



Leibniz
Gemeinschaft



UMR Plante-Microbe-Environnement (Dijon, France)

and

**Leibniz-Institut für Gemüse- und Zierpflanzenbau
(Großbeeren, Germany)**

**Abstracts of the
Second annual joint meeting**

**6 September 2011
Embassy of the French Republic
Berlin, Pariser Platz 5**



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Methodologies

Mycorrhizosphere Research at the IGZ: Methodological advances in AM fungal extraradical mycelium studies

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Increased nutrient uptake of plants via the extraradical mycelium (ERM) of AM fungi is the most direct contribution these fungi make to plant growth. This process can lead to more intensive foraging of the growth substrate. Investigations into ERM development and function in soil are technically difficult and therefore still scarce. To overcome this challenge, we applied different compartmented planting systems which permit the separate study of intact ERM. In this contribution, we will give a short overview on applications of this methodology.

The application of “omic” approaches to distinct cell types of mycorrhizal roots to determine reprogramming processes during the arbuscular mycorrhiza symbiosis

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The development of AM symbiosis is an asynchronous process and the use of mycorrhizal roots for down stream analysis can mask cell-specific variations. Therefore we developed a cryotome/ laser capture microdissection (LCM)-mediated protocol to isolate distinct cells from mycorrhizal root tissue. The LCM technique was combined with state-of-the-art “omic” approaches to gain insight into mycorrhiza-associated reprogramming events. Cell-type specific RNA was employed for large scale transcript profiling by using GeneChip® Medicago Genome Array. The obtained dataset will be used for the functional characterization of mycorrhiza-dependent regulated candidate gene.

Furthermore comparative analysis of distinct cell-types was achieved using powerful analytical tools such as nHPLC-MS/MS and GC/TOF-MS for the identification of mycorrhiza-induced metabolites and proteins. We were able to find clear differences in symbiosis-dependent primary metabolism and protein accumulation in *M. truncatula* roots.

The combination of LCM with transcriptomics, proteomics and metabolomics can be successfully applied to understand cellular processes during the symbiotic interactions with a high spatial resolution. In general, region-specific information makes it possible to study other specific biological phenomena in a systems biology context.

Diversity

Plant Growth Promoting Rhizobacteria that are naturally associated with *Arabidopsis thaliana*

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The mechanism of plant growth promotion by rhizobacteria is poorly understood. We isolated various species of plant growth-promoting rhizobacteria (PGPR) that are naturally associated with *Arabidopsis thaliana* to investigate the molecular mechanisms that are involved in plant growth-promotion. We analysed the impact of these bacteria on growth and development of the host plant under various growth conditions and nutrient availability. Different physiological and biochemical parameters were determined and will be discussed here to characterise the interaction.

Compositional divergence and convergence in arbuscular mycorrhizal fungal communities

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Neutral models provide predictions about beta-diversity. This is valuable for organisms that are challenging to study under natural conditions (e.g. AMF). An extensive literature search allowed us to synthesise the information of data sets with the minimal requisites for creating a dynamic hypothesis in terms of beta-diversity. We calculated the first estimates of neutral parameters for several AMF communities from different ecosystems. Communities either diverge or converge with respect to compositional dissimilarity expected under neutrality, which informs on different assembly dynamics.

Molecular studies of diversity of populations of arbuscular mycorrhizal fungi

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Arbuscular mycorrhiza fungi (AMF) which form a mutualistic symbiosis with most terrestrial plants are not only important for natural ecosystems, but also for agricultural production. The aim of this study is to characterize fungal populations at different geographic scales in order to better understand the processes influencing AMF taxonomic and functional diversity and to determine the environmental factors affecting AMF populations, especially in the context of agricultural management. The mitochondrial large subunit gene of ribosomal RNA will be used as molecular marker to resolve population structure of *Rhizophagus irregularis* (formerly known as *Glomus intraradices*). Different sampling approaches are planned, combining sampling of geographically distant sites across France and field sites affected by different environmental factors such as disturbance, fertilization and agricultural management.

Functions of common mycorrhizal networks

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Extraradical fungal hyphae formed by arbuscular mycorrhizal fungi have many functions related to their ability to form extensive interconnected networks. Of these many functions, we focus on stabilization of soils due to improved soil aggregate formation by hyphae, and increased rates of information flow between organisms due to transport of organic infochemicals along hyphae. Aggregate formation is increased in the field and greenhouse by mycorrhizal fungi, with strong additional effects of hyphal grazers such as collembola. Transport of allelochemicals along hyphae greatly increases the bioactive zone of allelopathic plants and may be very important for plant-plant interactions in natural environments. Common mycorrhizal networks clearly play pivotal roles in many ecosystem processes.

