Final Technical Report
Executive Summary

Micro-N-fix

Research supported by the FP5 Quality of Life Programme

Microbial fixation of atmospheric nitrogen for staple food crops

Contract number: QLK5-CT-2002-00791

Non-confidential

MicroNfix details

Title of the project: **Microbial fixation of atmospheric nitrogen for staple food crops**		
Acronym of the project: **Micro-N-fix**		
Type of contract: **RTD collaboration project**		Total project cost **€ 2,458,584**
Contract number **QLK5-CT-2002-00791**	Duration **48 months**	EC contribution **€ 1,518,306**
Commencement date **01-10-2002**	End date **30-09-2006**	

PROJECT COORDINATOR

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Key words (5 maximum)
Nitrogen, fixation, bacteria, microencapsulation, inoculant

World Wide Web address
http://www.micro-n-fix.org

List of participants

1	**University of Hohenheim Institute of Plant Nutrition (330)** Role: coordinator, greenhouse trials, investigation of mechanisms of nitrogen fixation and plant growth promotion by rhizobacteria, fermentation via subcontractors	Germany	Coordinator
2	**Bio-gen Applied Soil Research Company Kft** Role: fermentation, development of compost carriers, pot and field trials	Hungary	Contractor
3	**Ecowork Laboratories Consulting GesmbH** Role: isolation and characterisation of plant growth promoting rhizobacteria, measurement of nitrogen fixation, tube tests	Austria	Contractor
4	**Ecole Nationale d'Ingénieurs des Techniques des Industries Agricoles et Alimentaires** Role: development of encapsulation and seed coating techniques, production of encapsulated inocula and coated seeds for pot and field trials	France	Contractor
5	**Dalgety Arable Research** Role: field trials, end user	UK	Contractor
6	**AGRON, Agrochemicals Development and Marketing, Ltd** Role: field trials, end user	Israel	Contractor

MicroNfix objectives and expected achievements

Inorganic fertilisers and chemical crop protection agents have throughout the past 50 years become the mainstay of staple crop production throughout Europe and the rest of the world. Although the use of these products has given impressive yield improvements, this has been at the expense of increasing environmental problems, including soil degradation, loss of biodiversity, nitrate contamination of groundwater and a significant contribution to global warming from nitrous oxide emissions. The production of inorganic fertilisers is also energy intensive with a significant consumption in fossil fuel which is unsustainable. Organic farming is the main alternative for crop production but this requires large volumes of compost or manure fertiliser.

MicroNfix will research the use of inoculants containing non-GM, free-living plant growth promoting rhizobacteria (PGPR) to act as biofertilisers, with the potential to replace some or a large majority of inorganic fertiliser input required for non-leguminous, staple crops, including wheat and maize. For organic farming, the concept will have the benefit of improving the nutrient value of each unit volume of organic fertiliser.

Rhizobium type strains are well known for their symbiotic relationship with legumes, supplying fixed nitrogen to the plant through nitrogenase activity, and inoculants are always used in legume cultivation. Up until recent times, no equivalent nitrogen fixing bacteria were known to interact directly with non-legumes, which provide of course a major proportion of the population's diet. In the last two decades, various microbial strains such as different *Azospirillum* species have been discovered which can potentially fix nitrogen for non-legumes, though the plant growth promotion effect may also be due to excretion of plant hormones or other phytostimulatory effects. The relationship with the plant is not symbiotic as in legumes, but the microorganisms are closely associated with the root systems, or in some cases, are found living inside plant tissues (endophytes). MicroNfix will screen rhizobacteria for popular varieties of wheat and maize, but will be particularly focused on researching methods for optimal application of the bacterial inoculants so that a maximum number of bacteria are active within the rhizosphere. This emphasis has been neglected in previous work.

To improve survival of the inoculants, it is important that an appropriate carrier is developed. Most nitrogen fixing rhizobacteria are non spore-formers and are relatively vulnerable to adverse conditions. A microbial inoculant must be preserved during storage and application, and a carrier material must provide physical protection and nutrients so that the microorganisms can survive. It is also important that the material is easily handled with common farm equipment. Currently, most inoculants are delivered in liquid form, with a short shelf life of less than 3 months.

