Contract: 036314

MICROMAIZE

Management of Plant Beneficial microbes to balance fertiliser inputs in maize monoculture

Instrument: STREP

Thematic Priority: Food quality and safety

Publishable Final Activity Report

Period covered: From 01/11/2006 To: 31/01/2010

Date of preparation: 30/03/2010

Start date of project: 01/11/2006 Duration: 39 Months

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Project coordinator organisation name: CNRS Revision: 1

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1.1 Summary Description of project objectives

1.1.1 Project Summary

The objective of MicroMaize was to develop and validate integrated farming practices based on phytostimulation and biofertilisation to enable a reduction of chemical fertiliser usage in maize monoculture. Maize is a strategic target as it is often grown in a non-sustainable way in Europe, and it has been imported from Mexico in relatively-recent times, without its entire guild of root-associated beneficial microbes. The project focused on synergistic consortia of multifunction plant-beneficial microbes comprising the two PGPR *Azospirillum* (nitrogen fixation and modulation of plant hormonal balance) and *Pseudomonas* (phosphate solubilisation and phytohormone modulation), and the arbuscular mycorrhizal fungus *Glomus* (nitrogen and phosphorus mineralization/acquisition).

Novel research tools including micro-array technology, biosensors and plant metabolomic profiling were used to design consortia and monitor plant-microbe interactions in situ. The establishment of introduced and indigenous plant-beneficial microbial consortia in the maize rhizosphere was sought by inoculation and by management of the indigenous microbial community via the choice of maize genotypes, respectively. The novel microbial management strategies developed in this project were integrated with current farming practices in maize monoculture. They were tested validated at different field locations throughout Europe (and in Mexico) under real-life conditions, and agronomic effects on crop yield, yield quality and food safety (mycotoxins) were quantified. Microbial innovation was disseminated to farmers and other end-users via demonstration actions and various dissemination modes. These goals were achieved by a partnership including three top academic centres, two technical institutes, two SMEs, one OTH and an INCO partner who is currently managing the largest successful inoculation program for maize worldwide.

1.1.2 Project objectives

The first project objective was the development of a plant-microbe interaction toolbox, both by customizing recent molecular methods to target specific plant-beneficial microbes and designing novel methods and tools (WP1). Indeed, the lack of such a toolbox is one of the reasons behind our insufficient knowledge of beneficial plant-microbe interactions and the lack of established sustainable microbial management practice currently available for maize monoculture. This toolbox was planned to be used in combination with established methodology at the different stages of the project, i.e. to improve our knowledge of the physiology and ecology of plant-beneficial microbes, to assist in the selection of inoculant consortia and maize cultivars, and to monitor the functioning of plant-microbe interactions in the greenhouse and in the field.

The second project objective was to design microbial consortia in which *Azospirillum*, *Pseudomonas* and *Glomus* AM fungal strains were combined (WP2), in line with the promising results now available in the literature and partner labs on the efficacy of various inoculant consortia. First-generation microbial consortia were to be selected by greenhouse screening and second-generation consortia after further analysis of molecular interactions.

The third project objective was to develop inoculant products, so that microbial consortia could be field tested under real-life technical and economic conditions (WP3). In terms of biosafety, the plant-beneficial strains used belong to non-pathogenic microbial taxa and have been shown to lack detrimental ecological effects when used as inoculants in the field. However, the most promising consortium validated in the project was to be checked for lack of toxicological effects. In addition, novel inoculant technology was to be developed to optimize production and formulation of plant-beneficial consortia, so as to facilitate their use under current agronomic conditions and enhance their efficacy on the crop.

