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León (Spain)
16th to 18th June 2015

XV National Meeting of the Spanish Society of Nitrogen Fixation
IV Portuguese-Spanish Congress on Nitrogen Fixation

The bridge between biotechnology, agriculture and environment

ABSTRACTS BOOK

Editors:
Fernando González-Andrés
Rebeca Mulas

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León (Spain) / From 16th to 18th June 2015

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Keynote lectures

Commercial applications and plant growth promotion abilities of *Azospirillum brasilense.*

Okon Y.

Department of Plant Pathology and Microbiology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot campus, Israel.

The inoculation of soybeans with Bradyrhizobium japonicum mainly using liquid inoculants has developed into a widely utilized commercial practice. About 40 million ha of soybeans are currently inoculated in South America. In the past 15 years, all over the world but mainly in Latin America, commercial inoculant companies have developed and tested in the field, inoculants containing free-living plant growth promoting rhizobacteria (PGPR) such as Azospirillum, Pseudomonas, Gluconacetobacter and Herbaspirillum, generally nominated as biofertilizers. The Azospirillum genus comprises free-living, plant growth-promoting, nitrogen-fixing bacteria found in the rhizosphere of plant roots. There have been ~300 reports of inoculation field experiments utilizing mainly Azospirillum brasilense liquid commercial inoculants, under varied climatic and soil conditions mainly in Argentina, Uruguay, Brazil and Mexico. In 2014, above ~2.5 million hectare of maize, wheat and other crops were inoculated with commercial inoculants of Azospirillum. To maintain high-quality inoculants, products are being approved and registered by government agencies, inoculant company associations and official agricultural research institutions. Successful inoculant products with 109 bacterial cells per ml, should posses a shelf life of 12 months. The main mechanism for plant growth promotion and yield increases by Azospirillum, has been reported to be in the range of 5-30% above non-inoculated fields mainly, at intermediate or adequate levels of N, P, K, microelements and water. Crop yield increase is derived from enhanced root development and efficiency in mineral and water uptake by roots. Production of indole-3-acetic acid and nitric oxide by the bacteria are recognized as important growth enhancing factors. In most Gramineae systems, the contribution of free-living biological nitrogen fixation to the N-nutrition of the plant does not seem to play a major role in plant growth promotion. Clear promotion effects on nodulation, nitrogen fixation and plant growth have been observed by Azospirillum and Rhizobium co-inoculation of legumes. Azospirillum was shown to promote in addition of root growth, the production of flavonoid signals by the legume, thus enhancing nodulation by rhizobia. Recent research has elucidated key properties of A. brasilense that contribute to its ability to adapt to the rhizosphere habitat and/or to promote plant growth. They include synthesis of auxin, nitric oxide, carotenoids, bacterial cell surface components, chemotaxis and the phenomenon of phenotypic variation. Storage and utilization of polyhydroxyalkanoate polymers are important for the shelf life of the bacteria in production of inoculants. Phase variation- or phenotypic variation- is one of the mechanisms by which microorganisms adapt to environmental changes. This phenomenon is characterized by the presence of a sub-population of the bacteria presenting a different phenotype relative to the major population. When plated on solid media, some A. brasilense Sp7 colonies were shown to possess a much more mucoid morphology, producing 7.5 to 8 times more exopolysaccharides (EPS) with different monosaccharide composition than the parental strain Sp7. The rate of appearance of this kind of variant colonies is of approximately 1 in 5,000, in agreement with the accepted rate for the phase/phenotypic variation phenomenon. The characterized EPS-overproducing variants were significantly more resistant to heat and UV-exposure than the parental strain and displayed genomic changes as seen by the different band patterns following ERIC-PCR, BOX-PCR and RAPD analyses. In plant inoculation experiments under greenhouse conditions, with maize, wheat, soybean and peanuts, these variants performed as similar as the parental strain. Therefore, EPS overproduction did not confer an apparent advantage to A. brasilense in terms of induction of plant growth promotion.

Recent chapters and books about the subject.

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Beta-rhizobial symbioses with legumes

James E.

The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

It has long been known that many species in the Leguminosae (Fabaceae) form a beneficial symbiotic interaction with N-fixing soil bacteria in the genus Rhizobium, as well as with other N-fixing bacteria in the Alphaproteobacteria class, such as Bradyrhizobium, Azorhizobium, Ensifer (Sinorhizobium), and Mesorhizobium (members of these genera are collectively termed “rhizobia”). In the last 14 years there has been increased focus on a newly-described group of bacteria that can nodulate legumes. These are not related to conventional “rhizobia” in the Alphaproteobacterial genus Rhizobium, but are all in the Betaproteobacteria, and are thus sometimes termed “Beta-rhizobia” (Gyaneshwar et al. 2011). The early studies of Beta-rhizobia suggested that they might have a close affinity with the large legume genus Mimosa (Mimosoideae), which contains >500 species, with Brazil as their main centre of radiation. This affinity has been demonstrated for Mimosa spp. in the Cerrado and Caatinga, which were almost exclusively nodulated by Burkholderia. Interestingly, relatives of Mimosa in the “Piptadenia Group” which is also centred in Brazil, were recently shown to be nodulated preferentially by Burkholderia genotypes similar (or identical) to those nodulating Mimosa (Bournaud et al. 2013). Therefore, based upon these studies it is reasonable to suggest that Brazil is a major centre of symbiotic Burkholderia diversity.

The legume-nodulating burkholderias are closely related to plant-beneficial, often diazotrophic, endophytic and environmental Burkholderia species and strains, but not to the phytopathogenic/pathogenic group of burkholderias (including the “B. cepacia complex”; Suarez-Moreno et al. 2012). It is considered that the legume-nodulating burkholderias may have originated from the environmental/endophytic ones. In the specific case of Mimosa, it is estimated to have emerged at approx. 30 mya, and then to have radiated within a number of centres in the Americas, but most notably in the acidic soils of the Cerrado and Caatinga biomes of Brazil, resulting in >200 species residing within them, many of which have been shown to be nodulated by a wide range of Burkholderia genotypes, some new and some already described (Bontemps et al. 2010; dos Reis Junior et al. 2010). It is thus reasonable to suggest that the ancestral Mimosa spp. and their relatives in the tribe Mimosae (Bournaud et al. 2013) encountered their ancestral Burkholderia symbionts in these biomes, and the two partners then diversified and coevolved with each other, thus resulting in the high diversity of both symbiotic partners now evident in both biomes (Bontemps et al. 2010). Similarly, in the acidic soils of the Cape Core Subregion (the Fynbos) of South Africa several genera of endemic legumes in the Papilionoideae (e.g. Cyclopia, Lebeckia, Rhynchosia, Podalyria) have diversified and evolved to nodulate with a group of non-Mimosa-nodulating Burkholderia spp. that possess nod genes that are phylogenetically closely-related to B. tuberum STM678 (Gyaneshwar et al. 2011; Lemaire et al. 2015). Indeed, the number of symbiotic species of Burkholderia has increased concomitantly as new legume symbiont diversity studies are regularly and continually being published, and this number will undoubtedly continue to increase as more centres of diversity (particularly those in the tropics and sub-tropics) are investigated.

Cupriavidus is closely related to Burkholderia, but unlike the latter genus it contains very few N-fixing species. Cupriavidus (syn. Ralstonia) taiwanensis was one of the first described Beta-rhizobial species, and has now been isolated from nodules on invasive M. pudica and M. diplotricha in many countries in SE Asia (China, India, Taiwan, Philippines) and Australasia (Papua New Guinea, New Caledonia), where it may often predominate as symbionts of these species (Gyaneshwar et al. 2011; Gehlot et al. 2013). Its predominance in some environments appears somewhat surprising, as M. pudica and M. diplotricha also have the potential to nodulate with Burkholderia, and the latter will outcompete C. taiwanensis in lab-based experiments (Elliott et soils.al. 2009). On the other hand, under particular soil conditions, such as when N-levels are increased from close to zero, the competiveness of C. taiwanensis is increased relative to Burkholderia (Elliott et al. 2009), and this most likely explains why it is frequently found in nodules on Mimosa growing in high(er) fertility neutral-alkaline

So from where does *C. taiwanensis* originate? Based upon its ability to nodulate *Mimosa*, the first places to look might be the centres of *Mimosa* radiation, such as the Cerrado and the Caatinga in Brazil, but, surprisingly, no *Cupriavidus* symbionts were found during the studies of Bontemps et al. (2010) and dos Reis Junior et al. (2010), which suggests that they have a different centre of origin to the *Mimosa*-nodulating burkholderias, and that unlike *Burkholderia* they may not have co-evolved with *Mimosa*. Indeed, this is supported by the fact that *C. taiwanensis* has a much reduced host range in the genus *Mimosa* when compared to *B. phymatum*, and also by the fact that most Brazilian species (including Cerrado/Caatinga endemics) cannot nodulate effectively (or at all in some cases) with *C. taiwanensis* (dos Reis Junior *et al.* 2010). It is possible that *C. taiwanensis* recently acquired its symbiotic ability from promiscuous *Mimosa*-nodulating burkholderias that were spread with invasive/widespread *Mimosa* spp. (such as *M. pudica*) throughout tropical and sub-tropical South America (Gyaneshwar *et al.* 2011), but most particularly in the coastal lowlands where they predominate. The recent description of other species of *Cupriavidus* that can nodulate legumes, such as *C. necator* in Brazil and Uruguay is interesting; it is known that *Mimosa*-nodulating *Burkholderia* generally prefer acidic soils, whereas symbiotic *Cupriavidus* prefer neutral-alkaline soils, so it is possible that *Mimosa* spp. that are endemic to neutral-alkaline non-Cerrado soils in South America are preferentially nodulated by *Cupriavidus* spp. Additional factors that may potentially favour *Cupriavidus* in the soils of metal-mining regions of South America are the presence of high levels of heavy metals, particularly of Zn and Cu. Indeed, a strong possibility that is now emerging as a candidate for a centre of *Cupriavidus* diversity is Uruguay (Cold et al. unpublished), where there are several endemic *Mimosa* spp. that appear to be nodulated by *Cupriavidus* strains in species that have previously not been shown to nodulate.

Fourteen years after the first description of Beta-rhizobia, they are now being shown to be highly diverse, and to comprise several groups (especially in the genus Burkholderia) that have affinities with tropical and sub-tropical American species in the Mimosoid tribe Mimoseae and in several South African endemic species in the Papilionoideae. The root nodulating ability of Betaproteobacteria may also be extended into new genera, such as Herbaspirillum and Achromobacter, but more conclusive data on their nodulation abilities are required to establish their status as true symbionts.

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Session 1

Ecology, diversity,
and evolution of Plant Probiotic
Microorganisms (PPM)

Analysis of the PGPB potential of bacterial endophytes associated to maize

Celador-Lera L[1], Menéndez E[1], Flores-Félix JD[1], Mateos PF[1, 2], Rivas R[1, 2]

[1]Departamento de Microbiología y Genética. Universidad de Salamanca; [2]Unidad Asociada de I+D Universidad de Salamanca (USAL)-CSIC (IRNASA).

Bacterial endophytes live inside plants for at least part of their life cycle because plants are very attractive as nutrient reservoirs for such bacteria. However, plants can need the presence of associated bacteria for their growth and adaptation to different ecosystems. This is the case of maize, which is a well-known cereal crop for hosting a great diversity of endophytic bacteria. Thus, microbes take advantage of the plant nutrients whereas plants receive benefits from associated bacteria, such as nitrogen and phosporous uptake (Hardoim *et al.,* 2008).

To study bacterial diversity, we isolated 69 strains of endophytic bacteria from stalk and root of maize (*Zea mays*) growing in a soil of Ciudad Rodrigo (NW Spain). Bacterial isolates were analysed by RAPD fingerprinting, allowing the differentiation among strains of the same species. Furthermore, we also obtained TP-RAPD profiles (Rivas *et al.,* 2001), which allow us to differentiate subspecies, by using two universal primers for 16S rRNA gene amplification. Our results showed the high diversity of isolates inside maize rhizosphere and endosphere. However, despite of the usefulness of RAPD and TP-RAPD profiles in bacterial diversity analysis, their suitability in taxonomy may be discussed due to the existence of different strains of the same species or subspecies. For this reason, 16S rRNA gene sequencing was carried out and subsecuently analysed.

Our results showed a great bacterial diversity of maize rhizosphere, including genera such as *Pantoea, Bacillus, Enterobacter, Serratia, Pseudomonas, Paenibacillus, Brevundimonas, Burkholderia, Leifsonia, Yokenella, Arthrobacter, Microbacterium, Erwinia, Staphylococcus, Curtobacterium* and *Streptomyces.* However, less grade of bacterial diversity was found in the endosphere, obtaining six genera: *Bacillus, Pantoea, Microbacterium, Sphingomonas, Agrococcus* and *Aerobasidium.* The CFUs average showed that bacteria preferably colonized plant parts closer to the soil than those of the steam tissues. This difference may be due to the existence of a transition zone, called rhizoplane, being able to accumulate a great number of different microorganisms, some of those may behave as endophytes, due to the possibility of colonizing new ecological niches, attracted by root exudates.

Moreover, we determined the ability of these strains to promote plant growth, performing *in vitro* PGPB mechanisms analysis: (i) phosphate solubilization, (ii) siderophores production.and (iii) IAA and/or precursors biosynthesis. These mechanisms confer to bacteria an important relevance for plant growth promotion. About 18% of the strains isolated had the ability to solubilize phosphate, 52.2% to produce siderophores and all of the strains were positive for indol-3-acetic acid (IAA) production.

Therefore, the infraespecific diversity of bacterial endophytes isolated from *Zea mays* is higher than we expected and these strains can promote plant development because of their abilities as PGPBs.

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High promiscuity of *Lotus corniculatus* in soils of northwest Spain.

Marcos-García M[1], Menéndez E[1], Cruz-González X[1], Mateos PF[1, 2], Rivas R[1, 2].

1Departamento de Microbiología y Genética. Universidad de Salamanca. 2Unidad Asociada de I+D Universidad de Salamanca (USAL)-CSIC (IRNASA).

Lotus corniculatus, commonly called birdsfoot trefoil, is a worldwide-distributed species, which tolerates a wide range of environmental conditions. Probably, this is the *Lotus* species most commonly employed for ecological restorations of soils affected by nutrient deficiency, salinity, drought, or contaminants. Root nodules are colonized by rhizobia establishing a metabolic cooperation between both partners, rhizobia reduce atmospheric nitrogen to ammonia, which is provided to the plant for further incorporation into organic compounds, and the plant provides with carbohydrates derived from the photosynthetic process to the bacteria, which can be used as carbon and energy sources (Escaray *et al*, 2012).

Little is known about the genetic diversity and phylogeny of rhizobia nodulating *L. corniculatus* in northwest Spain. Therefore, we have studied the bacterial diversity of *L. corniculatus* root nodules, collected in Carbajosa de la Sagrada (Salamanca, Spain). In this study, we isolated 90 strains from *L. corniculatus* nodules. Bacterial isolates were characterized and PGPR mechanisms, such as P solubilization, siderophores production and IAA biosynthesis, were tested. Also, cellulose and cellulase production and *in vitro* biofilm formation ability were determined. Our isolates presented high possibilities to be used as inoculants in *L. corniculatus* crops, as well as for other legume and non-legume crops. Approximately, 55% the strains belong to different species of the genera *Micromonospora, Dermacoccus, Arthrobacter, Methylobacterium, Acinetobacter, Lysinibacillus* and *Paenibacillus.* Interestingly, 45% of the isolates belong to different species of the genus *Mesorhizobium*, as *M. loti*, *M. tianshanense* or *M. ciceri*. It is well-known *M. loti* is the compatible symbiont of *L. corniculatus*. Previous studies have shown there is a high bacterial diversity in *L. corniculatus*, most of them belongs to the genus *Mesorhizobium* (De Meyera, *et al*., 2011). To the best of our knowledge, there are few studies which confirm those *Mesorhizobium* strains are able to renodulate *L. corniculatus* (Ampomah and Huss-Danell, 2011). Here we report a comparative analysis of nodulation ability and effectiveness by performing nodulation tests with our isolates, which belonged to the genus *Mesorhizobium*. Although all of the *Mesorhizobium* isolates tested were able to form N_2-fixing nodules, their symbiotic effectiveness was diverse.

Our results show an unexpected promiscuity in *L. corniculatus,* which is nodulated by other *Mesorhizobium* species different from the compatible symbiont *M. loti.*

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Genome-scale analysis of Pseudomonas fluorescens complex phylogeny reveals eight ecophysiologic groups and provides information about specific traits related to biocontrol and bioremediation

Garrido-Sanz D, Martín M, Rivilla R, **Redondo-Nieto M**.

Departamento de Biología. Facultad de Ciencias. Universidad Autónoma de Madrid.

The *Pseudomonas fluorescens* complex includes *Pseudomonas* strains described as plant growth promoting rhizobacteria (PGPR) with potential applications in biocontrol and biofertilization. So far, phylogeny of this group has been established according to 16S rRNA sequence, multilocus sequence analysis (MLSA) and phenotypic traits. However, frequent taxonomic rearrangements have resulted in a poor definition of the complex. In the last years, the genome of a large number of strains has been sequenced showing important genomic heterogeneity and providing information suitable for phylogenomic studies. Previous MLSA and phylogenomics have divided the *P. fluorescens* complex into several groups (Loper et al, 2012, Redondo-Nieto et al, 2013), but their species status have not been established yet.

Based on MLSA and phylogenomic analysis of 93 strains, we have divided the *P. fluorescens* complex into 8 groups. ANI and TETRA indexes agree with this division and strongly suggest the adscription of the strains to different ecophysiologic groups. Using the current GOLD standard for prokaryotic species definition, an ANI cut-off value of 95%, we can clearly differentiate 68 species. Nevertheless, an ANI cut-off value of 87,5% supports the existence of 8 groups named as following: *P. mandelii* (group I), *P. jessenii* (group II), *P. koreensis* (group III), *P. corrugata* (group IV), *P. fluorescens* (group V), *P. gessardii* (group VI), *P. chlororaphis* (group VII), and *P. protegens* (group VIII).

Cluster of orthologues analysis of the lineage specific genome for each of the groups has shown congruence between the phylogenomic determination of these groups and its putative phenotypes. When comparing all the selected strains the central core genome is composed by 1334 orthologues (~23% genome size average). These values rises up to ~59% when determining each group core genome. Lineage-specific genome for each group, that is, the specific orthologues within each group, corresponds to a 3,82% average. These analysis has shown that best strains for biocontrol are likely to correspond to *P. corrugata, P. chlororaphis* and *P. protegens*, while *P. jessenii, P. gessardi, and P. koreensis* strains appear more suited for bioremediation/rhizoremediation applications.

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Coexistence of N2-fixing and non-fixing rhizobia in the legume-Rhizobium symbiosis: explanations from modelling and experiments.

Marco DE.

Departamento de Matemática, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Ciudad Universitaria, CC 509, 5000 Córdoba, Argentina, and CONICET.

Mutualistic and symbiotic interactions impose challenging ecological and evolutionary problems. Their origin and persistence in nature is difficult to explain since the existence of exploitative, 'cheating' partners that could erode the interaction is common. Host (plant) sanctions against non N_2 fixing, cheating symbionts have been proposed as a force stabilizing mutualism in legume-*Rhizobium* symbiosis. Penalizations would include decreased nodular rhizobial viability and/or early nodule senescence in nodules occupied by cheating rhizobia.

I analyze the ecological and evolutionary stability of *Rhizobium*-legume symbiosis when "cheating" strains are present, using a combination of experiments and mathematical modelling. In different experiments, soybean plants were inoculated with two rhizobial strains, a cooperative, normal N_2 fixing strain and an isogenic non-fixing, “perfect” cheating mutant derivative that lacks nitrogenase activity but has the same nodulation abilities. Based on these experiments, a population dynamic model with and without the inclusion of plant host sanctions was developed.

No experimental evidence of functioning plant host sanctions to cheater rhizobia was found. Plant populations persist in spite of the presence of cheating rhizobia without the need of incorporating any sanction against the cheater populations in the model, under the realistic assumption that plants can at least get some amount of fixed N_2 from the effectively mutualistic rhizobia occupying some nodules. Inclusion of plant sanctions merely reduces the time needed for reaching plant population equilibrium and leads to the unrealistic effect of ultimate extinction of cheater strains in soil. Different factors were added to the model to resemble realistic field conditions. For example, competition for nodulation and co-occupation of the same nodule by strains with different fixation abilities are important sources of concern in cultivated legumes. The effect of plant stress was also included in the model. The potential consequences for agricultural practices like artificial inoculations when strains with different fixation abilities are present are discussed.

Endophite community of *Pantoea* genus in Guadalquivir Marshes rice paddies. Proposal of a *Pantoea ananatis* subsp. *oryzae* as a new subspecies of *Pantoea ananatis*

Megías E[1], Reis F[2], Márquez MC[1], Ollero FJ[3] y Megías M[1].

[1]Universidad de Sevilla. Departamento de Microbiología y Parasitología. [2]Embrapa Cerrados. [3]Universidad de Sevilla, Departamento de Microbiología.

The genus *Pantoea* belongs to the family *Enterobacteriacea* and it is worldwide distributed. Bacteria belonging to this genus have been commonly described as endophytes in rice plants. According to literature, several strains from this genus have beneficial effects in their hosts, either by direct effects (Plant-Growth Promoting activities) or indirect effects (acting as biocontrol agents). The main goal of this work is to determine the diversity of endophytes belonging to the genus *Pantoea* in rice paddies from Guadalquivir Marshes and to find strains with potential to promote plant growing. In order to do that, 150 strains were isolated from rice plants following an endophyte isolation method. DNA from all strains was isolated using a commercial kit (NucleoSpin® Tissue) and 16S rRNA gene was amplified for all strains using the universal primers 16SF27 and 16S1488R. PCR products were sequenced and analysed using *EzTaxon* platform. 26 strains were preliminarily identified as belonging to the genus *Pantoea* and were further examined using a polyphasic approach to properly classify the isolates within the genus. MLSA assay included the analysis of a concatenate sequence comprised by sequences from four housekeeping genes: *atp*D, *gyr*B, *inf*B and *rpo*B. Each individual sequence was also analysed. Phenotypic analysis consisted on API 50CH and API 20E test strips. The structural characteristics studied were acid composition and DNA base distribution. DNA-DNA hybridization was performed on selected strains using the competition procedure by Johnson (1994).

MLSA analysis showed that isolated strains were included in the following species: *Pantoea dispersa* (1 strain), *Pantoea deleyi* (1 strain) and *Pantoea ananatis* (6 strains). There was a group of 18 strains forming an independent clade closely related to *P*. *ananatis*. Phenotypic tests results resembled the phylogenetic grouping of the clades. In order to determine if the new clade defined a new species, structural characteristics were studied on selected strains. According to these results, there were no significant differences with *P. ananatis*. Finally, DNA-DNA hybridization results proved that the new clade wasn't a new species, being closely related to *P. ananatis*. However, due to their phenotypic differences and their phylogenetic position constituting a particular clade, we propose the definition of a new subspecies named *P. ananatis* subsp. *oryzae*, based on the plant it was isolated from. Thus, we propose that species *P. ananatis* should be constituted by the subspecies: *P. ananatis* subsp. *ananatis* and *P. ananatis* subsp. *oryzae*.

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Analysis of cultivable endophytic bacteria in roots of maize in a soil from León province in mainland Spain

Menéndez E [1], Ramírez-Bahena MH[2,3], Peix A[2,3], Tejedor C[1], Mulas R[4], González-Andrés F[4], Martínez E[1,3], Velázquez E[1,3].

[1]Departamento de Microbiología y Genética. Universidad de Salamanca. Spain, [2]IRNASA-CSIC. Salamanca. Spain, [3]Unidad Asociada Universidad de Salamanca- CSIC 'Interacción Planta-Microorganismo'. Salamanca. Spain, [4]Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad. Universidad de León. León. Spain.

Endophytic microorganisms constitute the plant microbiome that most directly can affect the growth and health of plants, due to the location of these microorganisms in inner tissues (Gaiero et al. 2013). The strict endophytic bacteria can be transferred from parental plants to their progeny through the seeds of the vegetative parts of the plants, whereas the falcultative endophytes can enter the plant along its life cycle (Reinhold-Hurek & Hurek, 2011). Only cultivable and facultative endophytes can be used as biofertilizers and also, they must be non-pathogenic for humans, animals or plants (García-Fraile *et al.*, 2012), making necessary their identification before their use in biofertilization schemes. In the present study, we isolated 18 endophytic bacterial strains from maize roots in a soil located at León (NW Spain), cultivated with maize. The strains isolated from maize displayed 15 RAPD different profiles, showing the high genetic diversity of the endophytic bacteria. Representative strains from each RAPD type were identified on the basis of the 16S rRNA gene sequencing that showed that most of isolates belong to gamma-Proteobacteria and specifically to the families *Enterobacteriaceae* and *Pseudomonadaceae* (genus *Pseudomonas).* Some of the isolated strains belong to species that can be pathogenic for humans, such as *Pantoea agglomerans*, *Pantoea ananatis*, *Enterobacter amnigenus*, *Enterobacter cloacae* and *Rahnella aquatilis* or for plants, such as *Pseudomonas corrugata* or *Pseudomonas brassicacearum*. These results showed the need of a correct identification of bacterial endophytes before selecting strains for biofertilizers design.

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Salt tolerance of rhizobial populations from contrasting environmental conditions: understanding the implications of climate change

Cardoso P[1], Freitas R[1], **Figueira E**[1].

[1]BiologyDepartment & CESAM, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal.

It is predicted that global climate change may alter environmental parameters such as rainfall distribution which in turn may alter the salinity of soils with unpredictable effects upon soil microbial populations. In the present work the tolerance to salinity of rhizobia, isolated from locations with contrasting climatic conditions, and the potential of strains to fix nitrogen symbiotically under saline conditions were investigated. Since plasmids may encode key genes related to growth and survival under environmental stress conditions, which will reflect on protein synthesis, both the plasmid and protein profiles were analysed. A multivariate statistical approach related salt tolerance to the origin of the isolates, identifying rainfall and water availability as a possible factor explaining the differences in salt tolerance displayed by rhizobial isolates. The classification analysis allowed the subdivision of isolates in terms of salt tolerance into extremely sensitive (<0.15 %), sensitive (0.15–0.6 %), moderately tolerant (0.9–1.5 %), tolerant (2.1–3.6 %) and extremely tolerant (≥5.4 %). Taken all together the tolerance of rhizobial populations to salinity is wide, changing spatial (different locations) and temporally (Alentejo late spring and late autumn). The high sensitivity of the legume-rhizobia symbiosis to salinity has long been recognized. Our study showed that salinity severely affected the efficiency of N_2 fixation and that the salt tolerance of strains impacted differently the symbiosis efficiency, under salt conditions. Inoculation with sensitive and extremely sensitive strains had higher impact (70 %) on symbiosis efficiency than tolerant strains, especially with extremely tolerant strains (40 %). These results prove that the salt tolerance of the microsymbiotic partner influences the symbiosis performance. It is generally accepted that the most sensitive symbiotic partner to salinity is the host, i.e. the legume. The results of our work are not in accordance to these reports, since all isolates from the three locations in northwest Portugal displayed higher sensitivity than the pea cultivar used in this work.

Results obtained in this work highlight that alterations of the present climate conditions leading to salinity osmotic increase in the soil may severely affect rhizobial populations either in free-living or in symbiosis with a legume host, evidencing that environmental conditions selecting for osmotic tolerance, also select for salinity tolerance. The salinity tolerance can only be achieved by metabolic changes, which are reflected in protein profile alterations. Our results suggest that rhizobial populations from southeast Portugal can withstand changes in soil water availability, but the populations from northwest Portugal cannot. If the predicted scenario of dry summers, described as inevitable in the context of global changes for southern Europe materializes, northwest populations will be more susceptible to changes in soil water availability. Our work illustrates the impact of the climate change predicted (lower rainfall,increased variability in water supply, increased salinity) on ecological systems with different salt sensitivities, highlighting the need to consider not only the nature of the climate change, but also the vulnerability of a given system when predictions of climate change impact are made.

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Identification of human pathogenic bacteria in maize roots by using MALDI-TOF MS methodology

Velázquez E[1], Menéndez E[1], Sánchez F[2], Valencia N[3], Pérez J[3], León-Barrios M[3], Pérez R[3], Garrido A[5], González JM[2,4].

[1]Departamento de Microbiología y Genética. Universidad de Salamanca. Spain, [2]Instituto de Investigación Biomédica de Salamanca (IBSAL). Salamanca. Spain, [3]Departamento de Bioquímica, Microbiología, Biología Celular y Genética. Universidad de La Laguna. Tenerife. Canary Islands. Spain, [4]Departamento de Bioquímica y Biología Molecular. Universidad de Salamanca. Spain. [5]Granja Agrícola Experimental. Área de Agricultura y Ganadería. Cabildo de Lanzarote. Canary Islands. Spain.

MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry) is a reliable method for bacterial identification mainly applied to date to clinical samples (Patel, 2015). Taking into account that bacterial endophytes can include pathogenic species (Rosenblueth and Martínez-Romero, 2006) and that all cultivable bacterial species causing disease in humans, animals or plants are included in the Biotyper 3.0 database, MALDI-TOF MS analysis can be an excellent tool for the recognition of pathogenic bacteria inhabiting plants. Therefore in this work we used this methodology to analyse a wide collection of endophytic bacteria isolated from maize roots in Canary Islands in order to identify the pathogenic species present in the microbiome of this plant. The results obtained showed the presence of several human pathogens, such as *Bacillus cereus*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pantoea agglomerans*, *Pseudomonas aeruginosa, Pseudomonas fulva*, *Pseudomonas mendocina*, *Pseudomonas mosselii* and *Staphylococcus epidermidis.* This unexpected high number of cultivable human pathogenic species among the isolated bacteria is a wake up call to researchers to perform carefully analyses of plant endophytes before using them as biofertilizers in order to avoid risks for both consumers and biofertilizer handlers (García-Fraile *et al.*, 2012). Our results showed that MALDI-TOF MS is an useful technique in the process of characterization of endophytic bacteria, allowing to discard strains belonging to pathogenic species that cannot be included in biofertilization schemes.

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Analysis of *in vitro* plant growth promotion mechanisms of endophytic bacteria isolated from maize and wheat roots in Northern Spain

Peix A[1,2], Menéndez E[3], Ramírez-Bahena MH[1,2], Flores-Félix JD[3], Tejedor C[3], Mulas R[4], González-Andrés F[4], Martínez E[2,3], Velázquez E[2,3].

[1]IRNASA-CSIC. Salamanca. Spain, [2]Unidad Asociada Universidad de Salamanca- CSIC 'Interacción Planta-Microorganismo'. Salamanca. Spain, [3]Departamento de Microbiología y Genética. Universidad de Salamanca. Spain, [4]Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad. Universidad de León. León. Spain.

Endophytic bacteria can promote plant growth through direct mechanisms such as production of the phytohormones indole acetic acid, the enzyme ACC deaminase involved in the metabolism of 1-aminocyclopropane-1-carboyclic acid (ACC), a precursor of ethylene, or the mobilization of nutrients such as nitrogen via nitrogen-fixation or phosporous via solubilization of soil insoluble phosphates. Also, some endophytic bacteria present indirect mechanisms of plant growth since they produce microbial inhibitory compounds such as siderophores, Fe^{+3} ion-chelating molecules, that inhibit the growth of phytopathogens in soils with low content of this ion promoting indirectly plant growth (Gaiero *et al.*, 2013). Some PGPR (plant growth promoting rhizobacteria) endophytic of maize have been isolated in America (Montañez *et al.*, 2012), but there are few data about the abilities as plant growth promoters of endophytic bacteria from cereals such as maize or wheat in other soils. Therefore, in the present work we analysed the *in vitro* mechanisms of plant growth promotion in endophytic bacteria isolated from roots of maize and wheat growing in two soils from Northern Spain. The results showed that several of these strains were able to grow in nitrogen-free media, to solubilize phosphate in media containing insoluble P sources, to produce ACC desaminase, indole acetic acid or siderophores. Therefore non-pathogenic endophytic bacteria present in cereal roots are good candidates to be used as biofertilizers.