Compatibility and Incompatibility

An apoplastic factor induces stomatal deregulation in *Vitis vinifera* leaves infected by *Plasmopara viticola*

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The obligate biotroph oomycete *Plasmopara viticola* is the causal agent of grapevine downy mildew and induces abnormal stomatal opening in infected leaves. Several arguments suggest that this deregulation is induced by a signal compound secreted by the oomycete or the plant during infection. To check this hypothesis, apoplastic fluids were obtained from control and *P. viticola* infected grapevine leaves and analysed with respect to their effect on stomata aperture. Only those obtained from infected leaves induced a significant increase. Further analyses hinted that the active compound is a protein of above 50 kDa and that o-glycosylation is essential for the activity. Nano-HPLC coupled to orbitrap mass spectrometry analyses revealed several candidate proteins responsible for this effect.

***Petunia hybrida*/Thielaviopsis basicola pathosystem for studying mycorrhiza-induced resistance in ornamental crop production**

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High chemical inputs associated with the development of industrial agriculture have become a major cause of environmental disorder, also in horticulture. Arbuscular mycorrhizal (AM) fungi represent a promising biological tool to turn resource-wasting into sustainable production. Since demand increases for ornamental crops produced under environmental-friendly conditions, we adopted *Petunia hybrida* as a system for basic and applied mycorrhizal research and established *Thielaviopsis basicola* as a pathosystem. *T. basicola* is a soil-borne fungal pathogen that causes black root rot and severe yield losses in many horticultural crops, including petunia. The ability of three different AM fungi to reduce root rot was tested: only *Glomus mosseae* efficiently suppressed the pathogen as compared to *Glomus intraradices* and *Gigaspora rosea*. The model system petunia/*G. mosseae*/*T. basicola* was therefore chosen for molecular investigations in order to identify genes involved in AM bioprotection using targeted approaches. RNA accumulation analyses could not identify genes which show a 'priming'-related expression pattern. Instead intrinsic up-regulation of a number of defence-related genes in mycorrhizal roots accompanied induced

resistance to the pathogen. Results are discussed concerning current hypotheses on the molecular basis for mycorrhiza-induced resistance.

Mycorrhizal effects on the interaction between sesame and broccoli roots in a model system

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The intra- and extraradical development of arbuscular mycorrhizal (AM) fungi may be affected by the presence of non-host plant roots. We studied this effect in a model intercropping system, using experimental boxes with three horizontal compartments. The AM host plant species sesame (*Sesamum indicum*) and the non-host plant species broccoli (*Brassica oleracea* var. *italica*) were grown with split roots, so that roots of two plants shared the central (combi) compartment, while the other part of each root system grew separately in an outer (solo) compartment. Combinations of sesame, broccoli and sesame and broccoli plants were cultivated in soil that was either mycorrhiza free (-M) or inoculated with *Glomus intraradices* (+M). Formononetin was applied to the (combi) compartment to study its effect on hyphae development.

At the time of harvest (nine weeks after transplanting), no effect of formononetin application was observed. Both sesame root colonization and extraradical hyphae development (extraradical mycelium dry weight and spore density) were negatively affected by the presence of broccoli in the (combi) compartment. The dry weight of sesame roots in the (combi) compartment was also decreased when broccoli roots were present. Mycorrhiza inoculation increased plant growth in sesame. Mycorrhiza also increased P and K concentrations in sesame shoots and alleviated the competition between two plant species to some degree. In contrast, the presence of mycorrhizal inoculum in soil had negligible effects on broccoli growth and nutrient uptake. In Brassicaceae, glucosinolates may affect the incompatibility between roots and AM fungi. Glucosinolate profiles and concentrations of individual glucosinolates were determined in broccoli leaves as well as in (solo) and (combi) roots. 3-Indolylmethyl glucosinolate in broccoli leaves was influenced by interactions of mycorrhiza and intercropping. All measured individual glucosinolates as well as total glucosinolates in (combi) roots were also affected by mycorrhiza or/and neighbour plant species, but a similar trend was not observed in (solo) roots. These results suggest that defence against AM fungi in non-host plants may be related to locally-induced changes in endogenous glucosinolate concentrations.

Calcium homeostasis in arbuscular mycorrhizal fungi: gene expression in compatible and incompatible interactions

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Interactions between arbuscular mycorrhizal fungi and plants begin with recognition and exchange of molecular signals which trigger morphological changes necessary for symbiosis. Evidence has shown that calcium is implicated in plant molecular responses leading to establishment and functioning of the mycorrhizal symbiosis but little is known about fungal genes involved in these processes. We selected seven fungal ESTs encoding Ca²⁺-related proteins from the transcriptome sequencing programme of *Glomus intraradices* which the laboratory is involved in (Tisserant et al. submitted). Fungal gene expression was monitored in pre-symbiotic stages and in a time-course study of developmental stages with compatible wild-type or incompatible mutant *Medicago truncatula* genotypes. Genes were expressed to different extents in spores and root interactions. Full length cDNA sequences of genes with interesting expression profiles and potentially involved in calcium homeostasis (intracellular signalling) are being cloned for functional analyses to gain insight into their role in the mycorrhizal symbiosis.