DALGETY's Arable Development Centre at Throws Farm, Essex, UK, site of several MicroNfix field trials

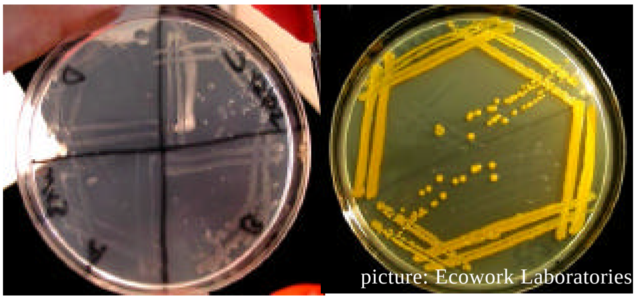
The following scientific and technological objectives were defined for MicroNfix:

- Screening, isolation, and characterisation of 2-3 new PGPR from bacteria sourced from existing stocks and new isolates. Growth on specific media to select for target functions, particularly N-tolerance.
- Quantification of nitrogen fixation and other beneficial effects of bacteria (e.g production of phytohormones) through rigorous isotopic studies and classical plant nutrition techniques, and investigation of the mechanisms causing enhanced plant growth.
- Screening of wheat varieties with analysis of root exudates which best attract and feed PGPR strains
- Research of compatible compost carriers to obtain equivalent nutrient value with 20% less volume of compost
- Research of innovative microencapsulation techniques for improved survival of PGPR during storage and functionality in the soil, also seed coating with polymer/cells suspension
- Production, testing and validation of inoculants in field trials in contrasting soil and climate zones in Europe
- Investigation of ecological impact of inoculants on local soil microflora
- Optimisation of strain selection and inoculant delivery modes based on analysis of results from field trials

MicroNfix results

The MicroNfix project has run for a total of 48 months, extended beyond the original 38 months. As one of the first actions, the commercial partners BIOGEN, DALGETY and AGRON prepared a specification of end user requirements. This highlighted that PGPR would need to provide a cost advantage to farmers to be attractive. Also the potential for improvement of protein content in grain was highlighted.

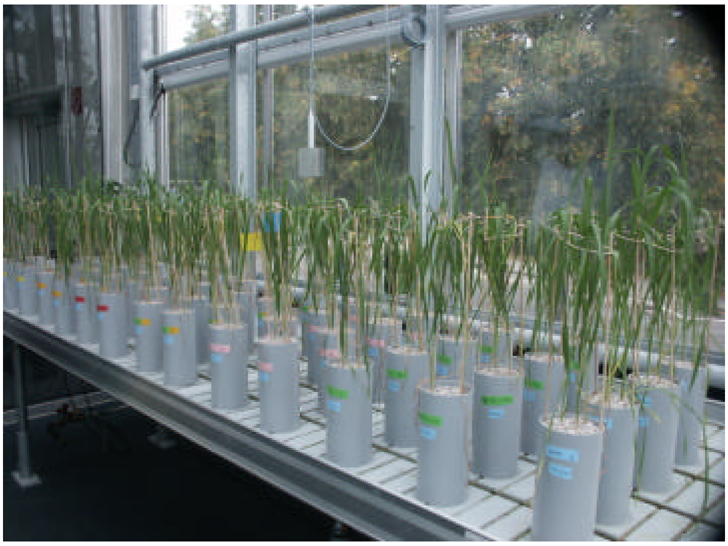
The research commenced with a screening programme of wheat roots and strain collections in Europe. Several partners were involved in collection of wheat roots from near their locations and these were sent to ECOLAB for isolation of PGPR. The selection of strains was performed on low nitrogen media in order to isolate N fixing bacteria. In the first year 52 strains were identified by ECOLAB and BIOGEN as being nitrogen fixing with potential as PGPR. Many of these strains were spore-formers, which may be more adept at survival during processing and inoculation in the soil.



Comparative growth of N_2-fixing bacteria on N-rich and N-free medium

In the meantime, a known strain of *Azospirillum brasilense* was selected as a positive control. BIOGEN was initially responsible for the fermentation of the first batch of *Azospirillum* for pot and field trials, but problems were experienced with contamination and damage in transit. The first year of pot trials (UHOH) and field trials (DALGETY, AGRON) returned no positive plant growth promotion effect with this inoculant. For later field trials, a reliable fermentation company was found and stringent quality control was put in place.

The partners developed several novel methods for testing of rhizobacteria, which were used in the project in addition to the established methods for nitrogen fixation, the acetylene reduction assay and ^{15}N dilution. ECOLAB designed a novel bio-test based on an ammonium-dependent GFP labelled fungus, and also a tube test that measured acetylene reduction and ^{15}N dilution with a germinating wheat seedling present in the tube. The last method was used to select for further study 4 leading strains.