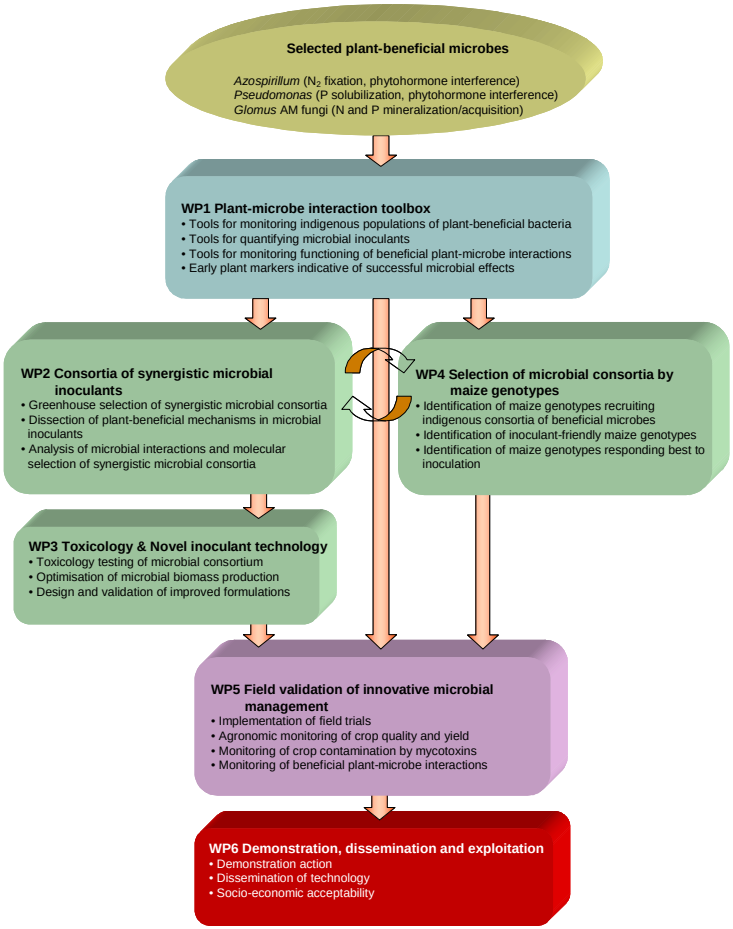
The fourth project objective was to identify maize genotypes that are the most efficient at interacting with beneficial microbes (WP4). Indeed, whilst outstanding plant-beneficial strains selected in laboratories have had the opportunity to be tested under different field conditions including on different cultivars, research to date indicates that the potential to maximize beneficial plant-microbe interactions by a more careful choice of cultivars is high. Besides the plant-beneficial effects of microbial inoculants, this is also relevant when considering (i) root colonization by indigenous plant-beneficial microbes and the positive effects of the latter on maize, as well as (ii) recent literature indicating that certain plant-beneficial inoculants can favour root colonization by fellow, indigenous plant-beneficial microbes. The focus on maize genotypes is also based on the rationale that several thousands different varieties are registered in Europe, providing breeders, seed distributors and farmers with an opportunity to select within each cultivar category the particular cultivars interacting best with maize-beneficial microbes.

The fifth project objective was to field test in various maize-growing regions of Europe and in Mexico the microbial consortia selected in the project (WP5). This was to take place in different soil and climatic conditions, with the possibility to select relevant microbial consortia according to pedo-climatic factors. A comprehensive monitoring of agronomic benefits was planned, and the actual functioning of beneficial plant-microbe interactions was assessed at one site by applying molecular ecology methods under field conditions. In addition, monitoring targeted the quality of maize products including the issue of mycotoxin contamination.

The sixth project objective was to disseminate project results (WP6). A multi-target approach was chosen to disseminate project results and communicate on possibilities of practical implementation of microbial inoculation. This key activity is in fact in-built in the project as the consequence of the partnership that has been assembled in MicroMaize and that includes several academic centres that will ensure scientific dissemination (via university training, publications, web sites and seminars), as well as industrial

partners (SMEs) and technical institutes already in direct contact with farmers and other end-users for technology transfer (via demonstration trials, training of personnel, technical newsletters, web sites, etc.).

1.2 Project Organisation


Selected plant-beneficial microbes
Azospirillum (N2 fixation, phytohormone interference)
Pseudomonas (P solubilization, phytohormone interference)
Glomus AM fungi (N and P mineralization/acquisition)
WP1 Plant-microbe interaction toolbox
• Tools for monitoring indigenous populations of plant-beneficial bacteria
• Tools for quantifying microbial inoculants
• Tools for monitoring functioning of beneficial plant-microbe interactions
• Early plant markers indicative of successful microbial effects
WP2 Consortia of synergistic microbial inoculants
• Greenhouse selection of synergistic microbial consortia
• Dissection of plant-beneficial mechanisms in microbial inoculants
• Analysis of microbial interactions and molecular selection of synergistic microbial consortia
WP4 Selection of microbial consortia by maize genotypes
• Identification of maize genotypes recruiting indigenous consortia of beneficial microbes
• Identification of inoculant-friendly maize genotypes
• Identification of maize genotypes responding best to inoculation
WP3 Toxicology & Novel inoculant technology
• Toxicology testing of microbial consortium
• Optimisation of microbial biomass production
• Design and validation of improved formulations
WP5 Field validation of innovative microbial management
• Implementation of field trials
• Agronomic monitoring of crop quality and yield
• Monitoring of crop contamination by mycotoxins
• Monitoring of beneficial plant-microbe interactions
WP6 Demonstration, dissemination and exploitation
• Demonstration action
• Dissemination of technology
• Socio-economic acceptability

1.3 Contractors involved

Role *	No.	Participant name	Participant short name	Country	Date enter project	Date exit project
CO	1	UMR CNRS 5557 Ecologie Microbienne	CNRS	France	M1	M36
CR	2	Agrauxine	AGRX	France	M1	M36
CR	3	National University of Ireland in Cork	UCC	Ireland	M1	M36
CR	4	Swiss Federal Institute of Technology	ETH	Switzerland	M1	M36
CR	5	Agricultural Research Institute, Hungarian Acad. of Sciences	Ari-Has	Hungary	M1	M36
CR	6	National University of Mexico	UNAM	Mexico	M1	M36
CR	7	Symbio-M	Symbio	Czech Republic	M1	M36
CR	8	Arvalis	Arvalis	France	M1	M36
CR	9	Alma Consulting Group	Alma	France	M1	M36

* CO = Coordinator, CR = Contractor

1.4 Work performed and end results

1.4.1 WP0

Leader: Alma

WP0 objectives

This Work Package dealt with the project management and the consortium animation. The objectives were to implement a technical, financial and administrative management on a day-to-day basis in order to follow-up and monitor the program work progress. This Work Package was divided into four tasks that are: financial management, technical reporting, day-to-day follow-up and consortium animation. Two partners were involved in this Work Package: CNRS and ALMA

Financial aspects (CNRS and ALMA)

- Distribution to the partners of three prefinancings.
- Creating suitable templates for the financial reporting.
- Distribution of these templates to the partners in order to establish the state of the costs and of the manpower at each period for internal and external use.
- Collection and analysis of the costs spent for each period of the project.

