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DE SALAMANCA



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IRNASA  
INSTITUTO DE RECURSOS NATURALES  
Y AGROBIOLOGÍA DE SALAMANCA

SALAMANCA, DEL 3 AL 5 DE JULIO DE 2024



XIX  
**SEFIN**  
CONGRESS



40<sup>+1</sup>

Salamanca '24

**BeMiPlant**  
Beneficial Plant-Microbe Interactions

**Book of Abstracts**

## ORGANIZERS



Sociedad Española de Fijación del Nitrógeno (SEFIN)



Universidad de Salamanca (USAL)



Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC)

## INSTITUTIONAL PARTNERS



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The communications compiled in the present "Book of Abstracts" have been reviewed by the Scientific Committee and selected to be presented at the XIX SEFIN Congress and II BeMiPlant.

## PREFACE/WELCOME

Dear friends,

We are pleased to welcome you to the XIX Congress of the Spanish Society for Nitrogen Fixation (SEFIN), which we host in Salamanca July 3 to 5, 2024.

A conference is always an exceptional opportunity for meeting and communication among colleagues. Previous editions have been characterized by what a mathematician would describe as a bijective function: each registered colleague is a friend. Certainly, the SEFIN congress is for everyone and for all its members, because each member is a master beam of it. Furthermore, this group of friends has grown in recent years with the participation of our dear Portuguese colleagues, who organized the successful 1<sup>st</sup> BeMiPlant congress, of which this congress is a continuation.

To the joy of meeting again, we will add the commemoration of the 40<sup>th</sup> anniversary (one year late imposed by the covid-19 pandemic) of the creation of SEFIN and its first congress, which was organized by Prof. Rodríguez-Barrueco and held in Salamanca, June 8 to 10, 1983. Most of those who would later become our scientific mentors were present at that meeting. Those teachers laid the foundations of what would be a successful scientific society, our Spanish Society of Nitrogen Fixation (SEFIN).

Therefore, it is a reason for satisfaction that in 2024, we can continue honoring the hard work started four decades ago. The XIX SEFIN / II BeMiPlant congress will be an excellent opportunity to do so. Thus, we have a new opportunity to share scientific ideas, to show some of our most recent achievements, and to actively participate, especially the youngest members of the Society, for whom our congress should be a magnificent training platform that facilitates learning and the dissemination of their scientific findings.

Collaborations have always been the hallmark of SEFIN members. In this sense, the organization of this congress is a clarifying example of this type of cooperation, since the organizing committee is made up of members from two different institutions: the University of Salamanca (USAL) and the Institute of Natural Resources and Agrobiology of Salamanca (IRNASA), belonging to the Higher Council for Scientific Research (CSIC). On behalf of both institutions, we want to thank the Spanish Society for Nitrogen Fixation (SEFIN) for the trust placed in us to organize this new, very special edition in Salamanca.

Without further ado, it is a pleasure to invite you to participate in the XIX SEFIN / II BeMiPlant congress which, as in previous editions, will be enjoyable and productive, both on the scientific level and in the social aspect. We are waiting for you in Salamanca!

The Organizing Committee.

# Organizing Committee

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2002 - Tomás Ruiz Argüeso

2006 - Manuel Megías Guijo

2010 - Eulogio Bedmar Gómez

2015 - Eustoquio Martínez Molina

2019 - Manuel Becana Ausejo

## Scientific Program

Wednesday, July 3<sup>rd</sup>

4:30 p.m.-6:00 p.m.

Registration and poster placement

6:00 p.m.

Welcome

Pedro F. Mateos and Mariano Igual – Presidents of the local organizing committee

César Arrese Igor – President of SEFIN

José Miguel Mateos Roco – Vice Chancellor of USAL Research

Mar Siles Lucas – IRNASA-CSIC director

6:30 p.m.

Opening Conference

“Genomic-derived insights into the metabolism and evolution of rhizobia”

George diCenzo, Queen's University, Canada

8:00 p.m.

Welcome cocktail

Thursday, July 4<sup>th</sup>

9:00 a.m.-11:00 a.m.

**Session 1. Diversity and ecology in plant-microorganism interactions**

*Chairs: José David Flores-Félix (USAL) and Ricardo Soares (INIAV)*

**9:00-9:20h S1-O1.** D. Garrido-Sanz (University of Lausanne)

“Sequential propagation of the wheat rhizobiome reveals the contribution of soil and seed-borne bacteria”

**9:20-9:40h S1-O2.** P. van Dillewijn (EEZ-CSIC)

“Volatilomic profile of *Sinorhizobium meliloti* and its effects on the soil and plant microbiome”

**9:40-10:00h S1-O3.** A.S. Santos (Institute of Chemical and Biological Technology (ITQB)-NOVA)

“Innovative approach to foster beneficial bacterial strains for tomato growth enhancement: Biostimulation by algae extracts”

**10:00-10:20h S1-O4** Z. Saati-Santamaría (University of Salamanca)

“Unveiling global microbiome signatures in natural and agricultural soils”

**10:20-10:40h S1-O5** C. Frade (IRNASA-CSIC)

“How do edaphic microorganisms respond to different livestock and pasture management practices in dehesa?”

**10:40-11:00 a.m.** *Discussion and conclusions of session 1*

11:00 a.m.-11:30 a.m.

Coffee break

11:30 a.m.-1:30 p.m.

**Session 2. Agronomic impact of FBN and phytoremediation**

*Chairs: Clarisse Brigido (MED-UÉvora) and Daniel Garrido Sanz (University of Lausanne)*

**11:30-11:50h S2-O1** M. Montoya (Autonomous University of Madrid)

“A synthetic bacteria community (SynCom) promotes plant growth in tomato (*Solanum lycopersicum*) through its use as an inoculant”

**11:50-12:10h S2-O2** I. Rebelo-Romão (Institute of Chemical and Biological Technology (ITQB)-NOVA)

“Evaluation of native and non-native, seed-isolated bacteria as biotreatment to increase drought resilience in maize (*Zea mays* L.)”

**12:10-12:30h S2-O3** C. Brígido (MED-UÉvora)

“Introduction of exogenous AMF species alters the diversity and functionality of AMF communities in cowpea”

**12:30-12:50h S2-O4** G. Costas-Fernández (CIALE-USAL)

“Priming mechanisms in the *Trichoderma* -wheat system: Indirect defense against Powdery Mildew”

**12:50h-13:10h S2-O5** A. Díez-Méndez (Catholic University of Ávila)

“*Bacillus licheniformis* A.1 isolated from ashes of fire-affected forest soil in Avila: implications for ecosystem recovery”

**1:10 p.m.-1:30 p.m.** *Discussion and conclusions of session 2*

1:30 p.m.-3:30 p.m.

Lunch time

3:30 p.m.-5:00 p.m.

**Session 3. Climate change, biotic and abiotic stress in plant-microorganism interactions**

*Chairs: Alexandra Diez-Méndez (UCAV) and Antonio José Fernández González (EEZ-CSIC)*

**15:00-15:20h S3-O1** JI Vilchez (Institute of Chemical and Biological Technology (ITQB)-NOVA)

“Domestication caused a “Lost in translation” problem for microbiota beneficial interaction: From root exudates to inheritance”

**15:20-15:40h S3-O2** J. Purswani (University of Granada)

“Social Biofertilizers: Rapid social consortia selection”

**15:40-16:00h S3-O3** R. Roca-Couso (University of Salamanca)

“Bacterial volatiles as biofungicides. A new tool against phytopathogenic fungus *Botrytis cinerea* “

**16:00-16:20h S3-O4** M. Abdullah (University of Navarra)

“Impact of Arbuscular Mycorrhizal Fungi (AMF) on vegetative growth, yield, and fruit quality of tomato (cv. Moneymaker) growing under salinity”

**16:20-16:40h S3-O5** A. Romero-Bazán (ICA-CSIC)

“Post-fire temporal dynamics of soil properties and fungal communities in *Pinus pinaster* Ait. Forests”

**16:40-17:00h** *Discussion and conclusions of session 3*

5:00 p.m.-6:00 p.m.

Coffee Break and Poster Session

6:00 p.m.-7:00 p.m.

SEFIN Assembly

Free time

8:30 p.m. Guided visit to Salamanca

Friday, July 5<sup>th</sup>

9:00 a.m.-11:00 a.m.

**Session 4. Molecular biology of plant-microorganism interactions**

*Chairs: Bruna de Sousa (UPM) and Alvaro Alonso-Caballero (UAM)*

**9:00-9:20h S4-O1** SK Guedes-García (EEZ-CSIC)

“Regulation of *Sinorhizobium meliloti* nitrogen fixation genes by antisense RNAs

**9:20-9:40h S4-O2** A. Alonso-Caballero (Autonomous University of Madrid)

“Mechanosensing and motility in plant-colonizing *Pseudomonas* species”

**9:40-10:00h S4-O3** JN Soldek (Center for Plant Biotechnology and Genomics, UPM-INIA/CSIC)

“Metal-binding protein RLV\_3444 is a component of a symbiotically relevant zinc ABC transporter system in *Rhizobium leguminosarum*”

**10:00-10:20h S4-O4** L. Ruiz-Sáez (EEZ-CSIC)

“Genetic factors involved in the production of a Mixed-linkage  $\beta$ -glucan (MLG) in rhizobia”

**10:20-10:40h S4-O5** P. del Cerro (University of Seville)

“Understanding the molecular basis of *Sinorhizobium fredii* HH103-soybean compatibility conferred by bacterial secreted proteins”

**10:40-11:00h** *Discussion and conclusions of session 4*

11:00 a.m.-12:00 a.m.

Coffee Break and Poster Session

12:00 p.m.-2:00 p.m.

**Session 5. Symbiotic nitrogen fixation**

*Chairs: Pablo del Cerro (US) and María Isabel Rubia (UPNA)*

**12:00-12:20h S5-O1** P. Ayala-García (University of Seville)

“Analysis of extracellular membrane vesicles from *Rhizobium tropici* CIAT 899 in the presence and absence of the inducing- flavonoid apigenin”

**12:20-12:40h S5-O2** F. Pérez-Montaño (University of Seville)

“Unrestricted intimate relationship: proteomic analysis of extracellular membrane vesicles from bacteroids”

**12:40-13:00 S5-O3** CN Jacott (University of Seville)

“Harnessing soybean genetic diversity and rhizobia effectors to enhance nitrogen-fixing symbioses”

**13:00-13:20h S5-O4** MI Rubia (Public University of Navarra)

“Several UMAMIT plant amino acid transporters are required for efficient symbiotic nitrogen fixation in *Medicago truncatula*”

**13:20-13:40h S5-O5** RM Esquinas-Ariza (EEAD-CSIC)

“Metabolome and hormone profiling of hemoglobin glb1-1 and glb2-1 mutants in *Lotus japonicus*”

**1:40 p.m.-2:00 p.m.** *Discussion and conclusions of session 5*

2:00 p.m.-3:30 p.m.

Lunch time

3:30 p.m.-4:30 p.m.

Presentation and Conference of the person awarded with  
the Antonio Palomares Prize

“Choices vs. decisions: complex decision making in rhizobia”

Carmen Sánchez-Cañizares (University of Oxford)

4:30 p.m.-5:30 p.m.

Closing conference

“Rhizobial infection, a journey into plant cells: insights into transcellular entry mechanisms  
in legume roots”

Fernanda De-Carvalho-Niebel, INRAE-CNRS, France

5:30 p.m.- 6:00 p.m.

Closing ceremony

Free time

9:00 p.m.