Transport and Metabolism

Analysing the molecular background of AM symbiosis

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Most plants live in symbiotic association with arbuscular mycorrhizal (AM) fungi. Since phosphorus is one of the most difficult nutrients for a plant to acquire and very critical for plant growth, the enhanced phosphorous nutrition is probably the most important benefit plants gain during AM symbiosis. However, there is growing evidence that a variety of other nutrients can be taken up via the symbiotic uptake pathway. Laser Capture Microdissection coupled with transcriptome analyses identified a set of plant transporter genes, which are strongly induced in arbuscule-containing cells but also in non-arbusculated cells of mycorrhizal roots. In addition we used deep sequencing of RNA isolated from arbuscule-containing cells and non-symbiotic fungal structures to identify fungal transporter genes, which are specifically expressed in arbuscules.

Regulation and function of *Mtha1* encoding a proton pump expressed in arbusculated cells

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Active transport of nutrients across biological membranes is driven by proton gradients formed by H⁺-ATPase. The *Medicago truncatula* gene *MtHA1* encodes such a proton pump and is expressed in cells colonised by arbuscules of mycorrhizal fungi. In order to study the function of the gene, transgenic plants with transposon insertions in *Mtha1* have been investigated concerning plant biomass, mycorrhization, P uptake and the RNA accumulation of several arbuscule-specific genes. Results underline the role of *Mtha1* for mycorrhizal functioning and for the expression of other genes.

Calcium homeostasis in arbuscular mycorrhizal fungi: gene expression in compatible and incompatible interactions

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Arbuscular mycorrhizal (AM) symbioses represent mutualistic relationships in plant roots, occurring in all plant lineages. Generally, 80% of land plants are engaged in mycorrhizal symbioses with fungi of the phylum Glomeromycota (Smith and Read, 2008). The exploration of surrounding soil done by the extraradical mycelium makes of the AM fungus a functional extension of the plant root system, providing enhanced nutrients availability to the plant in exchange of part of the photosynthates. These exchanges improve plant nutrition and increase growth and resistance to biotic and abiotic stresses of the host-plant (Smith & Read, 2008). In the last decade, more evidence has accumulated about how the use of mycorrhizal fungi might contribute to decrease fertilizer and pesticide use in agriculture and forestry.

The efficiency of mycorrhizal systems depends mainly on the ability of the fungal symbionts to uptake nutrients available in organic and/or inorganic forms in the soil and to move them (or related metabolites) to the symbiotic root. Therefore, a better understanding of the nutrients' transport mechanisms of the AM fungus might greatly help to identify easy-to-screen markers of a functional AM interaction.

An *in silico* screening of the EST database, generated by the International consortium for genome and transcriptome sequencing and annotation of *Glomus intraradices*, has been done using different keywords to search transport-related expressed genes (transportome). Among over 25 thousands contigs, 527 present in their IDs at least one of the keywords used.

Further analyses will allow a better understanding of the *G. intraradices* transportome for a further selection of genes, which might be used as fungal functional markers of the mycorrhizal interaction.

Carbohydrate assimilation and transport in mycorrhizal tomato plants

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Arbuscular mycorrhizal fungi are heterotrophic organisms depending on external carbohydrate sources and receive up to 20% of photoassimilates of the plant symbiotic partner. This is at least partially compensated, because mycorrhizal plants show increased photosynthetic activities and concentrations of soluble sugars. In frame of the project detailed

analyses have been carried out concerning parameters of carbon assimilation, the photosynthetic apparatus and of genes involved in carbon transport. Special attention was pointed to the tomato gene *LeSUT2* putatively encoding a sucrose transporter.

Effects of Plant Growth Promoting Rhizobacteria on primary plant metabolism

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We analysed effects of *Pseudomonas sp.* G62 on plant growth, gene expression and sugars in the host plant *Arabidopsis*. Inoculation with *Pseudomonas sp.* G62 promoted plant growth under various growth conditions. Microarray analysis revealed rapid changes in transcript levels that are similar to sugar starvation although plants were not depleted from soluble sugars. The expression of several of these genes remained stable over weeks, but appeared differentially regulated in roots and shoots. The attempt by the plant to overcome the assumed carbohydrate depletion could support the catering of carbon to the bacterium and may as well stimulate plant growth.