Technical reporting (CNRS)

- Creating templates for MICROMAIZE Newsletters.
- Creating suitable templates for the Activity Report distributed to the Work Package leaders taking into account the EC request.

- Creating template for deliverables.
- Collection of the data and consolidation of the Interim and Periodic Activity Reports.
- Consolidation of the deliverables.

Day to day follow-up (ALMA)

- Establishment of a partner list in order to gather the details (phone and e-mail address) of the participants. This partner list was regularly updated.
- Nomination of the members of the Steering Committee.
- Nomination of the Work Package leaders.
- Nomination of the Management team (Dissemination and exploitation manager, Risk Manager, Project office).
- Collecting the Forms A signed by all partners.
- Signature of the Consortium Agreement by all partners.
- Organisation of all the meetings by creating agenda and logistic document.
- Submitting the first amendment of the European Contract to the European Commission regarding the integration of the special Clause 23 for the partner CNRS.

Consortium animation (ALMA)

- Building up the MICROMAIZE Communication Platform “MYNDSPHERE” with e.g. official documents, deliverables, details of the partners, etc.
- Creation of the MICROMAIZE logo and of the Project Information Brochure.

1.4.2 WP1

Leader: ETH

WP1 objectives

Beneficial plant-microbe interactions remain poorly understood, largely due to a lack of adequate methods. In WP1, the objective was to assemble a molecular toolbox to enable comprehensive analysis of key microbial interactions and plant-beneficial effects, with a focus on both indigenous and inoculated plant-beneficial microbes. This included the tailoring of validated state-of-the-art methods to enable the quantification of bacterial inoculants (PCR methodology) and the monitoring of plant-microbe interactions (based on biosensors and RT PCR for beneficial genes, and 33P/15N radioactive isotopes for plant nutrient uptake measurements). In addition, innovative methods were to be developed for high-throughput monitoring of indigenous plant-beneficial bacteria (based on a 16S rRNA taxonomic microarray), direct quantification of AM fungal inoculants, and early identification of successful microbial effects on the plant (based on enzymatic and metabolomic fingerprints of maize). These new tools were then to be used, in combination with established methodology, to address the objectives of the other WP of the project.

Tools for monitoring indigenous populations of plant-beneficial bacteria

A range of 16S rRNA microarray probes have been designed for *Azospirillum* spp., for plant-beneficial fluorescent *Pseudomonas* (at various taxonomic levels) and other PGPR taxa (*Bacillus*, *Enterobacter*, *Azotobacter*, etc.). These probes allow a high-throughput monitoring of indigenous plant-beneficial bacteria.

Tools for quantifying microbial inoculants

Real-time PCR methods have been developed to enable quantification of the candidate bacterial inoculant strains (i.e. 3 *Azospirillum* and 3 *Pseudomonas*) in the rhizosphere of maize and in field soil. An internal standard was included to circumvent differences in DNA extraction efficiencies between samples. By targeting particular regions of the genome we were able to identify a marker system allowing distinguishing the inoculant genotype of *Glomus intraradices* BEG 158 from several other genotypes of the same AM fungal

species. It is the first time that it is possible to monitor an AM inoculant in soil. In addition we developed a real-time PCR toolbox for monitoring the native communities of AM fungi.

Tools for monitoring functioning of beneficial plant-microbe interactions

A biosensor was developed to assess the expression of the plant-beneficial gene *acdS* (ACC deaminase activity) in *P. fluorescens* F113. The biosensor was constructed by placing the *acdS* promoter upstream from the gene *egfp* (coding an autofluorescent protein) and validation was done by fluorescence monitoring and by semi-quantitative RT-PCR. When the F113Rif P_{acdS}-*egfp* strain was inoculated on pre-germinated seeds of the maize hybrid PR37Y15, fluorescent cells were detected in several parts of the root system, demonstrating that maize roots may exude enough ACC to induce the ACC biosensor.

The *ipdC* gene of the 3 *Azospirillum* inoculants is involved in the synthesis of the phytohormone indole acetic acid (IAA). Biosensors *gusA-ipdC* were developed but allowed to visualized *ipdC* gene expression on maize roots mainly under sterile conditions. A RT-PCR protocol allowed to detect *ipdC* expression in the rhizosphere of maize planted in natural soils of different textures, except in soil with high clay content.