**“Casino del Tormes” Gala Dinner**

## Communications list

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- S1-O2.** Volatilomic profile of *Sinorhizobium meliloti* and its effects on the soil and plant Microbiome. van Dillewijn, P.\*, Bernabéu-Roda, L.M., Soto, M.J. **11**
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- S1-P-01.** Deciphering the metabolic potential of *Ensifer* spp. in bioremediation. Montero-Calasanz, M.d.C.\*, Barcia-Piedras, J.M., Cubo, M.T., Espuny, M.R., Camacho, M. **15**
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- S1-P-03.** Fungal microbiome of wild strawberry (*Fragaria vesca*) for the improvement of cultivated strawberry production. Zabalgoceazcoa, I.\*, Barroso E, Vázquez de Aldana, B.R. **17**
- S1-P-04.** Optimization of a new methodology to study the seed microbiology of maize (*Zea mays* L.). Gomes, J.\*, Silva, D., Rebelo-Romão, I., Kasa, F., Vílchez, J.I. **18**
- S1-P-05.** *Pseudomonas putida* KT2440 type VI secretion systems mediate adaptation to the rhizosphere. Vázquez-Arias, D.\*, Civantos, C., Durán, D., Bernal, P., Rivilla, R., Martín, M. **19**
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- S1-P-08.** From salty soils to scientific breakthroughs: *Halocnemum strobilaceum* and its novel plant-growth promoting bacterial species. Romano-Rodríguez, E.\*, Navarro-Torre S., Mateos-Naranjo E., Flores-Duarte N.J., Rodríguez-Llorente I.D., Pajuelo E., Redondo-Gómez S. **22**

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- S2-O2.** Evaluation of native and non-native, seed-isolated bacteria as biotreatment to increase drought resilience in maize (*Zea mays* L.). Rebelo-Romão, I\*, Gil, T, Gomes, J., Sousa, A., Teixeira, R., Kasa, F., Katamadz, A., Vergara-Diaz, O., Vicente, R., Vílchez, J.I. **32**
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- S2-P-02.** Design of a synthetic community for the bioremediation of hydrocarbon polluted soil. Carrera-Ruiz, L.\*, Montoya, M., Durán-Wendt, D., Redondo-Nieto, M., Martín, M., Rivilla, R. **37**
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- S2-P-13.** Promoting soil vitality: impact of cover crops on soil enzymatic activity in intensive agricultural systems. Denysov, A.\*, Barradas, A., Nunes, A.P., Godinho, M.C., Fareleira, P.\* **48**
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- S2-P-15.** A bacterial consortium for inoculation of annual ryegrass in pasture systems. Fareleira, P.\*, Santos, A.M., Soares, R., Barradas, A., Videira e Castro, I. **50**

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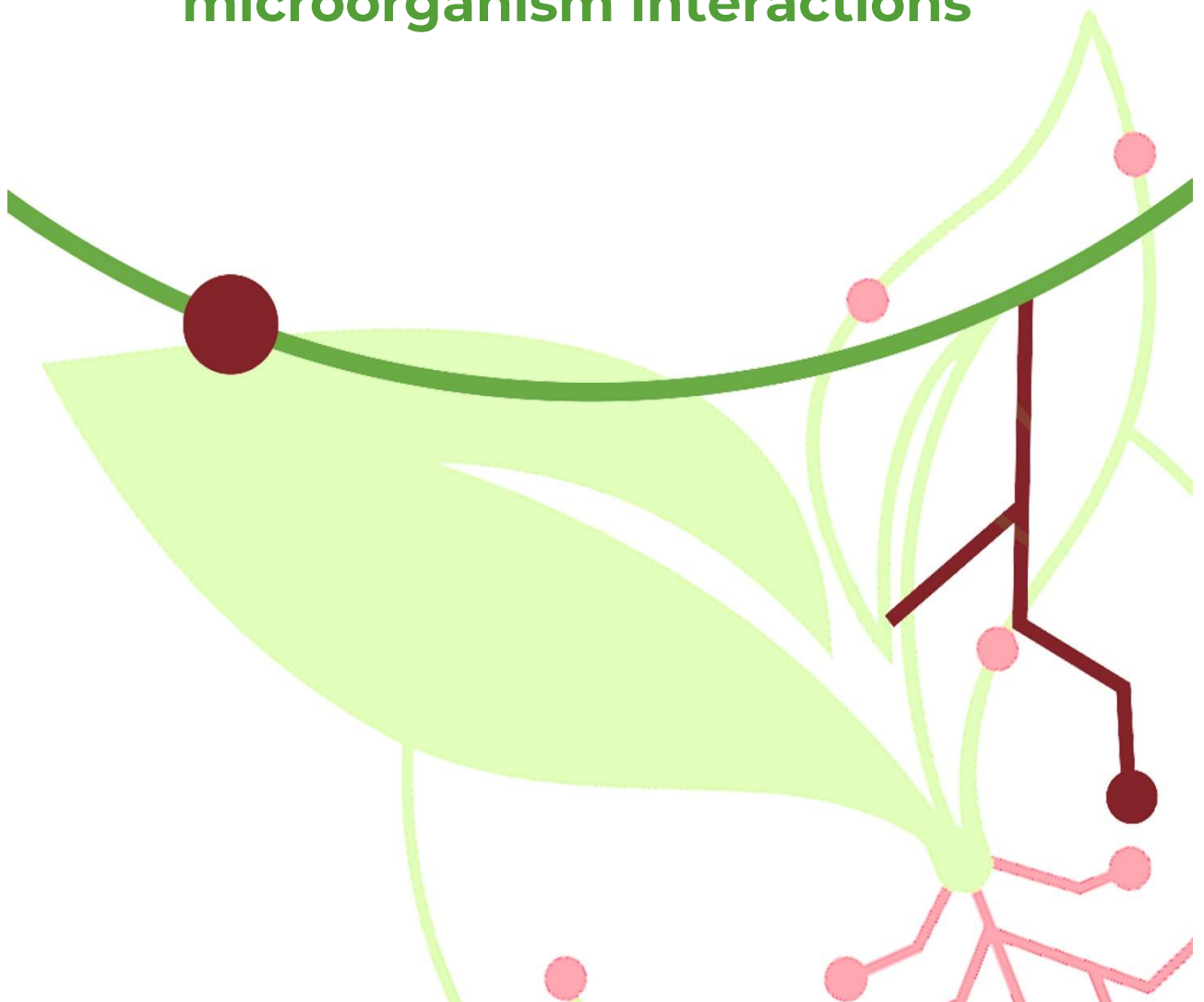
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## Session 1

# Diversity and ecology in plant-microorganism interactions





# Sequential propagation of the wheat rhizobiome reveals the contribution of soil and seed-borne bacteria

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## Abstract

Plant-associated microbiomes carry a wealth of functions that favour plant growth and health. Understanding the assembly of rhizosphere microbiomes holds promise for manipulating and utilizing them in agricultural applications. Nonetheless, the challenge of working with microbiomes of the rhizosphere—one of the most diverse environments on Earth—limits the depth of mechanistic studies. Here, we report the generation and characterization of a reproducible and species-rich wheat rhizosphere community (RhizCom) using a sequential propagation approach. This served to study the assembly of an initial soil microbiome on wheat roots, constrained by the plant root secretions as the sole carbon source. Our results show that bacteria belonging to *Proteobacteria* and *Bacteroidota* are the major groups that assemble in the wheat rhizobiome, and to a less extent also members of the *Firmicutes* and *Verrucomicrobiota*. Seed-borne rhizosphere bacteria (SbRB) collectively accounted for 51% of the total RhizCom, mainly represented by *Flavobacterium* and *Serratia*. Bacteria that assembled into the RhizCom comprised only the 4% of the original soil inoculum, but 85% of the SbRB. Functional characterization of these communities revealed near-complete coverage of the RhizCom and SbRB, while only 52% of the soil community, illustrating the enormous diversity of soil microbiomes. The wheat rhizobiome was enriched in functions related to host interaction, such as cell motility, sugar metabolism, biofilm formation, and functions involved in plant-beneficial interactions, such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, hydrogen cyanide, and alkaline phosphatase biosynthesis. SbRB were enriched in the transport and metabolism of different carbohydrates, specifically saccharides, while the soil microbiome was enriched in functions related to the metabolism of broader substrates and energy production. Our results demonstrate that obtaining diverse and reproducible natural communities can be successfully achieved by employing sequential microbiome propagation, offering a valuable tool for elucidating the intricate interactions between plants and their associated microbial communities [1].

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# Volatilomic profile of *Sinorhizobium meliloti* and its effects on the soil and plant microbiome

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## Abstract

Bacteria release a large number of small, volatile compounds (VCs) belonging to diverse chemical classes [1]. Bacterial VCs are known for their beneficial effects on plants where they can increase plant growth and/or resistance to biotic and abiotic stresses but they can also play important roles in microbe-microbe interactions by affecting growth and bacterial behaviors such as motility, biofilm formation, virulence, or stress and antibiotic resistance [1]. Despite the important biological activity of bacterial VCs on microorganisms, very little is known about their effects on soil microbial populations or the plant microbiome. The knowledge about the production and ecological role of VCs emitted by rhizobia is also limited. Only recently have data indicating a role for rhizobial volatilomes as infochemicals in bacteria and in inter kingdom communication with plants been found [2]. For instance, a methyl ketone produced by rhizobia was implicated in intermicrobial communication [3]. Here, the volatilomic profile produced by *Sinorhizobium meliloti* GR4 (SmGR4) and a long chain methyl ketone overproducing mutant, SmGR4fadA, is described. Soil and *Medicago truncatula* plants were exposed to the volatile blend produced by SmGR4 or SmGR4fadA. Their effect on bacterial populations in the soil, the rhizosphere and the root endosphere compared with control (medium only) conditions are shown and discussed.

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## Innovative approach to foster beneficial bacterial strains for tomato growth enhancement: Biostimulation by algae extracts

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### Abstract

Conventional agriculture has relied on chemical pesticides and synthetic fertilizers inputs, causing a depletion in farms and ecosystems, but also in plant-related microbiota, which are crucial for plant growth [1]. As a sustainable alternative management, algae extracts (AEs) and plant growth-promoting bacteria (PGPBs) have shown promising results [2]. However, the effects of AEs on soil microbiota are still poorly understood. Recent studies have suggested that AEs may increase the presence of beneficial microorganisms or enhance their plant beneficial traits [3]. In this context, our study aimed to use the microbial-modulating capacity of AEs to prospect for potential PGPB. To achieve this, we applied six algae extracts (*Gracilaria gracilis*, *Fucus vesiculosus*, *Bifurcaria bifurcata*, *Padina* sp., *Styopodium* sp. and *Enteromorpha* sp.) into a natural soil, and monitored changes in the population of culturable bacteria. By differential abundance analysis, we identify shIPs in bacterial populations, allowing the selection of 24 bacterial isolates that showed positive associations with AEs application. These isolates were then subjected to a series of biochemical assays to determine their plant-growth-promoting activities. Among the 24 isolates, the results demonstrated the presence of specific bacterial isolates with high biofilm formation (12.5%), high production of auxins (21%), antioxidants (17%) and siderophores (42%), as well as nitrogen fixation (54%), phosphate (42%) and potassium (58%) solubilization, and sulphur oxidation (25%). Following this characterization, 3 selected isolates (ID05, ID09 and ID13; under IP protection) were inoculated into tomato plants, in which the bacterial isolate ID09 resulted in significant improvements in agronomic traits, even under challenging soil conditions, such as high pH (soil pH 9.2) and salinity (150 mM). These findings underscore the potential of AEs as a novel and effective approach to identify and isolate microorganisms with unique beneficial properties for agricultural management.

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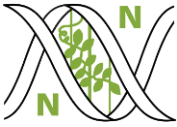
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# Unveiling Global Microbiome Signatures in Natural and Agricultural Soils

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## Abstract

Plant-microorganism interactions play a crucial role in natural and agricultural soils, shaping ecosystem health and productivity. In natural soils, these interactions contribute to nutrient cycling, disease resistance, and overall ecosystem stability. In agricultural soils, they are pivotal for nutrient uptake, disease management, and fostering soil fertility, highlighting their significance for sustainable crop production<sup>1</sup>. Also, it is known that the bacterial and fungal dynamics in soils are highly distinct<sup>2,3</sup>. Yet, our understanding of the distinctions between natural and agricultural soils remains limited, and existing microbiome surveys often lack spatial and climatic diversity, predominantly focusing on major crops like maize, wheat, and rice.