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Identification of rhizobial strains nodulating *Pisum sativum* in Northern Spain soils by MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) analysis

Martínez E[1], Sánchez F[2], Carro L[1], Flores-Félix JD[1], Martínez-Hidalgo P[1], Cerda E[1], González JM[2,3], Velázquez E[1].

[1*]Departamento de Microbiología y Genética. Universidad de Salamanca. Salamanca. Spain, [2]Instituto de Investigación Biomédica de Salamanca (IBSAL). Salamanca. Spain, [3]Departamento de Bioquímica y Biología Molecular. Universidad de Salamanca. Spain.

MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry) is a reliable method for bacterial identification mainly applied to date to clinical isolates (Patel, 2015). In year 2011 we extended by first time the database Biotyper 2.0 to include the species from family *Rhizobiaceae* (Ferreira *et al.*, 2011) and now we have added to this database several recently descibed species of genus *Rhizobium.* In this work we used by first time MALDI-TOF MS and the new extended database for identification of strains nodulating *Pisum sativum* L. (peas), a legume very appreciated due to the high nutritional value of its seeds for human and animal feeding. The strains were isolated in soils from Valladolid, Salamanca and León, three provinces of Castilla y León where the 30% of the total Spanish production of peas is located. In the soil from Valladolid *Pisum sativum* is commonly cultivated, in the soil from Salamanca the traditional crop is *Lens culinaris* and in León soil *Pisum sativum* has been never cultivated. The results obtained after MALDI-TOF MS analysis showed a correct identification, with score values higher than 2.0, for most of the strains analysed which were identified as *Rhizobium leguminosarum* and *Rhizobium laguerreae*, a recently described species originally isolated from nodules of *Vicia.* The results of this work confirmed MALDI-TOF MS as a reliable technique for identification at species level of fast-growing rhizobia being able to differentiate between sister species that are not distinguishable on the basis of 16S rRNA gene analysis as occurs in the case of *R. leguminosarum* and *R. laguerreae* (Saïdi *et al.*, 2014).

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Pyrosequencing reveals differences in bacterial endophyte communities from roots of maize grown in the Quechua region of the Peruvian Andes

Correa-Galeote D[1], Arone GJ[2], Bedmar EJ[1].

1 Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Agencia CSIC. Granada. 2 Departamento de Ciencias Agrícolas, Universidad Nacional de Huancavelica, Acobamba (Huancavelica, Peru).

Endophytes are organisms able to colonize plant organs and their infection is generally inconspicuous. Beneficial endophytic bacteria are of particular interest in promotion of plant growth and control of plant disease. Maize (Zea mays L.) is after rice and wheat the third most important agronomic crop in terms of world production. In the Quechua region of the Peruvian Andes, native peasants use small (200-10,000 m2) plots called “chacras” to grow maize, pea, wheat, potatoes, etc. Genetic and archaeological data indicate that maize spread out from Mexico reaching the Andean highlands some 4,000 years ago (Perry et al. 2006). Since then, maize is the staple diet of the Quechua natives who continue growing it as did their ancestors, mostly without chemical fertilization and no irrigation, and yet chacras maintain a sustainable production for years. Previous studies have assessed endophytic bacterial diversity in maize roots, but none of them provided an analysis of bacterial diversity estimated by using massive parallel 16S rRNA gene tag sequencing. Here, we analyse the composition of the bacterial endophytic communities inhabiting inside roots of maize plants grown in fallow soils and maize-cropped soils from the Quechua maize belt.

Lateral roots were taken from maize plants grown in 2 chacras with fallow soil and other 2 chacras with maize-cropped soil. DNA was extracted from 500 mg of root tissue as indicated earlier (Correa-Galeote et al. 2013). PCR amplification of the hypervariable V4-V5 regions of the 16S rRNA gene was performed with an 8 base pair key-tagged sequence joined to universal primers U519F and U926R (Baker et al. 2003). For each sample, amplicons were generated in several replicate PCRs. Amplicons of the same treatment were pooled to reduce per-PCR variability and purified. After purification, the samples were combined in equimolar amounts and subjected to pyrosequencing with the Genome Sequencer Titanium GS-FLX 454 system. After cleaning of the sequences using the RDP Project, operational taxonomic units (OTUs, 3% sequence divergence) were estimated using MOTHUR and a taxonomic description of each OTU was assigned by the comparison of these sequences with the previously deposited in NCBI database. Statistical analyses were run using XLSTAT.

The number of OTUs from root extracts of maize plants grown in fallow soil and maize-cropped soil were 80 and 140, respectively, with 35 OTUs in common, of which 15 OTUs represented more than 60% of the bacterial sequences and were used for statistical analyses. NMS assays of the relative abundance of the 15 OTUs from each sample indicated the existence of a high variability between samples from the fallow soil and that samples from maize-cropped soils had much more similarity between them. These results suggest that maize plants somehow select their endophytes from the total bacterial populations in the plant rizosphere. Regardless of the precedence of the samples, either from fallow or maize-cropped soils, major OTUs found were related to genera Burkholderia, Sphingomonas, Herbaspirillum, Dyella, candidatus Glomeribacter gigasporarum, Bradyrhizobium, Variovorax, Methylophilus and Streptococcus. According to the Levene test ($\alpha = 0.10$), except for genera Burkholderia and candidatus G. gigasporarum, relative abundances of sequences corresponding to the remaining seven genera were higher in plants grown in maize-cropped soils.

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Study of bacteria diversity in different zones of the Lebrija marshes

Vasseur M[1], Barcia-Piedras JM[1], Redondo-Gómez S[2], Rodríguez-Llorente ID[3], Aguado A[1], **Camacho M**[1].

1 IFAPA, Centro Las Torres –Tomejil. Ctra Sevilla-Cazalla,km 12.200. Alcalá del Río, 41200. Seville (Spain), 2 Facultad de Biología. Universidad de Sevilla. Avda Reina Mercedes s/n, 3 Facultad de Farmacia. Universidad de Sevilla. Profesor García Gonzalez 2.

Saline environments resulting from periodic seawater inundation occur on coasts worldwide. Only a few highly salt-tolerant species (halophytes) are able to survive and flourish in coastal salt marshes and salt steppes. Depending on tidal and evapotranspirational regimes, such plants may experience salinities two- to three-times higher than seawater for protracted periods (Redondo-Gómez *et al*., 2010). In the European Union, the Directive 92/43/EEC (European Commission, 1992), also called ‘‘Habitats Directive’’ (HD), was created to respond to a continuing deterioration of European natural habitats and an increasing number of seriously threatened wild species. It remains the single most important European Union instrument for safeguarding biodiversity. Salt marshes are not only “HD” priority habitats but also badly in need of protection. From the 1970s onward, the extent of salt marshes in Spain has been dramatically reduced due to the heavy pressure from man-induced activity (agricultural practices and tourist development). *Arthrocnemum macrostachyum* is an evergreen halophilous shrub typical of the Mediterranean salt marshes. It can tolerate occasional flooding and frequently occurs in tidal and inland salt marshes in the South of Spain (Gómez Mercado *et al*., 2014).

Plants live in close association with an enormous diversity and abundance of soil microorganisms that interact with roots and in many cases can promote the plant growth and fitness of plants (Berendsen *et al*., 2012). Microbial communities under natural vegetation may specifically be adapted to their host plant and the local soil conditions.

A research project was initiated at Lebrija marshes (SW Spain), using *A. macrostachyum* plants, aimed at i) recovering for plant cultivation saline regions of poor or no productivity and ii) providing alternative crop production in areas which are limited by salinity. In a first step, the microbial biodiversity present in the rhizosphere of *A. macrostachyum* in their natural habitat is being studied using the PCR-DGGE technique. So that, the main goal of this work has been to study the microbial communities in the Lebrija marshes and highlight differences between zones with different saline irrigation water of the marsh as well as the microbiota changes due to the seasons, not only in the rizosphere of *A.macrostachyum* plants, but also in non rizospheric soil.

Subsequently, the most abundant bacteria in the rhizosphere will be isolated and those possessing a plant growth promoting activity will be selected, and will be used, individually or in consortium, to coinoculate *A. macrostachyum*. The purpose is to optimize the plant development.

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Diversity of medic rhizobia in Egypt is marked by dominance of two genetic types

El Batanony NH[1], Castellano-Hinojosa A[2], Correa-Galeote D[2],**. Bedmar EJ**[2]

[1*] Environmental Studies and Research Institute (ESRI), University of Sadat City, Sadat City, Menoufiya, Egypt. [2] Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC. Granada, Spain.

Nitrogen (N) is the most significant yield-limiting element in many agricultural production systems. External inputs of N to agriculture may come from inorganic fertilizers, the production of which is heavily dependent on fossil fuels. Bacteria belonging to the family *Rhizobiaceae* fix atmospheric dinitrogen (N_2) through symbiotic association with legumes. Globally considered, about 44-66 million tons of nitrogen is biologically fixed annually, which provides nearly half of all requirements used in agriculture. Due to its considerable agricultural and environmental significance, the legume-symbionts are being extensively used as an alternative of synthetic fertilizers to supply nitrogen requirements of plants in agro-ecosystems.

Medic plants, *Medicago*, *Melillotus* and *Trigonella*, play an important role for forage and medicinal products in Egypt. However, establishment of medics in pasture systems is truly a challenge as different biotic and abiotic stresses can affect both the plants and their N_2-fixing symbiotic partner. With the aim to estimate the natural medic rhizobia diversity in relation to its geographical location, a countrywide diversity study was conducted. Twenty four bacterial strains, 12 from species of the genus *Melilotus*, 8 from *Medicago* and 4 from *Trigonella* were isolated from extracts of nodules taken from healthy, wild-grown plants in different locations in Egypt. The nearly complete sequence of the 16S rRNA gene from each strain revealed that 24 strains were members of the family *Rhizobiaceae* of the Alphaproteobacteria and that the remaining 4 strains belonged to non-rhizobial strains. Among the *Rhizobiaceae*, 21 strains classified into genus *Ensifer* and 3 belonged to genus *Rhizobium*. The ML phylogenetic tree and EzTaxon-e analysis inferred from the 16S rRNA gene sequences indicated that strains NHBM3B, NHBM5, NHBM10, NHBM10B and NHBM13 from *Melilotus (Mel) indicus*, NHBM16 from *Mel. messanensis*, NHBM18 from *Mel. siculus*, NHBM23 from *Medicago (Med). intertexta* and NHBM24 from *Med. polymorpha* clustered with *E. medicae* WSM419^T with identity values higher than 99.0%. Strains NHBM9, NHBM12 and NHBM14 from *Mel indicus*, strain NHBM17 from *Mel. messanensis*, strain NHBM19 from *Mel. siculus*, strain NHBM22B from *Med. intertexta*, and strains NHBM26 and NHBM27 from *Med. laciniata* grouped with *E. meliloti* LMG6133T with identity values higher than 99.0%. Strains NHBTR69, NHBTR70, NHBTR72 and NHBTR74 isolated from *T. maritima* also clustered with *E. meliloti* LMG6133^T with identity values higher than 99.0%. In addition to genus *Ensifer*, strains NHBM21 NHBM25 and NHBM29 were isolated from nodules of *M. sativa*, *M. polymorpha* and *M. laciniata*, respectively, that showed 100% identity with *R. huautlense* SO2^T type strain.

Amplification of the *nodC* gene from representative strains of the rhizobial species isolated in this study indicated that those of genus *Ensifer* had more than 99% identity with the corresponding *Ensifer* type strains, *E. meliloti* LMG 6133^T and *E. medicae* WSM419^T, and that those of genus *Rhizobium* were similar to *R. huautlense* S02^T with identity values of 100%.

In addition to rhizobial species, 4 strains were also isolated from nodules of *M. indicus* whose members belonged to genera *Paenibacillus* (strains NHBM4 and NHBM6), *Brevibacillus* (strain NHBM7) and *Variovorax* (strain NHBM15), respectively.

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Molecular and phenotypic diversity of root nodule bacteria isolated from *Phaseolus vulgaris* in Peru

Zúñiga-Dávila D[1], Gonzales-Medina E[1], Ormeño-Orrillo E[1,2], Martínez-Romero E[2]

[1] LEMYB, Universidad Nacional Agraria La Molina, Lima, Peru, [2] CCG, Universidad Nacional Autónoma de México, Cuernavaca, Mexico

Root nodule bacteria from *Phaseolus vulgaris* grown in the Central coast of Peru were obtained from field-collected nodules. Isolates were divided in two groups based on their growth rates, group I showed visible growth after 48 h of incubation in yeast extract mannitol medium while group II were extra-fast growers producing colonies after 24 h. All isolates grew in lactose yeast extract agar but only those of group II produced 3-ketolactose which is a characteristic of biovar 1 agrobacteria (Sakane et al 1995). 16S rDNA gene sequencing of selected group II isolates confirmed their affiliation with *Agrobacterium*. One group II agrobacterial isolate was tested for symbiotic ability and showed to induce nodulation of *P. vulgaris* although nodules were small and lacked leghemoglobin. On the other side, most group I isolates induced nodulation of *P. vulgaris*. Phylogenetic analysis of the *rpoB* gene revealed that the majority of group I isolates which induced effective nodulation were closely related to *Rhizobium phaseoli* while a few isolates, all unable to induce nodulation, clustered with *Neorhizobium galegae*. Growth in different media and at different pH, temperature, and NaCl concentration, as well as phosphate solubilisation ability and production of indolic compounds were evaluated for all group I rhizobia. All isolates were able to solubilize phosphate and to produce indolic compounds but were unable to growth at 40 °C. Isolates clustering with *N. galegae* tolerated up to 2% NaCl while those related to *R. phaseoli* did not growth above 0.5% NaCl. Tolerance to acidity (pH 4) and high temperature (37 °C) was heterogeneous among *R. phaseoli*-like isolates. Plant growth promotion of *P. vulgaris* was observed even with isolates which were unable to induce nodulation.

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Characterization of phosphate solubilizing rhizobacteria associated with pea (*Pisum sativum* L.) isolated from two agricultural soils

Ramírez-Bahena MH[1,2], Velázquez E[2,3], Laguerre G[4], Peix A[1,2]

[1]IRNASA-CSIC. Salamanca. Spain, [2]Unidad Asociada Universidad de Salamanca- CSIC 'Interacción Planta-Microorganismo'. Salamanca. Spain, [3]Departamento de Microbiología y Genética. Universidad de Salamanca. Spain, [4]INRA, UMR Symbioses Tropicales et Méditerranéennes, Montpellier, France.

Pisum sativum L. (pea) is one of the most widely cultivated legumes worldwide that in addition of nitrogen, which can be supplied by rhizobial endosymbionts, requires phosphorous which has specific effects on N_2 fixation and nodule initiation, growth, development, and metabolic function (Habtegebrial and Singh, 2006). Therefore, in the present work the occurrence of efficient phosphate solubilizing bacteria (PSB) in the rhizosphere of pea and their genetic diversity were investigated grown in two French agricultural soils with distinct physico-chemical features. The results showed that the PSB represented approximately 5-8% of the cultivable rhizobacteria and sixteen strains able to solubilize phosphate were isolated in both rhizospheres. The isolated strains were phenotypically characterized using API 20NE system that showed they are phenotypically diverse. In order to select strains for 16S rRNA gene sequencing, the strains were analysed by TP-RAPD fingerprinting showing that they formed 10 genetic groups corresponding to the genera *Bacillus*, *Pseudomonas, Burkholderia,* and *Rhizobium*. The strains were closely related to *Bacillus thuringiensis*, *Bacillus toyonensis*, *Burkholderia caledonica*, *Pseudomonas azotoformans, Pseudomonas baetica*, *Pseudomonas frederiksbergensis, Pseudomonas jessenii, Pseudomonas lutea* and *Rhizobium grahamii.* As expected, the strains of genus *Rhizobium* were also able to nodulate *Pisum sativum* L. and the genus *Pseudomonas* was the most frequent PSB in the two rhizospheric soils analysed. These results confirmed those of previous studies showing that *Bacillus*, *Burkholderia*, *Pseudomonas* and *Rhizobium* are the most commonly found PSB in soil (Käempfer, 2007).

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Acacia macracantha nodulating rhizobia and nodule structure

Cordero I, Ruiz-Díez B, Coba T, Lucas MM, Rincón A, **Pueyo JJ.**

Instituto de Ciencias Agrarias, ICA-CSIC, Madrid, Spain.

Acacia macracantha Willd. is a valuable tree legume that grows in a variety of environments, often under severe abiotic stress. Thirteen bacterial isolates were obtained from nodules of *A. macracatha* grown in soils from different locations in Peru. Sequencing of the 16S rRNA gene revealed that they belonged to the genera *Ensifer* and *Rhizobium,* and phylogenetic analyses of the symbiotic genes *nodC* and *nifH* allowed relating them to rhizobial strains of American origin. Rhizobial inoculation had a positive effect on acacia seedlings, which correlated with the symbiotic effectiveness of the isolates. The structure and ultrastructure of *A. macracantha* nodules elicited by the isolates were analysed. Most strains formed elongated indeterminate nodules with good nitrogen-fixing activity. The nodule cortex and central zone presented distinctive characteristics, which included tannin deposits in some of the nodule cortex cell layers, intermingled meristematic and freshly infected cells in the nodule apical part, and non-infected cells with dense vacuole content in the nodule tip. The ultrastructural analysis showed unusually large mitochondria and complex mitochondrial structures in mature and senescent infected cells. This is, to our knowledge, the first report on *A. macracantha* nodulating bacteria and nodule structure. Our results provide the basis for the formulation of effective inoculants to be used in ecological restoration programs.

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Ensifer meliloti ORT12 and *E. medicae* SF3.41 *nolG* mutants are less resistant to heavy metals but are more competitive for the nodulation of *Medicago sativa*

Alías-Villegas C[1], Bellogín RA[1], Camacho M[2], Cubo T[1], Temprano F[2], Rosario Espuny M[1].

[1] Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Avda. Reina Mercedes 6, 41012-Sevilla. Spain, [2] Centro Las Torres-Tomejil (IFAPA), Apartado Oficial, Alcalá del Río, Sevilla. Spain.

Bacteria have developed diverse multidrug resistance system to the active extrusion of drugs and heavy metals from cells by multidrug resistance efflux pumps (MDR pumps). Five families of MDR pumps have been described; one of them is the resistance-nodulation-cell division (RND) family. The RND pumps are multicomponent systems consisting of an inner membrane transporter (IMP), a membrane fusion protein (MFP), and an outer membrane factor (OMF). These three components are essential for pump function and are assembled to form a large complex spanning both the inner and outer membranes (Eda et al 2011).

The strains of this study, *Ensifer medicae* SF3.41 and *E. meliloti* ORT12, were isolated from nodules of *Medicago marina* from the sea dunes in San Fernando (Cádiz) and marshes close to the joint estuary of the Tinto and Odiel rivers (Huelva), respectively. The last one is a very polluted area, with high concentrations of Co, Cr, and Ni. This is partially due to the fact that the Tinto and the Odiel rivers drain the Iberian Pyrite Belt, one of the most important mining areas of southwestern Europe, with extremely high concentrations of heavy metals (Davis, R.A. et al., 2000). Both strains can grow with relatively high level of heavy metals, and the presence of, at least, two components of MDR pumps have been detected. One of them is the *nolG* gene (sma0875) of *E. meliloti* wich belongs to an operon constituted by *nodM-nolFG-nodN* genes of a RND complex, whose expression is activated by luteolin, (Baev et al. 1991). *nodM* gene codify for a glucosamine synthetase, the function of NodN protein is not yet known. NolG (SMa0875) is the IMP and NolF (SMa0876) is the membrane fusion protein (MFP) that connect the transporter with the third component in the system, which is an outer membrane factor (OMF) codified by a gene which is not ligated to the system.

In order to know the role of the *nolG*, we have constructed mutants in this gene by insertion of a *lacZ::Gm* cassette in both strains, *E. medicae* SF3.41 and *E. meliloti* ORT12. Phenotypic studies showed that *E. medicae* SF3.41 *nolG* mutant was less tolerant to Cu, Zn, Cd, and Co in comparison with the wild type, however, the *E. meliloti* ORT12 *nolG* mutant had the same resistance level facing heavy metals that its corresponding wild type strain. On the other hand, both *nolG* mutants showed a higher swimming motility in Bromfield medium (0.24% agar) after 5 days of growth at 28ºC, and were more competitive for the nodulation in *Medicago sativa* plants when they were coinoculated with their respective wild type strains.

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Effect of vegetable buffers on denitrification activity and microbial community structure in olive tree cultivation soil

Pesciaroli C[1,2], Rodelas B[2,3], García MJ[1], Herrero P[4], Fernández B[5], Fernández R[5], Contreras V[5], Ruiz C[6], González-Martínez J[7], García FJ[7], González-López J[2,3], Osorio F[1].

[1]Department of Civil Engineering, University of Granada, Spain, [2]Institute of water research, University of Granada, Spain, [3]Faculty of Farmacy, University of Granada, Spain. [4]Paisajes del Sur S.L., [5]Bonterra Iberica S.L., [6]Technical assistance for LIFE+ EUTROMED project supervision, [7]Department of Environment, Granada County Council.

The eutrophication of surface waters, due to the accumulation of nitrates, represents an environmental problem of enormous importance (Carpenter et al. 1998). One of the main causes of this type of pollution is the excessive application of nitrogen fertilizer. The aim of this work was to study the effect of a buffer-technology (vegetable buffers) on the concentration of nitrates from agricultural sources in runoff water (Blanco-Canqui et al., 2006; Yuan et al, 2009). For this purpose, a set of vegetable buffers were installed in a designated olive tree cultivation area (municipal area of Deifontes, Granada). After the setup of the experimental plots, water samples were analysed to measure physico-chemical parameters and concentration of pollutants (nitrates, nitrites, phosphates, etc.) both in buffer-technology treated plots and in control plots. The results obtained in treated areas showed a reduction of contaminants of about 50% compared to controls. Soil samples were collected in the same sites to determine denitrification capacity. The high denitrifying activity registered in soil, suggested that high levels of nitrates in the soil produced an increase of activity, resulting in loss of nitrogen that cannot be used by plants. An extensive study of microbiota in soil was performed using tag-pyrosequencing techniques. At class level, the dominant microorganisms were Actinobacteria, a class that is highly represented in agricultural soils and is well known for its important role in biodegradation processes of macromolecules. A comparative analysis of biodiversity in soils of treated and control plots, suggested that microbiome was similar in all conditions tested. To confirm this result, a comparative statistical analysis of genus biodiversity in all samples was performed. Also in this case, the statistical study confirmed that there was no evident differentiation among the various areas analysed. This result was particularly important since it demonstrated that the presence of the treatment did not produce significant changes in soil microbial communities leading to the development of biogeochemical cycles that provide fertile soil.

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Symbiotic efficiency of *Bradyrhizobium* strains isolated from *Cajanus cajan* in Dominican Republic

Díaz-Alcántara CA[1], Araujo J[1], Marcano IE[1], Vicioso AF[1], Pimentel A[1], Velázquez E[2], González-Andrés F[3].

[1]Facultad de Ciencias Agronómicas y Veterinarias, Universidad Autónoma de Santo Domingo, República Dominicana, [2]Departamento de Microbiología y Genética. Universidad de Salamanca. Spain, [3]Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad. Universidad de León. León. Spain.

Cajanus cajan L. Millsp. (pigeonpea) is a popular vegetable crop in Caribbean countries, grown for its green peas obtained from the pods harvested at the green immature stage (Gooding, 1962). This is the case in Dominican Republic, where it is a horticultural crop known as guandul (Cedano, 2006). *Cajanus cajan* establishes symbiotic relationships with fast and slow growing rhizobia in different continents and there are some data about the species nodulating this legume in Trinidad Island in the Caribbean sea (Ramsubhag *et al.*, 2002). Nevertheless, the symbiosis *Cajanus cajan*-rhizobia has been poorly studied up to date and there are few data about the symbiotic effectiveness of rhizobia nodulating this legume. In this work we analysed the symbiotic efficiency of several slow-growing strains isolated from nodules of *Cajanus cajan* in Dominican Republic. The effectiveness experiments were carried out in hydroponic conditions and the results showed significant differences in the nodulation ability, in the dry weigth of shoots and in their N an P contents among the isolated strains. Some of these strains were able to form up to 45 nodules per plant and originated plants with a dry weigth higher than 1250 mg, whereas other strains formed less than 10 nodules per plant originated plants with a dry weight lower than 500 mg seven weeks after inoculation. No significant differences were found in the N fixed percentage between plants inoculated with these last strains and uninoculated non-fertilized plants, whereas significant differences were found with respect to the higher infective ones. Therefore, taking into account that the nodulation of *Cajanus cajan* in Dominican soils is naturally poor, biofertilization of guandul with native and highly effective strains is a good practice to increase the production of this legume in Dominican Republic without addition of nitrogen biofertilizers.

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Diversity of Lotus spp. root nodule bacteria: cultivated versus wild species

Soares R[1], Lorite MJ[2], Figueira E[3], Sanjuan J[2], Videira I[1].

[1] Laboratório de Microbiologia do Solo, UEISSAFSV, INIAV, Oeiras, Portugal. [2] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Granada, Spain. [3] Departamento de Biologia & CESAM, Universidade de Aveiro, Portugal.

Lotus is a cosmopolitan legume plant genus with approximately 150 annual and perennial species. It includes several species of high agronomic value, i.e., *L. uliginosus*, which are used world-wide as forage due to their potential growth over a large variety of soils. It also includes less studied wild species, such as *Lotus parviflorus*. It has been long thought that *Lotus* spp. would only establish specific symbiosis, with either intermediate growing mesorhizobia or with slow-growing bradyrhizobia. However, recent studies have shown that other genera of rhizobia can also establish specific symbiosis with various *Lotus* spp. (Lorite *et al*., 2010).

In this work the diversity, phylogeny and host range of 44 root nodule bacteria (rnb) (13 isolated from *L. parviflorus* and 31 from *L. uliginosus*) were studied. The diversity was evaluated by REP- and ERIC-PCR amplification. The phylogeny was examined using the sequence of the 16S rRNA gene, of the housekeeping genes,*rec*A, *atp*D and *gln*II, and also the symbiotic genes, *nod*C and *nif*H. Host range specificity assays were performed using *L. parviflorus*, *L. uliginosus*, *L. tenuis* and *L. corniculatus* as host plants, being inoculated with the rnb isolates. The results obtained by ERIC- and REP-PCR fingerprints showed that *L. parviflorus* isolates were much more diverse than those of *L uliginosus*. However, only one isolate of *L. parviflorus* was confirmed to nodulate, producing nitrogen-fixing efficient nodules in both *L. parviflorus* and *L. uliginosus,* and was classified in the *Bradyrhizobium canariense* species. The other isolates didn't nodulate any of the *Lotus* species tested and belong to different genera, most of them out of the *Rhizobiaceae* family. However, it was found that some of these isolates were able to colonize the nodules only when mixed with a bradyrhizobia nodulating strain. Inversely, almost all *L. uliginosus* isolates nodulated the *Lotus* plant hosts tested and were nitrogen-fixing efficient only with *L. uliginosus* and *L. parviflorus*. These isolates were closely related to the *Bradyrhizobium* sp. and *B. japonicum* bv. *genistearum* strains, similar to previous reports by Lorite *et al*. (2012) and Batista *et al*. (2013) for *L. uliginosus* isolates from Portugal and Uruguay, respectively.

Several plant probiotic bacterial (PPB) activities, such as phosphate solubilization, siderophore production, cellulose and pectin hydrolysis, and antagonism against *Phytophora cinnamomi* and *Botryosphaeria corticola*, were also tested. *In vitro* results indicate that only a small percentage of isolates (about 7%) displayed these other activities related to plant growth promotion.

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Genetic diversity of bacterial endophytes isolated from maize roots in Lanzarote (Canary Islands).

León-Barrios M[1*], Menéndez E[2], Valencia N[1], Pérez J[1], Garrido A[3], Pérez R[1], Velázquez E[2].

[1*]Departamento de Bioquímica, Microbiología, Biología Celular y Genética. Universidad de La Laguna. Tenerife. Canary Islands. Spain, [2]Departamento de Microbiología y Genética. Universidad de Salamanca, [3]Granja Agrícola Experimental. Área de Agricultura y Ganadería. Cabildo de Lanzarote. Canary Islands. Spain.

Maize endophytic bacteria have been analyzed in several studies, mainly those with ability to fix atmospheric nitrogen (Riggs *et al.*, 2001; Roesch *et al.*, 2007). In some of these studies enhancement of maize yield was found in the absence of fertilization with nitrogen compounds after inoculation with strains of *Klebsiella pneumoniae* and *Pantoea agglomerans* isolated from maize roots (Riggs *et al.*, 2001). In the present study we characterized endophytic bacteria from roots of maize plants growing in an agricultural area of Lanzarote (Guatiza). Endophytic bacteria were isolated as previously described (Kuklinsky-Sobral et al., 2004) from the roots of four plants. Twenty nine strains were selected based on the colony morphology. The genetic diversity of the 29 isolates analyzed by RAPD fingerprinting distinguished 23 RAPD types. Partial sequences of strains representative for different RAPD types showed the presence within the maize roots of different genera and species belonging to Alpha- and Gammaproteobacteria, Firmicutes and Actinobacteria. Some of these bacteria were able to grow in free-nitrogen media and presented several *in vitro* plant growth promotion mechanisms including phosphate solubilization, siderophore and indol acetic acid production.

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Session 2

Genetics, genomics
and proteomics of PPM
and their associated plants

A C-terminal region of the *Bradyrhizobium japonicum* regulator $FixK_2$ is crucial for protein activity and protease recognition

Cabrera JJ[1], Fernández N[1], Bonnet M[2], Hennecke H[2], Bedmar EJ [1], **Mesa S [1].**

[1*] Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, Granada, Spain. [2] Institute of Microbiology, ETH-Zurich, Zurich, Switzerland

$FixK_2$ is a key regulator that controls a large number of genes required for the anoxic and microoxic, endosymbiotic and nitrogen-fixing life styles of the α-proteobacterium *Bradyrhizobium japonicum* (1). $FixK_2$ is an unusual member of the CRP/FNR family of bacterial transcription factors, because it is active *in vitro* without an additional effector molecule and is regulated posttranslationally by the oxidation of its singular cysteine residue (2). $FixK_2$ is also regulated by proteolysis, both by general degradation by the $ClpAP_1$ chaperone-protease system (3) and by specific cleavage. In the latter control, we observed that a truncated variant is always co-purified together with N-terminally His_6-tagged $FixK_2$ proteins. Mass spectrometric analysis revealed that this truncated form is a C-terminally cleaved derivative (between V220 and L221), which lacks the last twelve amino acids. Likewise, this shorter $FixK_2$ species is also present in cells of *B. japonicum*. Remarkably, the recently solved $FixK_2$ structure in complex with DNA revealed that the C-terminus of $FixK_2$ is surface-exposed, and therefore a target for proteolysis (4). We have constructed and characterized a series of protein variants with modifications within the C-terminus of $FixK_2$: (i) truncated derivatives, (ii) proteins with amino acid exchanges at the cleavage site, and (iii) chimeras with a different amino acid sequence but with similar secondary structure. Our results showed that the C-terminal stretch of twelve amino acids plays a crucial role in $FixK_2$ proteolysis, protein folding, and DNA binding capacity and activity.