A biosensor was developed to measure changes in bio-available phosphate in the rhizosphere resulting from phosphate solubilisation by *Pseudomonas*. In *P. fluorescens* F113, the promoter of a phosphate-responsive gene was fused to the GFP gene. The biosensor assay is able to distinguish between soil samples with ‘high’ or ‘low’ phosphate levels.

Phosphate transporters of the inoculants *Glomus intraradices* and *Glomus mosseae* were cloned and used to develop a real-time RT PCR assay for quantification of target mRNA, so as to estimate P acquisition from the soil solution by the AM fungi. The tool was developed for maize grown in greenhouse in an autoclaved soil-sand mixture.

Both ^{33}P radioisotope and ^{15}N stable isotope were successfully used to quantify fluxes of P and N from the soil to maize plants as affected by mycorrhizal inoculation. The cultivation system employed spatial separation of plant roots from isotope-labeled soil patches, accessible only to the AM fungal hyphae through fine meshes (30 µm). These experiments showed high relevance of mycorrhizal symbiosis for P nutrition of the maize plants.

Plant response to microbial inoculants

A metabolomic method to monitor the beneficial effect of microbial inoculants has been developed. Specific secondary metabolites can be used to assess maize responses in agronomic field situations. In contrast, the applicability of specific enzymatic activities in root extracts was shown for maize growing in river sand but not in field soil.

1.4.3 WP2

Leader: UCC

WP2 objectives

The objectives of WP2 were to design synergistic consortia of plant beneficial inoculants for biofertilisation and phytostimulation of maize. First-generation microbial consortia for field trials were selected at the start of the project following a greenhouse screening in which selected plant-beneficial inoculants were combined and tested based on plant-beneficial effects. Scientific bottlenecks concerning poorly-understood plant-beneficial mechanisms were removed by acquiring relevant molecular knowledge, which was done by applying molecular tools to study microbial interactions in consortia. Second-generation microbial consortia were selected by molecular selection, based on inoculant compatibility in the rhizosphere and synergistic plant-beneficial traits.

Greenhouse selection of synergistic microbial consortia

No potential antagonistic interaction was found between inoculants strains, except an inhibitory effect on AMF spore germination of *Glomus mosseae* JJ 964 when inoculated with certain *Azospirillum brasilense* strain.

Greenhouse screening and selection determined that bacterial inoculation gave interesting results, often positive, but the hierarchy between inoculation treatments was often not very pronounced and could fluctuate according to the experiment considered, the maize cultivar, and the plant parameters being measured. Three bacterial combinations were then tested with *Glomus* strains.

The last experiments, in which *Azospirillum*/*Pseudomonas*/*Glomus* inoculations were tested with different soils and growth durations, did not give the same results, even though one consortium fared well in both. Again, the hierarchy between the different *Azospirillum*/*Pseudomonas*/*Glomus* consortia was not very pronounced.

Heavy labeling of key plant nutrients, 33P and 15N, found only weak (and indirect) evidence for mineralization of organic nutrients, with modest contribution of microbial inoculation in this system, perhaps because the native population contribute to nutrient mineralization/immobilization processes. Therefore, the inoculants may still work and mineralize organic nutrients, but the contrast between inoculated and non-inoculated treatments is not great. Inoculant microbes (possibly the AMF) help accessing remote soil zone, and they appear important for P nutrition of maize in the experimental system. There is a possibility that some incompatibility between the three microbes supplied to the soils may limit the appearance of major effects on the plants. Therefore, analyzing dual mixtures and/or to search for more mutually compatible microbes would be helpful.

Dissection of plant beneficial mechanism in microbial inoculants

The identification of mechanisms by which *Pseudomonas* increases phosphate availability to maize directed demonstrated that *Pseudomonas* spp. differ in both the amounts and types of organic acids they produce, which results in a range of capacities to solubilise inorganic phosphate. Unlike the other *Pseudomonas* spp., which produced mostly 2-ketogluconic acid from glucose metabolism, *P. fluorescens* F113 and Pf153 produced mostly gluconic acid. In addition, there are few studies where genes other than those directly involved in organic acid production have been implicated in the inorganic phosphate solubilisation process. We have demonstrated that mutation of the *dsbA* gene results in a decrease in swimming motility, a significant decrease in inorganic phosphate solubilisation on NBRI agar plates, but no significant changes in the level of phosphate liberated, pH decrease or gluconic acid production in broth-based assays. It is tempting to speculate that DsbA plays a role in the formation of active Gcd enzyme by facilitating disulfide bond formation. However, the differences in solubilisation phenotype in solid and liquid medium could point to the effects of *dsbA* mutation on motility as being equally important. Furthermore, we demonstrate that carbon source and plant derived rhizosphere components such as seed exudates affect the expression of *gcd* and *pqqB* (involved in organic acid production) at the transcriptional level in *P. fluorescens* F113. Of the carbon sources tested, gluconic acid was the strongest inducer of *gcd*, whereas

glucose was the strongest inducer of *pqqB*. Both of these carbon sources are major constituents of sugarbeet root exudates which suggests that F113 is well suited for phosphate solubilisation in the sugarbeet rhizosphere from which it was originally isolated.