Here we present a comprehensive analysis of soil microbiomes from 32 countries, encompassing 1921 soil samples (n=439 natural; n=1482 agricultural). Our study provides unprecedented insights into understudied tree species such as *Quercus ilex* (Spanish Oak) (n=225), *Olea europea* (Olive) (n=160) and *Pistaccia vera* (Pistachio) (n=145), amongst others. As part of our analyses, we focused on the effect of fertilizers and pesticides on bacterial and fungal crop communities. For example, our findings reveal that these agricultural inputs exert a stronger influence on bacterial composition ( $p=0.001$ ) compared to diversity, which is just significantly affected by pesticides ( $p=0.03$ ) in the overall crop comparison. However, the effects vary when analyzed on a crop-by-crop basis. Furthermore, we elucidate differences in diversity and composition between natural and agricultural soils. For instance, bacterial taxa such as *Acidobacteriaceae*, *Acidothermaceae*, and *Frankiaceae* are enriched in natural soils, whereas *Micrococcaceae*, *Azospirillaceae*, and *Rhodobacteraceae* dominate agricultural soils.

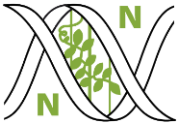
Overall, we show the intricate interplay between soil bacterial and fungal communities, highlighting their responsiveness to environmental dynamics and agricultural practices. Our insights deepen our understanding of soil biodiversity and the factors shaping bacterial and fungal composition, offering valuable clues for achieving more sustainable crop production amidst the challenges posed by climate change.

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## Acknowledgements

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## How do edaphic microorganisms respond to different livestock and pasture management practices in dehesa?

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### Abstract

Soil microorganisms perform key ecosystem functions such as soil carbon sequestration, nutrient cycling and the maintenance of soil fertility [1,2]. Silvopastoral systems, such as dehesa, extend widely and hence play a crucial global role in the provision of environmental services. Most dehesas are located on low-productive land, leading to opposed management practices, such as abandonment or use intensification through livestock management and/or pasture improvement. However, very little is known on how these practices influence the structure and activity of soil microbial communities [1,3]. Here, we have studied the microbiological structure, using PLFAs and targeted metagenomics, and functionality, by means of enzymatic activities, of soils in nine dehesas under five different pasture and livestock management practices. We found that young legume sowing (LJ) and rotational grazing (RO) exhibited higher microbial biomass and lower nutritional stress than abandonment (AB). Moreover, AB, LV and LJ soils showed higher bacterial diversity (Shannon) compared to RO and continuous grazing (CT) soils. In contrast, protist diversity was larger in RO soils. Meanwhile, fungal diversity was not altered by the different managements. Microbial community structure and composition was also affected by agro-livestock practices. Regarding functionality, major differences were found for  $\beta$ -Glucosidase activity, which was higher in LJ than in LV soils. Overall, our results demonstrate that microbial community composition and activity are shaped by management regime.

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### Funding

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## Deciphering the metabolic potential of *Ensifer* spp. in bioremediation

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### Abstract

More than 6.24% of the European agricultural area already has toxicity problems related to agricultural practices. Phytomanagement of contaminated soils was presented as a feasible solution and in line with the principles of circular economy. In this sense, it has been shown that *Medicago* spp. (*Leguminosae*, *Fabaceae*) are perfect candidates for phytomanagement, thanks to their high phytoextractive potential, their growth rate, their greater adaptability to stressed environments, their ability to grow in soils contaminated by heavy metals, and, of course, their ability to establish a symbiotic relationship for nitrogen fixation with nitrogen-fixing bacteria. However, heavy metal contamination can seriously affect the alfalfa-rhizobium symbiosis. Therefore, proper selection of well-adapted symbionts is required to maximise the benefits of the use of alfalfa in the remediation of contaminated sites.

The aim of this work was to perform a comparative genomic study of 65 genomes of *Ensifer* sp. to elucidate the metabolic mechanisms that underline their potential stress tolerance. Our results revealed significant genomic differences between members of the genus with respect to the biosynthesis of nodulation factors and protein secretion systems, indicating host-specific nodulation, and provided insights into strong environmental adaptations. Heavy metal resistance genes (HMRs) and complete clusters related to resistance to arsenic, zinc, cadmium, cobalt, chromium, mercury and copper, as well as genes involved in detoxification, were frequently found.

### Funding

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# The olive rhizosphere microbiome across the Mediterranean Basin

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## Abstract

The complex and co-evolved interplay between plants and their microbiota is crucial for the health and fitness of the plant holobiont which also have implications on the fruit production. At the same time, the plant microbiome is also influenced by the soil properties, agricultural managements of the crops and climatic conditions. The olive tree cultivation has economic, social and historical importance in the Mediterranean Basin since 5,000 years B.C. In this work, we have analyzed 52 olive orchards from 15 provinces in 5 countries of the Mediterranean Basin: Portugal, Spain, Morocco, Italy and Greece. The olive orchards were subjected to either traditional, organic or hedgerow cultivation systems, under different climatic and pedological conditions. All orchards were sampled on May 2023 at or just after full bloom. A total of 472 samples from olive root rhizosphere were subjected to MiSeq sequencing for ITS2 and 16S *rRNA* gene amplicons. We obtained more than 40 million raw reads for both bacteria and fungi which yielded 11,460 ASVs for bacterial and 5,411 ASVs for fungal communities. The preliminary analysis of the beta diversity established two clearly distinctive groups: a) samples from Spain and Morocco, and b) samples from Portugal, Italy and Greece. This picture was also present in the taxonomic analyses that showed a dominance of Proteobacteria in the rhizosphere of olive trees from Portugal, Italy and Greece while in Spain and Morocco Actinobacteria was the predominant bacterial phylum. A similar image was obtained for the fungal communities, where Ascomycota was the most abundant phylum in all countries. However, the phylum Mortierellomycota had a relative abundance of 33 % in Morocco and the Glomeromycota had 7 % in Spanish soils, while both phyla were clearly diminished or absent in the other countries.

## Funding

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# Fungal microbiome of wild strawberry (*Fragaria vesca*) for the improvement of cultivated strawberry production

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## Abstract

*Fragaria x ananassa*, the cultivated strawberry, is derived from the spontaneous hybridization of two American species: *Fragaria chiloense* x *Fragaria virginiana*. These ancestral species were imported to Europe, where the hybridization event occurred in the 18<sup>th</sup> century [1]. The geographical dislocation of the parental species from its original habitat might have caused a loss of their native microbiome. As a result, the full potential of modern strawberry plants might be limited, because microbial symbionts often perform functions useful for their host plants.

*Fragaria vesca*, the wild European strawberry, is an ancestor of the parental species of *Fragaria x ananassa* [1]. *Fragaria vesca* occurs in different natural habitats in Europe, and its microbiome might perform functions useful for habitat adaptation. Knowledge on the microbiome of this wild ancestor might represent a useful resource to restore the microbiome, and improve the performance of modern strawberry cultivars.

We present the results of a preliminary survey of culturable strains of fungi isolated from roots of *Fragaria vesca* in different locations in Spain. The assemblage obtained consists of about 90 different taxa, mostly ascomycetes. Niche functionality and other characteristics of this set of fungi are presented in this work.

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# Optimization of a new methodology to study the seed microbiology of maize (*Zea mays* L.)

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## Abstract

The study of seed microbiota, both metagenomics and culturomics, face the uncertainty of the current methodologies' accuracy on this field. Precise resolution in a source with so limited and hypervariable microbial populations is really determinant, as any minimal contamination really impacts on the results [1]. Moreover, the surface-sterilization approach remarkably depends on the seed characteristics: different structures and morphologies in seeds require different approaches. In order to ensure the best resolution, in this study we aimed to optimize an effective method to discern seed microbiota using maize as model seeds, and adapting previous methodologies [2]. Considering not to impact on germination rate, and that our previous experience indicated a notable proportion of the seed microbiota is located close to the surface, our procedure included several steps of evaluation. Firstly, the seeds underwent surface washing and abrasion to remove the outer coat and any environmental debris, then we evaluated several treatments of ethanol and bleach, as well as different exposition time, in order to find the less harmful combination, using changes in culturable populations as indicator. Without conditioning germination as well, the best treatment in terms of registered biodiversity and population number corresponded to 5-min of 70% ethanol. After three wash cycles, a bulk soaking overnight was performed to enhance germination. Subsequently, the seeds germinated for 5 days, followed by root sampling for population analysis. Therefore, we found a combination that allowed us to discern ascertain populations, which would enhance the description of new strains reliable for future bioinoculants, as well as for metagenomic analysis.

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# ***Pseudomonas putida* KT2440 type VI secretion systems mediate adaptation to the rhizosphere**

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## **Abstract**

The Type VI secretion system (T6SS) is a bacterial nanomachine involved in interbacterial competition. Attacking bacteria release toxins inside prey cells, inhibiting the growth and/or killing competitors in a contact-dependent manner, providing fitness advantages. The biocontrol agent *Pseudomonas putida* KT2440 efficiently uses the T6SS as a mechanism to protect plants from deleterious phytopathogens<sup>1</sup>. This strain encodes three type VI secretion systems (K1-, K2- and K3-T6SS) but only the K1-T6SS has been proved to be functional and to have antibacterial activity up to date<sup>1</sup>. Here, we study the functionality of the K2- and K3-T6SSs by testing the capacity of single, double and triple T6SS mutants to kill different preys in competition assays. The triple mutant was less competitive than the mutant lacking only the K1 system specially when the prey was a plant pathogen. These data indicate that the K2- and/or K3-T6SS might be active in *P. putida* natural niches in the presence of real competitors. An increasing number of studies are showing the relevance of T6SS in modulating complex polymicrobial communities, especially in the gut<sup>2</sup>. Here, we study the role of *P. putida* T6SSs in shaping microbial communities in its habitat, the rhizosphere. First, we investigate the ability of the wild type strain and the T6SS mutants to colonise the rhizosphere of tomato plants growing in agricultural soil by the wildtype and the T6SS mutants, as previously reported<sup>3</sup>. Secondly, we analysed the microbiome present in the rhizosphere of these plants inoculated with the aforementioned strains. We observed that all T6SS mutants have a lower capacity to colonise the rhizosphere when compared to the wild type strain. In accordance with that, the Principal Component Analysis (PCA) of the tomato plants microbiota showed two clearly differentiated groups. On the one hand, the microbiota of plants inoculated with the wildtype strain showed that it was able to outcompete foes in a T6SS dependent-manner; on the other hand, the microbiota of plants inoculated with the T6SS mutants were unable to do that. These results suggest that the T6SSs of *P. putida* are functional in the rhizosphere, in the presence of competitors, modulate this polymicrobial community and are instrumental for *P. putida* colonization of the plant roots.

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## Study of endophytes of the endemic species *Astragalus paposanus* as plant growth promoters

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### Abstract

Plants growing in extreme conditions show high ecological fitness. This ability is partially attributed to the complex interactions within the plant microbiome [1]. It is widely acknowledged that microorganisms confer advantageous effects on growth and resilience to stress conditions in plants [2]. Consequently, the study of those microbiomes can be a source of plant growth-promoting (PGP) bacteria.

Endophyte strains isolated from both roots and shoots of *Astragalus paposanus*, an endemic species from Atacama Desert, were investigated for putative plant growth-promoting properties and stress tolerance traits. We studied not only their tolerance to high salt concentrations and extreme temperatures, but also their ability to grow collectively in a consortium. We selected 8 candidates and design 2 synthetic communities (SynComs), which were tested in *Astragalus* sp. (as the original species) and in *Brassica napus* (as an example of agronomic crop of interest) to evaluate the ability of those bacteria to promote plant growth in presence and absence of stress conditions.

Despite the thermotolerance of the bacteria and the observed enhancements in the growth of some plants, no significant effects were observed respect to the control plants when a thermic stress was applied, under the temperatures tested. However, the results reveal the potential of two individually applied bacteria and a four-bacteria SynCom to confer advantageous effects on plant development under stress-free conditions. These findings offer a preliminary selection of bacteria with PGP properties useful for future studies with potential application in agronomic crops of interest.