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Genomic analysis of three *Bradyrhizobium* geno(species) nodulating Lima bean (*Phaseolus luna*tus L.) in Peru

Ormeño-Orrillo E [1,2], Zúñiga-Dávila D[1], Rey L[3], Durán D[3], Imperial J[3], Ruiz-Argüeso T[3], Martínez-Romero E[2].

[1*] LEMYB, Universidad Nacional Agraria La Molina, Lima, Peru, [2] CCG, Universidad Nacional Autónoma de México, Cuernavaca, Mexico
[3] CBGP, Universidad Politécnica de Madrid, Madrid, Spain.

The Lima bean (*Phaseolus lunatus*), also known as pallar, ibes, garrofón or butter bean in Peru, México, Spain and USA, respectively, is the second most economically important species of *Phaseolus*. Peru is a centre of origin and domestication of Lima bean. This crop is cultivated mainly in the Central coast of Peru under a subtropical arid climate. In contrast to the common bean (*Phaseolus vulgaris*) which forms nodules with fast growing *Rhizobium* strains, the Lima bean forms nodules with slow growing bacteria of the *Bradyrhizobium* genus (López-López et al. 2013, Ormeño-Orrillo et al. 2006). We found strains of *Bradyrhizobium yuanmingense* and of three novel *Bradyrhizobium* genospecies inside *P. lunatus* nodules in Peru (Ormeño-Orrillo et al. 2006). Strains of the three novel genospecies were characterized by showing an extra-slow growing phenotype (generation time > 10 h^{-1}) and strong alkali production in yeast extract mannitol medium. Two of the novel genospecies were recently named as *Bradyrhizobium paxllaeri* and *Bradyrhizobium icense* (Durán et al. 2014). *B. paxllaeri* strains dominate nodule occupancy followed by those of *B. icense* and then the third and yet-unnamed genospecies. With the aim to gain insights into this differential competitive ability, we sequenced the genome of one representative strain of each species.

Sequencing was performed with the Illumina HiSeq or MiSeq platform and genome assembly with the SPAdes program. Gene prediction and automated annotation was performed with Prokka and RAST. Annotation of genes putatively involved in competitiveness was manually curated. Assemblies had from 55 to 175 contigs, with N50 sizes > 131 kb. Genome sizes of *B. paxllaeri* and *B. icense* were similar (8.2 Mb) and larger than that of the third genospecies (7.8 Mb). Preliminary analysis revealed differences between *B. paxllaeri* and the other two genospecies such as more genes for type IV pilus and two *nodA* genes. A comparative genomic analysis of *P. lunatus* symbionts will be presented at the meeting.

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Regulatory *nodD1* and *nodD2* genes of *Rhizobium tropici* strain CIAT 899 and their roles in the early stages of molecular signaling and host-legume nodulation

Del Cerro P[1], Pérez-Montaño F[1], Rodríguez-Carvajal MA[3], Gil-Serrano A[3], Megías M[1], Ollero FJ[1], Hungria M[2].

[1]Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Sevilla, España. [2]Embrapa Soja, Londrina, Paraná, Brasil. [3]Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla. Sevilla, España.

Nodulation and symbiotic nitrogen fixation are mediated by several genes, both of the host legume and of the bacterium. The rhizobial regulatory *nodD* gene plays a critical role, orchestrating the transcription of the other nodulation genes. *Rhizobium tropici* strain CIAT 899 is an effective symbiont of several legumes with an emphasis on common bean (*Phaseolus vulgaris*) and is unusual in carrying multiple copies of *nodD*, the roles of which remain to be elucidated.

Phenotypes, Nod factors and gene expression of *nodD1* and *nodD2* mutants of CIAT 899 were compared with those of the wild type strain, both in the presence and in the absence of the nod-gene-inducing molecules apigenin and salt (NaCl). Differences between the wild type and mutants were observed in swarming motility and IAA (indole acetic acid) synthesis. In the presence of both apigenin and salt, large numbers of Nod factors were detected in CIAT 899, with fewer detected in the mutants *nodC* expression was lower in both mutants; differences in *nodD1* and *nodD2* expression were observed between the wild type and the mutants, with variation according to the inducing molecule, and with a major role of apigenin with *nodD1* and of salt with *nodD2*. In the *nodD1* mutant, nodulation was markedly reduced in common bean and abolished in leucaena (*Leucaena leucocephala*) and siratro (*Macroptilium atropurpureum*), whereas a mutation in *nodD2* reduced nodulation in common bean, but not in the other two legumes.

Our proposed model considers that full nodulation of common bean by *R. tropici* requires both *nodD1* and *nodD2*, whereas, in other legume species that might represent the original host, *nodD1* plays the major role. In general, *nodD2* is an activator of nod-gene transcription, but, in specific conditions, it can slightly repress *nodD1*. *nodD1* and *nodD2* play other roles beyond nodulation, such as swarming motility and IAA synthesis.

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Unravelling the universe of small RNA regulators in the legume symbiont *Sinorhizobium meliloti*

Jiménez-Zurdo JI, Robledo M.

*Grupo de Ecología Genética de la Rizosfera, Estación Experimental del Zaidín (CSIC)

High-throughput transcriptome profiling has uncovered large and heterogeneous populations of small noncoding RNA species (sRNAs) with potential regulatory roles in bacteria. These sRNAs act mostly by protein-assisted base-pairing with target mRNAs to fine-tune post-transcriptional reprogramming of gene expression underlying bacterial responses to changing environments. Riboregulation impacts virtually any physiological process, and has been shown to largely influence virulence of pathogenic bacteria. Here, we review our incipient knowledge on the structure, conservation and function of the noncoding transcriptome of the α-rhizobia *Sinorhizobium meliloti*, the nitrogen-fixing symbiotic partner of alfalfa and related medics.

Several RNAseq-based surveys in *S. meliloti* have shown abundant transcription from hitherto regarded as noncoding intergenic regions (IGRs), strikingly high numbers of mRNA-derived RNAs and pervasive antisense transcription of protein-coding genes (Jiménez-Zurdo *et al*. 2013). sRNAs encoded within IGRs constitute the most extensively studied group of bacterial RNA regulators. These sRNA molecules (~50-200 nt in length) are differentially expressed and modulate translation and/or turnover rates of *trans*-encoded target mRNAs by short and discontinuous antisense interactions within or outside their 5'-UTRs (Untranslated Regions). In enteric model bacteria, the widespread RNA chaperone Hfq is usually required for the function and/or stability of the *trans*-sRNAs.

S. meliloti expresses a functional Hfq homolog that influences major rhizobial stress and symbiotic traits e.g. metabolism, quorum sensing, flagella biosynthesis or nitrogen fixation (Torres-Quesada *et al*., 2014). However, RNAseq profiling of Hfq RNA ligands from bacteria subjected to a number of abiotic stresses identified as Hfq-bound a minor fraction (14%) of the at least 600 *trans*-sRNAs encoded by the *S. meliloti* genome (Torres-Quesada *et al*., 2014). Among symbiotic rhizobia, regulatory sRNAs have been functionally characterized only in *S. meliloti* to date. The *trans*-sRNAs AbcR1 and AbcR2 are examples of Hfq-dependent sRNAs whereas EcpR1 does not bind Hfq (Torres-Quesada *et al*., 2014). We will provide insights into the transcriptional regulation and activity mechanisms of these sRNAs for the targeting and control of multiple mRNAs involved in nutrient uptake (AbcR1/2) and cell cycle progression (EcpR1; Robledo *et al*., 2015) in *S. meliloti*.

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Antisense transcription of symbiotic genes in *Sinorhizobium meliloti*

Robledo M[1,2], Jiménez-Zurdo JI[2], Becker A[1].

[1]LOEWE Center for Synthetic Microbiology and Faculty of Biology, Philipps-University Marburg, Germany. [2] Grupo de Ecología Genética de la Rizosfera, Estación Experimental del Zaidín (CSIC), Granada.

The α-proteobacterium *Sinorhizobium meliloti* is the symbiont of various forage legumes including alfalfa (*Medicago sativa*). Its genome is composed of a single chromosome and two megaplasmids, pSymA and pSymB (Galibert et al, 2001). pSymA megaplasmid encodes most of the symbiosis related genes required for the formation of nitrogen-fixing nodules on alfalfa roots (Barnett et al, 2001). Recently, a RNA-seq approach delivered ~ 17,000 experimentally mapped transcriptional start sites (TSS) that were assigned to both protein-encoding genes and non-coding transcripts (ncRNAs) from the three replicons. Considering the individual sizes of the three replicons, there is an over-representation of trans- (40.9%) and cis-encoded antisense (23.9%) ncRNAs (asRNAs) on pSymA (Schlüter et al, 2013). Therefore, it is tempting to speculate that processes relevant to symbiosis are modulated by ncRNAs, especially those related to bacterial adaptation during the transition from free-living to host-dependent conditions, e.g. induction and colonization of root nodules and rhizobial differentiation to nitrogen-fixing bacteroids. However, nothing is hitherto known about the biological relevance of ncRNAs in symbiosis.

The presence of 3,720 asRNAs suggests considerable antisense transcription in *S. meliloti* (Schlüter et al, 2013). Furthermore, TSS of asRNAs are particularly represented within a 275-kb pSymA region including the symbiotic genes. To further investigate the role of asRNAs in posttranscriptional regulation of these genes, in this work we focused on ncRNA candidates transcribed antisense to the coding regions and/or the 5'- or 3'-UTRs (Untranslated Regions) of protein-encoding genes required for the synthesis of nodulation factors and the nitrogenase components. We have confirmed transcription of seven of these candidates by independent RNA gel blot analysis and monitored their expression in different biological conditions i.e. stress shifts, luteolin and plant root exposure, microaerobiosis, and in nodules. Hybridization signals corresponding to small RNA transcripts (≤120 nt) were reliably detected. The two largest asRNA candidates showed more than one hybridization signal, suggesting that they are processed. Interestingly, some transcripts accumulate differentially in response to different environmental factors, and seem to repress *nif* genes under conditions in which functional nitrogenase is dispensable, suggesting regulatory functions. Furthermore, *M. sativa* plants showed slight differences in nitrogen fixation ability respect to the control when inoculated with genetically modified *S. meliloti* strains overexpressing one of these asRNAs. These experiments provided the first evidences of antisense riboregulation in rhizobia in response to diverse abiotic and symbiotic conditions.

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Symbiotic characterization of *Sinorhizobium fredii* HH103 *nolR* and *nodD2* mutants with *Lotus japonicus* GIFU and *L. burttii*.

Rodríguez-Navarro D[1], Acosta-Jurado S[2], Fernández J[2], Vinardell JM[2], **Ruiz-Sainz JE**[2].

[1]IFAPA, Centro Las Torres-Tomejil. Apartado Oficial 41200, Alcalá del Rio, Sevilla, Spain, [2]Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes nº6, 41012-Sevilla, Spain.

Sinorhizobium fredii HH103 is a fast-growing bacterium that nodulates soybeans (*Glycine max*) and many other legumes. The genome sequence of HH103 is deposited in public databases (Vinardell et al. 2015). This broad hosts-range bacterium only forms ineffective pseudonodules with *Lotus japonicus* GIFU. In *L. burttii*, however, nitrogen fixing nodules are formed (Sandal et al. 2012).

We have investigated the symbiotic phenotype of a collection of *S. fredii* mutants affected in different surface polysaccharides (SP), such as EPS (exopolysaccharides), LPS (lipopolysaccharides), capsular K-antigen polysaccharides (KPS), and cyclic glucans (CG). All the EPS, LPS, and KPS mutants analyzed retained the capacity to nodulate *L. burttii*, although in some cases the bacterial symbiotic capacity was reduced. CG mutants only formed pseudonodules on *L. burttii* roots. None of the *S. fredii* HH103 SP mutants showed any symbiotic improvement with *L. japonicus*. Other mutants affected in substitutions decorating the terminal N-acetylglucosamine residue of *S. fredii* HH103 Nodulation-factors showed symbiotic phenotypes with *L. japonicus* and *L. burttii* that were similar to those described above.

Here we show that, surprisingly, *S. fredii* HH103 *nodD2* and *nolR* mutants formed truly (infected) nodules with both *Lotus* species. The number of nodules formed in *L. burttii* plants inoculated with the *nodD2* mutant was higher than in those inoculated with the *nolR* one. *L. japonicus* GIFU nodules induced by mutant SVQ548 (*nolR*) clearly fixed more nitrogen than those induced by mutant SVQ554 (*nodD2*). Nitrogenase activity was detected by ARA in nodules occupied by the *nodD2* mutant, albeit at very low activity. Both mutants were faster nodulators with *L. burttii* than its parental wild type strain HH103 Rif-r. Competition for nodulation experiments are on course.

Two other independent *nodD2* (called SVQ514) and *nolR* (SVQ517) mutants showed the same symbiotic phenotype with *L. japonicus* and *L. burttii*. Thus, the symbiotic improvements shown by *nodD2* and *nolR* mutants are due to the *nodD2* or *nolR* mutations rather than to the occurrence of an undetermined secondary mutation. This conclusion is also supported by the fact that reintroduction of the *nodD2* gene in SVQ554 (or *nolR* in SVQ548), abolishes the formation of nitrogen fixing nodules in *L. japonicus* GIFU.

Transcriptomic analyses showed that the *nolR* gene represses the transcription of many genes in the presence of genistein. The list of genes repressed by *nolR* includes genes of symbiotic significance. The fact is in accordance with previous reports showing that *nolR* affects a wide range of symbiotic signals (Vinardell et al. 2004).

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Functional studies of the *S. fredii* HH103 MucR1 transcriptional regulator.

Acosta-Jurado S[1], Murdoch PS[2], Rodríguez-Carvajal MA[3], Navarro-Gómez P[1] Ruiz-Sainz JE.[1], Zehner S.[4], Göttfert M[4], Vinardell JM[1].

[1] Departamento de Microbiología and [2] Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Biología, Universidad de Sevilla. [3] Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla. [4] Institut für Genetik, Technische Universität Dresden

The transcriptional regulator RosR was firstly studied in *Agrobacterium tumefaciens* (Close *et al*., 1985) and orthologues are present in several bacteria as *Sinorhizobium meliloti* (*mucR*)*, Rhizobium leguminosarum* bv. trifolii (*rosR),* and *A. radiobacter (rosAR).* In rhizobia*, RosR/MucR* is a key protein involved in EPS synthesis (Janczarek, 2011). In *S. meliloti* MucR regulates positively and negatively the production of EPS I and EPS II respectively. In *R. leguminosarum* bv. trifolii inactivation of *rosR* results in a substantial decrease of EPS production and ineffective symbiosis with clover . RosR/MucR are small proteins (approximately 15,7 KDa) with a zinc finger motif which confers the ability to regulate target gene expression by direct binding to the promoter region of these genes. This includes its own promoter region since MucR1 shows negative autoregulation. Genome sequencing of *S. fredii* HH103 revealed that this strain harbors two copies of *mucR*, *mucR1,* located on the chromosome, and *mucR2,* contained in the pSym (Vinardell *et al*., 2015). These two genes are quite similar, showing 84 and 75% of identity at the nucleotide and aminoacid level, respectively. RosR/MucR binds to specific inverted sequences (MucR-box or RosR-box) which are present in the promoter region of target genes. However, this sequence is poorly conserved among the different genes regulated by MucR/RosR.

In this work, we have obtained *S. fredii* HH103 mutants in *mucR1* (SVQ706 = HH103ΔmucR1) and *mucR2* (SVQ719 = HH103 *mucR2*::*lacZ*-Gm^R), as well as a double mutant Δ*mucR1 mucR2*::*lacZ*-Gm^R (SVQ720). Inactivation of *mucR1*, but not that of *mucR2*, led to impaired EPS production (determined by NMR). HH103 *mucR1* mutants were also affected in attachment to abiotic surfaces, showing increased biofilm capacity with regard to their parental strain. Regarding to symbiosis, inactivation of *mucR1* affected negatively *S. fredii* HH103 symbiotic performance with soybean Williams. Although the number of nodules formed was higher than when the wild type strain was used as inoculant, those nodules were white and plants showed clear symptoms of nitrogen deficiency. We have also proved that *S. fredii* MucR1 autoregulates negatively its own expression. At present, we are carrying out electrophoretic mobility shift assays (EMSA) to determine the specific sequence to which MucR1 binds.

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Identification by MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) analysis of rhizobia nodulating *Phaseolus vulgaris* in soils with different cultivation history

López LI[1], Sánchez F[2], Ramírez-Bahena MH[3,5], González JM[2,4], Peix A[3,5], Velázquez E[1,5].

[1*]Departamento de Microbiología y Genética. Universidad de Salamanca. Spain, [2]Instituto de Investigación Biomédica de Salamanca (IBSAL). Salamanca. Spain, [3]IRNASA-CSIC. Salamanca. Spain, [4]Departamento de Bioquímica y Biología Molecular. Universidad de Salamanca. Spain. [5]Unidad Asociada Universidad de Salamanca- CSIC 'Interacción Planta-Microorganismo'

Phaseolus vulgaris L. is a legume commonly cultivated in León province where the IGP "La Bañeza" comprises several areas, one of them located near the Orbigo river bank and called "La Vega". In this area, fields irrigated by canals are the most commonly cultivated, nevertheless in the last decade with the introduction of the sprinkler irrigation system, large areas of rainfed soils have been converted to irrigated cultivation. In the present work we identify strains nodulating *P. vulgaris* in rainfed soils converted to sprinkling irrigated areas in Riego de la Vega (León) by using MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry). Recently we extended our database of family *Rhizobiaceae* (Ferreira *et al.*, 2011) in order to include several type strains of recently described species from rhizobia as well as other strains isolated from nodules of different legumes. The strains from this study were isolated in two rainfed soils, one of them converted to irrigated soil one year before sampling and cultivated with common bean (soil 1) and the other converted five years before sampling and cultivated with common bean in rotation with sugar beet and maize (soil 2). The results obtained after MALDI-TOF MS analysis showed that the strains isolated from soil 1 matched with more than 2.0 (correct identification in the case of fast-growing rhizobia) with strains from the groups I and II of the species *R. leguminosarum* (Mulas *et al.*, 2011), being more abundant those matching with the strain RPVR24 (group II) than those matching with the strain RPFV18 (group I), both isolated in a soil cultivated for more than 20 years in the same location. However almost all strains isolated from soil 2 matched with more than 2.0 with a strain (RPVR32) also isolated in the soil cultivated for 20 years, but belonging to a third phylogenetic group related to *R. leguminosarum* (García-Fraile *et al.*, 2010) that was not found in the soils analysed by Mulas *et al.* (2011). These results showed that MALDI-TOF MS is not only a reliable technique for identification at species level of fast-growing rhizobia, but also for differentiation of subspecific groups, which is very useful for ecological studies.

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First transcriptomic analyses of *Sinorhizobium fredii* HH103.

Vinardell JM[1*], Navarro P[1], Pérez-Montaño F[1], Acosta-Jurado S[1], Jiménez I[1], Ollero FJ[1], Ruiz-Sainz JE[1], López FJ[1].

[1*] Department of Microbiology, Faculty of Biology, University of Seville. Avda. Reina Mercedes 6, 41012-Seville (Spain), jvinar@us.es

Sinorhizobium fredii is a rhizobial species that has an extremely broad host-range (more than 100 genera of legumes are nodulated) that includes plants forming determinate nodules, such as *Glycine max*, as well as plants developing indeterminate nodules, such as *Glycyrrhiza uralensis* (Margaret *et al*. 2011). Among the different *S. fredii* strains known, three are the most studied so far: NGR234, USDA257, and HH103. In fact, genomic information is available for all of them and very recently we have reported the manually annotated genome of HH103, which is composed of 7 different replicons (Vinardell *et al*. 2015). An intriguing difference between these three strains lies in their symbiotic behaviour with soybean, one of the most important crops worldwide: NGR234 does not induce the formation of Fix$^+$ nodules on soybean, USDA257 only does it on both wild and non-commercial (Asiatic) varieties of soybeans, and HH103 nodulates effectively both Asiatic and the commercial American soybeans. As in other sinorhizobia, HH103 genes involved in Nod factor production are located on the so-called symbiotic plasmid (= pSym, pSfHH103d).

In a previous work, Perret et al. (1999) defined nineteen different *nod*-boxes (NB) in the symbiotic plasmid of NGR234. Interestingly, 4 of these NB are not present in HH103 (what accounts for the differences in the sets of Nod factors produced by these strains), and there are differences between these two strains in the genes located under the control of other 4 NB. Moreover, the symbiotic relevance of a number of HH103 genes under the control of NB remains to be studied. In this work, we will present studies (RNAseq and *q*PCR) of the expression of these genes in the presence and absence of genistein, an effective *nod* gene inducer in HH103. These studies have been performed not only with the wild strain HH103 but also with several mutants affected in symbiotic regulatory genes: *nodD1*, *nodD2*, *nolR*, and *ttsI*. Thus, an expression map of the *S. fredii* HH103 whole genome in the presence of genistein will be provided, with especial emphasis on genes whose expression is NB-dependent. The role of NodD1, NodD2 and NolR in the expression of NB-dependent genes (and that of TtsI on T3SS-related genes) as well as the interconnections between these regulatory genes will also be assessed.

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RNA-seq analysis of *Rhizobium tropici* CIAT899 genome in the presence of apigenin and salt.

Ollero FJ[1], Del Cerro P[1], Jiménez-Guerrero I[1], Megías M[1], Pérez-Montaño F[1].

[1]*Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain.*

Rhizobium tropici CIAT899 is a rhizobial strain that nodulates some legumes such as *Phaseolus vulgaris*, *Macroptilium atropurpureum* and *Leucaena leucocephala.* This bacterium produces a broad variety of nodulation factors in the presence of inducer flavonoids (Garcia *et al.,* 1996). Interestingly, CIAT899 is also able to produce these key signal molecules under abiotic stress such as salinity (Estévez *et al.*, 2009; Guasch-Vidal *et al.*, 2013). Hence, in this work we have analyzed by RNA-seq the relative expression of the whole CIAT899 genome when the bacteria was grown in the presence of either apigenin (3.7 µM) or NaCl (300 mM).

Results showed that in the presence of both apigenin and salt the transcription of genes that encode proteins implied in the synthesis of Nodulation factors, synthesis of indol acetic acid, and in nitrogen fixation (all of them located in the symbiotic plasmid) were induced. Moreover in both cases, the transcription of genes that encode proteins of the phosphonate metabolism and the synthesis of the type IV pilus (located on the chromosome) were repressed.

On the other hand, in the presence of salt but not in the presence of apigenin we found that several cellular processes, such as protein transport, mainly ABC transporters and membrane pore proteins, and vitamin and cofactor synthesis, were also induced. Genes that coded for these functions were located in the chromosome. By contrast, some genes, located in a cryptic plasmid, that code proteins implied in bacterial capsule production were transcriptionally inhibited only in the presence of salt.

Thus, data shown in this work suggest that both apigenin and salt modulate (activating or repressing) the expression of some genes present in the CIAT899 genome that were crucial for a successful symbiotic process. In the case of bacterial cultures grown in the presence of salt, many other cellular processes were affected, maybe due to a response to the osmotic stress generated by this molecule.

In general, apigenin seems to activate only the transcription of the nodulation genes but repress other physiological process. However, salt stress seems to induce the transcription of symbiotic functions, including the activation of the *nif* genes, and also other physiological process in CIAT899.

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Isolation of salt-tolerant mutants of *Mesorhizobium ciceri* strain Rch125 and identification of genes involved in salt sensitiveness

Moussaid S[1], Nogales J[2], Munoz S[2], Berraho EB[1], **Aurag J**[1], Sanjuan J[2].

1-Laboratory of Microbiology and Molecular Biology, Faculty of Sciences, University Mohammed V, Avenue Ibn Battouta, BP 1014, Rabat, Marocco. 2-Departamento de Microbiologia del Suelo y Sistemas Simbióticos, Estacion Experimental del Zaidin, CSIC. C/ Prof. Albareda 1, E-18008 Granada, Spain

Salinity stress is known to be one of the most serious factors limiting crops productivity. In legumes, salt affects in particular the establishment and functioning of the legume-rhizobium symbiosis and consequently the yield. This is particularly the case of the *Cicer arietinum* - *Mesorhizobium* symbiosis, which is drastically affected at different stages of the nodulation process and nitrogen fixation capacity (Mhadhbi et al 2007). *Mesorhizobium* strains are generally good nitrogen fixers but in contrast are very sensitive to salt stress (Maatallah et al 2002). Therefore, obtaining mesorhizobium strains tolerant to high salt concentrations may be a promising approach to improve symbiosis and plant growth under saline conditions. Salt-tolerant mutants were obtained by random Tn*5* mutagenesis of the strain Rch 125, a *Mesorhizobium ciceri* salt-sensitive isolate obtained from root nodules of chickpea plants growing in soil samples collected from an arid region of Morocco. Genes disrupted in salt-tolerant derivatives were identified and encoded diverse hypothetical functions, including an ABC-transporter permease, a chromate-resistance protein or a protein involved in riboflavin biosynthesis. A fourth group included protein of unknown function. In some cases direct involvement of the mutations in acquisition of salt-tolerance was demonstrated after genetic complementation. Inoculation tests performed under controlled non saline conditions, demonstrated that the salt-tolerant mutants preserved their symbiotic properties and some showed higher nodulation efficiency compared to plants inoculated with the wild type strain.

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Molecular determinants of negative regulation of the *Bradyrhizobium japonicum* transcription factor $FixK_2$

Fernández N[1], **Cabrera JJ**[1], Salazar S[1], Parejo S[1], Lindemann A[2], Hennecke H[2], Bedmar EJ[1], Mesa S[1].

[1] Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, Granada, Spain. [2] Institute of Microbiology, ETH-Zurich, Zurich, Switzerland

In the facultative soybean endosymbiont *Bradyrhizobium japonic*um, the $FixK_2$ protein plays a crucial role in a complex regulatory network which controls a large number of genes necessary for anoxic, microoxic and symbiotic growth (1). $FixK_2$ is a CRP/FNR-type transcription factor, ubiquitous proteins which respond to a wide spectrum of metabolic and environmental cues. Expression of the *fixK*$_2$ gene is activated by the superimposed two-component regulatory system FixLJ in response to low oxygen (at or below 5% O_2 in the gas phase) and repressed by its own product (directly or indirectly). $FixK_2$ is also regulated at posttranslational level by oxidation and proteolysis (2).

The mechanism how $FixK_2$ exerts a negative feed-back on its own expression remains yet enigmatic. In *Sinorhizobium meliloti* and *Caulobacter crescentus*, FixT-like single domain response regulators (3) are involved in the repression of genes encoding FixK-type proteins via an interaction at the FixLJ level. A *fixT*-like gene (bll2758), located between *fixLJ* and *fixK*$_2$, was also identified in *B. japonicum*. This gene is a direct target for $FixK_2$, however the Bll2758 protein turned out not to be involved in the negative control of *fixK*$_2$ gene (4, and references therein). Next, we rationalized that other transcription factors whose genes are activated by $FixK_2$ might be involved in the negative auto-regulation of *fixK*$_2$ expression. Therefore, we performed a functional mutagenesis of two transcription factor genes (bll2109, bll3466) which code for members of the CRP/FNR family of regulators and analysed *fixK*$_2$ expression in microoxically-grown *B. japonicum* cells (0.5 % O_2). Where *fixK*$_2$ gene expression was increased in the Δ*fixK*$_2$ background, that observed in both Δbll2109 and Δbll3466 strains was similar to the wild-type strain. Recently, we noticed that negative auto-regulation of *fixK*$_2$ expression occurs also in oxically-grown cells. Further, this repression was more pronounced in stationary than in early exponential phase, both in oxic and microoxic conditions. However, Bll2758, Bll2109 and Bll3466 did not play a role in such control either.

In conclusion, the mechanism underlying the negative feed-back on *fixK*$_2$ expression is still unknown. Our work has revealed an unexpected species-specific differences in the design of an oxygen-responsive signalling network despite the fact that the regulatory modules (FixL, FixJ, FixT, FixK) are conserved.

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Session 3

Plant-microorganisms
interactions

Boron deficiency causes incorrect N-Glycosylation and Unfolded Protein Response (UPR) in rhizobia-legume symbiosis.

Valiente R[1], Abreu I[1], Cuevas BF[1], Reguera M[1], Orus MI[1], Bolaños L[1], Bonilla I[1].

[1] Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid. c/ Darwin 2. Madrid. 28049. Spain.

Boron (B) is an essential micronutrient in plants. Our group described a special requirement of B for a correct development of the rhizobia-legume symbioses. The fact that all the steps of the symbiotic relationship are highly dependent of the nutrition of the microelement, made certainly improbable that the only B function recognised in plants, namely, the dimerization of Rhamnogalacturonan II pectin at plant cell wall can explain such strong dependence. Our current hypothesis is that other molecules containing *cis*-diol groups, like glycoproteins, can form complexes with B essential for the progression of the rhizobia-legume symbiosis. Because during nodule organogenesis an intense synthesis of new glycoproteins occurs, we are studying the existence of a B-glycoprotein nexus.

In the present work we analysed the time-course synthesis of Mannose Rich N-Glycoproteins (MRNG) and Complex N-Glycoproteins (CNG), synthesized in the Endoplasmic Reticulum (ER) and Golgi Apparatus (GA) respectively, in three symbioses: *Pisum sativum* and *Rhizobium leguminosarum* bv. 3841; *Medicago sativa* and *Ensifer meliloti* 1021; and *Glycine max* and *E. fredii* HH103. Our results indicate a clear accumulation of MRNGs and CNGs in both B-deficient root and nodules. This suggests a requirement of B during glycosylation and/or protein trafficking, because altering the machinery for N-glycan remodelling machinery or the secretory pathway would lead to the observed accumulation of N-glycoproteins.

A preliminary analysis by HPLC-MS/MS of an enriched fraction of MRNGs derived from B-sufficient or B-deficient *P. sativum* nodules shows differences in several proteins involved in protein folding and quality control machinery. We analysed unfolded protein response (UPR) at both transcriptomic and protein level. We detected an increase in expression of UPR reporter genes as BiP chaperon, calreticulin and PDI (Protein Disulphide Isomerase) which coincides with an accumulation of several isoforms of BiP proteins revealed by anti-BiP western blot.

To our knowledge, this is the first time that a biochemical characterization of N-glycosylation and its relationship with B nutrition is performed in the rhizobia-legume symbioses. Future work in this and other biological models should be drive to confirm the role of B related with N-glycan synthesis/trafficking and to identify B-glycoprotein complexes.

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Study of ASUPs produced by *Alnus glutinosa* and their implication in *Frankia* nutrition.

Carro L[1,2], Pujic P[1], Alloisio N[1], Fournier P[1], Boubakri H[1], Normand P[1].

1 Université Lyon 1 CNRS UMR 5557 Ecologie Microbienne Villeurbanne (France), 2 Departamento de Microbiología y Genética, Edificio Departamental Lab 209 Universidad de Salamanca (Spain).

Frankia is a nitrogen fixing actinobacterium which establishes a root symbiosis with dicotyledonous plants collectively called actinorhizal. Although trophic exchanges are known to occur between the partners, the identity of the compounds that are exchanged are still unknown. Antimicrobial peptides have recently been described as important components in symbiotic processes, being able to control the localization or the morphology of symbiotic bacteria.