Application

INOCULUMplus

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INOCULUMplus is a spin off from the UMR Plant-Microbe-Environment which aims to develop arbuscular mycorrhizal inocula and associated services for their optimal use in order to promote new agricultural practices based on biotools rather than chemicals. Technologies involved: open and *in vitro* production of inoculants, methods of inoculation, assessment of viability and efficiency of arbuscular mycorrhizal fungi, molecular probes for tracing inoculants in the field. These technological developments are associated to educational activities towards food producers and consumers (www.inoculumplus.com).

Mycorrhiza fungi in Germany – Applications for today and tomorrow

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The application of mycorrhiza fungi is manifold: the symbiosis between plant and fungus is known to improve plant growth, tolerance against drought and bad soil conditions as well as pests and diseases. The benefits of the microorganisms are interesting for all fields of applications in garden- and landscaping as well as agriculture. Still the production of mycorrhiza inoculants has to face the unpredictable effectiveness of products in some applications. Our scientific projects therefore investigate the mechanisms of the symbiosis to utilize it e.g. in agricultural systems worldwide. We also focus on the optimization of production as well as formulation of products.

Mitochondrial genotyping of isolates of the arbuscular mycorrhizal fungus

"*Glomus intraradices*" from the isolate collection of the International Bank of

Glomeromycota

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The culture collection of the International Bank of Glomeromycota (BEG) provides an important service to scientific community and industry by acting as a repository for well-defined AMF isolates which are made available to the public. The most widely-used species

has been referred to as "*Glomus intraradices*", and was recently renamed *Rhizophagus irregularis*. This species has emerged as a model organism for experimental research and is the subject of the first AMF genome to be sequenced. The only genetic marker that can currently be used to distinguish strains of *R. irregularis* in field settings is the mitochondrial large subunit (mtLSU). In addition, genetically distinct strains of this species have been demonstrated to possess different symbiotic properties. In order to trace and recognize the different BEG isolates of this species, we characterized them genetically using the high-resolution molecular marker mtLSU. The results showed that the seven isolates we characterized from the collection corresponded to seven different mtLSU haplotypes.

A multigene phylogenetic study of *Tuber aestivum/uncinatum*

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Tuber aestivum and *Tuber uncinatum* share a complicated common history from a taxonomic point of view. Indeed, different previous studies have shown contradictory conclusions. For some authors, *Tuber aestivum* and *Tuber uncinatum* are one species and for others, they are two different species. In those studies, few phylogenetic markers have been used, mainly the ITS marker. However, it has become the standard in phylogenetic studies to combine several genes to obtain a more robust phylogenetic representation. In our study, we used 9 molecular markers and sampled fruiting bodies from different European populations including 14 *Tuber* "*aestivum*" types and 14 "*uncinatum*" types. The results show some genetic differences between samples for some of the analyzed sequences. However, no genetic differentiation that would support the separation of two different species was observed.

Arbuscular mycorrhizal fungi and rhizostabilisation of highly heavy metal contaminated orphan mining soils in southern France

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Arbuscular mycorrhizal fungi (AMF) are known to play a key role in plant tolerance to heavy metal (HM) stress. In the framework of the ANR (Agence Nationale de la Recherche) project "SyMetal", we are investigating the AMF diversity in soil and roots of the spontaneous vegetation present in extremely HM-polluted orphan mining soils in the region of Saint-Laurent-le-Minier, in southern France, and their potential for rhizostabilisation. Preliminary results, obtained using morphological and molecular methods targeting the AMF large

subunit (LSU) rDNA, showed a low presence of AMF in the roots of the analyzed plants and dominance of a few species, mainly belonging to the *Glomus* genus. Some of the identified taxa have already been reported in HM-rich environments, suggesting a specific AMF functional diversity. Experiments under greenhouse conditions are being performed in parallel in order to study the potential role of AMF under extremely high HM levels.



QualiRedFruits: A European effort to develop new agricultural practices for quality production for red fruits enriched in healthy compounds*

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The QualiRedFruits project is co-funded by the European Commission under the 7th Framework Programme. It brings together 7 SMEs and 5 universities and research centers, and deals with the competitiveness of raspberry production and market. The aims of the project are to create a new market of raspberry with higher quality and to improve the already existing one. This will be done by developing innovative cultural practices respectful of the environment – natural plant defense elicitor treatment and biotisation with beneficial microorganisms including arbuscular mycorrhizal fungi (AMF) – and identifying raspberry varieties with higher AOM content. Molecular markers will be developed for the identification of beneficial microorganisms and for the assessment of plant sanitary status. A cryo-preservation protocol will be developed for germplasm long-term conservation of varieties with higher quality. These approaches will be tested from *in vitro* culture to the field. Finally, recommendations for quality production of raspberry to SMEs will be drawn.

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