The *in silico* analysis of the promoter region of the *acdS* gene of *P. fluorescens* F113 evidenced potential binding sites for Lrp, CRP and FNR regulators. This led us to check whether ACC deaminase activity and/or *acdS* gene expression differed according to the carbon source (including glucose; CRP regulation), the amino acid (including leucine; LRP regulation) and the oxygen level (FNR regulation), which are relevant conditions in maize rhizosphere. Using different methods, the ACC deaminase activity was shown to be down-regulated by the presence of leucine, up-regulated in presence of anoxic conditions and unaffected by the type of carbon source. The effect of maize genotypes on ACC deaminase activity of F113 was also characterized.

Analysis of microbial interactions in consortia

Molecular analysis showed that there were clear interactions between the inoculant microbes themselves and with the indigenous microbial communities, too. It seems that some combinations of AMF and bacteria bring greater benefits than the mycorrhiza alone. The nutritional benefits for triple microbial combination (as for the total nutrient contents in the plants) were significantly lower than for some dual mixtures. This means that in spite of substantial complementarities between members of the inocula, it does not necessarily mean that the most highly diverse mixture will do better than a less diverse mixture. On the other hand, results of isotopic labeling indicated generally greater benefits of multi-microbial inoculation as compared to the single-microbe inoculations. Therefore, further analyses of dual and triple mixtures appear very important, as selection of compatible microbes may need to be done under relevant soil conditions in the future (and not *in-vitro* only) and the triple mixture must potentially be scrutinized for possibly antagonistic effects. Specific formulations (microbial blends, isolates with different properties) for particular soil conditions may need to be adopted. The assessment of plant metabolomic response to consortium inoculation showed distinct patterns concerning the biosynthesis/accumulation of secondary compounds for roots. Variation was due to microbial factors (as differences were found between inoculation treatments), as well as to experimental factors based on comparison of same treatments in separate experiments (due to differences in soil, growth condition and/or maize growth stage, and to their modulation of microbial effects). The results

confirm the usefulness of maize secondary markers to discriminate inoculation treatments and to better understand plant-beneficial effects.

1.4.4 WP3

Leader: Agrauxine

WP3 objectives

The objective was to develop inoculant products based on the microbial consortia designed in the project. First, the most promising microbial consortium was subjected to standard toxicology testing, in line with the regulatory requirements for biofertilisers for agriculture specified in EU Directive 2001/36/EC. Second, novel inoculant technology was developed to obtain pre-commercial inoculant products suitable for subsequent field testing under relevant farming conditions in this project. In addition to optimizing microbial biomass production, this will include the design of formulation for storage and field application.

Toxicology testing of microbial consortium

An *Azospirillum*/*Pseudomonas*/*Glomus* consortium was processed to start the registration process as a bio-fertilizer. The assessment for the AFSSA Dossier (i) included toxicology studies (Phycher Laboratory), (ii) physical & chemical analysis (Laboratoire départemental de Laon), and (iii) microbiological analysis (Laboratoire départemental de Laon). Toxicology tests (acute oral toxicity, acute intraperitoneal toxicity, acute pulmonary toxicity and pathogenicity, eye/skin irritation) indicated that the consortium had no negative effect.

Optimisation of microbial biomass production

Optimisation of microbial biomass production was performed under laboratory conditions and is now feasible at a small pilot level. Culture media for each bacterium have been defined. Thus, bacterial biomass production for 2008' and 2009' field trials was performed. Quality control on products sent to the field partners for sowing gave satisfactory results in both years, but quality control on products received after sowing from field partners gave poor results in 2008.

Glomus inoculum was produced successfully for the field experiments. Quality control was performed on the mycorrhizal inoculum produced for 2008' and 2009' field trials, based on infectivity in MPN plant tests. Results indicated that the minimum number of propagules required for successful establishment of mycorrhizal colonization with plant roots was satisfied.

Design and validation of improved formulations

Novel inoculant technology was developed to optimize formulation for plant-beneficial consortia. A new seed-coating formulation for AMF was tested, but results of plant mycorrhization were not satisfactory. The best results were obtained when using a combination of two formulations. One formulation is a bacterial seed coating, whereas the other bacterium and the fungus are formulated together using a distinct solid carrier delivered directly in the furrow, in the vicinity of seeds.

1.4.5 WP4

Leader: CNRS

WP4 objectives

In this project, work was done with selected plant-beneficial microbes to enhance maize growth, and the fluctuation due to different plant genotypes was considered in this workpackage. Thus, the objective of WP4 was the identification of maize genotypes that interact efficiently with indigenous and/or inoculated plant-beneficial microbes. This was done by taking into consideration the ability

of certain plant-beneficial bacteria to produce metabolites that increase root exudation, which in turn is likely to enhance microbial colonization of roots (and should improve the efficacy of microbial consortia). The assessment made use of methods developed in WP1 and focused both on microbial colonization of roots as well as plant-beneficial effects. A range of maize varieties and hybrids were considered.