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## Impact of Fire on Rhizobia Associated with *Lotus corniculatus* in Salamanca Province, Spain

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### Abstract

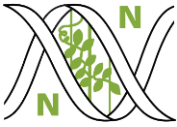
In Europe, 450,000 hectares/year are burned in wildfires. However, the medium- and long-term impacts of these fires lead to soil erosion, which in turn results in reduced soil fertility, limiting its potential for recovery [1]. In this context, legumes can serve as a valuable tool for restoring soil, given their pioneering characteristics and potential to improve soil fertility [2]. Using different soils, both burned and unburned, from the province of Salamanca, the legume *Lotus corniculatus* was employed as a trap plant by planting 15 sterilized seeds. After 40 days, root systems were cleaned, and rhizobia from the nodules of *L. corniculatus* were isolated. A total of 13 isolates were obtained from unburned soils, while 17 were obtained from burned soils. MALDI-TOF was employed to analyse infraspecific diversity and putative identification of the isolates by comparing them with a database containing various genera of *Rhizobia*, resulting in a preliminary identification of the isolates within the genera *Mesorhizobium* and *Bradyrhizobium* for unburned and burned soil isolated strains respectively. Following spectral grouping, representative strains from each group were selected for sequencing of the 16S rRNA gene, the *atpD* gene, the *glnII*, and the *nodC* gene (used for determining symbiovarieties). These results confirmed the obtained taxonomic affiliation and showed that *Mesorhizobium* strains could be a putative new species, meanwhile strains from burned soil were identified as *B. cytisi*. Next, the hydroponic assays were performed using sterile vermiculite as substrate and watered with N-free nutritive solution, being measured the shoot dry weight four weeks post-inoculation. Isolates of the genus *Mesorhizobium* and some isolates belonging to *Bradyrhizobium* were capable of nodulating and fixing-nitrogen in symbiosis with *L. corniculatus* showing that under post-fire conditions, *Lotus* adapts its endosymbiotic communities and allows to select strains adapted to these conditions to design revegetation strategies.

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### Funding

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# From salty soils to scientific breakthroughs: *Halocnemum strobilaceum* and its novel plant-growth promoting bacterial species

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## Abstract

*Halocnemum strobilaceum* is a widespread hyperhalophyte desert plant with notable antioxidant, antimicrobial, insecticidal and phytoremediation properties. The remarkable stress resistance of halophytes is largely attributed to their unique associated microbial community [1]. Research shows that bacterial endophytes confer plant growth promotion, metabolic enhancement, and stress resistance benefits to their host plants. However, the underlying mechanisms of plant-endophyte interactions and their role in salinity tolerance remain poorly understood [2]. This study investigated the uncharted endophytic bacterial community in *Halocnemum strobilaceum* stems from Huelva, Spain. We isolated 74 halotolerant and halophilic endobacteria, all exhibiting in vitro plant growth-promoting (PGP) traits, including IAA and siderophores production, biofilm formation, K solubilization and high salinity tolerance (up to 200 mM NaCl). Sequencing of the 16S rRNA gene revealed some isolates with low identity percentages compared to known bacterial species (<98.7%) [3]. Further genome sequencing, pairwise genome comparisons and digital DNA-DNA hybridization (dDDH) unveiled three novel PGP-endobacteria species, TE3, EE7 and GE22 strains, belonging to the Gram-negative bacteria *Kushneria* and *Vreelandella* genera (ANI<95-96%, dDDH<70%) [4].

To assess the salt stress mitigation capacity of endophytes in plants, we formed two consortia from six PGP-endobacteria isolates: a Gram-negative consortium (TE3, EE7, and EE4 strains, C1) and a Gram-positive consortium (*Bacillota* phylum strains, C2). We conducted a greenhouse trial with *Medicago sativa* L. (alfalfa), using inoculated and non-inoculated treatments (n=24). Alfalfa performance was evaluated by measuring shoot and root length, fresh and dry biomass, and physiological parameters as gas exchange and photosystem II efficiency. Additionally, a comparative transcriptomic analysis was performed to understand differential gene expression in alfalfa induced by the two inoculums.

Our findings highlight the potential of halophyte plants as a source of novel PGP endophytic bacteria and provide insights into their interaction mechanisms with host plants, suggesting innovative applications in sustainable agriculture and phytoremediation.

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## Funding

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# Unraveling beneficial plant-bacteria interactions: Integrating amplicon and shotgun metagenomics with PGP features for the design of bacterial consortia

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## Abstract

The complex interplay between plants and microorganisms in agricultural soils is crucial for ecosystem health and productivity [1]. Here we study the diversity and dynamics of several agricultural soils by employing an integrated approach microbiome analysis alongside with lab-based Plant Growth-Promotion (PGP) tests. The final goal is to design a synthetic community to be inoculated as a biofertilizer improving the stability of native microbial communities [2]. We selected 4 crop rotation agro-systems to sample soil (rhizospheric and bulk) of wheat and vetch crops preceded by wheat, vetch or lentil. Firstly, we combined an 16S rRNA metabarcoding approach, which was employed to elucidate the diversity and structure of the soil bacteriome, and a shotgun metagenomics analysis, which provided insights into the functional dynamics of these agricultural soil microbiomes. On the one hand, we found that genera such as *Rubrobacter*, *Skermanella*, *Microvirga* and *Bacillus* were represented in all the sample types with a high abundance. On the other hand, we found that the diversity of bacterial PGP genes decreased with the sampling time, being maximum in the bulk soil samples and minimum in the rhizospheric soil samples when the plant was more developed. Secondly, we carried out an isolation process followed by a PGP analysis focused on nutrient assimilation. We used these results to design four microbial consortia (one of them based on PGP results, and the rest based on core genera under different criteria) which were employed for an *in planta* assay with the initial plant species (wheat, vetch and lentil). Surprisingly, our results showed that consortia formed by isolates belonging to the core microbiome (attending both, amplicon and shotgun massive sequencing) were the ones that promoted plant growth to a greater extent. Our results support the use of these core microbiome consortia as future biofertilizers.

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# Soil bacterial communities associated with black truffle producing *Quercus ilex* L.

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## Abstract

The black truffle, *Tuber melanosporum* Vitt., is one of the most highly appreciated edible fungi worldwide. It naturally occurs in countries around the Mediterranean basin, forming ectomycorrhizal symbiosis with different tree species, notably the holm oak. The truffle's mycelium exerts a strong allelopathic effect on the soil, creating the "brûlé", an area around the host tree devoid of vegetation.

Our objective was to study the bacterial communities associated with black truffle-producing holm oaks, and to assess the impact of the brûlé on them.

For this, four *Quercus ilex* L. trees were selected both in plantations and wild forests, at three sites representative within the natural distribution area of black truffles in Spain [1]. For each tree, three compartments were analysed: rhizosphere, inside and outside the brûlé. Roots and bulk soil samples were collected, rhizospheric and soil DNA extracted, and the 16S gene sequenced by Illumina MiSeq. Bioinformatics analysis of sequences was conducted by DADA2 and the SILVA database within the R software.

The results will elucidate (a) the extent to which soil bacterial communities respond to the brûlé effect and (b) whether specific bacteria emerge in the different soil compartments and truffle producing systems. According to literature [2], we anticipate lower diversity and distinct species composition of bacterial communities inside than outside the brûlé. Moreover, the presence of bacteria common to all soil compartments is expected, but especially bacteria unique and/or indicative to each compartment.

Gaining insights into the changes induced in soil bacterial communities by the presence of *T. melanosporum* mycelium will enhance our understanding of the ecology and interactions of microbial communities in black truffle producing systems.

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## Microfluidic tools to investigate the rhizosphere

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### Abstract

Movement is required for bacteria to colonise the rhizosphere, but we know very little about bacterial mobility in the rhizosphere because observation in soil is challenging due to the opacity of soils and the problems on repeatability and variability of experiments. The aim of this work is to develop technologies for characterising the role of cell motility on movement of bacterial populations in soil. We have developed various microcosm systems for live observations of bacterial movement in porous medium. The porous environments for bacterial cells cultures are based on transparent soil substrates which enable co-culture with plants [1]. Microfluidic devices were also developed when better control of pore size distribution is needed. Finally, we also developed paper-based microfluidics for in situ extraction of root exudates which acts as powerful chemo attractant to the bacteria [2]. Using *Bacillus subtilis*, a flagellated bacteria we have observed that bacterial populations are able to move as a flock [3], and future work is now focusing on understanding the conditions under which collective movements of bacteria occur in soil.

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# Analyses of wheat rhizoplane bacterial communities in soils with diverse crop history

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## Abstract

Despite bacterial biofertilizers show promising features in lab-based tests, lose effectiveness when they are applied to agrosystems. This lack of success might be related to shifts in the dynamics of soil bacterial communities when a bacterial inoculant is applied, amongst other factors [1]. Including intimately root-associated bacteriome members in the biofertilization products confer advantages to overcome stresses [2]. A plethora of studies described the crops' bacteriome composition and their plant promotion and protection activities, specially in agroeconomic important crops, such as wheat. However, there is a gap of knowledge referring to how those bacteriomes assemble and attach to roots. Here, we focus on wheat rhizoplane bacterial communities as a model to understand rhizoattachment and biofilm colonization in complex community assemblies. First, we set up a plant trap experiment including 2 wheat varieties, 4 soils with different histories (2 with wheat presence under different agropractices and 2 without previous wheat presence and with/without metal toxicity) and 2 timepoints. We analyzed the bacterial community on the rhizoplane by amplicon sequencing. Data reveal that wheat rhizoplane communities are highly diverse, showing significant differences among the different soils and timepoints but not between varieties. It was detected that after 15 days of experiment the genera *Acinetobacter*, *Psychrobacillus* and *Pantoea* were most abundant, while after 30 days they were *Exiguobacterium*, *Asticcaulis* and *Cellvibrio*. The core bacteriome, genera found in all the samples, is composed by 15 genera belonging to 4 bacterial phyla (*Actinomycetota*, *Bacillota*, *Pseudomonadota* and *Bacteroidota*) and an archaean phylum (*Thermoproteota*). The most abundant genera are *Pantoea*, *Pseudomonas*, *Streptomyces* and *Solirubrobacter*. Wheat plants collected showed no signs of decay or stress. These results on rhizoplane bacteriome taxa will help in the design of biofertilizers based on complex communities of efficient root-colonizing bacteria.

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## A high diversity of rhizobial genera is present in holm oak tissues as endophytic bacteria in a Northern Spain dehesa forest

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### Abstract

The dehesa forest is a unique Mediterranean ecosystem based on pasturelands with autochthonous trees mainly different oak species, that harbours a huge biodiversity and have been exploited as agroforestry systems for millions of years, for which the natural and economic value is outstanding. The climate change scenario is affecting dehesa ecosystems by different biotic and abiotic factors, and strategies for enhancing its resilience and conservation are essential. In this context, maintenance of biodiversity is critical and studies have to be done to understand the microbial diversity of this particular ecosystem. Among plant-associated bacteria, rhizobia play an essential role in the dehesa taking part in the nitrogen-fixing symbiosis of different pasture and shrub legumes, and also associated to some ectomycorrhizal fungi. During a study focused on the holm oak (*Quercus ilex* subsp. *ballota*) bacterial microbiome in Salamanca (Northwest Spain) by metabarcoding, we analysed the occurrence of ASV taxonomically assigned to rhizobial genera present both in bark and wood tissues. A high diversity of rhizobial genera was found in both tissues, some of them present only in wood such as *Allorhizobium*, *Bradyrhizobium* and *Ensifer*, and other genera were present in bark and wood, including *Neorhizobium*, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Microvirga*, with different abundances depending on genus and tissue. We are currently trying to isolate these rhizobial strains in appropriate culture media for further characterization and more studies will be necessary to understand their dynamics and ecological role in the dehesa forest ecosystems

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# The influence of sustainable practices and tree cover on the diversity of Archaea in Spanish Dehesas

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## Abstract

The Dehesa ecosystem represents the largest area of wooded grassland in Europe. In the context of Climate Change, it is essential to understand the influence of this ecosystem on global C dynamics. Soil microorganisms play key roles in nutrient cycling and fertility maintenance. In particular, Archaea are important players in both C and N cycling and directly influence greenhouse gas emissions [1]. In this work we have investigated the diversity of soil Archaea, using metagenomics, in nine Spanish dehesas under 5 different livestock and pasture management practices. We also considered the influence of tree cover as trees act as ecosystems engineers [2]. The results obtained revealed that archaeal diversity (Shannon) was higher in soils under abandonment, young and old legume sowing (AB, LJ and LV, respectively) than in rotational and continuous grazing (RO and CT), and the differences were statistically significant ( $p < 0.05$ ) in AB and LV soils compared to RO soils. Conversely, tree cover had no significant effect on archaeal diversity. However, both treatment and habitat (under or outside tree canopy) significantly shaped the structure and composition of archaeal populations. For instance, members of the family *Methanosarcinaceae* were more abundant outside the tree canopy. Overall, our results show that archaeal communities are influenced by both management practices and tree cover in dehesa ecosystems.