Pot trials of candidate PGPR strains at UHOH

UHOH carried out pot trials with the leading wild type strains, as well as testing a number of GM strains of *Azospirillum* with wheat and model plants to investigate plant growth promoting mechanisms of rhizobacteria. However, only one trial has shown significant differences between treated and untreated pots. It may be that preferential interactions between rhizobacteria and wheat genotype are important. In field trials by DALGETY and AGRON, some significant differences have been seen with some of the strains. Also some significant differences have been seen in the tube test of ECOLAB. Due to the lack of reproducibility, the partners have not yet progressed to measuring the impact of the inocula on local microflora.

UHOH has also investigated preferences of the rhizobacteria for different plant species and wheat varieties through chemotaxis assays testing root exudates from the plants. These have indicated in some cases a preferential plant-microbial interaction. ECOLAB have also worked to label the studied rhizobacteria with GFP to enable observation of root colonisation and easy detection by PCR. Attempts have been made to develop a PCR method to detect the strains by targeting distinctive gene sequences but these have not proved specific enough. Also ECOLAB have commenced the sequencing of the genomes of the leading strains.

BIOGEN concentrated on the development of compost carriers. It was initially hoped that the PGPR inocula could be co-fermented with the compost but it was realised that the composting temperature would be too high to permit survival of the rhizobacteria. Aerobic and anaerobic composts have been tested and one of each selected for pot and field trials. Some positive effects on plant growth promotion were observed in pot trials of the compost-inocula combinations. BIOGEN also developed a solid silicate carrier that was efficient at stabilising *Azospirillum*.

ENITIAA developed encapsulation of rhizobacteria, producing large capsules suitable for drilling alongside seed. The selection of encapsulation materials has been optimised for the desired physical properties and cost, and survival of bacteria in the capsules is excellent. Encapsulated inocula have been tested in pot and field trials, with interesting results. These indicate that encapsulation may potentiate any plant growth promoting effects. Also ENITIAA have worked on seed coating, which was indicated by the end user partners as being the most desirable formulation for ease of application by the farmer. There have been some difficulties in obtaining seed coatings with a good survival rate of rhizobacteria, and it has been not so easy to optimise the physical properties. By the end of the project some batches of coated seeds are giving better results, but they are not yet reproducible.

picture: ENITIAA

Coated seeds with alginate prepared by drum coating

Combine harvester used in AGRON field trials

MicroNfix potential impact

The MicroNfix project has isolated 52 strains of rhizobacteria, four of which are considered promising as candidate PGPR. These have been tested positively in experiments based on a new development of the acetylene reduction assay, as fixing atmospheric nitrogen in the wheat rhizosphere. Also, excretion of phytohormones has been confirmed for at least one of the candidate PGPR strains. However, confirmation of a plant growth promotion effect in terms of increased yield in pot or field experiments has been mainly elusive, with differences only found in some of the studies.

However, MicroNfix has put in place some of the process technology that would be necessary to commercialise PGPR inocula, in particular reliable fermentation of the strains and an effective encapsulation method at reasonable cost. Still there are many aspects where further research is required. The precise conditions where plant growth promotion can be observed still need to be defined, a reliable method of seed coating has not yet been achieved, and reproducible effects need to be established in pot and field trials before PGPR inoculants can be commercialised. The cost of PGPR inocula needs to be competitive with N fertilisation, though it is recognised that the cost N fertilisers is likely to continue to rise over the next few decades with increasing limits on the supply of natural gas and oil feedstocks.

Most of the MicroNfix partners are to participate in a further research project "RHIBAC" to be funded by the EU FP6 programme in the period 2007-2010. This project will use genetically modified PGPR to understand better the processes of rhizosphere colonisation and plant growth promotion effects. The specific factors influencing the interaction of PGPR with different wheat genotypes will be investigated. It will also provide the opportunity for large scale field trials should effective PGPR inocula be developed.

The RHIBAC project includes additional partners from Europe, Turkey and South America, thus allowing the testing of additional PGPR from these regions, and extending the possibilities for exploitation of results. Should reproducible, positive results be generated in RHIBAC, building on the work of MicroNfix, the partners will be well placed to develop innovative PGPR inocula for introduction to arable farmers in many parts of the world.

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