Effect of different maize genotypes on rhizobacterial community composition

A greenhouse experiment was performed in which 10 inbred lines and 2 maize hybrids were compared, in the absence of inoculation. Rhizosphere DNA of seedlings was studied using a 16S taxonomic microarray to assess the composition of the bacterial community. The total rhizobacterial community displayed a clear rhizosphere effect when compared with bulk soil, and significant differences in rhizobacterial community composition were also found when comparing certain inbred lines. Differences were of less importance when considering particular probes targeting taxa containing PGPR strains, especially with the *Azospirillum* and *Pseudomonas* genera. In contrast, limited differences were found in rhizobacterial community composition when comparing hybrids and parental lines.

Functional effect of different maize genotypes on beneficial bacteria

Experiments were carried out to assess differences in amino acid exudation according to maize genotype, including in presence of microbial inoculants. Root exudates were obtained from seedlings grown in a glass bead system. Chemical analysis showed that exudates were very different between the two maize varieties tested. *Pseudomonas* inoculation had a strong impact on amino acid exudates, and the impact differed when a 2,4-diacetylphloroglucinol mutant was used. The effects of the wild-type and of the mutant were not the same when another maize variety was studied. Thus, *Pseudomonas* and its metabolites have the potential to influence root exudate efflux of amino acids, but the plant genotype plays an equally significant role, highlighting how important it is to match the plant genotype to plant growth-promoting strains for optimal plant growth promotion.

A joint experiment was performed in non-sterile farm soil to assess colonisation of nine *Zea mays* genotypes by *Pseudomonas*, *Azospirillum* and *Glomus* inoculated as a three-component consortium. Inoculants were monitored by real-time PCR analysis at 6

weeks after inoculation. Whereas the *Azospirillum* inoculant survived poorly with all maize lines and was below detection limit at sampling, the *Pseudomonas* inoculant survived well, and its qPCR levels varied mostly according to the implementation or absence of chemical fertilization. Effects of inoculation were more significant on qPCR counts of indigenous or total *Glomus* genotypes than the inoculated *Glomus* strain. Overall, differences were found between maize genotypes when considering *Pseudomonas* and *Glomus* inoculants, and these differences depended on (i) the inoculant considered (i.e. *Pseudomonas* versus *Glomus*) and (ii) soil fertility level (i.e. fertilised or not).

Responses of different maize genotypes to inoculation

The same experiment in non-sterile farm soil was used to characterize the effect of maize genotype and inoculation on shoot biomass at 6 weeks, and maize genotypes responding best to inoculation (and/or chemical fertilization) were identified. Shoot data indicated that (i) biomass differed from one genotype to the next, regardless of whether fertilizer was applied, and that (ii) chemical fertilization improved shoot biomass of non-inoculated plants for 6 of the 9 genotypes. Inoculation had no effect on shoot biomass in the absence of chemical fertilization. When chemical fertilizers had been added, inoculation improved shoot biomass for 2 maize genotypes. Therefore, maize growth in response to inoculation depends both on plant genotype and soil fertility level (i.e. fertilized versus non-fertilized soil).

1.4.6 WP5

Leader: Arvalis

WP5 objectives

The objective was to field test microbial strains and inoculation procedures designed in the project in various maize-growing regions of Europe and in Mexico, so as to encompass a variety of soil and climatic conditions. A comprehensive monitoring of agronomic benefits was to be performed, ranging from yield analysis to the assessment of mycotoxin contamination of maize kernels. In addition, the actual functioning of beneficial plant-microbe interactions was to be assessed at one experimental site, by applying molecular ecology methods under field conditions. The final goal was to assess whether the phytostimulatory effect of microbial inoculants on maize allowed to reduce N and P fertilisation levels while maintaining a high level of production.

Implementation of field trials

During the two last years of the project, 10 field experiments were carried out in Europe (4 in Hungary, 2 in the Czech Republic, 1 in Italy, 3 in France), and 2 field experiments in Mexico. In Hungary, 2 of the experiments were carried out in an old long-term trial with 5 NPK rates applied since 1961. Except the trial of Mexico where a local variety was sown, all the others were sown with a commercial hybrid widely used in Europe and chosen according to its growth response to microbial inoculation in previous pot experiments. Three strains of *Azospirillum*, two strains of *Pseudomonas*, and two *Glomus* inoculants were assessed in consortia. Maize was inoculated the days of sowing and was studied at 3 fertilisation rates in 2008 and 2 rates in 2009. All experiments were carried out until grain harvest.

Agronomic monitoring of crop quality and yield

Monitoring of agronomic performance was implemented in all 12 field trials. Results indicated that there were only few effects of inoculation on growth, nutrition and yield of maize. The most important effects on grain yield were observed in the trial carried out in France in 2008 in a sandy soil, and in the fertility trial performed in Hungary. In the two trials, the fertilisation had a significant effect on grain yield but microbial inoculation had only a significant and positive effect at the lowest fertilisation rate in the sandy soil. However, quality control evidenced that certain inoculation products displayed low microbial counts. In 2009, there was not any significant positive effect of inoculation on grain yield in any of the trials, but some positive effects on the biomass at silage harvest were observed in the trial carried out on the clayey soil in France and in the two trials in Hungary. In many trials, the effects of fertilisation

were not pronounced either. In none of the trials there was a marked interaction between the effects of the fertilisation and inoculation. In conclusion, the 12 field experiments showed that inoculation with *Azospirillum*, *Pseudomonas* and mycorrhizae was rarely beneficial for maize production. Therefore, it is not recommended to seek a reduction in NP fertilisation using the consortia and inoculation technology used in this project, even though mineral fertilisation itself did not always have a significant effect on agronomic parameters.