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## Funding

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## Rhizosphere microbiome conditioned by biotic stress affects plant growth and defense in tomato and sunflower

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### Abstract

Plant root exudates contribute to the assembly of a plant-specific microbiome in the rhizosphere [1]. This plant-specific assembly affects plant development and tolerance to stress [2]. However, exudates are not only plant-specific, but also affected by phytohormonal changes in response to stresses [2]. Several studies have found that abiotic and biotic stresses modify microbial communities, as plants recruit specific taxa that are beneficial in stress conditions [3, 4]. This is referred to as cry-for-help response. Among biotic stresses, insect herbivory, as a driving source of cry-for-help, and its outcomes remain little explored. We hypothesized that herbivory by leaf-chewing insects in tomato and sunflower produce distinct fungal and bacterial communities in a natural soil. Furthermore, the outcomes of these communities in plant growth and defence are hypothesized to be plant-specific. To test these hypotheses we conditioned the microbiome in the natural Dehesa de Salamanca soil with infested and non-infested tomato and sunflower plants. The rhizosphere microbiome was collected, analysed and used as inoculant in the subsequent herbivory bioassay; in which we grew new plants in soils inoculated with the microbial communities and infested them. Plant performance was assessed studying changes in biomass and plant nutrient content, while insect performance was assessed with larvae biomass and survival. We found that the main factor driving the rhizospheric microbiome was plant species. The effect of herbivory on bacterial and fungal community structure was less pronounced in sunflower than in tomato. Furthermore, outcomes on plant growth and tolerance to biotic stresses were also plant specific. Sunflower grown in herbivore-induced microbiome showed more tolerance to herbivory, but tomato plants showed a better overall resistance to the plague. In conclusion, recruitment of microbes via the cry for help response to insect herbivory and its effects on plant performance and tolerance are highly dependent on the plant species.

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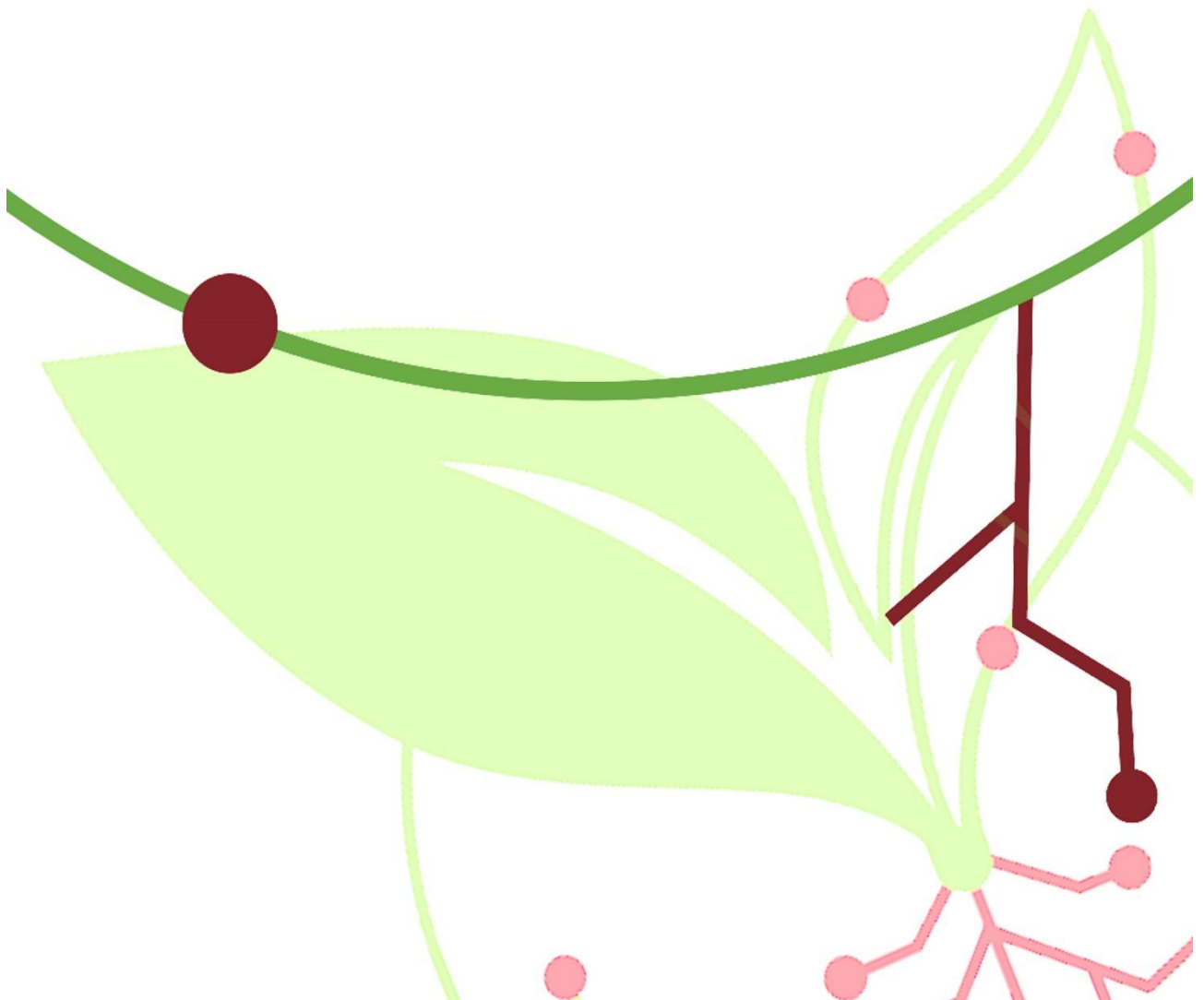
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## Session 2

# Agronomic impact of FBN and phytoremediation





# **A synthetic bacteria community (SynCom) promotes plant growth in tomato (*Solanum lycopersicum*) through its use as an inoculant**

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## **Abstract**

Agricultural intensification depends on the input of inorganic and organic fertilizers to sustain food production [1]. However, the use of synthetic chemical fertilizers is associated with important environmental problems such as the contamination of agricultural soils, water and the production of greenhouse gases that pollute the atmosphere [2]. Therefore, it is necessary to move towards sustainable agricultural production by adopting new alternatives that reduce the pollution associated with the application of chemical fertilizers without penalizing yields [3]. In this sense, the study of the interaction of plant growth-promoting rhizobacteria (PGPR) with agricultural crops has led to the development of biofertilizers or bioinoculants as an environmentally friendly alternative fertilization strategy [4]. In this context, this study aimed to design and construct a bacterial synthetic consortium and determine its ability to be used as biological fertilizer. Additionally, we analyzed the effect of its application on growth and yield of the tomato plant. Finally, we attempted to obtain information on the plant response derived from the inoculation of the bacterial consortium. Our metagenomic results reveal the biodiversity and metabolic functional of SynCom and the presence of multiple gene clusters involved in plant growth promotion and disease control in many of the strains of the bacterial consortium. We have also tested the stability of the SynCom in the rhizosphere and its effect on the rhizospheric soil bacterial community. Transcriptomic analysis of tomato root and aerial part also provided information on the expression of genes associated with the establishment of beneficial interactions between the SynCom and tomato plant. Since the SynCom interact efficiently with the tomato plants, they trigger a beneficial effect on the fruit yield. These results show the potential of this SynCom as a plant growth promoting inoculant.

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# Evaluation of native and non-native, seed-isolated bacteria as biotreatment to increase drought resilience in maize (*Zea mays* L.)

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## Abstract

Maize, an essential crop for human consumption, animal feed, and economic progress, is facing increasingly harsh weather conditions that are limiting its growth potential, along with other key crops [1]. Innovative treatments using microorganisms have shown promise in boosting plant growth and combating stress. One such novel approach involves harnessing bacteria found in seeds for biotreatment, suggesting a potential co-evolution process that enhances effectiveness due to pre-adaptation [2]. Our study sought to assess the effectiveness of native maize seed microbiota compared to those from different species in alleviating drought stress. We analyzed the seedborne microbiota of a common maize variety in Portugal to utilize it as a biotreatment for two other varieties, selecting the strain *Pseudomonas fulva* MB as the most promising candidate. As a non-native control strain, we used the *Stenotrophomonas maltophilia* MS-M1 isolated from wild alfalfa seeds, previously described as drought tolerance enhancer. The results indicate that both maize varieties exhibited improved growth when treated with either individual or combined strains, showing enhanced plant height and weight under varying irrigation levels. Productivity in terms of grain yield also increased significantly, with the drought-sensitive variety experiencing a 17% to 40% increase and the most tolerant variety seeing a 25% to 55% increase. While the native strain *P. fulva* MB showed slightly better performance overall, the consortium treatment excelled under specific irrigation conditions for certain production traits, highlighting the efficacy of seed microbiota as biotreatments for enhancing crop resilience to drought stress. These findings point towards the potential benefits of leveraging seed-isolated strains, both native and non-native, to optimize agricultural solutions in the face of changing climate patterns.

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# Introduction of Exogenous AMF Species Alters the Diversity and Functionality of AMF Communities in Cowpea

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## Abstract

Salinity in arid and semi-arid regions is rapidly increasing due to climate change and human activities [1]. The use of inoculants containing beneficial microbes, such as arbuscular mycorrhizal fungi (AMF) and rhizobia, offers a promising alternative for enhancing plant production in these areas [2]. This study investigates the effects of common agricultural practices, specifically microbial inoculation and crop rotation, on cowpea growth and its interaction with soil microbes under both non-stressed and salt-stressed conditions. Experiments were conducted in a greenhouse using non-sterilized soil, with and without NaCl supplementation. Inoculants included the *Bradyrhizobium yeanmingense* BR 3267 strain and a commercial AMF mixture (Endoplant Riego). Additionally, cowpea growth was assessed following the succession of buffelgrass (*Cenchrus ciliaris*) with or without prior soil disturbance. We evaluated plant and symbiotic parameters, nutrient content in leaves, and AMF and root nodule communities through DNA metabarcoding. Under non-stressed conditions, inoculation with AMF and/or the BR 3267 strain significantly increased cowpea biomass and enhanced N and P content in leaves. Although salinity negatively impacted cowpea growth, it did not significantly affect symbiotic parameters. Moreover, the increase of AMF propagules available through the inoculation of commercial AMF in the soil at buffelgrass sowing played a crucial role in mitigating the effects of soil tillage and salinity on cowpea growth. Interestingly, bacterial communities in the root nodules were more influenced by AMF communities than by rhizobia inoculation. The benefits of commercial AMF are likely due to changes in the biological composition and functionality of the AMF communities associated with cowpea. Overall, this study demonstrates that microbial inoculation and crop rotation are effective strategies for improving cowpea growth and mitigating the adverse effects of salinity.

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# Priming mechanisms in the *Trichoderma*-wheat system: Indirect defence against Powdery Mildew

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## Abstract

Wheat stands as the second most significant crop for human and animal consumption. However, wheat production faces significant challenges due to pathogen-caused diseases, among which powdery mildew (*Blumeria graminis* f. sp. *tritici*) significantly impacts high-yielding genotypes, causing approximately 5–14% of total crop yield losses. Aligned with the European Green Deal, which gradually limits fungicide use, promotes the use of resistant varieties, and implements biocontrol techniques such as beneficial microorganisms like *Trichoderma* spp., sustainable agricultural practices can be achieved, while reducing fungicide dependency<sup>1</sup>. *Trichoderma* spp., renowned for its direct and indirect biocontrol properties, induces priming against pathogens by means of mechanisms that allow the plant to grow or defend itself according to its needs. While the effects of induced resistance against rhizospheric pathogens, necrotrophic or hemibiotrophic foliar pathogens are widely understood, *Trichoderma*-mediated resistance to biotrophic fungi affecting above-ground parts is still much less studied<sup>2</sup>. In this work, we tested the indirect defence capacity of the *Trichoderma harzianum* T34, *T. simmonsii* T137 and *T. asperellum* T140 strains against powdery mildew in a panel of wheat cultivars challenged with seven geographically diverse mildew races. Specific *Trichoderma* strains can either up-regulate or down-regulate the basal host's defence response during these powdery mildew-*Trichoderma* interactions, hinting at competition among both fungi. We further show that there is a bivalent effect in this three-player (wheat-mildew-*Trichoderma*) interaction, including both induction and reduction of virulence, with hypersensitivity responses (HR) observed depending on the combination of strain, race, and genotype. Therefore, our data reveal that the interactions most likely rely on yet unknown genetic loci, present in the fungi or the plant. Our findings suggest a high level of specificity, demonstrating both positive and negative biocontrol effects of *Trichoderma* in the wheat-powdery mildew pathosystem.