The first days after plant-microorganism contact are the most active for communication between both partners, so we studied the up-regulated genes in *Frankia*-Actinorhizal plant system after 7 and 21 days of contact by microarray analysis (Hocher *et al*., 2011). Among the most over-expressed genes, defense peptides were identified. The fold-change values were also confirmed by qRT-PCR. One of these peptides was Ag5 (Carro *et al*., 2015), the most up-regulated peptide after seven days of contact with the bacteria. Evaluation of *in vitro* effects on *Frankia* strains were tested: capacity of growth, respiration activity, nitrogen fixation capacity, morphological changes, membrane permeabilization, etc. Immunolocalization of Ag5 was made and found to be specifically attached to the bacteria vesicles (specialized cells for nitrogen fixation). Most of these *Alnus* symbiotic up-regulated peptides (ASUP) are expressed specifically in nodules and are supposed to be in control of symbiotic *Frankia* cells. Some important compounds for this kind of symbiosis, in which carbon and nitrogen are exchanged, have been shown able to pass through the modified membrane (i.e glutamate and glutamine). On the plant side, a dicarboxylate transporter, AgDCAT1, has been shown to be specifically expressed in nodules and to be localized in symbiotic cells (Jeong *et al.*, 2004). Its ability to transport malate, fumarate, succinate and oxaloacetate has been showed in heterologous host. However, no specific transporters for these compounds have been detected in *Frankia* symbiotic transcriptome. One of the most reliable compounds supplied by the host according to transcriptomics and metabolomics of *Frankia* in symbiotic cells and *Frankia in vitro* are tricarboxilic acids. As some of these compounds are not directly used *in vitro,* some tests to verify if the membrane permeability provided by Ag5 ASUP allows them to cross was evaluated. In the evaluation of these and other organic acids, another significant change has been observed in the symbiotic structures for nitrogen fixation *in vitro*, some of the compound used generate bigger vesicles, similar to the ones developed inside the host.

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Bacterial factors involved in *H*ost-dependent symbiotic efficiency (Hse) in the *Rhizobium*-legume interaction.

Sanz-López A[1], Rubio-Sanz L[1,2], De la Torre N[1], Brito B[1], Palacios JM[1].

[1]Centro de Biotecnología y Genómica de Plantas (CBGP) y Departamento de Biotecnología, Campus de Montegancedo. 28223 Pozuelo de Alarcón, [2]Departamento de Química y Tecnología de Alimentos. ETS de Ingeniería Agrícola, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, Ciudad Universitaria s/n 28040 Madrid.

Rhizobium leguminosarum bv. viciae is an endosymbiotic bacterium capable of establishing effective associations with legumes belonging to different genera, including *Pisum, Lens, Vicia* and *Lathyrus*. Several host-dependent symbiotic bacterial phenotypes have been described for this bacterium when associated with different members of this cross-inoculation group, namely pea and lentil: i) hydrogenase expression, leading to recycling the hydrogen produced by the nitrogenase, occurs in pea nodules but not in lentil (Brito et al., 2008); ii) nickel transport required for hydrogenase synthesis occurs differently when bacteroids are induced in lentil vs. pea nodules (Brito et al., 2010); and iii) the interaction between mechanisms of nickel uptake and efflux are different in both hosts (Rubio-Sanz et al., 2013). These differences suggest that legumes in the same cross-inoculation group can modulate differently the microsymbiont's behaviour during the symbiosis. The study of these differences may help to better understand the involvement of the plant in the complex interaction between the two components during symbiosis.

Random mutagenesis of *R. leguminosarum* strain UPM1137 had led to the identification of a mutant (UPM4239) showing a host-dependent symbiotic phenotype. The mutant is symbiotically deficient in pea plants, in which only white, ineffective nodules containing mainly undifferentiated cells are induced, whereas normal, effective nodules are obtained in symbiosis with lentil plants. In contrast, UPM1137 wild-type strain fixes nitrogen efficiently in symbiosis with both lentil and pea plants. A *R. leguminosarum* bv. viciae genomic library has been introduced into the mutant, and pea plants were inoculated "en masse" with the transconjugants. UPM4239 derivatives bearing complementing cosmids have been isolated from red nodules obtained from this complementation assay. The analysis of these cosmids has delimited a 20-kb DNA region, whose analysis through transposon mutagenesis is currently underway. Results of this analysis will be presented in the communication.

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Type VI secretion systems of *Bradyhizobium* nodulating lupines

Pacheco A[1], Pastor V[1], Durán D[1], Palacios JM[1], Imperial J[1,2], Ruiz-Argüeso T[1], **Rey L**[1].

[1] Centro de Biotecnología y Genómica de Plantas (CBGP) and Departamento de Biotecnología (ETS de Ingenieros Agrónomos). Universidad Politécnica de Madrid. Pozuelo de Alarcón 28223 Madrid (Spain), [2]CSIC-Madrid (Spain).

The *Rhizobium*-legume symbiosis is highly specific and depends on several molecular signals produced by both partners. Some of these signals are bacterial proteins named effectors that are translocated into the plant cells by secretion systems similar to contractile nanomachines also called injectisomes (Deakin and Broughton, 2009). The injectisomes puncture and deliver the effectors into the target cell. One of these nanomachines, known as type VI secretion system (T6SS), was discovered recently and is reminiscent of phage injection machinery (Records, 2011). The role of these systems in legume endosymbiotic bacteria is mostly unknown, and this work presents the initial study of T6SSs from different bradyrhizobia. T6SSs have been identified in draft genomic sequences from *Bradyrhizobium* strains isolated from *Lupinus* spp. thriving in the Iberian Peninsula. In all cases, the genes encoding T6SSs were grouped and showed, in most cases, a high degree of conservation among genes encoding the structural components of the system. *Bradyrhizobium* sp. strain ISLU101 isolated from *L. angustifolius*, contains two clusters of genes involved in the formation of T6SS. One of such systems, designated as T6SS-1, contains 17 genes and shows a high degree of conservation regarding genes of *B. diazoefficiens* USDA110. The other one, T6SS-2, contains 16 genes flanked by insertion element sequences. Amino acid similarity between equivalent proteins encoded in both clusters is only about 40-50 %. A phylogenetic analysis based on the concatenation of sequences of several T6SS proteins was performed, and results indicate a clear separation of T6SS-2 from most rhizobial T6SSs. ISLU101 T6SS mutant derivatives in genes *impO*, *impC1* and *impC2* were generated by single homologous recombination of amplified internal fragments from the respective genes cloned into the suicide vector pK18*mobsac*. The symbiotic behaviour of mutants was examined with *L. angustifolius*. Results showed no effect of *impC1* and *impC2* mutations, while the *impO* mutant generated smaller plants with a mixture of white/red nodules. These results suggest that T6SSs may play a role in the *Bradyrhizobium*-lupines symbioses.

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Sinorhizobium fredii HH103 differentiation into bacteroids in nodules of the IRLC legume *Glycyrrhiza uralensis* does not involve endoreduplication but does imply an alteration of the O-antigen.

Crespo-Rivas JC[1*], Guefrachi I[2], Mok KC[3], Villa-Ecija JA[1,2], Acosta-Jurado S[1], Ruiz-Sainz JE[1], Taga ME[3], Mergaert P[2], Vinardell JM[1].

[1] Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, C.P. 41012, Sevilla, Spain.[2] Institut des Sciences du Végétal, Centre National de la Recherche Scientifique UPR2355, 91198 Gif-sur-Yvette, France.[3] Department of Plant and Microbial Biology, University of California, Berkeley, Berkeley, CA 94720, USA.

Rhizobia are soil α- and β-proteobacteria able to establish symbiotic associations with many leguminous plants. This symbiosis leads to the formation of nodules, which are specialized organs on roots of legumes in which bacteria reduce N_2 to ammonia. A traditional classification divides nodules into two different types, determinate and indeterminate. First studies on bacteroid differentiation led to the general conclusion that in indeterminate nodules, but not in determinate ones, rhizobia suffered a terminal differentiation process. Later studies, however, showed that several rhizobial strains do not suffer such an irreversible differentiation process in their interaction with indeterminate-nodule forming legumes (Ardissone *et al*., 2011). At present, three different bacteroid differentiation processes, which depend on the particular clade to which any particular legume belongs, are known. Several reports indicate that in rhizobial species that nodulate IRLC (inverted repeat-lacking clade) legumes, such as in the interaction *Sinorhizobium meliloti-Medicago*, bacteroid differentiation is driven by an endoreduplication event that is forced by host NCR (nodule-specific cysteine rich) antimicrobial peptides and requires the participation of the bacterial protein BacA (Mergeaert *et al*., 2006). In this work we have studied bacteroid differentiation of *Sinorhizobium fredii* HH103 in three host plants: *Glycine max*, *Cajanus cajan* and the IRLC legume *Glycyrrhiza uralensis*. Flow citometry and microscopy analyses of bacteroids as well as confocal microscopy studies carried out in nodules showed that *S. fredii* HH103 bacteroids, regardless the host plant, presented DNA contents and cellular sizes similar to those of free living bacteria. Inactivation of *S. fredii* HH103 *bacA* neither affected symbiosis with *Glycyrrhiza* nor increased bacterial sensitivity to *Medicago* NCRs. Finally, HH103 bacteroids isolated from *Glycyrrhiza*, but not those isolated from *Cajanus* or *Glycine*, showed an altered lipopolisaccahride. Electrophoretic analyses suggest that this modification might affect the O-antigen. In pathogens such as *Salmonella*, variations of the O-antigen have been related to the enhancement of the resistance to antimicrobial agents (May and Groisman, 2013). Maybe *Glycyrrhiza*, but not *Glycine* or *Cajanus*, might produce factors responsible for triggering these changes on *S. fredii* HH103 LPS. Our studies indicate that bacteroid differentiation in the symbiosis of *S. fredii* HH103 with the IRLC legume *Glycyrrhiza* does not follow the pathway stated for the model symbiosis *S. meliloti-Medicago*.

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Effective colonization of spinach root-surface by *Rhizobium*

Jiménez-Gómez A[1], Menéndez E[1], Flores-Félix JD[1], García-Fraile P[2], Mateos PF[1,3], Rivas R[1,3].

[1] Departamento de Microbiología y Genética. Edificio Departamental de Biología. Universidad de Salamanca, [2] Institute of Microbiology ASCR, Laboratory of Fungal Genetics and Metabolism, Videnska 1083, 142 20 Prague 4, Czech Republic, [3] Unidad asociada de I+D Universidad de Salamanca (USAL)-CSIC (IRNASA) (España).

Plant Growth Promoting Rhizobacteria (PGPR) are able to promote plant growth and increase crop productivity. For a successful and effective bacteria-plant association, microorganism colonization and its arrangement and abundance on plant root surfaces are very important.

In this study, we analyzed the ability of the strain PEPV12, isolated from *Phaselous vulgaris* nodules and identified as *Rhizobium sp*., to colonize spinach (*Spinacia oleracea*) root surfaces and evaluated three parameters of *in vitro* plant growth promotion: i) siderophores production, ii) phosphate solubilization and iii) indole acetic acid production, as well as tested some nutritional parameters. PEPV12 colonies presented a yellow-orange halo indicative of siderophore production and also, this strain produced indole-3-acetic acid (IAA) at a final concentration of 0,132 mg/L. Phosphate solubilization was not detected.

In order to study colonization, we analysed the ability of PEPV12 to form aggregates, which allow root adherence. Moreover, since cellulose has an important role in the adhesion to the root surface, forming the matrix where bacteria are embedded, we tested *in vitro* cellulose production. In relation with this parameter, biofilm *in vitro* formation was analysed, as typical biofilms improve the colonization, increasing the protection and nutrients availability to the microorganisms (Ramey, et al., 2004). The results showed that this strain colonises effectively and adheres to abiotic surfaces.

Root colonization and adherence events were observed by fluorescence microscopy. GFP-tagged PEPV12 strain was inoculated on spinach seedlings, which were observed daily with a fluorescence microscope, showing a gradually increased attachment to root surfaces and hair roots. Interestingly, we observed morphologic changes in root hairs such as deformations and redirections at the tip in early stages of plant development of PEPV12 inoculated plants, in contrast with uninoculated plants.

Furthermore, we proceeded to evaluate *in vitro* plant development after inoculation of spinach seedlings with the strain of this study, showing significant differences in length development of aerial parts, respect to uninoculated plants.

Our results showed that *Rhizobium sp.* PEPV12 actively and successfully colonises spinach root surfaces, producing changes in root hairs and an increase of plant growth, suggesting its potential as a biofertilizer for *Spinacea oleracea*.

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Effect of cadmium on the symbiosis of *Ensifer meliloti* and *E. medicae* with *Medicago sativa*.

Del Saz M[1], Lara-Dampier V[1], Alías-Villegas C[1], Bellogín RA[1], Cubo T[1], Camacho M[2], Temprano F[2], **Espuny MR**[1].

[1] Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Avda. Reina Mercedes 6, 41012-Sevilla. Spain. [2] Centro Las Torres-Tomejil (IFAPA), Apartado Oficial, Alcalá del Río, Sevilla (Spain)

Cadmium (Cd) is not an essential element for plants; on the contrary, it is highly toxic to plant growth in the environment. It is taken up by the roots and partly translocated to the shoot after a prolonged exposure. Cd decreases growth rate by affecting various aspects of plant physiology, such as reducing carbon assimilation, increasing oxidative stress, nitrogen metabolism, etc. The accumulation of Cd within soil, sediments, and the aquatic environment is of concern because following uptake by plants or other life forms, Cd can be passed on throughout the food chain. It is therefore important to develop methods of rehabilitating Cd polluted soils.

Alfalfa (*Medicago sativa*) is one of the most important crop plants in the world. Alfalfa can be used for the removal of heavy metals due to its rapid growth, high biomass and suitability for nutrient stress habitats. At the same time the use of metal tolerant rhizobial species into metal contaminated soils has improved the vegetative growth, nitrogen-fixing efficiency, yields, and grain quality of various legume crops. In addition, the metal tolerant rhizobia has been found to profoundly reduce the accumulation of toxic metals in plant organs, and, consequently in grains. Rhizobia, in addition of fixing atmospheric nitrogen and to tolerate high concentrations of diverse metals, can synthesize plant growth promoting substances, could therefore, be used as inoculants for improving the productivity of legumes in metal-contaminated soils.

A collection of 30 isolates of *Medicago marina* nodules, mostly belonging to *E. meliloti* except one that was *E. medicae*, were analyzed for their ability to tolerate heavy metals as Cd, Zn, Co and Ni. Most of isolates were able to grow at concentrations of these metals that are considered as resistance values for bacteria. In addition, all of them shared the presence of genes that codify for components of heavy metal extrusion systems: *cadA* (a P_{IB}-type ATPase) and *nolG* (RND family of transporters). Some of these isolates were selected to analyze the potential to facilitate the growth of alfalfa plants in the presence of Cd. With this aim, firstly, it was determined which Cd concentrations (ranging between 0.5 to 3 mg/L of $CdCl_2$) in the nutrient solution let a suboptimal develop of alfalfa plants. Secondly, two of the *M. marina* isolates (*E. meliloti* ORT12 and *E. medicae* SF3.41) and their respective *nolG* mutant derivatives were selected to inoculate alfalfa plants to study the effect of Cd on the symbiosis in the absence of combined nitrogen. The plant tests were performed in Rigaud & Puppo solution solidified with agar (0.8 %) in test tubes with different diameters depending on the assay. It will be analyzed the kinetics of the nodulation, the number of nodules developed and the shoot dry weight at the end of the plant test

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Symbiotic role of the *Rhizobium*-specific type 3 secretion system effectors NopL and NopP, and identification of the novel effector NopI secreted by *Sinorhizobium* (*Ensifer*) *fredii* HH103.

Jiménez-Guerrero I[1], Pérez-Montaño F[1], Cubo T[1], Ollero FJ[1], **López-Baena FJ**[1].

[1*]Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Avenida de Reina Mercedes, 6. 41012, Seville (Spain).

Some Gram-negative phytopathogenic bacteria use the type 3 secretion system (T3SS) to deliver proteins, called effectors, directly into the cytoplasm of the host cells. These effectors suppress plant defense responses to promote infection and growth of the pathogen (Galan and Collmer, 1999). The T3SS has also been found in some symbiotic rhizobial strains and the secreted effectors, collectively known as nodulation outer proteins (Nops), are involved in host-range determination and symbiotic efficiency. Synthesis and secretion of Nops is controlled by the T3SS transcriptional regulator TtsI whose transcription is NodD- and flavonoids-dependent (Deakin and Broughton, 2009).

Sinorhizobium (*Ensifer*) *fredii* HH103 is a broad-host range bacterium able to nodulate dozens of legumes including soybean, which is considered its natural host plant. This bacterium secretes at least eight proteins through the T3SS in response to inducer flavonoids (Rodrigues *et al*., 2007). Two of these effectors, NopL and NopP, are specific to rhizobia and have no homologues in plant pathogens. NopL is phosphorylated by plant kinases and its function could be the modulation of host MAPK signaling or impair function of MAPK substrates. Inactivation of *nopL* induces the formation of necrotic areas in bean nodules, suggesting a possible role in suppression of premature senescence. NopP is also phosphorylated by plant kinases but its exact function in symbiosis is still unknown. However, inactivation of the *S. fredii* HH103 *nopP* gene causes an increase in the number of nodules formed in soybean (Deakin and Broughton, 2009).

In this work, we described for the first time a new *Rhizobium*-specific effector, which we have called NopI. This effector, like NopL and NopP, was *Rhizobium*-specific and could be of great importance in the symbiosis with soybean and *Vigna unguiculata*. Besides, while inactivation of *nopL* or *nopP* was beneficial for symbiosis with these plants, the absence of both NopL and NopP was detrimental, suggesting that these effectors could exert complementary functions in the symbiotic process. We also confirmed that the expression of both *nopL* and *nopI* was regulated by inducer flavonoids and by the transcriptional regulators NodD1 and TtsI. In addition, translocation of NopL to the cytoplasm of soybean root cells was confirmed by the adenylate cyclase assay.

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Mesohizobial strains as plant probiotic microorganisms (PPM) in cherry tomato plants

Pérez-Yépez J[1], González-Rivero O[1], Real J[2], Hernández M[2], León-Barrios M[1].

[1]Departamento de Bioquímica, Microbiología, Biología Celular y Genética, Universidad La Laguna, [2]IPNA-CSIC, La Laguna

It is widely known that several rhizospheric bacteria are able to colonize plant roots, promoting health and nutrition to the plants through several direct and/or indirect mechanisms (Picard *et al*., 2008). These bacteria, named Plant Growth Promoting Bacteria (PGPB) or Plant Probiotic Microorganisms (PPM), a more currently used term, can be used as an alternative to chemicals in agriculture crops.

Rhizobia are soil bacteria mainly known by their capability to establish a nitrogen-fixing symbiosis with leguminous plants. Some rhizobia, however, have also been involved in plant growth promotion through other mechanisms different to nitrogen nutrition (Yanni *et al*., 2001).

In this study we have assessed several direct plant-growth promotion properties, indole acetic acid (IAA) production, siderophore synthesis and phosphate solubilization, in a collection of mesorhizobial strains belonging to different species or genospecies isolated from root nodules of a wild chickpea, *Cicer canariense*, a legume endemic to the Canary Islands. The results showed that IAA was present in all isolates but the production levels varied greatly depending on strain. Siderophore synthesis was detected in 10% of isolates belonging to different species. Phosphate solubilization was, with few exceptions, restricted to isolates of species *Mesorhizobium ciceri.*

Two mesorhizobial strains, CCANP14 and CCANP122, which gathered at least two promotion properties, were selected to be inoculated in tomato var. cherry seedlings. Moreover, a root-nodule endophytic strain of *Bacillus cereus*, LCAn8, which had previously probed to promote plant growth, was also tested for comparison. Five weeks after inoculation, the plants were harvested and parameters as shoot length, root length; shoot fresh weight and shoot dry weight were measured. All the three strains promoted the growth of the tomato plants in comparison to uninoculated plants. Although *B. cereus* LCAn8 strain produced a good development of the plants, however, due to the potentially harmful effect of this species for human health (Lotte *et al.*, 2008), mesorhizobial strains as CCANP14 and CCANP122 were considered as better and safer biofertilizers, because, so far, rhizobia have never been reported to be noxious to human, plant or animals.

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Boron essentiality in *Ensifer meliloti*: requirement for EPS synthesis, abiotic stress tolerance and rhizobia-legume symbiosis.

Quijada NM[1], Abreu I[1], **Bonilla I**[1], Bolaños L[1].

[1] Dpto. Biología, Facultad de Ciencias, Universidad Autónoma de Madrid. c/ Darwin 2. Madrid. 28049. Spain.

Mineral nutrient unbalance is a common and worldwide problem when performing both intensive and sustainable agriculture. The issue is well addressed in intensive agriculture, although studies give prevalence to macronutrients on micronutrients. However, there is lack of information on mineral nutrition in sustainable agriculture, particularly in the use of the rhizobia-legume symbiosis and its requirements for micronutrients. Boron (B) has been reported essential for rhizobia-legume symbioses, observing defects in nodule organogenesis and function when the microelement is absent. Because B is an essential element for plants, the failure of the symbiosis has been traditionally attributed to effects of B deficiency on the legume partner. Meanwhile, very little input has been paid on soil living rhizobia.

In a previous work, we described a beneficial role of B for capsule production in several laboratory rhizobia species, which could partially explain defects in plant infection and nodule development. Therefore we analyzed the effects of B deprivation on exopolysaccharide (EPS) production and on key genes involved in regulation of succynoglycan (EPS-I) and galactoglycan (EPS-II) synthesis in *Ensifer meliloti*. Boron starvation led to an 80% reduction of EPS production in *E. meliloti* strain 1021 that produces EPS-I, but only to a 50% in strain 8530, able to synthesize both types of EPS. mucR, that plays a key role in positive regulation of EPS-I and negative regulation of EPS-II synthesis, was overexpressed in B-deficient strains. Meanwhile, exoY, the gene encoding the enzyme that catalyses the initial step of EPS-I synthesis, was downregulated in *E. meliloti* 1021 but induced in 8530. Moreover emmA, that negatively regulates EPS-I, was repressed in B-starved 1021 but not in 8530. These results point to a low production of EPS-II and an increase of EPS-I synthesis in B-deprived *E. meliloti* 8530, that was supported by increased calcofluor staining. Accordingly with low production of EPS II, biofilm formation was reduced in B-deprived 8530 strain. Moreover, B-starved *E. meliloti* 1021 was more sensitive than 8530 to detergent and H_2O_2 exposure, and both strains were more tolerant to salinity when grew in the presence of B.

Overall results suggest that B content in soils can alter the synthesis of rhizobia cell surfaces polysaccharides, which compromise both the ability to stablish symbiosis and the tolerance to abiotic stresses. In our opinion bacterial mineral nutrition should be taken into account as important factor in developing inoculants for a sustainable agriculture.

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Functional Characterization of a Chitinase class III (CgCHI3) and a Glutathione S-transferase (CgGST) involved in *Casuarina glauca*- *Frankia* symbiosis

Graça I[1,2], Liang J[1,2], Guilherme M[3], Ferreira-Pinto MM[1], Melo AP[1], Ribeiro-Barros AI[1,2], Pereira AS[3].

[1*]PlantStress&Biodiversity, Centro de Ambiente, Agricultura e Desenvolvimento (BioTrop), Instituto de Investigação Científica Tropical, I.P. (IICT), Av. República, Quinta do Marquês, 2784-505 Oeiras, Portugal, [2]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. República, Quinta do Marquês, 2780-157, Oeiras, Portugal, [3] UCIBIO, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal.

The actinorhizal plants are key elements of natural ecosystems. Besides their high adaptability to extreme harsh environments, most actinorhizal plants have the capacity to obtain high levels of nitrogen due to their ability to establish symbiosis at the root level with nitrogen fixing bacteria of the genus *Frankia*. The symbiosis is an ontogenic process that requires a highly coordinated sequence of events. One of such mechanisms is the induction of defense-related genes, whose main role during the symbiotic interaction remains to be elucidated. In this context, we have studied two defense-related proteins involved in the actinorhizal symbiosis established between *Casuarina glauca* and nitrogen fixing bacteria (N_2) *Frankia*, namely a class III chitinase (CgCHI3) and a gluthatione S-transferase (CgGST) (Fortunato et al 2007; Santos et al 2010). The study consisted in the cloning, overexpression and subsequent biochemical and biological characterization of the recombinant proteins, in order to understand their function during the symbiotic process. For that, the cDNA-ORFs of CgCHI3 and CgGST were cloned into vectors pET-28b and pET-21c, respectively, and overexpressed in *Escherichia coli* BL21(DE3) for the production of recombinant proteins. Despite being produced as protein aggregates, CgCHI3 showed endochitinase, ß-1,3 glucanase, ß-1,4 glucanase and lysozyme activity, as well as the capacity to inhibit the growth of pathogenic bacteria (*E. coli* BL21 and K12, *Paracoccus denitrificans and Bacillus subtillis*). It displayed no antifungal activity (*Colletotrichum gloeosporioides*, *Botrytis cinerea, Trichoderma viride* and *Fusarium oxysporum*) and did not affect the growth of symbiotic bacteria (*Frankia* and rhizobia) or the performance of nodulation factors. The results suggest that CgCHI3 is probably a multifunctional protein involved in the infection process, specifically in the lysis of the cell wall during intracellular penetration or the formation of the infection thread or any other modification of the cells in order to accommodate the symbiotic bacteria. The recombinant CgGST was produced in the soluble form and also proved to be functionally active, showing activity for the substrate 1-chloro2, 4-dinitrobenzene (CDNB), indicating that this protein may be involved in cellular detoxification processes and in response to oxidative stress. To date it has not been described any chitinase with β-1,3 and β-1,4-glucanase activity, so this may be the first case. Besides its importance in the symbiotic process, this protein may also be appreciated in view of the potential biotechnological applications. Regarding the GST, with less commercial value, but no less important from a fundamental point of view, it will be interesting to deepen the functional analysis of studies to better understand its role in symbiosis.

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Optimisation of the immunodetection of tagged-LYK3 Nod-factor receptor in *Medicago truncatula* root extracts

Magdalinin M, **Larrainzar E.**

Dpto. Ciencias del Medio Natural, Universidad Pública de Navarra, 31006 Pamplona (Spain)

One of the mechanisms mediating the legume-rhizobium symbiosis is the molecular recognition at the plasma membrane of signalling molecules called Nod factors. In the model legume *Medicago truncatula* two Nod-factor receptor kinases have been described: NFP and LYK3. They are plasma membrane-localised proteins present at very low amounts in root tissues and even using antibodies specific against these proteins, detection is challenging.

In the current work we employ *M. truncatula* transgenic lines expressing a tagged version of LYK3 that allows immunodetection of the protein using anti-HA antibodies (Haney et al., 2011). In order to optimize the immunodetection protocol in root tissue, a range of extraction conditions and detection methods has been tested. Upon differential centrifugation, the majority of LYK3 was found to precipitate at speeds at which plasma membrane markers remain soluble. Unexpectedly, LYK3 followed the pattern of markers for the endoplasmic reticulum and vacuole, suggesting that it may be also present in larger endomembrane structures. Taking advantage of the immunoprecipitation tag, concentration columns were shown to be effective for the enrichment of the protein, leading to an increase in the signal detected. Interestingly, LYK3 signal was reproducibly identified at a range of molecular sizes: not only at the size corresponding to the full-length protein, but also at lower molecular weights. This observation suggests the existence of post-translational mechanisms (e.g., cleavage), with possible implications in signalling.

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Microbiological characterization of a semi-arid meadow soil seeded with different proportions of orchard grass and alfalfa.

Medina M[1], Valverde A[2], Díaz-Raviña M[3], Santa-Regina I[4], **Igual JM[4].**

[1] Grupo Grica, Facultad de Ciencias Agrarias, Universidad de Antioquia, AA 1226 Medellín, Colombia, [2] Centre for Microbial Ecology and Genomics (CMEG), University of Pretoria, Pretoria 0028, South Africa, [3] Instituto de Investigaciones Agrobiológicas de Galicia (IIAG-CSIC), Avda. de Vigo s/n, 15780 Santiago de Compostela, Spain, [4] Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), C/ Cordel de Merinas 40-52, 37008 Salamanca, Spain.

Soil is a pivotal component of the environment and the maintenance of soil quality is critical for ensuring the sustainability of the biosphere. The use of fertilizers has increased at a faster rate than global food production. Modern agriculture is dependent on phosphorus derived from phosphate rock, which is a non-renewable resource. It is needed to improve P acquisition efficiency for both low input and intensive agroecosystems. However, few studies have focused on cereal-legume interactions with regard to soil P. The objective of this work was to study the effects of grass-legume mixtures and phosphorus fertilization on soil microbial community structure and biomass [determined by fatty acid methyl ester (FAME) profiling] as well as on the soil nitrogen (N) mineralisation rate. A field experiment including mono-cropping and intercropping of alfalfa (*Medicago sativa* L.) and orchard grass (*Dactylis glomerata* L.) at different densities and at two levels of phosphorus fertilization was established in 2008 and soils were monitored in spring, summer and autumn of 2009 and 2010.

PERMANOVA analyses showed highly significant (P<0.01) effects of both year and season of sampling on the soil FAME profiles indicating that the microbial community underwent compositional shifts over seasons and time. Soil microbial biomass and the relative abundance of saprophytic fungi were the microbial parameters most affected by seasonality, while the relative abundance of actinobacteria was higher in the second than in the first year. The highest soil N mineralisation rate was observed in the spring of the first year of sampling (2009), declining sharply thereafter. When soil samples were pooled by season, the type of plant cover greatly affected soil microbial community structure in the three seasons (P<0.01), while the level of phosphoric fertilization did not have a significant impact (P>0.4). The soil microbial community structure in orchard grass monocrops was very different from that of the other plant cover treatments. Soil microbial biomass and the relative abundance of mycorrhizal fungi were higher in soils with predominance of orchard grass, while relative abundances of saprophytic fungi, actinobacteria and Gram positive and Gram negative bacteria increased as the cover of alfalfa increased. Soil N mineralisation rates also tended to increase with increasing the density of alfalfa plants, with the highest and lowest values being observed in the alfalfa and orchard grass monocrops, respectively.

When alfalfa was intercropped with orchard grass, even at low seeding ratio, it deeply influenced the soil microbial community structure, however phosphorous fertilisation had no detectable effect on it. In soils with alfalfa, the relative abundance of Actinobacteria tended to increase with time, which may be due to a progressive soil enrichment in such bacteria since they are commonly associated with legume nodules (Martínez-Hidalgo *et al*., 2014). Also, alfalfa plant densities of 25 percent or higher increased soil N mineralisation and soil N-NO_3 contents with respect to orchard grass monocultures. Therefore, grass-legume intercropping is advantageous for semi-arid meadows.

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Characterization of surface motility in *Sinorhizobium meliloti*: regulation and role in symbiosis

Bernabéu-Roda LM, Soto MJ.

Departamento de Microbiología del Suelo y Sistemas simbióticos, Estación Experimental del Zaidín, CSIC, 18008 Granada, España.

The ability to move provides bacteria with considerable advantages including the capacity to approach and colonize preferred hosts. Some types of surface translocation, such as swarming, are closely linked to the virulence of pathogenic bacteria. Within rhizobia, it is generally accepted that flagella-mediated motility is not essential for nodulation or nitrogen fixation although it is thought to be important in the initial stages of the symbiosis, affecting bacterial infectivity and competitiveness. Nevertheless, this conclusion is based on results obtained mostly with genetically undefined mutants defective in swimming motility. Recent reports have shown that the alfalfa symbiont *Sinorhizobium meliloti* can exhibit different modes of surface translocation and there is evidence that suggests the existence of different control mechanisms over this type of motility among strains (1, 2). The molecular mechanisms involved and the role played by the different modes of surface translocation in the establishment of symbiosis are largely unknown. To gain insights about these issues, we have characterized the surface translocation shown by two *expR*- deficient *S. meliloti* reference strains (Rm1021 and GR4) by analyzing the behavior of a set of genetically defined mutants on new and more permissive conditions for *S. meliloti* surface spreading. In addition, we investigated the symbiotic phenotype of two non-flagellated mutants with different surface motility phenotypes.