Monitoring of crop contamination by mycotoxins

There was no effect of inoculation on *Fusarium* contamination of the cobs in the inoculation trials of the project. Further assessment was done in 2009, at the French field site selected for molecular analyses. First, natural *Fusarium* spp. infection was studied. No DON-producing species (*Fusarium graminearum* or *F. culmorum*) could be identified, whereas *F. verticiloides, F. proliferatum* and *F. subglutinans* were found. In parallel, DON and other B-trichothecenes (nivalenol, 15-ADON and 3-ADON) were not found by HPLC-UV analysis of kernels. Second, artificial *F. graminearum* infection was performed at flowering and was successful based on Fusarium symptoms on cobs and *F. graminearum* isolation and PCR confirmation. Inoculation did not have a significant effect on Fusarium infection parameters.

Monitoring of beneficial plant-microbe interactions

One of the field experiments carried out in France in 2009 served for further investigations based on molecular technology. PGPR inoculant monitoring at 16 days and 5 weeks showed that the *Azospirillum* strain in the *Azospirillum* treatment or in the consortium treatments had fallen below the detection limit, whereas *P. fluorescens* inoculants were found at 5 log per g or higher. Colonization of roots by *G. claroideum* at the first sampling was higher when inoculation was performed, and depended on the *Pseudomonas* strain inoculated. Effects were minor at the second sampling. Data on the expression of phosphate transporters of *G. intraradices* and *G. mosseae* did not show any statistically significant difference between treatments for both harvests.

Mesh bags containing soil labelled with ^{15}N and ^{33}P were buried around plants. 11 weeks later, no significant effect could be seen between the treatments on leaf dry matter production, phosphorus or nitrogen contents, and on transfer of labelled phosphorus and nitrogen from the bags to the leaves.

HPLC data of maize secondary metabolites, at 16 days after sowing, were subjected to discriminant PCA. This evidenced three main groups, i.e. (i) the non-inoculated control and *Azospirillum* treatment, (ii) the three-component consortium, the three two-component consortia, and (slightly away) the *Glomus* treatment, and finally (iii) maize inoculated with *Pseudomonas*. Thus, single inoculation already gave significant differences (except for *Azospirillum*) with control plants. Two and three-component consortia resulted also in clear differences, but it is interesting to note that consortium composition had a negligible effect. The most discriminant compounds were identified by chemical means.

1.4.7 WP6

Leader: Agrauxine

WP6 objectives

The objective was to communicate on the principle, technology and results of MicroMaize, as well as disseminate the technical and scientific findings of the project. Dissemination was to target the scientific community, regulatory authorities, the general public, the farming community and its partners. Possible technology dissemination and socio-economic acceptability of MicroMaize innovative microbial management were considered.

Demonstration action

Demonstrations at field trials were organized for farmers as well as representatives from private companies, public agricultural organisms, cooperatives and farmers associations in the two largest maize-growing European countries i.e. France and Hungary. A poster describing MicroMaize project and results was shown, and the MicroMaize brochure was distributed.

Dissemination of project results and technology
A plan for using and disseminating the generated knowledge was developed during the project. Dissemination was performed at several different levels, which included (i) publications in professional press and farmers newsletters, especially concerning results from field trials, (ii) seminars during farmers' and technical meetings, (iii) scientific presentations to students, (iv) scientific conferences and research publications targeting the research community, (v) discussions with seed companies and cooperatives to explore the potential for further technology development, (vi) the preparation of a project information brochure, and (vii) a joint meeting with EU STREP project RHIBAC for mutual information and exploring possibilities of collaborations.

Socio-economic acceptability
A report on the socio-economic acceptability of inoculation technology, as developed in MicroMaize, was prepared during the project.

1.5 Project relation to the state of the art

Soil microbes and beneficial plant-microbe interactions
The soil ecosystem is heavily colonised by a highly diverse community of microorganisms. The soil volume in contact with plant roots (termed the rhizosphere) is of particular interest, since it is a hot spot of microbial colonization and activity, where certain microbial populations play key plant-beneficial roles noticeably by protecting the plant from pathogens, fine-tuning plant hormonal balance, and/or improving the availability of nutrients to the plant [Kent and Triplett 2002; Somers et al. 2004]. Our knowledge of the

rhizosphere microbial community has drastically improved in recent years due to new methodology [e.g. DeAngelis et al. 2009], and MicroMaize has contributed to this through the development of new 16S microarray probes [Kyselkova et al. 2009], of PCR tools to monitor *Glomus* species, and the consideration of maize genetic diversity.