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## ***Bacillus licheniformis* A.1 isolated from ashes of fire-affected forest soil in Avila: implications for ecosystem recovery**

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### **Abstract**

*Bacillus licheniformis*, a Gram-positive bacterium, exhibits a wide distribution across diverse environments, including soil and plant rhizospheres [1]. It is known for its tolerance to various stressors such as osmotic stress [2] and high salinity concentrations [3]. Additionally, diverse studies have demonstrated its abilities to act as plant growth promoting bacterium [4,5]. The spore-forming capability endows *B. licheniformis* to withstand extreme conditions, including those encountered in wildfires. In 2021, Avila province experienced one of the most devastating fires in its history, with 12,000 hectares being destructed which was conducted by human negligence. Our study, aimed to explore the capabilities of one of the bacteria isolated from ashes collected from that fire, *Bacillus licheniformis* A.1, focusing on its survival rate under different stress and its potential role in ecosystem recovery. To evaluate its survival capacity under fire-related stress conditions a high temperature assay was carried out, as well as determining anaerobic growth rate. Additionally, its plant growth promoting mechanisms were tested *in vitro*. Our results demonstrated the strain's capacity to thrive within temperature ranges of 45°C to 200°C and its ability to growth under anaerobic conditions. Furthermore, we conducted a soil recovery assay using burned soil samples, monitored over a 60-day period, and assessed for diverse parameters. Encouragingly, our results indicated that strain A.1 significantly contributed to soil recovery and facilitated enhanced plant development, suggesting its potential utility in burned soil rehabilitation efforts. These findings highlight the exceptional abilities of *Bacillus licheniformis* A.1 under extreme conditions. Its thermotolerance and plant growth-promoting capabilities hold promise for advancing ecosystem recovery initiatives. Further investigations are necessary to unravel its specific molecular mechanisms resilience. Thereby, facilitating its strategic deployment in ecological restoration endeavours.

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# Polyethylene microplastics from agricultural mulch films: A threat to growth promoting abilities of bacteria?

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## Abstract

Climate change, fuelled by human activities such as fossil fuel consumption and industrialization, poses a global challenge, exacerbated by a predicted population increase to 9.8 billion by 2050, with far-reaching environmental consequences [1]. Extreme weather events and declining food crop yields highlight the need for long-term productivity solutions [2]. New agricultural techniques seem promising, with plant growth promoting bacteria (PGPB) developing as an environmentally benign alternative to chemical fertilizers, increasing agricultural output and disease control while protecting the environment [3]. Plastic mulching on the other hand has transformed farming by reducing initial drying phases, maintaining appropriate moisture levels, lowering soil temperatures, reducing seedling mortality, and boosting crop stand, all of which contribute to increased yields [4]. However, its disadvantages include the accumulation of plastic waste in soil as a result of its breakdown, with poorly known effects to the soil bacteria. To assess the impact of plastic particles on bacterial growth, which is critical for evaluating the efficacy of bacterial biostimulants in plastic-contaminated soils, a series of tests were conducted at a concentration of 1% (W/V) polyethylene, the predominant material in plastic mulching films, in microplastic form at various size intervals. The growth of five bacterial strains recognized for their growth-promoting capabilities was assessed in laboratory tests, with a parallel investigation carried out at the same concentration using a different polymer with biodegradable characteristics, considering that such alternative materials should also be examined to determine their potential environmental impact. The most resilient strain was tested further in soil with plastic particles to determine the impacts on its survival, biochemistry and PGPB capabilities. Auspicious results were obtained. This study compares traditional plastics and emerging biodegradable materials and discusses the potential of using bacterial inoculants in conjunction with plastic mulching agriculture, as a route of achieving sustainable food production and security.

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# Design of a synthetic community for the bioremediation of hydrocarbon polluted soil

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## Abstract

Petroleum-based products are a natural resource used mainly as a source of energy. Accidental releases of these products can cause environmental problems such as soil contamination [1]. Therefore, degradation and removing the petroleum pollutants is an important goal. Bioremediation of Total Petroleum Hydrocarbons (TPHs) can be performed through techniques based in bioaugmentation and phytoremediation. TPH are a complex mixture of hydrocarbons and their biodegradation requires inoculation with mixtures of microorganisms. Here we describe the design and testing of a synthetic bacterial community (SynCom) for the recovery of TPHs polluted soils. The SynCom was developed through the isolation of bacteria from an enrichment culture, obtained by growing bacteria present in hydrocarbon polluted soil, using diesel as the sole carbon and energy source. Culturomics were used to isolate bacteria from this consortium and isolated bacteria were identified through 16S RNA gene sequencing. Based in principles of functional redundancy and phylogenetic diversity, six strains were isolated and identified as *Pseudomonas* (2 strains), *Achromobacter*, *Delftia*, *Novosphingobium* and *Rhodococcus*. Metagenomic analysis of the constructed SynCom allowed us to assemble the genomes (MAGs) of the six strains with a completion from 85 to 100 %. Functional assignment of the metagenome showed the presence of genes encoding enzymes implicated in aliphatic and (poly)aromatic hydrocarbons degradation. However, some pathways were absent in the SynCom and we decided to include the previously isolated *Rhodococcus sp.* WAY2 [2]. We also tested the stability of the SynCom trough serial cultivation, with its composition persistent over time. We are currently testing this SynCom for the bioremediation of TPH polluted soils at the microcosm and field scale.

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## Phytoremediation of copper and zinc-contaminated soils through the combined use of *Medicago* spp and associated bacteria

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### Abstract

Soil pollution by heavy metals, such as copper and zinc, is an environmental problem growing exponentially mainly due to anthropogenic causes. The use of hyperaccumulator plants for these metals, such as some species of *Medicago*, is presented as an effective strategy in their decontamination. Furthermore, their combined use with plant growth-promoting bacteria (PGPBs) can increase their bioavailability for plants, as well as improve their tolerance to metal toxicity and promote plant growth. This study focuses on the ability of several *Medicago* species to accumulate copper and zinc in their tissues, facilitating their extraction from the soil. For this purpose, three species of *Medicago* (*M. sativa*, *M. polymorpha*, and *M. truncatula*) have been used, and an assay has been conducted to determine their tolerance to these metals. Subsequently, four bacteria, previously isolated from the nodules of these plant species, have been characterized based on their growth-promoting properties (production of auxins and siderophores, and phosphate solubilization) in the presence and absence of copper and zinc. Finally, the bacteria's ability to promote plant growth in the presence/absence of these metals has been studied. The data obtained establish *M. sativa* as the most sensitive species to heavy metals and show all species are more sensitive to zinc than to copper. Regarding the effect of bacteria, identified by 16S rDNA gene sequencing as *Pseudomonas atacamensis* and *Bacillus altitudinis*, it is observed that strain P13 (*B. altitudinis*) is the strain that, in most cases, improves plant tolerance to metals. However, *M. truncatula* species is the one that better responds to the different PGPBs.

### Funding

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## Phosphate solubilising bacteria and their agronomic application

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### Abstract

Phosphorus (P) is an essential macronutrient for plants, being involved in major functions. This nutrient often limits crop growth and productivity, and high yields are dependent on strong chemical fertilization [1]. A substantial part of the added phosphate may, depending on the characteristics of the soil, become unavailable to plants [1,2]. Therefore, solutions such as phosphate solubilising bacteria (PSB), capable of readily solubilise P and make it available to plants, will be a great advantage, since crop growth may be promoted with lower production costs, while contributing to a more sustainable agriculture [3]. This study aimed to test the effect of PSB inoculation on the growth of maize plants in two soils with different pH (7 and 8). For that, bacteria from the laboratory repository were screened for their phosphate solubilising capacity. Maize seeds were inoculated with the nine strains evidencing the best solubilising capacity and the effect on plant growth, biochemistry and physiology were determined. Results evidenced that PSB inoculation increased the levels of P in plant shoots (over 50% at pH 7 and 20% at pH 8) and other macronutrient levels (Ca, N, K, Mg, Fe and Zn). Bacterial strains increased plant growth (around 10% in length and until 50% in weight) at both pH. The germination time was decreased by some PSB strains at both pH. PSB strains also increased the chlorophyll content and some strains decreased membrane damage and induced antioxidant mechanisms, namely catalase activity. These results thus demonstrate the ability of bacteria to increase phosphate nutrition in plants and confirm PSB inoculation as an effective and sustainable methodology for increasing crop growth and productivity, relieving crop production from the dependence on inorganic phosphate fertilisation.

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## N-SAFE a patent pending technology to reduce N losses by leaching

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### Abstract

Nitrogen loss through leaching is a major problem in agriculture. Nitrate is not retained by soil colloids, it is highly mobile and prone to leaching, reaching groundwater and surface water and causing the problem of water pollution. Ammonium ions, on the other hand, are adsorbed by soil colloids, thus avoiding leaching. [1]. Therefore, a major strategy to reduce nitrate leaching is to use chemical inhibitors of nitrification to reduce the rate of conversion of ammonium ions to nitrate [2]. However, there are some concerns about the environmental impact of chemical nitrification inhibitors. N-SAFE is a composition of micro-organisms and fertilisers containing them with the aim of reducing nitrate leaching from agricultural soils. The microorganisms contained in N-SAFE are Gram-positive that simultaneously meet the following conditions: i) they are non-denitrifying strains, which is a key feature to avoid N loss as N<sub>2</sub> or NO<sub>x</sub>; ii) they reduce nitrates to ammonium ions by the Assimilatory Nitrate Reduction to Ammonia (ANRA) pathway; iii) they exhibit PGPR properties on crops. The metabolic pathway exhibited by the strains selected for N-SAFE is ANRA, rather than the dissimilatory pathway (DNRA) [3], which requires low oxygen concentrations. N-SAFE strains do not carry the marker gene for DNRA (*nrfA*) [3], but the genes for ANRA. Under in vitro conditions, the strains contained in N-SAFE reduced more than 10% of the nitrate present in the growth media to ammonium in 168 hours. In leaching column tests, ammonium nitrosulphate (ANS) fertiliser with N-SAFE technology reduced nitrate leaching by between 33% and 45% compared to the control with regular ANS, which is similar to the reduction achieved with chemical inhibitors of nitrification. In field trials, maize crops fertilised with ANS with N-SAFE technology increased yield by up to 6% compared with regular ANS and, in particular, improved the agronomic efficiency of nitrogen between 79% and 100%, similar to chemical nitrification inhibitors.

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## PGPR in the endless battle against *Botrytis cinerea*

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### Abstract

*Botrytis cinerea*, the causative agent of gray mold disease in plants, stands out as one of the most significant phytopathogens due to its ability to infect approximately 200 plant species, resulting in substantial global economic losses [1, 2]. Certain Plant Growth-Promoting Rhizobacteria (PGPR) exhibit the capacity to trigger Induced Systemic Resistance (ISR) in plants prior to pathogen invasion, thereby enhancing plant defense mechanisms [3]. By harnessing ISR-inducing PGPR, it becomes possible to achieve biocontrol of *B. cinerea* while simultaneously promoting plant growth, thereby reducing reliance on chemical fertilizers and antifungals.

The efficacy of three PGPR strains—*Peribacillus* (PG\_BC\_02), *Pseudomonas* (PG\_BC\_03), and *Azotobacter* (PG\_BC\_04)—in biocontrolling *B. cinerea* in tomato plants through the activation of ISR-associated genes and improving plant growth was evaluated. For this purpose, tomato plants were inoculated with the different strains at the root and subsequently infected with *B. cinerea* on the leaves. Control groups consisted of plants without inoculation and/or infection. The analyzed variables included aerial and root biomass produced by the plant, the extent of leaf area affected by infection, levels of malondialdehyde (MDA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as markers of oxidative stress, and expression of genes associated with ISR activation.