Our results show that Rm1021 can move over surfaces using at least two different types of motility: swarming and a flagella-independent mode of translocation. Of these, the latter is the most relevant as indicated by the phenotype shown by a *flaAB* mutant and is highly dependent on siderophore rhizobactin 1021 production. Surprisingly, this flagella-independent motility is abolished in an *flgE* mutant in which flagellum assembly is interrupted in an earlier stage than in the *flaAB* mutant. On the other hand, GR4 which moves over surfaces less efficiently than Rm1021, uses exclusively swarming as revealed by the non-motile phenotype of the *flaAB* derivative mutant. Intriguingly, in a GR4 *flgK* mutant, a flagella-independent surface translocation is triggered through an as yet unknown mechanism. In addition, we found that the *fadD* loss-of-function known to promote surface translocation in Rm1021 and GR4 (1, 2) promotes a flagella-independent type of motility in the latter strain while interfering with the surface movement of a Rm1021 *flaAB* mutant. Assessment of the symbiotic phenotypes of GR4-derivative *flaAB* and *flgK* mutants indicate that flagella-dependent motility positively influences the competitiveness for nodule occupation but is not essential for infectivity.

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Acknowledgements

This study unveils the complexity of surface motility in S. meliloti and suggests the existence of flagella-independent modes of surface translocation which seem to be controlled in a co-ordinated manner with the stage of flagellar assembly.

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Induction of systemic resistance by *Pseudomonas fluorescens* N21.4 and derived bioeffectors on growth, photosynthesis and protection against *Xanthomonas campestris* in tomato

Lucas JA[1], Boukerma L[1.2.3], García-Villaraco A[1], Bonilla A[1], Ramos-Solano B[1], **Gutierrez E**, Gutierrez-Mañero J[1].

[1]Universidad San Pablo CEU, Facultad de Farmacia, P.O. Box 67, Boadilla del Monte, 28668, Madrid, Spain. [2]Laboratoire National de Recherche en Ressources Génétiques et Biotechnologies, ENSA, Al Harrach 16131.Algérie. [3]Laboratoire de Protection et de Valorisation de Ressources Agro-Biologiques. ·Faculté SNV. Université Saad Dahleb, Blida 1. 09000, Algérie.

Plants are invaded by an array of pathogens of which only a few succeed in causing disease. The others pathogenic microbes are countered by a sophisticated immune network system possessed by the plants (Muthamilarasan and Prasad, 2013; Wiesel et al., 2014). Activation of plant immunity is largely depends on recognition of elicitors, known as conserved microbial features, called Microbe-Associated Molecular Patterns (MAMPs), Pathogen-Associated Molecular Patterns (PAMPs), and/or damage-Associated Molecular Patterns (DAMP) by transmembrane pattern recognition receptors (PRRs) in plant cells (Dangl and Jones, 2006).

This study aims to evaluate the ability of *Pseudomonas fluorescens* N21.4 and derived bioeffectors to protect tomato against *Xantomonas campestris* and find out the interrelationship between growth, protection, photosynthesis, oxidative stress enzymes and pathogenesis related proteins as markers of the systemic induction resistance. N21.4 caused significant suppression of bacterial spot disease in tomato by 40%-60%, stimulated growth by 17% in shoot dry weight and 40 % in root dry weight. Protection of tomato was associated with a transient increase of antioxidant enzymes activity. Guaiacol peroxidase (GPX) increased strongly after 24h of pathogen inoculation and SOD reached maximum level after 72h. Ascorbate peroxidase (APX) peaked after 48h as well as glutathione reductase (GR) activity showed a significant difference. The defense-related enzymes ß-1,3 glucanase (PR2), chitinase (PR3) achieved a height level 48h after pathogen challenge inoculation. N21.4 has not trigger polyphenoloxydase (PPO) activity. Nevertheless, N21.4 improved photosynthetic activity throughout an elevated total chlorophyll amount, a low ration chlorophyll a/b, an increased quantum efficiency of photosystem II (ϕ PSII), lowest value of minimum fluorescence (Fo) and induced change in non-photochemical quenching (NPQ). Big fraction bioeffector was most effective for disease reduction by 80% and maintains the same growth level as the healthy control. UV-killed N21.4 and small fraction bioeffector decreased dry root weight and ensure 50% of disease suppression. Our results suggest that N21.4 has primed tomato immunity; by inducing substantial metabolic and transcriptomic changes that are behind the mechanisms of systemic induced resistance, by early or late over-expression of reactive oxygen species scavenging enzymes and PR2, PR3. Structural componentes of cell surface of N21.4 are among determinant to trigger ISR and metabolic elicitor are more effective. Systemic induced resistance could be associated with growth enhancement or a decrease; that depends to the elicitor and the response of the plant, how it can manage its metabolism and allocating its resources. Enhancement of photosynthetesis and growth could have a key role in the tolerance of tomato against pathogen by saving some energy to cover immunity costs.

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The *Rhizobium*-specific type 3 secretion system effectors NopL and NopP secreted by *Sinorhizobium* (*Ensifer*) *fredii* HH103 are phosphorylated by soybean kinases and delivered to the nucleus of the host cell.

Jiménez-Guerrero I[1], Pérez-Montaño F[1], Medina C[2], Monreal JA[3], Ollero FJ[1], López-Baena FJ[1].

[1*] Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Avenida de Reina Mercedes, 6. 41012, Seville (Spain). [2] Departamento de Biología Molecular e Ingeniería Bioquímica. Universidad Pablo de Olavide. Centro Andaluz de Biología del Desarrollo (CABD). Ctra. Utrera, km 1. 41013, Seville (Spain). [3] Departamento de Fisiología Vegetal, Facultad de Biología, Universidad de Sevilla.

Pathogenic Gram-negative bacteria deliver effector proteins directly into the eukaryotic host cell via a specialized apparatus called Type 3 secretion system (T3SS). These effectors suppress plant defenses to promote disease but they can also be recognized by specific plant receptors that trigger a strong defense reaction to eliminate the pathogen (Pallen *et al*., 2003). The T3SS has also been found in some symbiotic rhizobial strains and the effectors secreted, involved in host-range determination and symbiotic efficiency, are also delivered to the interior of the host cells (Deakin and Broughton, 2009).

The broad host-range bacterium *S. fredii* HH103 secretes at least eight proteins through the T3SS. Two of these proteins, NopL and NopP, are phosphorylated by plant kinases, but their exact function in symbiosis is yet unknown (Deakin and Broughton, 2009). However, some results indicate that NopL could be involved in the modulation of the host MAPK signaling and in the suppression of premature senescence of nodules.

In this work, we studied the function of the *Rhizobium*-specific effectors NopL and NopP secreted by *S. fredii* HH103 in the symbiosis with soybean, which is considered its natural host plant. Both NopL and NopP were phosphorylated by soybean root kinases and the phosphorylation cascade was Ca^{2+}- and calmodulin-dependent. While the signaling pathway that culminates in the phosphorylation of NopL included ser/thr and MAPKK kinases, in the case of NopP this pathway was composed of ser/thr and tyr kinases but not MAPKK kinases.

Transient expression in *Nicotiana benthamiana* leaves of both *nopL* and *nopP* fused to YFP and further confocal imaging indicated that these effectors localized to the nucleus of the host cell and accumulate in nuclear foci, suggesting a possible role in plant gene regulation or responses to DNA stress. In this sense, the use of a yeast based array to determine functions of effectors indicated that NopP could be involved in microtubule-related processes and nuclear localization and migration. Finally, co-immunoprecipitation analyses of *N. benthamiana* NopL- and NopP-interacting proteins showed that NopL binds to proteins related to the plant immune response and also with calreticulin and NopP interacts with proteins related to nucleic acids (e. g. histone H4) or proteins related to plant immunity (GRAS2 transcription factors or cyclophilin 40).

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Different effects on *Vigna unguiculata* plants after the inoculation with strains from phylogenetically divergent *Bradyrhizobium* symbiovars

Silva LR[1,2,], Bejarano A[4], Andrade P[5], Velázquez E[4], Rivas R[4]

1* CICS-UBI-Universidade da Beira Interior, Covilhã, Portugal. 2 Instituto Politécnico de Castelo Branco, Castelo Branco, Portugal.3 LEPABE, Universidade do Porto, Porto, Portugal, 4. Departamento de Microbiología y Genética, Universidad de Salamanca, Spain. 5 REQUIMTE/. Laboratório de Farmacognosia, Universidade do Porto, Porto, Portugal.

The species *Vigna unguiculata* (cowpea) from the Tribe Phaseolae is indigenous to Africa where the Transvaal region is considered its evolutionary centre since the oldest varieties of this legume were found in this region (Paludosi and Ng, 1997). This species was introduced from Northern Africa into the South of Europe where this legume is currently cultivated in Mediterranean regions such as Extremadura (Spain), a warm region with acidic soils which is very appropriated for the cultivation of this legume and where it is very appreciated by the consumers. In a previous work we analysed the core and symbiotic genes of several rhizobial strains isolated from *V. unguiculata* nodules in a soil from Extremadura (Bejarano *et al.*, 2014). All of them belong to the genus *Bradyrhizobium* which was divided into groups I and II by Menna *et al.* (2009) on the basis of the 16S rRNA gene analysis. Most of *V. unguiculata* strains belong to the group I, whereas only one strain was classified into the group II. This last strain belongs to a new symbiovar (vignae) whereas all the remaining strains belong to the symbiovar genisteae, according to the results of the *nodC* gene analysis (Bejarano *et al.*, 2014). In the present work, we analysed the effect of the inoculation of strains VUPME29, representative from symbiovar genisteae, and VUPME10 (symbiovar vignae) in *Vigna unguiculata* yield and bioactive compounds production. The results showed that plants inoculated with the strain VUPME10 from symbiovar vignae have higher number of nodules per plant and dry shoot weigth than those inoculated with the strain VUPME29 from symbiovar genisteae. Also differences were found in the concentration of some bioactive compounds in leaves of *Vigna unguiculata* inoculated with these two strains. This is the first study about the effect of inoculation with strains from different symbiovars in the yield and the bioactive profile of a legume.

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Characterization of NopC, a type 3 secreted effector of Sinorhizobium fredii HH103.

Pérez-Montaño F[1], Jiménez-Guerrero I[1], Ollero FJ[1], López-Baena FJ[1].

[1] Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain.

Sinorhizobium fredii HH103 is a nitrogen-fixing bacterium able to nodulates many legumes, including soybean, which is considered its natural host plant. In several rhizobia, root nodulation is influenced by proteins, called Nodulation outer proteins (Nops), secreted through the type 3 secretion system (T3SS). This specialized secretion apparatus is a common virulence mechanism of many plant and animal pathogenic bacteria that delivers proteins directly into the eukaryotic host cells. These proteins are called “effectors” due to their action within the eukaryotic cell, where they interfere with signal transduction pathways and promote infection suppressing host defenses. In rhizobia, these proteins are involved in host-range determination and symbiotic efficiency (Deakin and Broughton, 2009). HH103 secretes at least eight Nops through the T3SS (Rodrigues et al., 2007; López-Baena et al., 2008). Some of them cannot be considered real effectors, since they are components of the extracellular appendages of the T3SS machinery. Interestingly, among the effectors secreted, there are *Rhizobium*-specific proteins, such as NopC, that do not have homologues in pathogenic bacteria. To date, no reports about the role in symbiosis of this putative effector protein have been published. In this work, we characterize for the first time the *S. fredii* HH103 *nopC* gene and confirm that its expression is regulated in a flavonoid-, NodD1- and TtsI-dependent manner. Besides, results indicate that the HH103 NopC have a positive effect on symbiosis with soybean and that this protein is delivered directly into *Glycine max* cv Williams nodule root cells by means of the T3SS machinery. All these results indicate that NopC can be considered a real effector secreted by *Sinorhizobium fredii* HH103.

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A *Rhizobium tropici* CIAT 899 *nolR*-like gene is involved in the regulation of the symbiotic process.

Megías M[1], Del Cerro P[1], Rodríguez-Carvajal MA[3], Gil-Serrano A[3], Hungria M[2], Ollero FJ[1].

[1]Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Sevilla, España. [2]Embrapa Soja, Londrina, Paraná, Brasil.
[3] Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla. Sevilla, España.

The establishment of the symbiosis between bacteria collectively known as rhizobia and their specific host legume plant, as well as the process of forming structures called nodules and biological nitrogen fixation resulting from this interaction, are highly complex events. This is a molecular dialogue that begins with exudation of plant flavonoids, which are recognized by rhizobia. When rhizobia are induced by these plant molecules, they synthesize lipochitooligosaccharides (LCOs), also known as Nod factors, responsible for launching the nodulation and nitrogen fixation processes. In this work we aim to elucidate the functional role in symbiotic processes of a new unknown transcription regulator belonging to the ArsR family that is located in plasmid pRtrCIAT899c, a cryptic plasmid of *Rhizobium tropici* CIAT 899, and its functional role in symbiotic processes.

Protein blast did not show equal identity in other known rhizobia species, but revealed similarity with the NolR protein (44-50% of identity) in different bacteria including CIAT 899. We propose to annotate this new gene as "*nrlC*". The *nrlC* mutant was obtained and then symbiotic phenotypes were evaluated. We found significant differences, mainly in nodulations assays, where the mutant is less efficient in nodulation than the wild-type strain in common bean. In addition, Nod factor profiles were carried out, showing different molecular decorations in comparison with the wild-type strain. Other symbiotic-related phenotypes such as EPS production and motility were analyzed and showed also significant differences.

We have characterized a new transcriptional regulator present in a non-symbiotic plasmid which mediates symbiosis directly or probably indirectly via other regulators in CIAT 899. Finally, we propose that this newly described regulator may modulate the expression of nodulation genes like the general transcriptional regulator NolR.

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Interactions between *Micromonospora* and arbuscular mycorrhizal fungi

Martínez-Hidalgo P. [1], García-Romera I. [2], Ocampo JA. [2]

[1*] Department of Microbiology and Genetics, University of Salamanca, Plaza Doctores de la Reina s/n, Salamanca, Spain. [2] Department of Soil Microbiology, Estación Experimental del Zaidín. Consejo Superior de Investigaciones Cientificas. Profesor Albareda 1. 18008. Granada-España.

Soil microorganisms are important to plant development and growth as they improve nutrient uptake, produce plant growth-stimulating substances and prevent pathogen attacks (Turner et al. 2013). The arbuscular mycorrhizal (AM) fungi are an important component of the soil microbial biomass. They are obligate symbiotic soil fungi that colonize the roots of the majority of terrestrial plants. The symbiosis is mutualistic based on bidirectional nutrient transfer between the symbionts. The plant benefits particularly through enhanced phosphorus, water and mineral nutrient uptake which often results in better growth. However, AM fungi not only improve plant growth but also can enhance soil structure, interact with other microbes and alleviate biotic and abiotic plant stress. There has also been stated that some soil microorganisms are known to promote the AM symbiosis (Adriano-Anaya et al., 2006) and have been named "mycorrhizal helper microorganisms".

Actinobacteria are particularly important and common components of the soil rhizosphere (McCarthy and Williams 1992). Studies on the interaction between actinobacteria and AM fungi are scarce and mainly centered on the *Streptomyces* genera.

Some *Micromonospora* strains are able to promote plant growth (Martinez-Hidalgo et al., 2014). *Micromonospora* activity against some fungal plant pathogens have been also found; these actinomycetes can suppress the development of plant disease by inhibiting the growth of plant pathogens.

Although *Micromonospora* improves plant growth and has antifungal activity against plant pathogens, the interaction between *Micromonospora* and AM fungi has not been studied before, therefore, the aim of the study was to work on the understanding of this interaction.

The results obtained allow us to conclude that: The *Micromonospora* strains ALFpr18c and ALFb5 did not adversely affect the colonization and development of the AM fungi *R. irregularis* and *F. mosseae* on plant root. *Micromonospora* strain ALFpr18c increased the beneficial effect of the AM fungi on shoot dry weight of alfalfa and tomato when the AM fungi were established on the roots. However, *Micromonospora* strains ALFb5 decreased the beneficial effect in some AM fungi-plant combination, therefore it is possible to use *Micromonospora* ALFpr18c combined with AM fungi to improve plant growth.

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Session 4

Inoculants for agriculture
and environmental science

Rhizobium as plant probiotic for non-legumes: effect on strawberry production in greenhouse conditions

Flores-Félix JD[1], Marcos-García M[1], Silva LR[2,3,4], Menéndez E[1], Martínez-Molina E[1,3], Mateos PF[1,5], Velázquez E[1,5], García-Fraile P[1], Andrade P[4], Rivas R[1,5]

[1]*Departamento de Microbiología y Genética, Universidad de Salamanca, Salamanca, Spain, [2]CICS-UBI-Universidade da Beira Interior, Covilhã, Portugal. [3]Instituto Politécnico de Castelo Branco, Castelo Branco, Portugal.[4] LEPAE, Universidade do Porto, Porto, Portugal, [5]Unidad Asociada Universidad de Salamanca -CSIC (IRNASA), Salamanca, Spain.

Last advances in biofertilization show the importance of plant probiotic bacteria to increase the yield and quality of crops (Spence *et al.*, 2012). These bacteria present different *in vitro* plant growth promotion mechanisms like nitrogen fixation, phosphate solubilization and production of siderophores and phytohormones. Nevertheless, bacteria used in plant biofertilization must be safe for human, animal and plant health (García-Fraile *et al.*, 2012). Therefore, selection of non-pathogenic bacteria is a necessary step in biofertilizers design based on plant probiotics, being the genus *Rhizobium* a good candidate for this purpose. Here we outlined the inoculation of a strain from this genus, PEPV16, isolated from *Phaseolus vulgaris* nodules (Flores-Felix *et al.*, 2014), in strawberry plants (*Fragaria x ananassa* cultivar Camarosa), since this rhizobial strain have several *in vitro* plant growth promotion mechanisms, like siderophore production, phosphate solubilization and IAA production. In the present work we showed that the inoculation with strain PEPV16 of strawberry seedlings produced an enhancement in first stages of plant development, including a significative increase in length roots and in secondary roots number. Microscopy studies confirmed its high ability to colonize the entire root surface regarding to uninoculated seedlings, as has been previously observed in other vegetables inoculated with this strain (Flores-Felix *et al.*, 2014). The inoculation with the strain PEPV16 in microcosms assays resulted in an increase of the main parameters indicative of plant production. The length and number of stolons, and the number of fruits and flowers increased in inoculated plants comparing to the uninoculated plants. Changes in the content on several organic acids with respect to uninoculated controls also were observed. These results confirmed that *Rhizobium* is a good plant probiotic ideal for biofertilization of plants whose fruits are raw consumed, such as strawberries, increasing their yield and quality and then improving the sustainability of this horticultural crop.

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How safe is your Plant Growth Promoting Bacteria?

Manzanera M, Vilchez JI, García-Fontana C, Aranda E, González-López J.

Institute for Water Research and Department of Microbiology. University of Granada, Spain.

Many microorganisms represent an alternative to transgenic plants in order to increase crop production. These microorganisms described as Plant Growth Promoting Bacteria (PGPB) by Kloepper and Schroth (1978), are able to colonize plants and to enhance their growth by different mechanisms. Some of these microorganisms could represent a real threat to humans, animals or plants health, however its use can easily granted on the grounds of being recommended as biofertilizers. Despite of the legislation on genetically modified food on several countries, little has been described on the biosafety tests needed for the use of wild type microorganisms applied to crops. Here we propose a set of tests and an evaluation system for a correct assessment of the different bacterial strains to guarantee the biosafety of their use. These tests include the analysis of the bacterial potential to alter plant growth (using pepper, tomato, corn and soybean), to evaluate the toxic effect of released compounds by the microorganism on light emission by Vibrio fischeri as indirect measurement of its metabolism, the effect of the bacterial cells themselves on the survival, reproduction and lifespan of nematodes (Caenorhabditis elegans), on annelids such as earthworms (Eisenia foetida), of arthropods such as lacewings (Chrysopela carnea) and colon ladybirds (Adalia bipunctata), as well as their pathogenicity on upper organisms such as mice (Mus musculus CD1). Having the results from these different tests we propose a scoring system to summarize the results and give a confidence value as safe microorganisms within the assays limitations.

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Rhizobial biofertilizers for ornamental plants

Rivas R[1,2], Escribano-Viana R[1], Flores-Félix JD[1], Mateos PF[1,2], Menéndez E[1].

[1*]Departamento de Microbiología y Genética. Universidad de Salamanca. Spain, [2]Unidad Asociada Universidad de Salamanca- CSIC 'Interacción Planta-Microorganismo'. Salamanca. Spain.

Plant growth promoting rhizobacteria, commonly called PGPRs, are one of the most important groups of rhizospheric microorganisms. This group includes free-living bacteria, endophytes and endosymbiotic bacteria, such as *Rhizobium* and *Frankia*, among others (Glick, 2012). PGPRs colonize plant roots, conferring several benefits to the plants through different mechanisms, which result in the improvement of plant health and development.

Despite of its classical involvement in root-nodule symbiosis with legumes, recent studies performed in our research group revealed the beneficial role of the genus *Rhizobium* as PGPR for several non-legume crops, such as vegetables or cereals (García–Fraile et al., 2012; Flores-Félix et al., 2013), supporting its inclusion in biofertilization schemes. However, studies based in the application of PGPRs as inoculants for ornamental crops are limited.

Ornamental crops have a high economic importance for several regions, being European Union in the first place of worldwide producers and Spain among the major producers of cut flowers. Due to their importance, in this study, we selected *Dianthus caryophyllus*, commonly known as carnation, and a rhizobial strain, PEPV13, which belonged to a collection obtained from *Phaseolus vulgaris* nodules, for the evaluation of the PGPR effects in this plant. Selected strain was identified as *Rhizobium sp*. and presented PGPR mechanisms; phosphate solubilization, siderophores production and IAA and precursors biosynthesis were positive in the selected strain.

In vitro assays showed an increase in carnation development in early steps of plant development, when plants were inoculated with PEPV13 respect to uninoculated plants. GFP-tagged *Rhizobium sp.* PEPV13 colonized effectively carnation root surfaces, producing typical biofilm structures. Interestingly, an increase in root hairs redirections was observed in PEPV13 inoculated plants.

To the best of our knowledge, this is the first study reporting the effects of *Rhizobium* in *Dianthus caryophyllus*, showing effective root colonization and an improvement in early stages of plant development.

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Selective immobilization of plant-growth-promoting- bacteria on inorganic carriers: Key considerations for choosing the most suitable carrier

Bejarano A, Mitter B, Preininger C

Austrian Institute of Technology, Department of Health & Environment, Bioresources Unit, Konrad-Lorenz-Strasse 24, 3430 Tulln, Austria.

Microbial biofertilizers and biocontrol agents are promising alternatives to resource consuming agrochemicals in sustainable agriculture. However, more research is needed in the field of inoculant production to find methods to improve formulations and their application in the field. Conventionally used solid and liquid formulations still encounter limiting factors, mainly due to poor bacteria survival during storage and field application (John et al., 2011). There is also lack of knowledge regarding how to evaluate the most suitable carrier in bacteria immobilization for a specific application. The ideal carrier should be inert, non-toxic, of porous structure, relatively cheap, easily available, and environmentally friendly and should provide a rough, irregular surface for colonization (Durham et al., 1994). On such type of carrier the whole external surface, cavities, crevices, irregularities and all pores are accessible for dense bacterial colonization. But for all that it is not known whether the main factors controlling the degree of bacterial adsorption are related to the capacity of the solid surface, or to physicochemical interactions, or to some combination thereof (Gordon and Millero, 1984). Therefore, the aim of our study was to get insight into the immobilization process itself and investigate how the type of carrier influences bacteria adsorption.

To address this challenge the plant-growth promoting strain *Burkolderia phytofirmans* PsJN was immobilized onto inorganic carriers such as talc, bentonite and silica. The particle sizes of the carriers ranged from 11 to 20 µm. The immobilization process was monitored over time. Carriers were incubated for 1, 3 and 24 hours. Surprisingly, 1 hour incubation was sufficient for bacteria adsorption and longer incubation times did not further enhance the adsorption rate. All carriers, talc, bentonite and silica adsorb acceptable numbers of viable cells (in order of 10^8 – 10^{10} CFU g^{-1} of carrier) after 1 hour of incubation of carrier with a proper volume of saturated bacterial suspension.

Next, the interaction between carrier and bacteria was studied as a function of the surface charge and hydrophobicity of the carrier, described by the zeta potential and the contact angle, respectively. The immobilization of bacteria onto carriers showed significantly positive correlation with carrier hydrophobicity, but not with its zeta potential. This indicates that the bacterial immobilization on mineral carriers is most likely controlled by the type of material used rather than the surface charge. Inoculated carriers were also characterized by Scanning Electron Microscopy. Finally, the effect of residual humidity on bacterial survival was studied. We found that the process of air drying rather largely reduced the viability of PsJN. Just in case of talc viable cells were recovered after drying, which evidence the better bacteria protection provided by talc compared with the other tested carriers.

These findings together with the stronger ability of this carrier to adsorb and protect bacteria make talc the most suitable carrier for *B. phytofirmans* PsJN. Hence, talc-based formulations will be further exploited and tested under greenhouse and field conditions regarding their efficiency to deliver bacteria to developing maize plants.

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Flooding and nitrate induce N_2O emission from soybean nodules

Tortosa G[1], Hidalgo A[1], Salas A[1], Bedmar EJ[1], Mesa S[1], Delgado MJ[1].

[1*]Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidin (EEZ), CSIC, E-419, 18080 Granada, Spain.

Nowadays, mitigation of greenhouse gas (GHG) emissions is one of the main scientific and politician concern. According to IPCC Fifth Assessment Synthesis Report (IPCC, 2014), agriculture, forestry and other land use (AFOLU) contribute to about 25% of the total direct GHG emissions. In agricultural soils, the GHG nitrous oxide (N_2O) is emitted by two main microbial processes, nitrification and denitrification, which convert synthetic nitrogen fertilizer applied to soils into N_2O. Denitrification occurs under low-oxygen conditions in which, nitrate is sequentially reduced to nitrogen gas according to the following chemical equation:

$$NO_3^{-} \xrightarrow{(a)} NO_2^{-} \xrightarrow{(b)} NO \xrightarrow{(c)} N_2O \xrightarrow{(d)} N_2$$

Biological nitrogen fixation by legume-rhizobia symbiosis could be an effective and environmental-friendly alternative to nitrogen fertilization and hence, to mitigate soil N_2O emissions. However, legume crops also contribute to N_2O emissions by providing N-rich residues for decomposition, and directly by some rhizobia that are able to denitrify. *Bradyrhizobium japonicum* is a gram-negative soil bacterium able to both, fix nitrogen in symbiosis with soybean and denitrify under free-living and symbiotic conditions. In this bacterium, denitrification depends on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes encoding nitrate[(a)]-, nitrite[(b)]-, nitric oxide[(c)]- and nitrous oxide reductase[(d)], respectively (Bedmar *et al.*, 2013). A better understanding of the environmental factors involved in the emission of N_2O from nodules will be instrumental for the development of strategies and management practices in agriculture, especially for mitigating release of N_2O from legume crops.

In this work, we have established an experimental methodology to measure *in vivo* N_2O emissions from nodulated roots using a close chamber technology coupled to electron capture detector-gas chromatography. Soybeans (*Glycine max L.* Merr., cv. Williams) were grown under controlled conditions in the presence or not of 4 mM nitrate and subjected to flooding. To investigate the contribution of denitrification in N_2O emission from nodules, soybean plants were inoculated with *B. japonicum* USDA110 and a *napA*[(a)], and *nosZ*[(d)] denitrification mutants.

While nitrate addition enhanced N_2O production from nodulated roots as compared to untreated plants, however nitrate and flooding produced a much greater induction of N_2O emission. N_2O production from nodules formed by the *napA* mutant was significantly lower than those produced by the wild-type strain. By contrast, nodules from plants inoculated with the *nosZ* mutant accumulated higher levels of N_2O compared to wild-type nodules. These results demonstrate that nitrate and flooding are important environmental factors for N_2O emissions from soybean nodules and that *B. japonicum* denitrification is involved in such emission.

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Inoculation with indigenous *Rhizobium* strains isolated from common bean (*Phaseolus vulgaris* L.) nodules increases the crop yield under conventional and conservation tillage systems in Northern Spain

Mulas D[1], Seco V[2], Casquero P[3], Velázquez E[4], González-Andrés F[1].

[1] Instituto de Medio Ambiente Recursos Naturales y Biodiversidad. Universidad de León. [2] Departamento de Ingenieríay Ciencias Agrarias. Universidad de León. [3] Escuela Superior y Técnica de Ingeniería Agraria. Universidad de León. [4] Departamento de Microbiología y Genética. Universidad de Salamanca.

Common bean (*Phaseolus vulgaris* L.) crops hold the potential to obtain higher yields by enhancing biological nitrogen fixation (BNF) with *Rhizobium*. In contrast to other legumes, the response of common bean to inoculation with *Rhizobium* has shown a lack of positive response in many cases (Rodriguez-Navarro et al., 2000), which has led to a limited use of rhizobial inoculants. The adaptation of bacterial strains to the rhizosphere conditions is a key factor in the success of any inoculant, especially in a promiscuous legume such as common bean.

This research aimed at increasing common bean crop yields with effective indigenous *Rhizobium leguminosarum*. Three highly effective *R. leguminosarum* strains (LCS0306, LBM1123 and ZBM1008, Mulas *et al.*, 2011) were separately inoculated in common bean in a field experiment. The experiment that was carried out under three environments and three tillage systems: conventional tillage (CT), no-tillage (NT) and a cover crop (CC).

The crop yield observed with the seed inoculation was significantly higher than the yield with a non-inoculated seed. Furthermore, under CT, the inoculation with *R. leguminosarum* LCS0306 produced higher yields than the nitrogen-fertilised crop, whereas yields under NT and CC proved not to be as high as those achieved under CT. The results in terms of grain yield were consistent with the analysis of the total biomass, the harvest index and the N content in seeds.

The findings related to the effect of different tillage systems suggest that conservation tillage (which includes NT and CC) is not the most appropriate for the inoculation of this crop under the edaphic and climatic conditions of Northern Spain. This is consistent with the controversy about the effects of tillage in BNF, because the soil parameters affected by the tillage may have in turn an effect in the BNF (Liu *et al.*, 2010).

The inoculation of common bean also provided the soil with a high-quality organic matter for the following season.

This is the first attempt to develop common bean inoculants in Europe based on indigenous microorganisms.

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Rhizobium as potential biofertilizer of *Eruca sativa*

Rubio-Canalejas A, Celador-Lera L, **Cruz-González X**, Menéndez E, Rivas R.

Departamento de Microbiología y Genética, Universidad de Salamanca, Spain

Eruca sativa, commonly known as rocket salad, is one of the leading leafy vegetables, belonging to the Brassicaceae family that also include other species of agricultural importance such as: broccoli, cabbage, cauliflower, mustard, turnip and radish, among others. Despite being native to the Mediterranean coast, it is widely cultivated and consumed in most of the world; therefore, its popularity is due to its peculiar flavor and nutritional value as its content in essential elements, carotenoids, vitamin C, fiber, flavonoids, etc., (Chou, 2003). In addition to the nutritional potential, different research has attributed some other properties, such as biofumigant, antidiabetes complement, antimicrobial, antiphlogistic, diuretic, aphrodisiac, antioxidant, antiulcer and many more.

