. Our further work aims to identify the gene encoding for the receptor protein, to understand its regulation, to get acquainted with the nature of the stimulus induced by lectin binding to the receptor, and the signal transduction mechanism which leads to the regulation of the nitrogen fixation. Further crucial problem remains the energy supply of the lectin enhanced nitrogen fixation.

The plant-bacterium communication model, presented in the dissertation, might give additions to understand other eukaryotic–prokaryotic signal transduction systems. The information between the partners is transferred by sugar moieties of the cell surfaces and oriented to target genes and functions in the cell. Beyond the nitrogen fixing associations and symbioses, this model may provide
additions to understand in more details the pathogenic plant-bacterium interactions, too.

Publications


Oral presentations


Posters