In the case of maize, the main symbiotic partners are arbuscular mycorrhizal (AM) fungi from the *Glomus* genus [Kabir et al. 1998], as well as plant growth-promoting rhizobacteria (PGPR) e.g. certain strains from *Azospirillum* and *Pseudomonas* [Dobbelaere et al. 2001; Bloemberg and Lugtenberg 2001]. Our understanding of these interactions is limited by a paucity of monitoring tools, and MicroMaize has contributed to fill this gap by developing quantitative PCR methods for inoculants strains [Couillerot et al. 2010; van Felten et al. 2010], especially for the first time for a *Glomus* strain.

Biostimulation and phytostimulation

Biofertilisation is carried out by plant-beneficial microbes that enhance nutrient availability in the soil [Riggs et al. 2001; Morrissey et al. 2002; Wu et al. 2005], noticeably biological nitrogen fixation by free-living bacteria e.g. the PGPR *Azospirillum* [Dobbelaere et al. 2001] and solubilization of phosphate or organically-bound phosphorus by PGPR and AM fungi [Kabir et al. 1998; Hamel 2004]. In this context, MicroMaize has contributed to a better understanding of P solubilisation by *Pseudomonas* PGPR.

In the case of phytostimulation, the microbe has a direct, positive effect on root ramification and growth, thereby enabling a better exploitation of the soil volume and thus indirectly enhancing uptake of mineral nutrients and water [Dobbelaere et al. 1999; Bloemberg and Lugtenberg 2001]. Phytostimulation results from microbial modulation of the plant hormonal balance. It involves the capacity of certain root-associated microbes (i) to produce phytohormones especially auxins [Dobbelaere et al. 1999], and/or (ii) to diminish plant production of the root growth-inhibiting hormone ethylene, which can be achieved by microbial deamination (i.e. consumption) of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) [Glick et al. 1998; Wang et al. 2000]. MicroMaize has contributed to a better understanding of the regulation of ACC deamination in *Pseudomonas* PGPR.

Biofertilisation and phytostimulation may take place naturally in the rhizosphere, but often the indigenous biofertilisers/phytostimulators are in insufficient numbers to act efficiently. However, new means have been proposed to control the composition and functioning of the indigenous soil microbial community [Chiarini et al. 1998; Russo et al. 2005]. Sometimes, this can

be achieved via the inoculation of crop plants with selected plant-beneficial microbes [Okon and Labandera-González 1994; Gianinazzi and Vosatka 2004]. Field results of inoculation trials have been highly variable in the past, but often these experiments have been carried out using only one strain in the inoculation product. Plant-beneficial microbes display significant compatibility with one another, and even synergistic effects [Marschner and Timonen 2005; Russo et al. 2005], making it important to test microbial consortia. MicroMaize has made a very significant contribution in this direction by assessing the plant-beneficial potential and biosafety of *Azospirillum Pseudomonas Glomus* three-component consortia, exploring procedures to enhance strain screening, and integrating the importance of maize genotype in determining plant response.

Assessment of beneficial effects

Plant-beneficial effects need to be confirmed in the field, under commercial farming conditions. MicroMaize conformed to this rule to a very large extent, by performing no less than 12 field trials over the last two years of the project. The specificity of the assessment done in MicroMaize was that in addition to standard agronomic monitoring, it included also the integration of biosafety issues related to mycotoxin contamination, as well as a molecular appraisal of interaction functioning in the field in terms of strain survival, inoculant effects and plant physiological response.

1.6 Impact of the project on the industry and research sector

The project MicroMaize will have several impacts on the research sector, which include:

- The development of new PCR tools to monitor inoculant survival ;
- The development of new fluorescent biosensors and the optimization of labelled nutrient monitoring to understand inoculant functioning ;
- The development of HPLC procedures to assess the physiological response of maize to inoculation ;

- New knowledge on the *Pseudomonas* plant-beneficial traits P solubilisation and ACC deamination ;
- New knowledge on the importance of matching microbial inoculants and maize genotypes ;
- New knowledge on the plant-beneficial potential of *Azospirillum Pseudomonas Glomus* three-component consortia ;
- New knowledge on the biosafety (i.e. lack of toxicology effects) of *Azospirillum Pseudomonas Glomus* consortium ;
- New knowledge on the functioning of *Azospirillum Pseudomonas Glomus* consortia under field conditions.

The project MicroMaize will have several impacts on the industry, especially for industrial partners of MicroMaize. This includes:

- The development of strain-specific tools enabling analysis of strains of commercial potential ;
- The identification of promising *Azospirillum Pseudomonas Glomus* three-component consortia ;
- The development of new formulations, which although not giving full satisfaction yet, will provide a starting base from which further improvement can now be sought ;
- New knowledge on the type of maize line responsive to microbial inoculation ;
- Toxicology assessment of promising *Azospirillum Pseudomonas Glomus* consortium, which is a prerequisite for registration and commercialisation ;
- Baseline information on the ecology of *Azospirillum Pseudomonas Glomus* consortia under agronomic field conditions.

2 SECTION II dissemination and use

Nb	Exploitable knowledge	Exploitable products or measures	Sector of Application	Timetable for commercial use	Patent of IPR protection	Owner and other partners involved
1	-	Microbial consortium	Agronomy	Not defined yet	-	Agrauxine, Symbio-m, ETH, CNRS