To maintain agricultural production, the use of chemical fertilizers, pesticides and herbicides have been occurring indiscriminate, which result in damage to soil, reducing biodiversity and producing adverse health effects (Selma et al., 2010). An effective alternative is the use of biofertilizers based in plant growth promoting rhizobacteria (PGPR) associations. These microorganisms colonize rhizosphere, producing an increase in the available concentration of minerals, in addition, some rhizobacteria have the ability to mimic the synthesis of plant hormones, by these and other characteristics they encourage the increased; germination, weight, yield and resistance to biotic and abiotic stress, (Chou, 2003). Among PGPRs, one of the groups that stand out are the rhizobia, which are able to fix nitrogen in symbiosis with legumes. However, recent research has shown its ability to support the growth of non-leguminous plants to make available certain elements to be used by the plant (Garcia-Fraile et al., 2012). The main aim of this study is to evaluate the potential use of *Rhizobium* sp. strain CRZM18R (isolated as radicular endophyte in *Zea mays* of Ciudad Rodrigo, Salamanca) as biofertilizer for *Eruca sativa*.

We performed different assays to demonstrate the potential of the strain CRZM18R as a PGPR. According to the obtained results, sequencing of CRZM18R 16S rRNA gene, classified this strains in the genus *Rhizobium*. This strain cannot solubilize phosphate under *in vitro* conditions. Moreover, is a microorganism that produced siderophores, although in minor quantities. However, it was excellent producer of the hormone indoleacetic acid (IAA) and its precursors. When this strain was inoculated in *E. sativa* plants, the shoot length and root number were increased, especially the radicular system experiments a 2-fold increase at 8 days post-inoculation compared with uninoculated plants. Therefore, our results support the possible inclusion of *Rhizobium* sp. strain CRZM18R in formulations as potential biofertilizer for *Eruca sativa* crops.

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Rhizobium cellulosyliticum as coinoculant enhances Phaseolus vulgaris (common bean) growth.

Díez-Méndez A[1], Menéndez E[1], García-Fraile P[2], Celador-Lera L[1], Mateos PF[1,3] Rivas R[1,3].

[1]Departamento de Microbiología y Genética, Universidad de Salamanca (Spain). [2] Institute of Microbiology Academy of Sciences of the Czech Republic, Prague, (Czech Republic), [3]Unidad asociada de I+D Universidad de Salamanca (USAL-CSIC) (IRNASA) (Spain).

The *Rhizobium*-legume symbiosis is a complex partnership, which implicates many factors. Nevertheless, bacterial colonization of the plant root surface and primary infection are considerated as key stages. In those processes, two molecules are mainly involved, cellulose and cellulases respectively, as described by Robledo *et al.* (2012). *Rhizobium cellulosyliticum,* isolated and identified in our research group (García-Fraile *et al.,* 2007), is a natural cellulose and cellulases overproducing strain. Thus, we hypothesize this strain is a potential "helper" of other symbionts during plant colonization and infection processes. In this study, we report the effect in *Phaseolus vulgaris* after co-inoculation of a nodulating rhizobial strain of *Phaseolus vulgaris,* which was also described as PGPR bacteria in tomato and pepper (García-Fraile *et al*., 2012) and a *Rhizobium cellulosyliticum* strain. In order to elucidate the combined effect between both strains, we designed greenhouse assays. These assays include a single inoculation with each strain, a co-inoculation with both strains and non-inoculated plants treatment in non-sterile peat (organic material 90% and ash content 10%). Chemical fertilizers were not added and plants were watered weekly. During the assay, chlorophyll content in the leaves at different growth stages was measured by spectrophotometry, as indicative of nutrient status of the plants. In order to evaluate the effect of each strain and the co-inoculation treatment, we analysed different parameters as nodulation plant growth and chlorophyll content. According to our results, a synergic effect can be postulated between the two *Rhizobium* strains, because co-inoculation improved *Phaseolus vulgaris* growth and exhibited the highest number of beans pods.

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Symbiosis of the endangered Lupinus mariae-josephae lupin especies: Successful “in situ” propagation with rhizobial inoculation

Durán D[1], Navarro A[2], Rey L[1,] Imperial J[1,3] , Ruiz-Argüeso T[1*].

[1]Centro de Biotecnología y Genómica de Plantas (CBGP) and Departamento de Biotecnología (ETSIA), Universidad Politécnica de Madrid, Campus de Montegancedo, Pozuelo de Alarcón (Madrid), Spain, [2]Centro para la Investigacion y Experimentación Forestal (CIEF), Generalitat Valenciana, Quart de Poblet, Valencia, Spain, [3]CSIC, Madrid, Spain

Lupinus mariae-josephae (Lmj) is a lupine endemism that is only found in soils from a small area in Valencia region, in Eastern Spain. This lupine thrives in alkaline soils with high pH, a unique habitat for lupines. In these soils, Lmj grows in just a few defined patches, and previous conservation efforts directed towards controlled plant reproduction have been unsuccessful. A legislative decree (70/2009, page 20156 Anex I) published in the el 'Diario Oficial de la Comunitat Valenciana' shows Lmj in a category corresponding, in the latest version of the Red List of IUCN (IUCN, 2012) (International Union for Conservation of Nature and Nature Resources), to an “Endangered” legume species not extinct in the wild. Most current IUCN criteria used to define rare, small-range legume species, are based on history of reproductive traits such as number of pods and seeds. We have previously shown that Lmj plants establish a specific root nodule symbiosis with bradyrhizobia present in those soils, and we reasoned that the paucity of these bacteria in soils might contribute to the lack of success in reproducing plants for conservation purposes. Greenhouse experiments using Lmj trap-plants showed an absence, or very low concentration, of Lmj-nodulating bacteria in ‘‘terra rossa’’ soils of Valencia outside of Lmj plant patches. No Lmj endosymbiotic bacteria were found in ‘‘terra rossa’’ or alkaline red soils outside the Valencia Lmj endemism region in the Iberian Peninsula or Balearic Islands. Among the rhizobia able to establish an efficient symbiosis with *L. mariae-josephae* plants, two *Bradyrhizobium* sp. strains, LmjC and LmjM3, were selected as inocula for seed coating. Two planting experiments were carried out in consecutive years under natural conditions in areas with edapho-climatic characteristics identical to those sustaining natural Lmj populations, and successful reproduction of the plant was achieved. Interestingly, the successful reproductive cycle was absolutely dependent on seedling inoculation with effective bradyrhizobia, and optimal performance was observed in plants inoculated with LmjC, a strain that had previously shown the most efficient behavior under controlled conditions. These results define conditions for *L. mariae-josephae* conservation and for extension to alkaline-limed soil habitats, where no other known lupine can thrive. Broadly speaking, the work singularly identified the rhizobial symbiosis as a factor affecting the conservation of legumes and often being exceedingly vulnerable to threats. Our results also indicate that seed inoculation with N_2-fixing, efficient Rhizobium strains is a strategy to consider in the conservation of endangered legume species

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Durán, D. Rey, L., Navarro, A., Busquetsc, A., Imperial,J., Ruiz-Argüeso, T., 2014 *Bradyrhizobium valentinum* sp. nov., isolated from effective nodules of *Lupinus mariae-josephae*, a lupine endemic of basic-lime soils in Eastern Spain. *Syst. Appl. Microbiol. 37, 336-341*

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Increased photosynthetic acclimation in nodulated alfalfa associated with arbuscular mycorrhizal fungi (AMF) and cultivated in greenhouse under elevated CO2

Goicoechea N[1], Baslam M[1], Erice G[2], **Irigoyen JJ**[1]

[1]Grupo de Fisiología del Estrés en Plantas (Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño), Departamento de Biología Ambiental, Facultades de Ciencias y Farmacia, Universidad de Navarra, C/ Irunlarrea, 1, 31008 Pamplona, Spain, [2]Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 1206W. Gregory Drive, Urbana, IL 61801, USA

Medicago sativa L. (alfalfa) can exhibit photosynthetic down-regulation when grown in greenhouse conditions under elevated atmospheric CO_2 (ECO_2) (Erice et al., 2006) probably due to the carbohydrate synthesis under ECO_2 exceeds the capacity to produce new sinks (sink limitation). This forage legume can establish a double symbiosis with nitrogen fixing bacteria and arbuscular mycorrhizal fungi (AMF), which may increase the carbon sink effect of roots. Our aim was to assess whether the association of alfalfa with AMF (M plants) can avoid, diminish or delay the photosynthetic acclimation observed in previous studies performed with nodulated non-mycorrhizal plants (NM). The plants of alfalfa (*Medicago sativa* L. cv. Aragón) (NM and M) were inoculated with *Sinorhizobium meliloti* 102F78 (The Nitragin Co. Milwaukee, WI, USA), and in the case of M plants also with the mycorrhizal inoculum AEGIS Endo Gránulo (Atens, Tarragona, Spain), a mixture of *Rhizophagus intraradices* and *Funneliformis mosseae*. When inoculation was established (four-week-old plants), plants continued growing during five additional weeks at 25/15ºC (day/night) temperature, 50/85% (day/night) relative humidity and natural light supplemented with artificial light providing a photosynthetic photon flux of around 500-600 μmol m^{-2} s^{-1} during 14 h, in two greenhouses one with ambient CO_2 concentration (395 ± 20 mmol mol^{-1}) and the other one with elevated CO_2 (710 ± 22 mmol mol^{-1}). Two harvests were done, one when plants were seven-week-old (2nd harvest) and two weeks later (3rd harvest, nine-week old plants). The results showed that mycorrhizal (M) alfalfa at the end of their vegetative period had lower carbon (C) discrimination than non-mycorrhizal (NM) controls, indicating photosynthetic acclimation under ECO_2 in plants associated with AMF. Decreased C discrimination was due to the acclimation of conductance, since the amount of Rubisco and the expression of genes codifying both large and small subunits of Rubisco were similar or slightly higher in M than in NM plants. Moreover, M alfalfa accumulated a greater amount of soluble sugars in leaves than NM plants, thus favouring a down-regulation effect on photosynthetic rates. The enhanced contents of sugars in leaves coincided with a reduced percentage of arbuscules in roots, suggesting decreased sink of carbohydrates from shoots to roots in M plants. The shorter life cycle of alfalfa associated with AMF in comparison with the NM controls may also be related to the accelerated photosynthetic acclimation in M plants. Further research is needed to clarify to what extent this behaviour could be extrapolated to alfalfa cultivated in the field and subjected to periodic cutting of shoots under climatic change scenarios. This work was supported by Ministerio de Economía y Competitividad (MINECO, BFU2011-26989). Thank A. Hernández (Atens, Tarragona, Spain) for kindly providing the mycorrhizal inoculum and to M. L. Fiasconaro, T. Kizildeniz and A. Urdiain for technical support.

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Induced Systemic Resistance could explain the reduction of black sigatoka incidence in banana plants inoculated with bacteria isolated from banana trees roots, in organic systems from Dominican Republic

Marcano IE[1], Díaz-Alcántara CA[1], Seco V[2].,Urbano B[3], González-Andrés F[4].

[1]Facultad de Ciencias Agronómicas y Veterinarias, Universidad Autónoma de Santo Domingo, República Dominicana, [2] Departamento de Ingenieríay Ciencias Agrarias . Universidad de León. [3]Departamento de Ingeniería Agrícola y Forestal. Universidad de Valladolid. Spain, [4]Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad. Universidad de León. León. Spain.

Black sigatoka caused by *Mycosphaerella fijiensis* (Morelet) is one of the main diseases affecting banana trees worldwide. It causes leaf spot and also premature maturation of fruit. The reduction of the functional leaf area produces also a reduction of the crop yield. The Induced Systemic Resistance (ISR) is a mechanism triggered by rizospheric bacteria which protects plants against pathogens. The ISR has been used for diseases control in several crops (Sing *et al.* 2011). The studies about ISR in banana have been focused to the control of *Fusarium oxysporum* (Fishal *et al*. 2010) and viral diseases, but not to the control of black sigatoka. Our research was focused on the prospection, characterization and evaluation of bacteria associated to the roots of banana trees *cv*. Cavendish in organic systems, in four regions of Dominican Republic, to be used like biofertilisers. After the isolation of autochthonous bacteria, they were identified by sequencing of the 16S rRNA gene, and the non-pathogenic isolates were tested in vitro for Plant Growth Promoting traits. The five isolates with an outstanding expression of such traits were used to inoculate the roots of banana plants, before transplanting for a field test. Unexpectedly, the treatments inoculated with two isolates from different species of the genus *Pseudomonas*, showed a significant reduction of the black sigatoka infection. The lower incidence of black sigatoka was also significantly correlated with higher banana yield. This result was interpreted as ISR phenomena. As a consequence of this positive and unexpected result, other 21 isolates from the collection, plus the five already selected ones, were tested in a new trial for black sigatoka control assessment. The selection of the 21 isolates was mainly based in the production of sideophore in vitro, as this trait has been related with the ISR (Djavaheri *et al.* 2012).This new test was carried out with banana plants grown in a growth chamber, which were inoculated with *Mycosphaerella fijiensis* (Morelet). The treatments consisted on the inoculation of the roots of banana plants with the selected isolates, just in the moment of transplanting them to 10 kg containers. Out of the 26 isolates, six of them, five from the genus *Bacillus* (close to *B. licheniformis, B. siamensis,* and *B. subtilis* ssp. *Inaquosorum*) and one *Rhizobium massiliae*, showed no significant differences in sigatoka infection (estimated as Severity Index, Morales Romero *et al.* 2011) compared with the control without sigatoka inoculation. The six mentioned isolates produced better results than the *Pseudomonas* isolates used in the field test. The sigatoka control have been tentatively assigned to ISR phenomena. This must be confirmed by further research, but could be exploited in the short term to control black sigatoka in organic banana production systems in Dominican Republic.

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Emission of nitrous oxide by agricultural soils as affected by nitrogen fertilizers

Castellano-Hinojosa A[1,2], Correa-Galeote D[1], González-López J[3], Bedmar EJ[1].

[1*] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Agencia CSIC. Granada, [2] Departamento de Microbiología, Facultad de Ciencias. Universidad de Granada, [3]Departamento de Microbiología, Facultad de Farmacia. Universidad de Granada

Nitrous oxide (N_2O) is one of the major greenhouse gasses with an estimated contribution of 10% to the anthropogenic global warming. Due to its radiative capacity and 150-year half-life in the atmosphere, its global warming potential is 300 times greater than that of the carbon dioxide (CO_2). Global concentration of N_2O has increased in the last decades and is still increasing in a rate of 0.25% per year. In the N cycle, denitrification is the biological process that reduces nitrates (NO_3^-) and nitrites (NO_2^-) to N_2 via the formation of nitric oxide (NO) and N_2O. Analysis of 80 genomes of denitrifying microorganisms indicates that more than 60% do not contain, or do not express, the *nosZ* gene, which encodes the enzyme nitrous oxide reductase responsible for reduction of N_2O to N_2. Accordingly, it´s a paradox that being denitrification the most important biological process by which environmentally-contaminant nitrates are removed during the N cycle, it is also a main mechanism responsible for the release of N_2O to the atmosphere. Urea, ammonium salts and nitrates are the main N-compounds used in agricultural practices for soil fertilization, and they largely contribute to production of N_2O. However, data on the contribution of those N-fertilizers to N_2O emissions in agricultural soils are scarce. Hence, the aim of this work was to determine the effect of the different forms of nitrogen fertilization, urea, ammonium and nitrates on N_2O emissions by soils with and without plant cultivation. Fallow soil for more than 5 years was taken near the locality of Motril (Granada), brought back to the laboratory, mixed independently using a concrete mixer with MAGRAMA-recommended 200 kg/ha of each urea, $(NH_4)_2SO_4$ and KNO_3, and used to fill containers of 5 kg capacity. A set of pots was used to seed tomato var. Pera. Unfertilized soil was used as a control. Pots were watered until maximum field capacity (20% of WFPS) once a week and kept under greenhouse conditions. Twelve hours after watering, 50 g soil aliquots were taken and analyzed for N_2O production by gas chromatography. For uncultivated soils, samples were taken every 15 days during the first two months and then once a month for the following 10 months. For soils cultivated with tomato plants, samples were taken as above for 4 months, when plants were at 10% fructification. Combined Kruskal-Wallis-Iman and Conover assays indicated that during the first two months, regardless of the presence or absence of plants, addition of either urea, ammonium or nitrate produced a significant increase in emission of N_2O as compared with the amount of gas produced by the unfertilized control (0.57 ± 0.20 nmol N_2O/g soil x h). During that time, production of N_2O by soils provided with urea was higher (6.01 ± 1.08 nmol N_2O/g soil x h; 5.27 ± 0.64 nmol N_2O/g soil x h for uncultivated and cultivated soils, respectively) than that in soils fertilized with ammonium (4.37 ± 1.28 nmol N_2O/g soil x h; 3.15 ± 0.41 nmol N_2O/g soil x h) and those amended with KNO_3 (2.42 ± 0.86 nmol N_2O/g soil x h; 1.18 ± 0.11 nmol N_2O/g soil x h). Two months after addition of the fertilizers there was a decline in N_2O emissions to reach a basal production. For uncultivated soils, basal production of N_2O determined during a 10-month period was ammonium (0.93 ± 0.43 nmol N_2O/g soil x h) > urea (0.17 ± 0.14 nmol N_2O/g soil x h) = nitrate (0.16 ± 0.07 nmol N_2O/g soil x h). However, N_2O basal production by cultivated soils during the 2-month period was higher in ammonium (0.96 ± 0.38 nmol N_2O/g soil x h) than in urea (0.33 ± 0.23 nmol N_2O/g soil x h), and urea higher than KNO_3 (0.05 ± 0.01 nmol N_2O/g soil x h).

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Agronomic parameters of soybean plants [*Glycine max* (L.) Merril] co-inoculated with *Azospirillum brasilense* and *Bradyrhizobium japonicum* by different methods of inoculation

Fukami J[1, 2], Abrantes JL[1], Nogueira MA[1], Hungria M[1], Megías M[3], Ollero FJ[3].

[1]EMBRAPA-Soja - Empresa Brasileira de Pesquisa Agropecuária (Lab. Biotecnologia do Solo, CP. 231, 86001-970, Londrina, PR, Brasil), [2]UEL - Universidade Estadual de Londrina (Dpto de Bioquímica e Biotecnologia, CP. 6001, 86051-990, Londrina, PR, Brasil), [3] Dpto. Microbiología. Fac. Biología. Universidad de Sevilla.

Inoculants based on *Azospirillum brasilense* have been used in Brazil in the past seven years as plant growth promoters (PGPR) for cereal crops. Previously, considering the benefits attributed to *A. brasilense*, experiments were performed to confirm the viability of co-inoculation of soybean [*Glycine max* (L.) seeds with *Bradyrhizobium* and *Azospirillum.* However, a main limitation relies on the compatibility of the bacteria with pesticides and other agrochemicals applied to the seeds at sowing season, pointing out the need of searching for alternative methods of co-inoculation. Experiments were performed under greenhouse conditions at Embrapa Soja, Londrina, State of Paraná, Brazil, using soybean cultivar BRS 317. Soybean seeds were inoculated with *B. japonicum* (strain CPAC 15) and different methods of co-inoculation with *A. brasilense* (strains Ab-V5 and Ab-V6, individually): directly on seeds, in-furrow or foliar spray. One control treatment consisted of soybean inoculated only with *B. japonicum*. At 35 days after emergence, the following parameters were evaluated: plant biomass [shoot dry weight (SDW)], nitrogen content (NC), shoot total nitrogen (STN), chlorophyll content (CC), nodule number (NN) and nodule dry weight (NDW). Data obtained were analysed by ANOVA (followed by Duncan post test, $p \leq 0.05$). Statistical differences were observed on the parameter of NDW with the application of Ab-V6 by foliar spray, resulting in an increase of 14% compared to the treatment inoculated exclusively with *B. japonicum*. This treatment also increased significantly SDW in comparison to the other co-inoculation treatments. Although no statistic differences were detected in the NC, the co-inoculation treatment with Ab-V6 applied as foliar spray was 34% higher than the control exclusively with *Bradyrhizobium*. In addition, we observed a positive effect of Ab-V5 inoculated directly on seeds, increasing NN by 32% in comparison with the single inoculation with *Bradyrhizobium*. In conclusion, the co-inoculation by foliar spray can represent a viable alternative for the traditional seed inoculation, avoiding the limitations related to the incompatibility with agrochemicals applied to the seeds at sowing season.

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Symbiotic performance of *Bradyrhizobium* isolates from Spain and the Andean region (Bolivia and Peru) with some cultivated lupin species *(Lupinus sp.)*

Rodríguez-Navarro D, Fernández-Perea J, Temprano F.,

[1] IFAPA-Centro las Torres. Carretera Sevilla-Alcalá del Río, km. 12,200. Alcalá del Río (Sevilla). Spain.

Lupins are legumes distributed in two areas around the world: the Mediterranean-African region (12-13 species) and the American region (more than 250 species). Four lupin species are actually cultivated as proteinrich grain legumes (Gladstones, 1998). *Lupinus albus, L. luteus* and *L. angustifolius* are originated in the Mediterranean basin and *L. mutabilis* has been cultivated in America by pre-Columbian cultures.

Lupinus can fix atmosferic nitrogen in symbiosis with soil bacteria of the genus *Bradyrhizobium*, so lupin crops do not need nitrogen fertilizers (Jarabo-Lorenzo, *et al.,* 2003). This fact is very advantageous for this crop from an economic and ecological viewpoint. In some occasions, as is the case of soils devoid of specific *Bradyrhizobium* populations, crops need to be inoculated with highly effective strains (Amarger and Duthion, 1983).

In this work, ten *B*radyrhizobium strains where obtained from nodules of wild lupins growing in soils of different Spanish provinces. In addition, another ten strains were isolated from soils of the Andes in Bolivia and Peru, using *L. mutabilis* as a trap host. The symbiotic properties (nodulation and nitrogen fixation) of all those isolates with cultivars of *L. albus, L. luteus, L. angustifolius* and *L. mutabilis* were tested in the greenhouse under controlled conditions.

All the isolates, either Spanish or Andean, nodulated *L. albus* and *L. mutabilis.* Similarly, all the isolates but one were competent to nodulate *L. luteus* and *L. angustifolius*. There was a strong interaction among the isolates and the *Lupinus* species for the nitrogen fixation efficiency (evaluated as shoot dry weight of plants). The *Bradyrhizobium* isolates from Spanish soils showed, in general, higher efficiency with *L. albus, L. luteus y L. angustifolius* than the isolates from Andean soils. On the contrary, *L. mutabilis* showed a higher nitrogen fixing capacity with the majority of the isolates from Bolivia and Peru. Remarkably, all Andean isolates were ineffective with *L. luteus*, whereas isolates from Spanish soils showed a high effiency with this species.

These results suggest that distinct inoculants, with the appropriate effective strains, should be used for *L. albus*, *L. luteus* and *L. angustifolius*, on one hand, and for *L. mutabilis* on the other hand.

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Symbiotic and agronomic characterization of bradyrhizobial strains nodulating cowpea, *Vigna unguiculata* (L.) Walp. in northern Peru.

Valdez RA[1], Soriano B[2], Prado G[3], Zavaleta D[3], Matsubara M[4], Zúñiga-Dávila D[4], Dion P[5], Valladolid A[6], Ruesta A[7], Bedmar EJ[1].

[1] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Granada, España. [2] Departamento de Microbiología y Parasitología, Facultad de Ciencias Biológicas, Universidad Nacional de Trujillo, Perú. [3] Universidad César Vallejo, Piura, Perú. [4] Laboratorio de Ecología Microbiana y Biotecnología, Departamento de Biología, Facultad de Ciencias, Universidad Nacional Agraria La Molina, Lima, Perú. [5] Universidad Laval, Quebec-Canada. [6] Asociación PROMENESTRAS TEX, Chiclayo, Perú. [7] Instituto Nacional de Innovación Agraria, Estación Experimental Vista Florida-Lambayeque-Perú.

Legumes are unique among the higher plants because of their ability to establish (N_2)-fixing symbioses with soil bacteria collectively referred as rhizobia. Based on the legume-rhizobia association, a low-cost and environmentally safe technology is the inoculation of leguminous plants with efficient N_2-fixing bacteria. Cowpea (*Vigna unguiculata*) is the most important grain legume in the Peruvian agricultural export market, with about 25.700 ha producing approximately 59.400 tn of grain valued 69 million dollars in period 2011-2014. To date, growth of cowpea depends on the use of synthetic nitrogen fertilizers that raise production costs and cause environmental problems. Because current knowledge on the rhizobial microsymbionts capable of nodulating cowpea is scarce, the aim of this study was the isolation and symbiotic, agronomic and genetic characterization of bacteria inside cowpea root nodules. They were taken from healthy cowpea plants growing in soils of Callería (8º 23' 22'' S-74º 31' 43'' W), Yarinacocha (8º 23' 55'' S-74º 31' 36'' W) and San Juan (8º 19' 01'' S-74º 36' 24'' W) located in the Ucayali region. Nodules were surface-sterilized and after homogenized in a drop of saline solution. Then, the resulting extract was streaked onto YEM medium supplemented with Congo Red and incubated at 30 ºC for 12 d. Colony forming units appeared in the medium were check for purity and used to inoculate cowpea var. CAU-9 seedlings. Plants were grown in Leonard jar assemblies under greenhouse conditions until 10% flowering. Efficiency (EFI) and effectiveness (EFV) of the strains were calculated using the equations: EFI = (PDW of inoculated plants/PDW of plants not treated with combined N) x 100 and EFV = (PDW of inoculated plants/PDW of plants treated with combined N) x 100, where PDW is the plant dry weight. The Tukey t-test ($p \leq 0.5$) was used for statistical calculations.

Out of the 80 strains tested, 50 nodulated cowpea and only 8 strains showed greater EFI and EFV than the controls. These superior strains were slow growers (5-7 days in YEM medium) and showed alkaline reaction in liquid YEM supplemented with bromothymol blue, which are well-known characteristics of genus *Bradyrhizobium*. Symbiotic performance (measured as grain yield) of the selected 8 strains was further examined in field trials carried out in agricultural soils of Paiján (La Libertad region) and Picsi (Lambayeque region), representing two main areas for cowpea production in Peru. For the Paiján site, increases in grain yield of the plants inoculated separately with each one of the 8 strains varied from 18% to 22% as compared with control plants (not N-treated, uninoculated plants). In soils from Picsi, however, increases in grain yield of the inoculated plants were not observed.

The nearly complete 16S rRNA gene sequence from each one of the 8 strains and pairwise alignments between globally aligned sequences showed they were closely related to members of genera *Bradyrhizobium* of the Alphaproteobacteria class. Five out of the 8 strains showed 99.8% similarity with *B. yuanmingense* CCBAU 10071^T and the remaining 3 strains had 100% similarity with *B. liaoningense* 2281^T. Taken together, these results indicate that strains isolated in this study could be used as biofertilizers for inoculation of cowpea plants in northern Peru.

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Biocontrol of *Fusarium oxysporum* f.sp. *phaseoli* and *Phytophthora capsici* with autochthonous endophytic bacteria in common bean and pepper in Castilla y León (Spain)

Barquero M.[1], Terrón A[2]., Velázquez E.[1], **González-Andrés F.**[2]

[1]Departamento de Microbiología y Genética. Universidad de Salamanca. Spain. [2]Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad. Universidad de León. León. Spain.

Fusarium oxysporum f.sp. *phaseoli* and *Phytophthora capsici* are soil pathogens which cause diseaes to common bean (*Phaseolus vulgaris*) and pepper (*Capsicum annuum*) respectively. The control of such diseases is commonly based in chemicals, although the increasing willingness of the consumers to buy products free from pesticides prioritizes the search for alternatives to chemicals. This is especially important in products with quality protection like Protected Geographic Indications (PGI), which are usually preferred by consumers concerned with the quality and healthiness of the food products. Some microorganisms associated with plants, show different bio control mechanisms against pathogens (Compant et al., 2005; Glick, 2012). The agricultural use of these mechanisms is a chief strategy as an alternative to chemicals (Gerhardson, 2002). In this work, we isolated and taxonomically identified, 122 endophytes from roots, 68 of them from common bean and the rest from pepper. The isolations were carried out in farms located in regions protected by a PGI: "*Alubia de La Bañeza – León*" in the case of common bean, and "*Pimiento de Fresno-Benavente"* in the case of pepper. Seven of the isolates controlled the growth of *F. oxysporum in vitro*, four the growth of *P. capsici*, and six more the growth of the two pathogens. These isolates were tested in plants of common bean and pepper, which were grown in hydroponic conditions and inoculated with *Fusarium oxysporum* f.sp. *phaseoli* and *Phytophthora capsici* respectively. Two isolates belonging to the species *Pseudomnas brassicacearum* and *Bacillus siamensis* respectively, controlled the *Fusarium* root rot in common bean, and even in the treatment inoculated with the pathogenic *Fusarium* plus the *Bacillus siamensis* isolate, the plant growth was higher than in the non-inoculated control. Moreover, two isolates belonging to *B. pumilus* controlled *P. capsici* in pepper plants, and also in the treatment inoculated with the pathogen (*P. capsici*) plus one of the *Bacillus pumilus* isolate, the growth was higher in the plants than in the non-inoculated control.

In trials carried out in microcosms conditions, common bean plants inoculated with the patogen *F. oxysporum*, plus an authochthonous *Rhizobium leguminosarum* strain and the isolate from *Bacillus siamensis*, showed a very weak attack from the pathogen, and the aerial biomass did not differ from the non-inoculated control. Regarding pepper, the co-inoculation with one of the *B. pumilus* isolates plus an autochthonous mycorrhiza, controlled *P. capsici* in the plants inoculated with the pathogen, at the same level than the chemical fungicide. This opens the door for the development of biocontrollers, alternative to chemicals, in these two crops.

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Microbial inoculants with autochthonous bacteria for biodiverse legume pastures in Portuguese agro-forestry ecosystems

Soares R, Arcos E, Ferreira E, **Videira I.**

Laboratório de Microbiologia do Solo, Instituto Nacional de Investigação Agrária e Veterinária (INIAV) Oeiras, Portugal

In Portugal, a strategy for improving pastures in the "montado" ecosystem has been developed (Ferreira and Castro, 2011). This strategy is based on the establishment of biodiverse permanent pastures including selected and improved plant species, in which inoculated legumes with high efficiency rhizobia have an important role. One key point to achieve the success of microorganisms like rhizobia as biofertilizers (inoculants) is the characterization and selection of the autochthonous strains by optimizing the interaction between legume species and rhizobia strains, which highlight the high efficiency in nitrogen fixation.

The aim of this work was to study the behavior of different annual clover species, usually present in biodiverse legume pastures (Crespo, 2006) inoculated with selected autochthonous *Rhizobium* strains and compare their efficiency in nitrogen fixation with commercial peat based inoculant. These strains were isolated from root nodules of *Trifolium subterraneum* plants grown in the field in the South of Portugal (Ferreira and Marques, 1992) and belong to the "INIAV Rhizobia Bacteria Collection". Besides nitrogen fixation, the native rhizobia bacteria were also screened *"in vitro"* for other important activities, such as the solubilization of mineral phosphate. For this work the methodology used was the following: First, a set of 10 *Rhizobium* strains were inoculated, separately, in plants of *T. subterraneum* cv. Clare growing in axenic conditions. Results obtained from shoot dry matter production of clover nodulated plants, allowed to differentiate *Rhizobium* bacteria according to their symbiotic effectiveness. Three strains were selected with this criterion (89 Ts2a, 123 Ts2a and 149 Ts2) and are being subject to a taxonomic identification based in the housekeeping genes. Four different species of clover plants, *T. subterraneum, T. incarnatum*, *T. suaveolens* and *T. vesiculosum* were inoculated with the selected *Rhizobium* strains and also with the commercial inoculant, which had about 10^9 bacteria g^{-1} of peat. Results of shoots dry weight indicated that the three selected native strains were highly efficient in nitrogen fixation (with values higher than those obtained with nitrogen addition control) especially with *T. suaveolens.* The strain 123 Ts2a was considered as having better performance than the commercial inoculant for *T. subterraneum, T. incarnatum* and *T. suaveolens,* being plants of *T. incarnatum* those with lower dry weight. However, for *T. vesiculosum* best results were obtained with strain 89 Ts2a. Mixed inoculation assays with these three strains and the different clover species are currently been performed. Results obtained for "*in vitro*" activities indicated that two strains (89 Ts2 and 123 Ts2a) can solubilize phosphate. Although the main feature required in rhizobia elite inoculants is the high efficiency in nitrogen fixation, as phosphorous is generally deficient in the Portuguese soils, the selected strains could also contribute to reduce the application of this nutrient.