The strain PG\_BC\_02 demonstrated the highest capacity to reduce lesions caused by *B. cinerea*, despite generating the smallest increase in biomass. On the other hand, strain PG\_BC\_03 also showed effectiveness in lesion reduction, although to a lesser extent than PG\_BC\_02, in exchange for a superior increase in biomass production. Lastly, strain PG\_BC\_04 promoted an increase in biomass but failed to mitigate the damage caused by the phytopathogenic fungus. Interestingly, the greatest biomass increments, compared to the uninoculated control, were observed in treatments infected with the fungus, highlighting the potential benefit of PGPR under stress conditions. Gene expression analysis indicated that strains PG\_BC\_02 and PG\_BC\_03 primarily employ ISR activation for their biocontrol action.

These findings contribute to expanding the pool of available strains for eco-sustainable biocontrol of *B. cinerea*.

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## Effect on pea growth of spore-forming bacilli isolated from pea nodules in consortia with *Rhizobium*

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### Abstract

Pea (*Pisum sativum* L.) is one of the most important legumes for human feeding in Europe considered as essential for an optimal transition to sustainable agri-food systems and diets [1]. Like other legumes, *P. sativum* establishes symbiosis with fast-growing rhizobia. These strains are accompanied in pea nodules by spore-forming bacilli which were identified through 16S rRNA gene analysis as belonging to the genera *Bacillus*, *Metabacillus*, *Oceanobacillus*, *Paenibacillus*, *Peribacillus*, *Priestia*, *Psychrobacillus*, *Terribacillus*. Several strains of these genera, some of them recently separated from genus *Bacillus* [2,3], present different *in vitro* mechanisms involved in plant growth promotion. Three of these strains isolated from pea nodules and presenting those mechanisms (phosphate solubilization, siderophore and indole acetic acid production), were inoculated on pea in hydroponic and microcosms conditions, alone and in combination with a *Rhizobium laguerreae* strain, highly effective on this legume. The hydroponic assays were performed using sterile vermiculite as substrate and watered with N-free nutritive solution, being measured the shoot dry weight and N percentage four weeks post-inoculation. The obtained results showed significant increases in these parameters when two of the three inoculated strains, those identified as *Peribacillus simplex* (formerly *Bacillus simplex*) and *Paenibacillus tundrae*, were inoculated in combination with the *Rhizobium* strain. We performed greenhouse microcosms experiments with these combinations obtaining significant increases in the shoot dry weight after six weeks of the inoculation comparing with the uninoculated control. These results showed that biofertilization with combinations of selected strains of rhizobia and nodule endophytic spore-forming bacilli can be a reliable eco-friendly agronomic practice.

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## Chromium tolerance in white lupin (*Lupinus albus*) and its microsymbiont *Bradyrhizobium canariense*

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### Abstract

Chromium (Cr), the seventh most abundant element in the Earth's crust, is widely used for many industrial processes, leading to a widespread contamination of agricultural soils with this highly phytotoxic element. *Lupinus albus* (white lupin) is a singular legume that can fix atmospheric N<sub>2</sub> by establishing symbiosis with rhizobia but can also develop cluster roots. These are bottlebrush-like clusters of rootlets associated with intense mobilization of P and other nutrients by inducing chemical changes in the rhizosphere [1]. They also have an important role in the immobilization of heavy metals [2]. The aim of this project is to identify Cr-tolerant ecotypes of *L. albus* and strains of its microsymbiont *Bradyrhizobium canariense* to be used as a phytoremediation tool in Cr-contaminated soils. A screening for Cr tolerance of 336 ecotypes of *L. albus* has been completed. Plants were watered with nutrient solution with or without Cr and fresh weight of the aerial part and leaf area were the parameters used to identify tolerant ecotypes. The capacity to accumulate Cr in the roots and shoots was analyzed in the most tolerant ecotypes, and bioaccumulation and translocation factors were calculated as a measure of their phytoremediation potential. Selected *B. canariense* strains were screened for their tolerance to Cr in solid and liquid cultures. In addition, the role of Cr in the appearance of cluster roots and their Cr immobilization capacity is being studied. In the next stage, the most tolerant plants will be inoculated with the most tolerant rhizobial strains to test the phytoremediation capacity of the resulting consortia.

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### Acknowledgements

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# Inoculation of white lupin with selected nodule-endophytic PGPR results on beneficial effects that compare to those of inoculation with symbiotic bradyrhizobia

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## Abstract

Legume nodules are often colonized by non-symbiotic bacteria. They accompany nodulating rhizobia and can have beneficial effects, as some of them display plant growth-promoting (PGP) properties that increase seed germination and plant growth, and enhance tolerance to biotic and abiotic stress. White lupin (*Lupinus albus*) is a legume crop that is gaining relevance as a plant protein source, and is considered as a suitable alternative to soybean [1]. Additionally, white lupin in association with its microsymbiont *Bradyrhizobium canariense* has been proposed as a heavy-metal phytoremediation tool [2]. Eleven nodule-endophytic bacteria were isolated from white lupin nodules. They belonged to the genera *Rhizobium*, *Ensifer*, *Pseudomonas* and *Bacillus*. Their PGP and enzymatic activities were tested *in vitro*. *Pseudomonas* sp. strains L1 and L12, displayed most PGP activities tested, and were selected for *in planta* assays. Inoculation with strains L1 or L12 increased seed germination, and provided the same positive effects on all plant growth parameters as did inoculation with symbiotic *B. canariense*, with no significant differences among treatments. In soils, inoculation with efficient nitrogen-fixing rhizobia must compete with rhizobia present in the soil and adapted to the edaphoclimatic conditions, which sometimes nodulate efficiently, but fix nitrogen poorly, leading to a low response to inoculation. In such cases, inoculation with highly effective PGPR might represent a feasible advantageous alternative to boost crop productivity.

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## Funding

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## Selected PGPR increase *Camelina sativa* growth by activating CO<sub>2</sub> fixation and transpiration and anticipate flowering

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### Abstract

*Camelina sativa* (Brassicaceae) is an oleaginose extremely resistant to drought with a good adaptation to low temperatures, making it suitable for semiarid regions. With a similar agronomic management to traditional cereals, good yields, allelopathic activity controlling weeds and resistance to pathogens, its cultivation surface is increasing. In addition to its use for animal foods, its used for sustainable fuel production [1]. Therefore, increasing plant yield is relevant for which purpose, plant growth promoting rhizobacteria (PGPR) from different origins are evaluated in the present work, in a greenhouse trial.

Among the 10 strains tested, all increased net carbon fixation and transpiration, resulting in significant increases in dry weight and anticipated flowering. As a general trend, photosynthetic pigments decreased suggesting a decrease in photooxidative stress and a better use of the absorbed energy as mechanism of action [2]; proline concentration was also increased speaking of antioxidant and drought protection mechanism. Strains *Pseudomonas kilonensis* Z10.21 and *Brevibacterium frigotolerans* TE59 achieved outstanding results and are candidates for future field trials.

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Camelina Company for providing *Camelina sativa* seeds.



## Effect of beneficial microorganisms on clubroot disease in brassicas

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### Abstract

The clubroot disease is caused by the obligate parasite *Plasmodiophora brassicae*. This pathogenic protist, transmitted through soil, poses a significant challenge to Brassica crops worldwide, causing deformation of the host root system and resulting in major yield and quality losses [1]. The best strategy to mitigate the economic impacts of the disease still consists of cultivating in areas where the pathogen is not present, using pathogen-free plants and cultivation substrates [2]. Strategies that encourage improvements in soil structure, texture, nutrition, and moisture content, when integrated with crop rotations and management, may reduce populations. However, they will not eliminate the disease [3]. The objective of this study was to test the effects of inoculating three bacteria, selected on the basis of previous studies, on the outcome of infection and disease severity in broccoli (*Brassica oleracea*). Two plant growth-promoting *Pseudomonas* sp. isolated from *Lolium multiflorum* and one *Lysobacter* sp. isolated from root nodules of *Lotus parviflorus* were chosen for this test. Methodologies included greenhouse bioassays with *B. oleracea* plants growing in pots containing soil from a farm in the West region of Portugal, with a history of brassica cultivation and where the soil is infected with the pathogen. The evaluated parameters, through a disease severity scale, number of leaves, and fresh and dry weights of the plants, showed positive results with the *Lysobacter* sp. and one of the *Pseudomonas* sp., compared to the non-inoculated control. Following these results, other tests are being carried out to continue evaluating the potential of these bacteria for the biocontrol of this disease.

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### Funding

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# The potential of legume seed biofertilizer to optimize symbiotic nitrogen fixation under climate change

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## Abstract

Inoculation of legume seeds included in biodiverse mixtures is an efficient strategy for introducing nitrogen-fixing bacteria (rhizobia) into the soil and rhizosphere of legumes. Selecting the best combination of nitrogen-fixing bacteria and host legume is essential to achieve more effective rhizobia-legume symbioses. Innovative inoculants can contain rhizobia strains selected for their ability to respond to biotic and abiotic stresses, reducing the negative impacts of these factors in agro-silvo-pastoral systems. In this work, we studied autochthonous nitrogen fixing bacteria isolated from root nodules of annual clovers grown in the south of Portugal, according to their effectiveness and antagonism against phytopathogenic oomycetes. We also evaluated the viability over time of the bacterial inoculant on the seed surface. Plant assays were performed under controlled environmental conditions to evaluate the symbiotic efficiency of rhizobia strains (individually and in consortium) with different *Trifolium* spp.. Inoculation experiments with annual clovers yielded the selection of five *Rhizobium* strains highly effective in nitrogen fixation. Since the *Rhizobium* strains did not show antagonistic activity against *Phytophthora cinnamomi* and *Phytophthora vexans*, a *Lysobacter* sp. strain, previously isolated from root nodules of *Lotus* spp. [1], was included in the consortium. The symbiotic effectiveness of this consortium was very high (> 75%). Assays with clover seeds inoculated with liquid cultures of *Rhizobium* containing Arabic gum as an additive (to improve stickiness and adherence to seeds) showed a decrease in the cell viability on the seed surface, from 10<sup>6</sup> to 10<sup>4</sup>, per gram of seed, at the end of 18 weeks. However, the symbiotic effectiveness remained high (65%). This consortium was used to inoculate seeds of annual clovers in a field assay. All the *Rhizobium* strains were recovered from the plant root nodules, showing good performance in the nodulation process. Our results form an important step in the implementation of more profitable and sustainable legume-rich biodiverse pastures using biofertilizers.

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# Promoting soil vitality: impact of cover crops on soil enzymatic activity in intensive agricultural systems

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## Abstract

In the context of soil health, cover crops have emerged as pivotal components of sustainable agricultural systems [1]. Beyond their well-known benefits in enhancing soil structure, soil organic matter, and weed suppression, the intricate interplay between cover crops and soil enzymatic activities warrants deeper exploration. Soil enzymes serve as vital indicators of soil health, reflecting microbial activity and organic matter turnover. Key enzymes such as dehydrogenase, beta-glucosidase, phosphatase, and arylsulfatase are essential to nutrient cycling and soil fertility [2]. For instance, dehydrogenase activity, quantified via spectrophotometry, provides insights into microbial respiration rates, while beta-glucosidase, phosphatase, and arylsulfatase activities indicate the potential for organic matter degradation and nutrient availability. This study aimed to evaluate the impact of cover crops on soil enzymatic activities, to assess their potential to improve the soil status in intensive horticultural systems. A field trial was installed at São João de Brito farm (Ribatejo, Portugal), testing the intercropping of maize (main crop) with two cover crop mixtures: Consociation 1 (legume-grass-brassica mix), and Consociation 2 (legume-grass mix). A plot without cover crops (spontaneous plants) was used as control. Triplicate soil samples were collected in each plot for two consecutive years, and analysed for dehydrogenase, beta-glucosidase, phosphatase, and arylsulfatase enzymatic activities using spectrophotometric assays. Consistently, the two cover crop mixtures exhibited higher enzymatic activities than the control in both sampling dates. Furthermore, Consociation 1 displayed enhanced activity across all enzymes compared to Consociation 2. The total enzyme activity index (TEi) [3]), integrating the activities of all evaluated enzymes, confirmed this response pattern. These results indicate that the cover crops mixtures, particularly Consociation 1, significantly enhanced soil microbiological activity and capacity for nutrient turnover, pointing to the improvement of the soil status. These findings underscore the importance of cover crop mixtures in promoting sustainable soil health in intensive agricultural systems.