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Plant growth promotion by phosphate solubilising *Pseudomonas azotoformans* isolated from annual ryegrass

Castanheira N, Muñoz O, Pais I, Semedo J, Scotti-Campos P, Castelo MA, Menino R, Martins JC, **Fareleira P**.

Instituto Nacional de Investigação Agrária e Veterinária, I.P., Av. da República, Quinta do Marquês, 2780-159 Oeiras, Portugal.

Annual ryegrass (*Lolium multiflorum* Lam.) is a fast-growing, cool season pasture grass that is broadly present in the "montado" (or "dehesa"), a typically Mediterranean agro-forestry-pastoral ecosystem adapted to poor productive areas in western Iberian Peninsula. The soils in "montado" are often shallow and affected by severe constraints, such as low levels of available phosphorus. A recent survey of annual ryegrass-associated bacteria in the Portuguese "montado" resulted in the disclosure of several strains with the ability to solubilise mineral phosphate (Castanheira *et al.* 2014). One of such strains, *Pseudomonas azotoformans* G1Dc10, was able to form large clearance halos on agar plates supplemented with insoluble tricalcium phosphate. Additionally, this strain was also able to produce siderophores and to stimulate the growth of annual ryegrass plants in gnotobiotic conditions. The aim of the present work was to deepen the information concerning this novel strain, specifically focusing on the phosphate solubilising activity and effects on plants, in order to explore its potential for use as plant inoculant in phosphorus-limited soils.

Inoculation assays of *L. multiflorum* in complete synthetic medium confirmed the stimulatory effect of strain G1Dc10 on plants biomass, resulting in average increases of 60% and 45% in root and shoot dry weights, respectively, relative to non-inoculated controls. Additionally, the chlorophyll and carotenoid contents of inoculated plants were also increased by 32% and 21%, respectively. Electrolyte leakage was reduced by 32% in inoculated plants, indicating increased membrane stability. Re-isolation of bacteria from inoculated plants confirmed the presence of strain G1Dc10 on the surface of the roots and in endophytic tissues of sterilized roots, stems and leaves, indicating both external and internal plant colonization. The potential of strain G1Dc10 to perform in P-limiting conditions was preliminarily evaluated in a small-scale pot experiment using a soil deficient in available phosphorus. In the absence of added phosphorus, inoculation of *L. multiflorum* with strain G1Dc10 resulted in increased root and shoot dry weights relative to non-inoculated plants. This result suggested that isolate G1Dc10 could be acting on the solubilisation of soil immobilised phosphates, increasing its availability to plants. In fact, the assessment of tricalcium phosphate solubilisation by liquid cultures of strain G1Dc10 revealed high rates of phosphate release into the medium (approx. 220 µmol P mg protein^{-1} in the first 24 h of incubation). However, plant inoculation also resulted in substantial biomass increases when the soil was supplemented with soluble phosphate, suggesting the possible involvement of mechanisms other than phosphate solubilisation. Similar results were obtained with *Trifolium subterraneum*, although in this case the stimulatory effects were detected only in the shoots. Further experiments involving a comprehensive analysis of plant physiological parameters and mineral composition (including plant phosphorus contents) will be necessary to obtain more information about the mechanisms involved and the potential of this novel strain to perform as plant growth promoter in P-deficient soils.

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Medicago truncatula composite plants and transgenic *Ensifer medicae* as a symbiotic tool to improve copper phytostabilization in contaminated soils

Caviedes MA[1], Pérez-Palacios P[1], Romero-Aguilar A[1], Delgadillo J[2], Doukkali B[1], Pajuelo E[1], Rodríguez-Llorente ID[1].

[1]Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, Profesor García González 2, 41012 Sevilla (Spain), [2]Microbiology Area, School of Post-Graduates, Montecillo Campus, Federal Road México-Texcoco, Montecillo 56230, (Mexico)

Copper (Cu) contamination in soils has a marked anthropogenic origin due to industrial activities. Because of its toxicity, legal requirements oblige industries to reduce Cu accumulation in wastewater and soils. Although Cu is involved in several crucial cell processes, this metal is one of the most toxic when its concentration reaches certain levels, causing a strong oxidative stress in the cell.

Rhizoremediation uses plant roots and their associated microorganisms to reduce mobility, solubility and/or inactivate toxic heavy metals through *in situ* rhizospheric processes. The uses of the rhizobia-legume symbiosis to phytostabilize heavy metal contamination from the soils have attracted considerable interest in the recent years. *Medicago truncatula* is one of the model plants for studying rhizobia-legume symbiosis, and molecular, genetic, proteomic and physiological studies have been centralized in its symbiosis with bacterial genus *Ensifer*. In order to use this symbiosis for bioremediation of heavy metal contaminated soils, it is necessary to select plants and bacteria resistant to these metals (Pajuelo *et al*., 2011).

Metallothioneins are metal-binding cystein-rich proteins with the ability to chelate metals and also it has been suggested some protection against oxidative stress being the cystein-rich residues which link ROS. The overexpression of seed specific *mt4a* metallothionein from *Arabidopsis thaliana* in vegetative tissues led to increased Cu tolerance and accumulation (Rodríguez-Llorente *et al*., 2010).

The aim of this work was to design a genetically modified symbiotic system with increased metal phytostabilization capacity. For this, *mt4a* gene from *A. thaliana* has been expressed in *M. truncatula* composite plants to increase Cu tolerance and/or accumulation. In addition, plants have been inoculated with a transgenic *Ensifer medicae* strain expressing the copper resistance genes *copAB* from a *Pseudomonas fluorescens* strain (Delgadillo *et al*., 2015). Composite plants were exposed to different Cu concentration (100 and 200 µM). The transgenic overexpression of a methallotionein in *M. truncatula* hairy roots and its effect on metal tolerance and accumulation has been studied for the first time.

Transgenesis was confirmed by PCR and GUS staining. Nodule number and length root were measured. Antioxidants enzymes activities (peroxidase, superoxide dismutase, ascorbate peroxidase and catalase) and membrane damage (through malondialdehyde (MDA) measurement) were recorded to evaluate differences in Cu tolerance between roots expressing *mt4a* and control roots. Heterologous expression of *mt4a* gene in roots of composite plants of *M. truncatula* improves Cu tolerance and nodulation and decreases oxidative stress. Inoculation with a genetically modified *Ensifer medicae* expressing bacterial copper resistance genes *copAB* further enhances Cu phytostabilization into plant root and nodules.

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Disease control and plant growth promotion (PGP) of selected bacterial strains in *phaseolus vulgaris*

Memenza M[1], Mostacero E[2], Camarena F[2], Zúñiga-Dávila D[1].

[1]LEMYB, Dpto. Biología, Facultad Ciencias, Universidad Nacional Agraria La Molina (UNALM), Lima, Perú.
[2]Programa de Leguminosas y Oleaginosas, Facultad Agronomía, UNALM, Lima, Perú.

The common bean (*P. vulgaris* L.) is original from the American continent and in Peru is one of the most consumed legumes due to its high content of proteins, fats, carbohydrates and minerals. Common beans occupy the largest cultivated area among legumes in Peru (30.800 ha). The yield is reduced by many phytophatogenic and nutritional aspects. Conventional crop management involves overuse of chemical pesticides and fertilizers which cause serious environmental and social problems. This situation, coupled with the effects of global warming, have increased the prevalence of diseases which diminish crop production up to 70%, threatening the food safety of the country. The use of rhizospheric bacteria, isolated from common bean plants, as microbial inoculants in the field, represents an alternative to protect the crop against diseases and to increase its productivity. The aim of this study was to evaluate in a field trial, the effect of *Rhizobium* (RE10 and RM02) and *Bacillus* (B02 and B15) strains to reduce the incidence of the disease caused by *Sclerotinia sclerotiorum* and to improve the yield of the common bean cv. Centenary. Such strains were previously tested *in vitro* and selected by their antagonistic capacity against the mentioned disease, and by its plant growth promoting activity (Calvo et al. 2010, Memenza and Zuñiga, 2015).

The field trial was located in Pachacamac, Lima, Peru, and consisted on 8 treatments: plants inoculated with only one strain, double inoculations, chemical control, and untreated plants. The experimental design was a complete random block design with three replicates. For the chemical control, different fungicides were applied on seeds and leaves. For each treatment, bacteria were first inoculated on the seeds, and thereafter inoculated three times more in the plants neck. The seeds were pelleted with bacterial suspension (10^8 CFU/ml) previously mixed with 1g of agricultural soil. The plants neck were sprayed with 6ml of $2x10^5$ CFU/ml, in three stages of crop growth. The following parameters were evaluated during the experiment: percentage of plants infected by the disease (%DI); percentage of germination (%G); at pre flowering: plant height, fresh and dry weight of aerial part and number of flower buds; at harvest: number of pods/plant (PP) and grain yield (kg/ha). At harvest time, all the inoculated treatments showed the lowest %DI caused by *S. sclerotiorum* with significant difference compared with untreated plants (14%). The best treatment was RE10+B02 (2%), followed by RM02 (3%) and B02 (4%) without significant difference compared with the chemical control (2%). The same strains also showed the highest %G. Treatment RE10+B02 also presented the highest yield (16 PP and 1717.8 Kg/ha) with significant difference compared with untreated plants (11 PP and 883.2 kg/ha). Therefore, the interaction between these strains showed an antagonistic activity against the pathogen in field conditions, in congruence with the results previously observed *in vitro* (Memenza and Zuñiga, 2015). Weather conditions with averages of 94.71% of humidity and temperatures between 13.67°C and 17.09°C had moderated influence in the disease development. In addition, plants responded well to bacterial inoculation despite being cultivated in an alkaline and saline soil with phosphorus deficiency. On the other hand, plants inoculated with *Rhizobium* strains presented between six to ten nodules per plant, while, untreated plants presented less than four.
This result shows the usefulness of PGP bacteria to control fungal disease and to improve the yield on bean crop in the field, reducing environmental pollution due to the use of chemical pesticides, and can contribute to the development of sustainable agriculture.

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Leaf application of *A. brasilense* on irrigated wheat, at different days after emergence

Silva PST, Freitas KC, Galindo FS, Teixeira MCM, Buzetti S[3], **Cassiolato AMR.**

UNESP-São Paulo State University, Ilha Solteira Campus. Avenida Brasil, 56, Ilha Solteira-SP, CEP 15385-000, Brazil.

Bacteria may improve plant growth via hormonal and/or nutritional effects, as for example some non-symbiotic diazotrophic bacteria. Beside the Biological Nitrogen Fixation, some Azospirillum species also can produce and release plant growth-regulating substances. Phytohormones can influence some root morphological traits, such as number and length of root hair and branching pattern. Plant inoculation with Azospirillum brasilense has been studied for several crops. However, there are few studies regarding foliar application, and even less about the most convenient moment for fir leaf application, to maximize plant benefits. The objective of this study was to evaluate the effect of leaf application of A. brasilense on wheat, at different days after emergence.

The soil used for the experiments was collected in the Brazilian Cerrado, and was classified as clayey Oxisol. The experimental design was a randomized block with six treatments and four replications, and the treatments were: control (no inoculation) and leaf application at 12, 24, 36, 48 and 60 days after emergence. A. brasilense strains Abv5 Abv6 (guarantee 2x108 CFU ml-1) was applied using a carbon dioxide pump, with a flow rate of 300 L ha1 at inoculum dose of 0.250 L ha-1 (liquid). Wheat cultivar CD 116 was chosen; the plant density was 13 plants m-1 plants, with plant spacing of 0.17 m on rows, and each plot consisted of 6 planting rows for study. Wheat grain production was determined using the whole plot plants. Soil samples were collected at the end of the crop cycle, at four different places per plot (0.0-0.10 m depth), forming one composite sample per plot. These samples were used for diazotrophic non-symbiotic bacteria enumeration.

A. brasilense inoculation did not produced statistical difference either in soil bacteria enumeration or in wheat grain yield, regardless of the application moment. Research with wheat has suggested that inoculation with nitrogen-fixing bacteria does not completely replace nitrogen fertilization. However, it can enhance absorption and utilization of available N (Saubidet et al., 2002), but this was not observed in this study. On the other side, a less susceptible host genotype to diazotrophic bacteria root colonization, may exhibit higher nutrients demand (Miyauchi et al., 2008), fact that could not be proved with this research results. These results may attribute to understand the relation between plant host and the most convenient moment for leaf inoculation application.

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Innovative liquid formulations for biofertilizers based in PGPR, using treated bio residues

Pastor R, **Mulas R**, González-Andrés F.

Instituto de Medio Ambiente y Recursos Naturales y Biodiversidad. Universidad de León. Avda. De Portugal, 41. 24009 León.

Formulation is a crucial aspect for the success or failure of a biofertilizer in the field (Xavier *et al.* 2004). The formulation of a biofertilizer consists on the active ingredient, corresponding in this case to the microorganism, plus a solid or liquid carrier, and several additives with different purposes as the protection or stabilization of the microbial cells (Herrmann and Lesuer, 2013). Another important aspect to develop biofertilizers is to produce them at the lowest possible cost. In this sense, the use of bio residues as substrate for the production of the bacteria, and like carriers, has been proposed. The main hindrance for the use of bio residues, is the presence of resident microbial populations, which must be eliminated because the biofertilizers must contain only selected microorganisms, and it is not acceptable the inclusion of natural populations, in addition of the PGPR microorganism. We work in different research project to valorize the effluent of the anaerobic digestion of vegetables industries, for agriculture. The goal of the research line is to transform this residue in a high added value product for agriculture.

The objective of this work was to analyze the possibility of using the anaerobic digestate as the substratum to grow the bacteria *Bacillus amyloliquefaciens*, previously selected as PGPR for pepper, in order to elaborate a liquid biofertilizer for this crop.

The first step was the selection of a carbonated protector for the bacterial cells. The compatibility of different polysaccharides was tested: carrageenan, alginate, locust bean, and carboxymethilcellulose. These substances have shown different degrees of compatibility with *B. amyloliquefaciens*, obtaining for carrageenan the best results.

The liquid effluent corresponding to the anaerobic digestate once it has been stabilized and autoclave sterilized (120 °C for 20 minutes) was adequate for *B. amyloliquefaciens* growth including a carbon source. Four carbon sources were tested (sucrose, glucose, mannitol and lactose all of them at 1 % w/v), all of them except for mannitol were used by the bacteria. Sucrose was selected for economic reasons because of its lower price compared with glucose and lactose.

After one week of incubation at 28 °C in the sterilized digestate supplemented with sucrose (1% w/v), the bacterial population was 4 x 10^8 UFC/ml, a concentration which is typically reached in semi-industrial or industrial fermentators (Trujillo-Roldan *et al*. 2013). At the end of the fermentation process carrageenan at 1% (w/v) was added as protector. The survival of the microorganisms was maintained over 10^8 UFC/ml for at least two months although longer periods are currently being investigated, and therefore the system proposed to elaborate biofertilizers is successful.

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Session 5

Physiology and biochemistry
of beneficial microorganisms
and associated plants.

Implication of homospermidine synthase in the response to salinity of *Rhizobium tropici* in symbiosis with *Phaseolus vulgaris*

Cobos-Porras L[1], Lluch C[1], Prell J[2], López-Gómez M[1].

[1] Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada, Campus de Fuentenueva s/n, 18071, Granada, Spain, [2] Soil Ecology, RWTH Aachen, Aachen, Germany

Root nodules of *Phaseolus vulgaris* contain a variety of polyamines some of which are nodule specific and synthesized by the rhizobia. Among these low molecular weight aliphatic compounds, homospermidine (Homspd) is the most abundant in nodules of *P. vulgaris* and the major cellular polyamine in different rhizobia species. Aditionally, 4-aminobutil cadaverine, only described before in nodules of *Vigna angularis* (Fujihara et al. 1995), was also detected in root nodules of *P. vulgaris* (López-Gómez et al. 2014). We hypothesize that the synthesis of both unusual polyamines in root nodules of *P. vulgaris* is catalyzed by Homospermidine synthase in the bacteroids utilizing cadaverine supplied from the host legume cells. In order to demonstrate the previous hypothesis, we generated a mutant strain of *Rhizobium tropici* CIAT899 impaired in the synthesis of homospermidine by the insertion of a Ω Spec cassette in the single copy gene for Homospd synthase (Rt899 *Hss*::Ω, Spc^r). This mutant strain was characterized as free living bacteria and in symbiosis with *Phaseolus vulgaris* under control and salt stress conditions, since polyamines and specifically 4-aminobutil cadaverine, has been shown to be involved in *Phaseolus vulgaris* root nodule response to salt stress.

The elution profile of polyamines in *R. tropici* wt and Rt899 *Hss*::Ω, Spc^r displayed the presence of Homspd as the main polyamine in the wt while in the mutant strain it was completely absent. A quantitative analysis of polyamines in both strains confirmed the absence of Homspd in the mutant strain together with an increment in the levels of common polyamines such as putrescine (Put), spermidine (Spd) and spermine (Spm). Under salt stress conditions the synthesis of Spd and Spm is favored in detriment of Put in the wt strain; however in the mutant strain, no accumulation of polyamines was induced. Growth curves reveal that Homspd is not required for normal growth in *R. tropici*, but under salt stress conditions, Rt899 *Hss*::Ω, Spc^r seems to be less tolerant.

Polyamines content in the citosolic and bacteroidal fractions of root nodules of *P. vulgaris* inoculated with the wt and mutant strain were analyzed, and neither Homspd nor 4-Aminobutil cadaverine were detected in the nodules containing the mutant strain. Those data support the synthesis of both uncommon polyamines by Homspd synthase in the bacteroids as previously hypothesized.

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A novel method to isolate native NifB-cofactor

Echavarri-Erasun C, Gonzalez E, Rubio LM.

[1*] CBGP-UPM, Campus Montegancedo, Pozuelo de Alarcón, 28223, Madrid

Shah et al (1994) described NifB-cofactor as the product of the NifB protein after accomplishing its *in vitro* purification in the *Klebsiella pneumonia* strain UN1217. Characterization of this small Fe-S cluster revealed its O_2-labile nature, greenish-brown appearance, EPR silence, and iron-only metal content. Later work showed that NifB-co was a FeMoco precursor that could be reconstituted *in vitro* using homocitrate, molybdenum and the NifEN scaffold protein (Curatti et al., 2007). Although the NifB-co structure is not fully understood, it was demonstrated that it comprises -at least- the 6Fe-9S core of FeMo-co coordinated with an interstitial light atom (George et al., 2008) that was later shown to be a carbon. Our inability to obtain fully homogeneous NifB-co preparations may be the reason why NifB-co structure is still eluding us, and it may have to do with sample alterations during the isolation procedure due to the effect of detergents and thiol reducing agents.

We have developed a new method to isolate intact NifB-co using a modified *K. pneumonia* UN1217 strain that overexpresses GST-tagged-NifX as a mean to hijack excess NifB-co. Interestingly, GST-NifX purifications present a greenish-brown colour due the presence of Fe-S cluster(s) which suggest that this strategy may be correct. Ethylene production after NifX-NifB-co reconstitution to FeMoco tested positive. We are currently testing NifX-NifB-co preparations for UV/visible, EPR, Mossbaüer, and protein crystallography to elucidate its structure.

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Headspace solid phase micro extraction and comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometer as a tool to detect bioactive volatile organic compounds synthesized by *Rhizobium leguminosarum*

Cardoso P[1], Rocha S[2], Freitas R[1], Figueira E[1]

[1]Departamento de Biologia & CESAM, Universidade de Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal. [2] Departamento de Química & QOPNA, Universidade de Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal

Bacteria are capable of producing a wide range of volatile organic compounds (VOCs). These VOCs belong to many chemical classes, for example fatty acid derivatives (hydrocarbons, alcohols, ketones and aldehydes), nitrogen containing compounds or volatile sulfur compounds, among other chemical classes. The majority of the existing literature on this subject is focused on bacterial VOCs with interest for the food industry. However, exciting results on the ability of rhizobacteria to produce VOCs that can interact with plants, with improvement of plant growth (Ryu et al., 2003), induction of systemic resistance (Ryu et al, 2004) and inhibition of growth of phytopathogenic fungi (Kai et al., 2009) have established VOCs as an important feature of plant growth promoting rhizobacteria (PGPR). Although it has already been reported that colonization by rhizobia can alter the emission of VOCs by the host plant, no data regarding the production of VOCs by rhizobia is available. Here we report a profiling of the VOCs produced by rhizobia utilizing comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometer (GCxGC-ToFMS) for the analysis of rhizobial samples extracted using headspace solid phase micro extraction (HS-SPME), we were able to detect the production of compounds with reported bioactivity in the literature, and which may have biotechnological interest. The combination of the high separation power of the GCxGC chromatography with the high sensitivity of time-of-flight spectrometer provide enough resolving power to detect compounds that elute early and hence cannot be detected with other technology. Moreover, since the extraction is performed using HS-SPME, there is no need for the use of solvents in the extraction procedure. The use of solvents limits the range of compounds that are compatible with the solvent used. On the contrary, HS-SPME allows a comprehensive extraction. Taken altogether, our results show the potential of this technique for the detection of bioactive compounds in rhizobia and possibly other PGPR, and indicate several compounds which deserve further attention, as they are reported has having bioactivity in other microorganisms.

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NifQ and NifO are essential to express nitrogenase activity in the presence of nitrate in *Azotobacter vinelandii.*

Jiménez-Vicente E[1], Barahona E[1], Wilcoxen J[2], Navarro-Rodríguez M[1], Buesa JM[1], Britt D[2], Rubio LM[1].

[1] Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Pozuelo de Alarcón, 28223 Madrid (Spain). E-mail: emilio.jimenez@upm.es, [2*] Department of Chemistry, University of California–Davis, One Shields Avenue, Davis, CA 95616 (USA).

In the presence of nitrate, *Azotobacter vinelandii* is able to assimilate nitrogen by using nitrogenase and nitrate reductase/nitrite reductase pathways simultaneously. Nitrogenase and nitrate reductase are Mo-enzymes containing FeMo-co and Mo-MGD at their active sites, respectively. In order to optimize the use of Mo, a scarce metal in nature, regulation of Mo distribution between both enzymes must be strictly controlled during nitrogen assimilation processes.

The *nifO* and *nifQ* genes are grouped together with *nifB*, *fdxN* and *rhdN* in one transcriptional unit. It has been shown that *nifO* and *nifQ* expression levels change antagonistically depending on the presence of Mo in the medium (Rodriguez-Quinones 1993). In addition, the *nifO* mutant exhibits a Nif- phenotype in the presence of nitrate, whereas *nifO* overexpression lowers nitrate reductase activity (Gutierrez, J.C. 1997). The *nifQ* mutant is unable to fix N_2 unless growth medium is supplemented with 1000-fold excess of Mo. Importantly, NifQ has been characterized as the physiological Mo donor to a NifEN/NifH complex during FeMo-co synthesis. (Hernandez, J.A. 2008).

We aimed to understand the relationship between NifO and NifQ during expression of nitrogenase activity in presence of nitrate in *A. vinelandii*. The *nifQ* mutant was unable to fix N_2 in the presence of nitrate, independently of the level of Mo in the medium. In contrast *nifQ* mutant showed enhanced nitrate reductase activity. Analysis of nitrogenase and nitrate reductase activities demonstrated that the *nifQ* overexpressing strain exhibited lower nitrogenase activity and higher nitrate reductase activity than wild-type when grown diazotrophically in the presence of nitrate, a phenotype similar to the *nifO* mutant (Gutierrez, J.C. 1997). An antagonist effect had been observed in the *nifO* overexpressing strain (Gutierrez, J.C. 1997). Simultaneous overexpression of both *nifQ* and *nifO* yielded nitrogenase and nitrate reductase activities similar to wild-type. The phenotype observed in *nifQ* overexpressing, but not in *nifOQ* overexpressing strain, points to NifO as candidate to preserve NifQ as Mo donor to nitrogenase when nitrate reductase is present.

Transcriptional expression analysis performed by RT-qPCR showed lower expression of nitrogenase structural genes in the *nifO* mutant. In contrast increased expression of nitrate and nitrite reductase structural genes was observed for both *nifO* mutant and *nifQ* overexpression strains.

Comparison between NifQ proteins isolated before and after addition of nitrate to the same culture of a *nifQ* overexpressing strain grown under diazotrophic conditions, showed NifQ cluster content alteration, resulting in decrease of $[Mo\text{-}3Fe\text{-}4S]^{3+}$ and increase of $[3Fe\text{-}4S]^{+}$ clusters. This effect of nitrate is consistent with the inability of NifQ to donate Mo for FeMo-co biosynthesis under nitrate reductase derepressing conditions.

These results revealed two Mo pathways to nitrogenase: one that can be sorted by a large excess of Mo in the medium, and a second pathway strictly dependent on NifQ and NifO that would be essential to maintain active nitrogenase while assimilating nitrate through the molybdoenzyme nitrate reductase.

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Salt stress tolerance in *Casuarina glauca* and its relation with nitrogen-fixing *Frankia* bacteria

Ribeiro-Barros AI[1,2], Batista-Santos P[1], Duro N[1,2], Da Costa M[1,2], Alves P[1], Graça I[1,2], Ramalho JC[1].

[1]PlantStress&Biodiversity, Centro de Ambiente, Agricultura e Desenvolvimento (BioTrop), Instituto de Investigação Científica Tropical, I.P. (IICT), Av. República, Quinta do Marquês, 2784-505 Oeiras, Portugal, [2]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. República, Quinta do Marquês, 2780-157, Oeiras, Portugal

Salinity is one of the most wide spread abiotic stress affecting agricultural productivity, with impacts in more than 800 million hectares worldwide. It is estimated that salt stress will cause the loss of more than 50% of arable land by the year 2050. A promising solution for the recovery of saline soils encompasses the use of actinorhizal plants, a group of perennial dicotyledonous angiosperms, mostly woody shrubs or trees, highly recalcitrant to extreme environmental conditions. These plants are also able to establish root-nodule symbiosis with N_2-fixing bacteria of the genus *Frankia*. However, it is not clear to which extent does the symbiosis contribute to the resilient capacity of this group of plants. In this context, we have initiated a multidisciplinary analysis to evaluate the mechanisms underlying salt tolerance in the model *Casuarina glauca* as well as to determine the extent of *Frankia* contribution to such capability. For that, *C. glauca* plants supplied with KNO_3 (KNO_3^+) or in symbiosis with *Frankia* (NOD^+) were subjected to increasing salt concentrations from 0 to 600 mM NaCl. Both KNO_3^+ and NOD^+ plants presented relevant salt tolerance (up to 400 mM NaCl). Analysis of the photosynthesis functioning, one of the first stress targets, revealed that the major impacts were mostly related to down-regulation events rather than to photo-damage. Additionally, the presence of *Frankia* did not enhance the plant capacity to overcome stress, although providing the required N supply that allowed the plant to express its tolerance ability. In fact, the symbioses was strongly affected by salt (from 200 mM NaCl onwards) with residual levels of nitrogenase activity at 400 and 600 mM NaCl, accompanied by an enrichment in $\delta^{15}N$ and $\delta^{13}C$ signatures, as well as by the down-regulation of the transcriptional activity of symbiotic genes. To unravel the global and integrated plant response, we are currently examining the effect of salt on the metabolome and proteome profiles of KNO_3^+ and NOD^+ plants. During the communication we will present the overall picture of the mechanisms underlying salt stress tolerance in *C. glauca*.

Acknowledgments

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MtNramp1 mediates iron supply to rhizobia-infected *Medicago truncatula* nodule cells

Tejada-Jiménez M[1], Castro-Rodríguez R[1], Kryvoruchko I[2], Lucas MM[3], Udvardi M[2], Imperial J[3], González-Guerrero M[1].

[1]Universidad Politécnica de Madrid. Centro de Biotecnología y Genómica de Plantas, Pozuelo de Alarcón, 28223 Madrid (Spain). [2]Plant Biology Division. The Samuel Roberts Noble Foundation. 2510 Sam Noble Parkway. 73401 Ardmore, OK. USA. [3]Consejo Superior de Investigaciones Científicas. Madrid. Spain. e-mail: manuel.gonzalez@upm.es

All known organisms need iron to accomplish important biological processes for life, ranging from gene transcription to respiration. Particularly in symbiotic nitrogen fixation (SNF) iron plays a critical role since the activity of key proteins involved in this process, such as nitrogenase, leghemoglobin, Fe-superoxide dismutase and other proteins involved in energy transduction, directly depends on the presence of iron as cofactor in their active centre. In the model legume *Medicago truncatula*, iron is delivered by the vasculature and released in the apoplast on the zone II of the nodule (infection/maturation zone). Then iron moves into rhizobia-infected cells and it is used in the synthesis of iron-containing proteins. Therefore, different iron transporters should mediate iron traffic through the plasma membrane of plant cells and the symbiosome membrane. However, no candidates were available to be responsible for iron transport across the plasma membrane from the nodule apoplast to rhizobia-infected cells.

In the present work, we have identified a *Nramp* member gene from *M. truncatula* (*MtNramp1*) as responsible for iron transport from nodule apoplast into rhizobia-infected cell. *MtNramp1* shows the highest expression in the nodule among the seven *Nramp* genes present in *M. truncatula* genome. Immunolocalization studies show that MtNramp1 is located in the plasma membrane of zone II nodule cells. A loss-of-function *nramp1* mutant presented impaired growth specifically under symbiotic conditions, concomitant with a lower nitrogenase activity compared to wild-type plants. This phenotype was rescued by the addition of iron to the nutritive solution or by complementation of a mutant with a wild-type *Nramp1* copy. Furthermore yeast complementation assays using mutant affected on iron transport pointed to a role of MrNramp1 in iron transport toward the cytosol. All together, these results point to a role of MtNramp1 in iron supply to nodule cells connected to SNF, and represent an important step toward the understanding of iron incorporation and homeostasis in plant nitrogen-fixing tissues.

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Increasing concentrations of manganese, nickel and silicon differentially affect nodulation and N_2 fixation rates in *Rhizobium*-nodulated *Vigna unguiculata*, *Vigna unguiculata* spp. sesquispedalis, *Vigna radiata* and *Phaseolus vulgaris*

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Izaguirre-Mayoral ML[1,2], Vasquez M[2], Kola M[1], Maseko ST[1], Dakora FD[1]

[1]Tshwane University of Technology, Department of Croip Science, Biological Nitrogen Fixation Laboratory, Pretoria, South Africa. [2]Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela

Current reports indicate that some legume species in symbiosis with N_2-fixing bacteria have an increased demand for micronutrient such as Mn, Ni and Si for greater growth and nodulation. At the plant level, Mn is required for the binding of rhizobia to root hairs, as a cofactor of enzymes involved in the catabolism of ureides in the leaves and of superoxide dismutase's enzymes, besides its direct participation as a component of the oxygen-evolving complex in the photosystem II. Mn also plays a regulatory function in the iron-uptake by soil free living rhizobial cells. Furthermore, Ni deficiency results in poor seed germination, reduced iron absorption by roots, delayed nodulation, disrupted ureide catabolism in leaves, accumulation of urea in leaves, hindered N remobilization, and lower hydrogenase activity in the nodules. Enhanced nodulation, cell and plant strength, increasing plant fitness in terms of pathogen resistance and the alleviation of abiotic stresses have been detected in plants grown in adequate levels of Si. Therefore, the aim of this investigation was to analyse the responses of *Rhizobium*-inoculated *V. unguiculata*, *V. unguiculata* spp. sesquispedalis, *V. radiata* and *P. vulgaris* to increasing concentrations of Mn (1, 10, 20, 40 and 60 μM), Ni (1, 5, 10, 20, 50, 100 and 200 μM) and Si (1, 3 and 5 mM). Seeds were pre-inoculated with commercial peat based rhizobial inoculants and sown in 1 kg sand irrigated with a N-free nutrient solution. Plants were grown in a green house and were harvested 30 days after germination. Results indicate enhanced growth and nodulation in twelve South African and one Venezuelan's *P. vulgaris* genotypes with increasing concentrations of Mn up to 60 μM. Increased growth and nodulation were also observed in *V. unguiculata* ssp sesquispedalis and *P. vulgaris* grown in 1 and 20 μM Ni, respectively. In contrast, the addition of ≤5 and ≤10 μM Ni did not affect the growth and nodulation of *V. radiata* and *V. unguiculata,* respectively. Toxicity was detected in *V. unguiculata* ssp. sesquispedalis and *V. radiata* grown in ≥5 μM Ni, *V. unguiculata* grown in >10 μM Ni, and *P. vulgaris* grown in >20 μM Ni. Concomitantly, the addition of ≤5 mM Si enhanced growth and nodulation in *V. unguiculata* ssp. sesquispedalis, *V. unguiculata* and *P. vulgaris*. In contrast, only 1 mM Si promoted growth and nodulation in *V. radiata,* followed by a decrease in plant growth and nodulation when grown in 3 or 5 mM Si. For all plants, there was an inverse relationship between growth and the leaf ureide concentrations. From the present results it can be concluded that Mn, Ni and Si promote the growth and symbiotic performance of *Rhizobium*-nodulated cowpea and common bean. However, the concentrations of each of those microelements that elicited maximum responses are species specific and must be determined before recommendations are made to farmers.

MtCOPT1 mediates copper transport to *Medicago truncatula* nodules

Senovilla M[1], Castro-Rodríguez R[1], Imperial J[1,2], González-Guerrero M[1].

[1]Universidad Politécnica de Madrid. Centro de Biotecnología y Genómica de Plantas, Pozuelo de Alarcón, 28223 Madrid. Spain. [2]Consejo Superior de Investigaciones Científicas. Madrid. Spain.

Copper is an essential oligonutrient. Its redox properties allow it to be a suitable cofactor for many proteins, such as cytochromes or superoxide dismutases. Copper is key for Symbiotic Nitrogen Fixation (SNF); For instance, bacteroids contain copper-dependent cytochrome oxidases that provide energy in the microaerobic environment within the nodule. Once copper is in the plant, it is delivered by the vasculature to the apoplast of zone II. From there, a plasma membrane transporter introduces this nutrient into the cell for copper-protein assembly. COPT family transporters mediate high-affinity copper transport towards the cytosol. Therefore, they are good candidates to introduce copper in nodule cells. From the 8 *COPT* family genes present in *M. truncatula* genome, *MtCOPT1* is the only one induced specifically in nodule. *MtCOPT1* can restore *Saccharomyces cerevisae Δctr1* capacity to uptake copper. Inmunolocalization and GUS fusion studies localize *MtCOPT1* in the nodule. Moreover, a Tnt-1-derived knockdown mutant line for *MtCOPT1* shows decreased nitrogenase activity when compared with the wild-type line. This activity is, at least, partially rescued when a wild-type copy of *MtCOPT1* gene is reintroduced. Taken together, all this data suggest an important role of *MtCOPT1* copper-mediated transport for SNF.

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Engineering a hydrogen biosensor: selection of overproducing nitrogenase variants for biohydrogen production

Barahona E, Rubio LM.

Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Pozuelo de Alarcón, 28223 Madrid (Spain).

Biologically-produced hydrogen (H_2) or "biohydrogen" is one promising source of renewable energy. A number of microorganisms are being studied as potential producers of biohydrogen through biophotolysis, indirect biophotolysis, photo-fermentations or dark-fermentations. Microorganisms produce H_2 by the activity of either hydrogenases or nitrogenases: Hydrogenase enzymes catalyze the reaction: $2H^+ + 2e^- \leftrightarrow H_2$ whereas nitrogenases catalyze the reduction N_2 with the following limiting stoichiometry: $N_2 + 8H^+ + 8e^- \leftrightarrow H_2 + 2NH_3$. In this work, we have coordinated aspects of both pathways to develop optimized biocatalysts for hydrogen overproduction using the following steps:

1. Engineering a hydrogen responsive genetic circuit in the purple non-sulphur nitrogen-fixing bacterium *Rhodobacter capsulatus* SB1003: *R. capsulatus* carries nitrogenase and hydrogenase enzymes able to produce H_2. It also carries a system to detect H_2 that is composed of three proteins: a H_2-sensor hydrogenase (HupUV), a histidine kinase (HupT) and a response regulator (NtrC-like transcription factor, HupR) (Vignais *et al*., 2005). In the presence of H_2, this sensor triggers expression of hydrogenase structural and biosynthetic genes. Taking advantage of this system, we have introduced a reporter gene under the control of hupS promoter and removed the uptake hydrogenase, generating a new biological-sensor strain capable of accumulating and detecting the presence of both exogenous H_2 and the H_2 produced by its own nitrogenase. This biotechnological tool allows us to obtain a measurable and proportional signal when H_2 is present in the cell.
2. Generating variants of the molybdenum nitrogenase structural genes *nifH*, *nifD* and *nifK*: we are using in vitro evolution techniques to perform random mutagenesis in these genes with a controlled mutation rate. The resulting variants were cloned under *nifH* promoter control into a broad-host-range vector (Kovach *et al*., 1995) optimized for diazotrophic conditions. Libraries obtained (around 4 x 10^6 clones) were introduced and expressed in the strain carrying the modified biological hydrogen sensor.

The suitable combination of both tools results in the development of a genetic circuit for the high-throughput screening of H_2 overproducing nitrogenase variants thus allowing detection and isolation of clones that present a significant signal increased, through the use of cell-sorting cytometry. Thus far, around 1500 clones have been successfully selected by this method, confirming the possibility of using the designed system to select hydrogen-overproducing enzymes.

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Differential response to cadmium stress of two cultivars of *Medicago truncatula* with different sensitivity to cadmium

García VS, Coba T, Lara-Dampier V, Granda-Bello C, Pueyo JJ, **Lucas MM.**

Instituto de Ciencias Agrarias, CSIC. Serrano, 115 bis, 28006-Madrid

Cadmium (Cd) is one of the most hazardous contaminants of soils and negatively affects crops growth. Cd enters the environment mainly through the metallurgical industries, waste incinerators and land applications of sewage sludge and of chemical fertilizers. It can be transferred to the food chain, being a risk for the human health. Cd can be taken up by plant roots and translocated to the plant aerial organs. Phytoremediation of Cd-contaminated soils with hyperaccumulator plants constitutes an environmentally friendly tool for soil cleaning. The selection of tolerant crop genotypes with low metal accumulation might be a promising approach to grow crop plants in contaminated soils with reduced Cd accumulation in plant edible organs. Therefore, the identification of tolerant varieties and the understanding of the mechanisms governing metal toxicity and plant responses are needed. The Cd toxicity mechanisms in plants are not completely understood, but reactive oxygen species overproduction is considered the origin of the damage following Cd exposure. Plant responses to Cd involve several signalling pathways and accumulation and cellular transport mechanisms, which seem to be species- and development stage-dependent. Legumes-rhizobia symbioses represent an interesting potential tool in soil bioremediation; besides symbiotic nitrogen fixation, they have the advantage of integrating microorganisms that may influence metal bioavailability. *Medicago truncatula* is a forage leguminous plant, a model plant to study genetic, cellular and physiological responses of legumes to heavy metals and could constitute a good candidate to be used in phytoremediation.

In this work we investigated Cd toxicity and tolerance mechanisms in a tolerant *M. truncatula* cultivar (CdT) and in a sensitive one (CdS), at different developmental stages (germination, seedlings, 15-day old and 24-day old plants). The cultivars were selected after screening 258 cultivars for Cd tolerance, following the methods described by García de la Torre *et al*. (2013). The CdT cultivar showed higher tolerance indices and Cd content, and lower Cd translocation factor than CdS. We analysed plant growth, gene expression (qPCR) and activity of enzymes involved in antioxidant defence (superoxide dismutase, catalase, ascorbate-glutathione cycle), and gene expression of enzymes involved in NADPH generation and glutathione and phytochelatin synthesis. The oxidative damage produced by Cd was estimated by lipid peroxidation. The CdT cultivar showed a better capacity to cope with Cd stress than CdS, with better germination rate and reserve mobilization in germinating seeds, a good antioxidant defence and lower oxidative damage both in seedlings and plants. Differences in nutritional status were also found between cultivars due to Cd treatment. The effect of Cd stress on nodulation of both cultivars was also studied. The nodulation kinetics and nitrogen-fixing activity were analysed. The results are discussed in the context of the different strategies displayed by the two cultivars under Cd stress. This information will be useful to understand Cd-tolerance, to establish physiological markers for legumes, and to evaluate suitability of this legume-*Rhizobium* symbiosis for phytoremediation purposes.

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Transpiration, metabolite transport and biological nitrogen fixation in well-watered nodulated soybean plants exposed to high vapour pressure deficit

Aldasoro J, García-Castro A, **Arrese-Igor C.**

Department of Environmental Sciences. Public University of Navarra. Campus Arrosadia. 31006 Pamplona (Navarra)

Legumes play a crucial role in sustainable agriculture by their ability to establish symbiotic relationship with soil bacteria allowing biological nitrogen fixation (BNF) to occur. These crops occupy approximately 15% of the cultivated land area (Graham and Vance, 2003), representing 27% of the global crop production, and about 33 % of the protein for human food (Vance, 2000). However, legumes are rather sensitive to adverse abiotic factors such as water stress, nutritional deficiencies, soil acidity, salinity, etc. ., being drought the most relevant. Hence, the study of the drought effects on BNF is of primary relevance in order to minimize crop damage. While soil drought has been extensively studied, little is known on atmospheric drought. The main objective of this work was to further understand the relationship between vapour pressure deficit (VPD) and BNF in well-watered, nodulated soybean plants (Glycine max (L.) Merr.). Plants were subjected to VPD variations by altering the relative humidity of the air surrounding the canopy. Transpiration, stomatal conductance, apparent nitrogenase activity (ANA), and the concentrations of metabolites in nodules were monitored. Data were taken at the onset of the treatment, and 4 and 8 hours thereafter.

High VPD caused a decrease in N-compounds concentration (ureides and amino acids) in nodules within 4 hours of treatment. This event may be the causative factor of the increased the BNF observed four hours afterwards. Moreover, changes in the concentration of carbon compounds were monitored. Thus, high VPD caused an accumulation of soluble carbohydrates, mainly sucrose, and a decrease of starch concentration in nodules, whereas malate concentration showed a transient decrease.

The overall results suggest a strong C/N interaction in the regulation of BNF in soybean nodules upon atmospheric water stress.

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Regulation of ML β-glucan synthesis in *Sinorhizobium meliloti*.

Baena I[1], Pérez-Mendoza D[2], Bonilla I[1], Rivilla R[1], Martín M[1], Sanjuan J[2], **Lloret J**[1].

[1] Departamento de Biología, Universidad Autónoma de Madrid, Madrid, Spain; [2] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Granada, Spain.

Sinorhizobium meliloti synthesizes two well-known exopolysaccharides, EPS I and EPS II, which play important roles in the free living and symbiotic life styles of this bacterium. Recently, it has been described the synthesis of a new type of bacterial exopolysaccharide by *S. meliloti*, a linear mixed-linkage β-glucan (ML β-glucan) formed by β-glucopyranoses linked by alternate 1→3 and 1→4 bonds (Pérez-Mendoza *et al*., 2015). The ML β-glucan, although not essential for neither the nodulation nor the nitrogen fixation processes, plays an important role in biofilm formation and root colonization. Quorum sensing regulates the synthesis of the polysaccharide and its production is enhanced by high levels of cyclic diguanylate (c-di-GMP). The ubiquitous bacterial second messenger c-di-GMP has been involved in a wide number of physiological processes, including biofilm formation, motility and exopolysaccharide production. c-di-GMP levels are controlled by proteins called diguanylate cyclases (DGC), which synthesize it from two molecules of GTP and contain a characteristic GGDEF domain, and specific phosphodiesterases (PDE), which hydrolyse it and contain either EAL or HD-GYP domains.

We have characterized a gene cluster located in the *S. meliloti* pSymB plasmid involved in regulation of ML β-glucan synthesis. RT-PCR and β-galactosidase gene fusion analysis showed that all six genes are transcribed as a single operon which is constitutively expressed. One of the ORFs codes for a protein with putative DGC and PDE activities. Despite this protein contains a non-consensus GGDEF domain, our data suggest that it is an active DGC and that this activity is regulated by the accompanying genes, which code for three hypothetical proteins, a methyltransferase and a putative membrane regulatory protein. Interestingly, several of these proteins contain functional domains conserved in proteins regulating the activity of σ^F and σ^B, the sigma factors responsible for the expression of genes involved in sporulation and stress response in *Bacillus subtilis* (Sharma *et al*., 2011). The construction of single, double and triple mutants and the consequent complementation studies has revealed that this operon codes for proteins involved in a signal transduction cascade regulating DGC activity in response to as yet unknown signals. In our working hypothesis, high c-di-GMP levels achieved after activation of the DGC would lead to activation of the ML β-glucan synthase BgsA. Ongoing protein-protein interaction assays will help us to unravel the role played by each of the components of this regulatory system.

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Glutathione peroxidases in legume nodules. Function, localization and post-translational regulation

Matamoros MA[1], Saiz A[1], Peñuelas M[1], Barja MV[2], Mulet JM[2], Moore M[3], James EK[4], Dietz KJ[3], **Becana M[1].**

[1] Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apartado 13034, 50080 Zaragoza, Spain; [2] Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera, 46022 Valencia, Spain; [3] Biochemistry and Physiology of Plants, W5-134, Bielefeld University, D-33501, Germany; [4] The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, UK

The cellular redox state must be tightly regulated to prevent oxidative damage and to permit optimal growth and development. Glutathione peroxidases (Gpxs) catalyze the reduction of H_2O_2 and organic hydroperoxides to water or the corresponding alcohols and may play additional roles in redox transduction and stress signalling. Plant Gpxs differ from their mammalian counterparts in that they contain cysteine instead of selenocysteine at the catalytic site and use thioredoxins as preferred electron donors (Herbette *et al.*, 2007). In this work, we have investigated two isoforms (LjGpx1 and LjGpx3) that are highly expressed in the nodules of the model legume *Lotus japonicus*. Their intracellular localization was determined using immunoelectron microscopy and fluorescence detection of the GFP-tagged proteins in *Arabidopsis thaliana* mesophyll protoplasts and *Nicotiana benthamiana* leaves. Both techniques suggest that LjGpx1 and LjGpx3 are present in the nucleus of nodule cells. In addition, LjGpx1 localizes to the plastids and LjGpx3 to the cytosol and endoplasmic reticulum. To get deeper insight into the function of the two proteins in the nodules, we carried out complementation studies with a yeast *Gpx* triple-deletion mutant. The results showed that LjGpx1 and LjGpx3 confer tolerance to different peroxides and protect cell membranes from lipid peroxidation.

LjGpxs contain thiol groups that are essential for catalytic activity. We tested the hypothesis that nitric oxide (NO), a key signal molecule that plays important roles in the legume–rhizobia symbiosis (Puppo *et al.*, 2013), regulates LjGpxs through *S*-nitrosylation. The biotin switch method (Jaffrey *et al.*, 2001) and streptavidin-agarose chromatography showed that LjGpx1 and LjGpx3 are susceptible to *S*-nitrosylation *in vitro* and *in vivo* after treatment with the physiological NO donor *S*-nitrosoglutathione. The *S*-nitrosylation of LjGpx3 was confirmed using the His-tag switch (Camerini *et al.*, 2007) followed by mass spectrometry, and the target residue was identified as Cys-85. *S*-nitrosylation causes inhibition of enzymatic activity and may contribute to NO-mediated intracellular redox signalling.

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Nitrate assimilation and nitric oxide detoxification in *Bradyrhizobium japonicum*

Salas A[1], Cabrera JJ[1], Lopez MF[2], Torres MJ[1], Lopez-Garcia SL[2], Mesa S[1], Bedmar EJ[1], Richardson DJ[3], Gates AJ[3], **Delgado MJ**[1].

[1]Estación Experimental del Zaidín, CSIC, Granada, Spain. [2]Instituto de Biotecnología y Biología Molecular (IBBM), Universidad Nacional de La Plata (UNLP), La Plata, Argentina. [3]School of Biological Sciences, University of East Anglia, Norwich, UK.

Bradyrhizobium japonicum is a soil bacterium that establishes nitrogen-fixing symbiotic associations with soybean plants, and may denitrify under free-living and symbiotic conditions. This bacterium is also able to grow with nitrate (NO_3^-) or nitrite (NO_2^-) as the sole nitrogen source. In contrast to other bacteria that assimilate nitrate, the genes *nasC* and *nirA* encoding the assimilatory nitrate reductase and nitrite reductase enzymes in *B. japonicum* are located at distinct *loci* on the chromosome. Whereas *nasC* is located in a gene cluster that encodes an ABC-type nitrate transporter, a nitrate/nitrite transporter (NarU), a flavoprotein (Flp) and a single domain haemoglobin (Bjgb), *nirA* clusters with genes for a nitrate/nitrite responsive regulatory system (NasTS). NasC and NirA are required for NO_3^- and NO_2^- assimilation, respectively, and Flp may function as electron-donor to NasC. In addition, *bjgb* and *flp* encode a nitric oxide (NO) detoxification system that functions to mitigate cytotoxic NO formed as a by-product of NO_3^-/NO_2^- assimilation. NasTS are involved in NO_3^-/NO_2^--dependent growth and in nitrate reductase and nitrite reductase activities. In this work, we have demonstrated that *narU*, *bjgb*, *flp* and *nasC* comprise a transcriptional unit. β-galactosidase activity of a *narU-lacZ* fusion showed that expression of these genes is repressed by ammonium or glutamate and induced by nitrate and nitric oxide. The involvement of the regulatory proteins NtrBC, NasTS, and NnrR in the expression of the *narU-bjgb-flp-nasC* operon has also been demonstrated. Expression analyses from a *nirA-lacZ* fusion revealed that while NtrBC and NasTS are involved in nitrate-dependent induction of *nirA*, NO or NnrR were not involved in *nirA* expression. Taken together, these results suggest that *narU-bjgb-flp-nasC* operon involved in nitrate assimilation and NO detoxification is subjected to a co-regulation in response to nitrate and NO, however, expression of *nirA* involved in nitrite reduction is not.

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Carbon flux under water stress in the soybean-*Bradyrhizobium japonicum* symbiosis

Rubia MI, Arrese-Igor C.

Department of Environmental Sciences. Public University of Navarra. Campus Arrosadia. 31006 Pamplona (Navarra)

Water stress is one of the main abiotic factors that affects crop physiology and is particularly relevant in legumes since biological nitrogen fixation is very sensitive to drought. This has been ascribed, among other factors, to a reduction in the flux of carbon within nodules due to a decrease in sucrose synthase activity (González et al., 1995). This was consistent with the long-known fact that nitrogenase-linked respiration declines previous to any effect in photosynthesis upon drought. Previous results of our group suggested the existence of a local rather than a systemic regulation of these responses (Marino et al., 2007; Gil-Quintana et al., 2013). However, the importance of the source-sink relationships and long-distance transport in these responses has been largely neglected.

In the current work, carbon fluxes at the whole plant level were measured. These include carbon source tissues (mature leaves), intermediate tissue (stems) and sink tissues (roots, nodules and young leaves) both under control and mild drought conditions in nodulated soybean plants (*Glycine max* L. Merr var. Sumatra). For this purpose, the upper surface of the youngest fully expanded leaf was labeled with ^{13}C-[U]-sucrose and, subsequently, its distribution was monitored. After one hour, the plant was separated into labeled leaf, sink leaf, other leaves, stem, roots and nodules. Then, the abundance of $\delta^{13}C$ was determined by elemental analysis - isotope ratio mass spectrometry.

Under control conditions 9% of the total labeled sucrose is transported to other tissues: 10% of this is found in stems, 17% in sink leaves, 23% in nodules and 42% in roots. Drought provokes a decrease in the transport of C from the labeled leaf to sink tissues with only 5% of the labeled sucrose transported. Interestingly, the main change observed is the reduction to the transport into nodules, which declines to only 4%. In contrast, the transport to the rest of sink tissues (roots and young leaves) is slightly increased. The implications of these results for understanding the regulation of source-sink relationships will be discussed.

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Possible reasons for tolerance to mercury of lupin plants inoculated with Hg-resistant and sensitive *Bradyrhizobium canariense* strains

Ruiz-Díez B, Quiñones MA, Fajardo S, Morcillo C, **Fernández-Pascual M**.

Instituto de Ciencias Agrarias. CSIC. Serrano, 115 dpdo. 28006 Madrid. Spain.

In previous works we demonstrated that the inoculation with a mercury-resistant bradyrhizobial strain (Ruiz-Díez *et al*., 2012) confers *Lupinus albus* plants the ability to grow under high concentrations of Hg and accumulate this heavy metal. Plant growth, photosynthetic efficiency and nitrogenase activity of plants inoculated with the tolerant strain were similar to control plants without mercury, while in those inoculated with de Hg-sensitive strain all parameters decreased significantly (Quiñones *et al*., 2013). The aim of this work was to elucidate the mechanism/s implicated in the acquisition of this tolerance. To do that, *L. albus* cv. G1 plants were inoculated with the Hg-tolerant strain, L7AH, and the Hg-sensitive, L3. Plants were watered with nutrient solution without nitrogen, containing different mercury concentrations (0-200 µM $HgCl_2$) and grown under controlled conditions for 6 weeks. Samples of leaves and nodules were collected to be processed for light and electron microscopy according de María *et al*., 2005. Furthermore, X-ray microanalyses, along with scanning electron microscopy were performed to detect the mercury presence and location in lupin nodules. Enzymes of the oxidative metabolism are being analysed and preliminary results show alterations in enzymatic activities associated to changes in Hg concentration.

Mercury application produced alterations in leaves and nodule structure, depending on the strain inoculated. Leaves ultrastructural observations of mesophyll cells from L7AH plants showed the chloroplasts located next to the cell wall, around the central vacuole, as in control plants, while in the chloroplast of L3 plants this location was lost and they remained floating in the cytoplasm of mesophyll cells, as if the tonoplast was broken. L3 chloroplasts showed a large increase in number and size of starch granules, which implies a big increase in chloroplast size. These modifications produced altered grana distribution with a totally disorganized thylakoid structure, and showed clear signs of degradation. The preservation of distribution and morphology of L7AH chloroplasts could be one reason why their photosynthetic efficiency remained unchanged after 200µM of mercury supply. Mercury exposure produced changes in L3 nodule ultrastructure, with evident signs of degradation, especially in bacteroids. However, only light alterations of nodule morphology were noticed in L7AH nodules. The X-ray microanalysis of nodules, along with scanning electron microscopy, gave us more information to the possible explanation of the mercury-tolerance of lupin plants inoculated with Hg-tolerant strain L7AH: the low entering of Hg in the infected area. While mercury is present in the nodules of plants inoculated with the Hg-sensitive strain, in both cortex and infected area, in nodules formed by inoculation of the tolerant strain only low levels of mercury in the outermost layers of cortex could be detected. The exclusion of mercury from the infected area and the conservation of the structure of bacteroids in nodules from plants inoculated with L7AH may be the cause of the maintenance of nitrogenase activity.

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Improved salt tolerance of *Mesorhizobium ciceri* by genetic modification of trehalose synthesis

Moussaid S[1], Dominguez-Ferreras A[2], Muñoz S[2], Aurag J[1], Berraho EB[1], **Sanjuan J**[2].

[1] Laboratory of Microbiology and Molecular Biology, Faculty of Sciences, University Mohammed V, Avenue Ibn Battouta, BP 1014 Rabat, Morocco.[2] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC. Prof. Albareda Nº 1, E-18008 Granada, Spain

Cicer arietinum (chickpea) is a legume very sensitive to salinity, and so are most of its rhizobial symbionts belonging to the species Mesorhizobium ciceri (Maatallah et al., 2002a,b). We observed that exogenous trehalose (i.e., added to the growth medium) can significantly improve growth of M. ciceri strain Rch125 under moderate salinity. In order to test if endogenous trehalose (i.e. synthesized by the cell) could also enhance salt tolerance, strain Rch125 was genetically modified with various trehalose biosynthesis genes from Sinorhizobium meliloti 1021 (otsA, treS, treY) and Mesorhizobium loli MAFF 303099 (otsAB). We found that overexpression of otsA or otsAB, but not treS or treY, significantly improved M. ciceri Rch125 growth in salt containing media. This growth improvement correlated with enhanced trehalose accumulation in otsA- and otsAB-modified cells, suggesting that increased trehalose synthesis via trehalose-6-phosphate can enhance bacterial salt tolerance. Chickpea plants inoculated with M. ciceri Rch125 derivatives carrying extra otsAB or otsA genes formed more nodules and accumulated more shoot biomass than wild type inoculated plants when grown in the presence of NaCl than wild type inoculated plants. These results indicate that improved salt tolerance of the bacterial symbiont can alleviate the negative effects of salinity on the host plant.

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Effect of osmoprotectant carbohydrates in nodules of two cultivars of *Phaseolus vulgaris* under salinity

Lluch C, Cobos-Porras L, López-Gómez M.

[1] Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada, Campus de Fuentenueva s/n, 18071, Granada, Spain,

Salinity is one of the major abiotic factors limiting global agricultural productivity, and it is estimated that one-third of the world's irrigated land are unsuitable for crops. Salt stress drastically affects the photosynthesis, the nitrogen metabolism, the carbon metabolism and the plant nutrition. Legumes are classified as salt-sensitive crop species and their production is particularly affected by salt stress because these plants depend on symbiotic N_2 fixation for their nitrogen requirement. The limitation of productivity is associated with a lower growth of the host plant, poor development of the root nodules and consequently with a reduction of the nitrogen-fixation capacity (López et al. 2008).

The presence of salt in the growth media often results in the accumulation of low-molecular mass compounds, termed compatible solutes or osmoprotectants. These compatibles solutes are low molecular weight substances non-toxic at high concentration and include mainly monosaccharides, disaccharides, polyols and amino acids (Palma et al. 2013). The accumulation of compatible osmolytes produces an osmotic adjustment (raise osmotic pressure) to counteract the high concentration of inorganic salts in the vacuole and the root medium. Another function of these accumulated compounds under salinity stress is to act as an energy sink or reducing power, such as C and N_2 source, or scavenging ROS.

In this work we have studied the changes induced by salinity in the content of some compatible osmolytes in root nodules of two different cultivars of *Phaseolus vulgaris* (Contender and Coco Blanc) inoculated with *Rhizobium tropici*. Specifically, the content of the polyols such as myoinositol and pinitol, as well as disaccharides and their relation with the adaptation of the symbiosis to salt stress was also evaluated.

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Study of diazotrophic bacteria isolated from rice fields of the Guadalquivir marshes.

Gallego-Parrilla JJ, Moyano-Bravo I, Megías E, Espuny MR, **Bellogín RA**.

Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Avda. Reina Mercedes 6, 41012-Sevilla. España.

Nitrogen (N) is the most limiting nutrient worldwide for plant productivity, which is specillay important in rice production. Losses of N fertilizer depend on nitrogen use efficiency, which can be affected by microbial N cycling that is mainly thought to occur in soil (Li et al. 2008). In order to attain sustainability of rice crop and to reduce the dependence of N mineral fertilizers, there is a need to use diazotrophic bacteria that can make biologically fixed nitrogen available for rice plants (Ladha et al., 2003). Rice production of the Guadalquivir marshes area (Sevilla, Spain) represents about 40% of the total Spanish crop. Free living bacteria able to fix atmospheric N_2 with additional plant growth promoting (PGP) abilities, could be developed as biofertilizers, which will contribute to a more sustainable agriculture, avoiding the employ of large quantities of chemical fertilizers.

The isolation of diazotrophic bacteria from rice fields located in the Guadalquivir marshes, was carried out with a repetitive enrichment method, inoculating in NFb and Burk semisolid (0.2 % agar) media. The colonies from test tubes with N_2 fixation activity (ARA+) were selected to investigate their BOX-PCR fingerprints and their diazotrophic and plant growth promoting (PGP) abilities (AIA, siderophore and ACC deaminase production and phosphate solubilisation). N_2-fixing isolates with more than one PGP abilities were selected to study the presence of enzymatic activities including protease, lipase, urease, amylase, chitinase, etc. and their NaCl tolerance.

Finally, two of the N_2-fixing isolates were selected for studying their contribution to the rice-plant development in different conditions. We assessed the N_2 fixation ability associated to the rice-plant rhizosphere and the plant shoot dry weight at the end of the assays. Results of these plant tests were not conclusive probably due to the very restrictive conditions used for plant growth (absence of combined nitrogen or the nature of the substrate used).

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Analysis of the interaction beween NtcA and *mcyAD* promoter using Surface Plasmon Resonance

Calvo-Beguería L, Ceballos L, Alias M, González A, Bes MT, Fillat MF, **Peleato ML.**

Departamento de Bioquímica y Biología Molecular, Instituto de Biocomputación y Física de Sistemas Complejos, Universidad de Zaragoza.

NtcA is a master regulator of nitrogen metabolism in cyanobacteria belonging to the CAP/CRP family (the catabolite activador protein or cyclic AMP receptor protein). It regulates the expression of a large number of genes, especially those involved in nitrogen metabolism, as well as other many aspects of cyanobacterial metabolism, such as carbon metabolism, photosynthesis, and stress responses. In the case of filamentous nitrogen fixing cyanobacteria, NtcA plays crucial roles at different stages of heterocyst differentiation (1, 2).

The interaction between NtcA and promoter regions of the mcy bidireccional promoter of the cluster involved in the microcystin synthesis from Microcystis aerugionsa PCC 7806 has been studied by SPR (Surface Plasmon Resonance), Biacore T200. NtcA was obtained as a recombinant protein with histidine-tag and purified accoding to [3]. Bidireccional mcyAD promoter was divided into two fragments, a 438 bp mcyA upstream sequence and a 331 bp mcyD gene upstream sequence. The 438 bp fragment contains at least 2 NtcA putative boxes, while the 331 bp fragment contains a single putative binding sequence [3]. SPR experiments were performed using a Biacore T200 sistem (Biacore AB, Uppsala, Sweden). Streptavidin sensor chip (SA chip) was used to study the binding of NtcA to promoter regions of the microcystin gene cluster (mcy operon). First of all, the biotinylated promotor was inmobilized using a concentration of 70 nM, with a flow rate of 2 ml/min and 300 seconds of contact time (464 RUs of ligand capture). After that, several concentrations of NtcA were injected (0-3000 nM) with a flow rate of 5 ml/min, 400 seconds of contact time and 1200 seconds of dissociation time. For the 438 bp fragment, NtcA exibit a Ka= 2,3 10-4 M-1s-1 and a Kd = 1, 68 10-7 M.

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