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## Response of soil microorganisms to the introduction of biodiverse cover crop mixtures in intensive maize cultivation

Varela, A.<sup>1\*</sup>, Pereira, P.<sup>1</sup>, Soares, R.<sup>1</sup>, Barradas, A.<sup>2</sup>, Nunes, A.P.<sup>3</sup>, Godinho, M.C.<sup>4</sup>, Videira e Castro, I.<sup>1,5</sup>, Fareleira, P.<sup>1,5</sup>

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### Abstract

In recent decades, intensified farming has led to excessive use of synthetic fertilizers and pesticides, disrupting the soil natural balances and reducing microbial biodiversity and functionalities. The introduction of cover crops may provide a range of environmental benefits, mitigating the negative effects of intensive cultivation practices and contributing to improve the soil status [1]. This study addressed the particular case of a field trial conducted at São João de Brito farm in the Portuguese region of Ribatejo, where biodiverse cover crop mixtures were intercropped with maize and evaluated for their effects on soil microorganisms. Two plots were cultivated with cover crop mixtures (Consociation 1 and Consociation 2) during the fall-winter season, preceding the maize crop. Both mixtures were composed of legumes and grasses, including clovers inoculated with rhizobia; Consociation 1 additionally contained brassicas. A plot without cover crops was used as control. Samples for evaluation of soil microorganisms were collected at the end of the cultural cycle of cover crops (soil samples) or maize (root samples). Both Consociation 1 and Consociation 2 induced small but significant increases in the geometric mean (GMea index; [2]) of culturable bacteria and fungi abundances in the soil. The Shannon diversity index of fungal genera was also significantly higher with cover crops, as well as the abundance of phosphate-solubilizing bacteria. The evaluation of soil rhizobia revealed very low native populations in the control plot and Consociation 1, but a notable increase in Consociation 2, indicating the advantage of this formulation in enriching the soil rhizobial population. Maize roots showed a significant increase in endomycorrhizal symbioses with Consociation 2, accompanied by a higher number of endomycorrhizal spores in the soil. These findings underscore the potential of biodiverse cover crop mixtures to positively influence soil microbial communities and plant-beneficial microorganisms.

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## A bacterial consortium for inoculation of annual ryegrass in pasture systems

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### Abstract

Annual ryegrass (*Lolium multiflorum* Lam.) is a pasture and forage crop widely present in biodiverse pasture mixtures cultivated in agro-silvo-pastoral systems (“montado” or “dehesa”) in the southern Iberian Peninsula. Previously, a set of five plant growth-promoting bacteria from the genera *Pseudomonas*, *Paenibacillus*, and *Rhizobium*, isolated from the rhizosphere and endosphere of annual ryegrass in “montado” soils, was characterized with a view to establish an inoculant consortium for annual ryegrass [1]. The present work evaluated this consortium regarding the effects on plants, inoculum delivery methods and performance in field conditions.

Inoculation of *L. multiflorum* seedlings in a synthetic medium confirmed the stimulatory effect of the bacterial consortium on plants biomass, resulting in average increases of 30% and 18% in root and shoot dry weights, respectively, after 6-7 weeks of incubation in a plant growth chamber. At the initial stage of seedling development, the consortium increased root branching, as well as the size and density of root hairs. Since seed coating is the most common method of delivering inoculants into biodiverse pasture mixtures, the viability of the bacteria on the seed surface post-inoculation was monitored over time, with various additives tested as protectants. *Paenibacillus* sp. showed consistent viability regardless of additives, while *Pseudomonas* sp. displayed marked sensitivity and decreased viability over time. The addition of 10% arabic gum best preserved the viability of *Pseudomonas* sp., while maintaining seed germination capacity and the plant growth-promoting effects of the inoculated bacteria. Including annual ryegrass seeds inoculated with this formulation in a biodiverse pasture mixture tested in the field resulted in average increases of around 20% in grass production. An increase in the abundance of phosphate-solubilizing bacteria in the soil was also observed. These results highlight the potential of this bacterial consortium for inoculating annual ryegrass, offering insights into improving sustainable agricultural practices.

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## Isolation of yeasts from horticultural soils in Portugal and screening for plant growth promoting traits

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### Abstract

Soil microorganisms are key players in soil biochemical and biological processes, essential for soil health and sustainable agricultural production. Plant growth-promoting microorganisms (PGPM) can stimulate plant growth through different mechanisms, such as the production of plant hormones or increasing nutrient availability. Additionally, some microorganisms protect plants from diseases by antagonizing plant pathogens. In intensive horticulture systems, the pressure for high production yields often leads to excessive application of fertilizers and phytosanitary products. In these systems, the use of PGPM-based microbial inoculants could contribute to more sustainable management practices. This study addressed a relatively unexplored group of soil microorganisms: yeasts. Thirteen yeast strains were isolated from several horticultural soils in Portugal and evaluated for their potential as plant growth promoters. The yeast isolates were taxonomically identified using the Biolog YT Microplate metabolic profiling. The production of indole acetic acid (IAA) was evaluated using a modification of the colorimetric method described by Pandi et al. (2019) [1]. Phosphate solubilization activity was assessed by the formation of translucent haloes around colonies on solid medium containing insoluble tricalcium phosphate [2]. The antagonistic activities of the yeasts were evaluated in plate confrontation assays [3] against various phytopathogenic microorganisms, including *Phytophthora cinnamomi*, *Diplodia sapinea*, *Sydowia polyspora*, *Pestalotiopsis pini*, *Macrophomina phaseolina* and *Aspergillus niger*. The results showed that isolates identified as *Candida tropicalis* and *Saccharomyces cerevisiae* produced significant amounts of IAA from tryptophan. *C. tropicalis* and *Pichia missouriensis* exhibited phosphate solubilization activity. Additionally, *P. missouriensis* and *C. tropicalis* demonstrated high antagonistic activity against *S. polyspora* and *M. phaseolina*. Due to the potential of the isolates to be used as PGPM, other plant growth-promoting traits will be tested. Based on these promising results, the potential applications of these yeast strains to increase the sustainability of intensive horticultural systems warrant further investigation.

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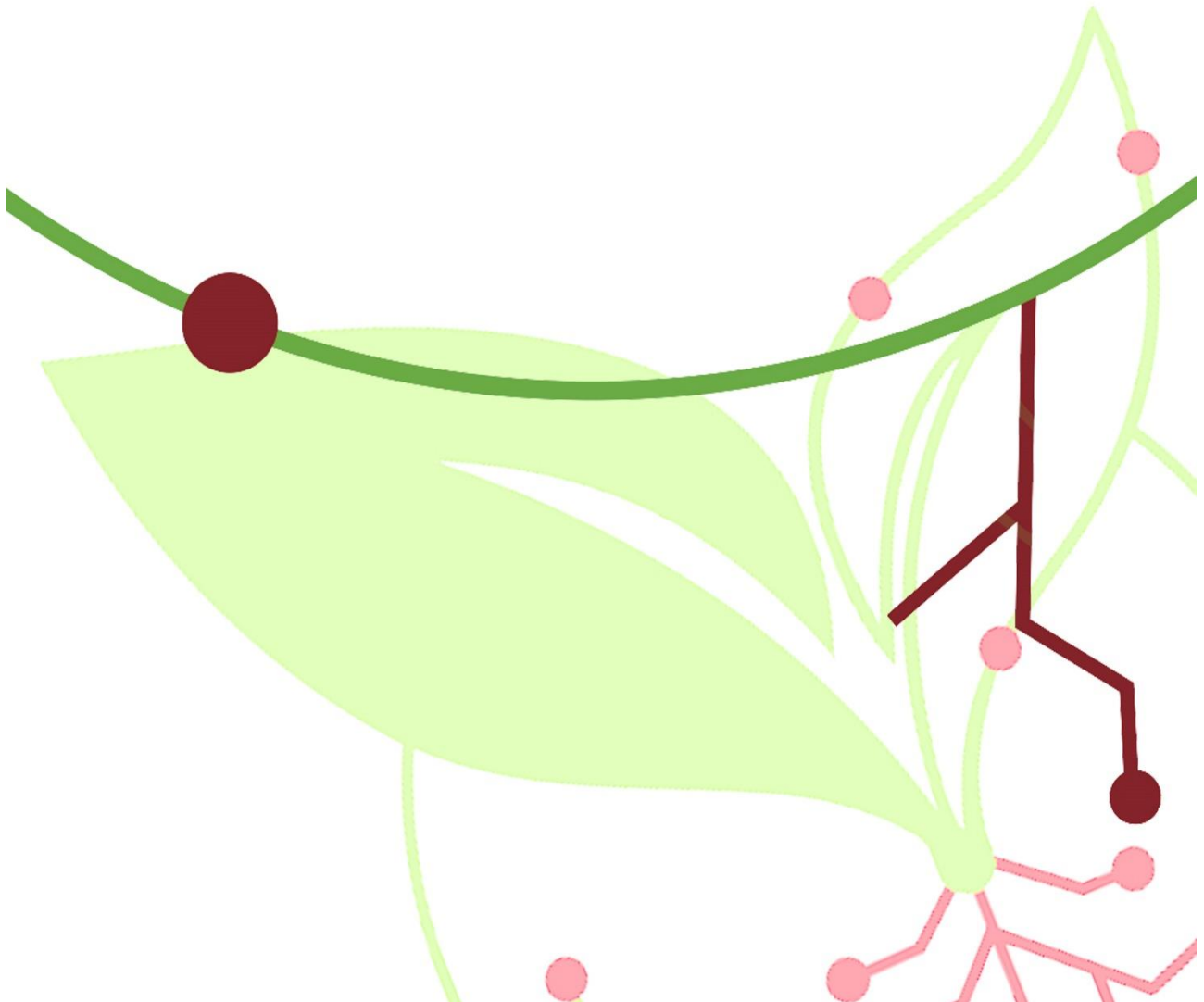
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## Session 3

# Climate change, biotic and abiotic stress in plant-microorganism interactions





## Domestication caused a “*Lost in translation*” problem for microbiota beneficial interaction: From root exudates to inheritance

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### Abstract

Domestication processes have led to increased crop sizes and production. Breeding processes have prioritized certain traits over others in the pursuit of higher production, more attractive colors or flavors, or better shapes. However, these varieties have become less resistant to environmental stressors [1]. In addition to genetic background, recent research has linked these phenomena to the weakening or loss of signaling pathways with beneficial microbiota that improve the plant's response to stress and provide better growth conditions [2]. These processes may also affect specific recruitment and transmission to subsequent generations, reducing pre-adaptation to the environment [3]. Through our studies with seeds, we have identified unique strains in cultivable microbiota of ancestors, landraces or wild relatives, absent or underrepresented in commercial varieties, that can improve crop responses to various stressors. Moreover, the analysis of signaling mechanisms has revealed differences linked to the presence or absence of different beneficial populations. With this, we have begun to develop new biotreatments based on the needs of current crops that can be reconstituted from better-adapted populations. Here we have some examples showing improvements in the productivity of corn, lentils, and beans under drought conditions through the use of a microbiota reconstitution strategy. This new avenue of research could help to fill gaps in beneficial microbial interactions in current agricultural systems and improve the biotreatments by a more precise design of the required inoculants.

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## Social Biofertilizers: Rapid social consortia selection

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### Abstract

Commercial biofertilizers have used single PGPMs, and although consortia based biofertilizers increase functional redundancy and stability, increasing species diversity increases microbial interaction complexity. Microbial interactions are ruled by high order interactions and require high number of tests for functional observations. The BSocial tool (<http://m4m.ugr.es/BSocial.html>)[1], which uses growth data of combinatorial populations, has been used to predict social behaviour in bioremediation with highest functional outcomes from a defined “social consortia” (species with positive/neutral behaviour). Biodegradation and growth are correlated when the substrate is the C-source [1,2], however, plant growth promoting traits (PGPT) produce enzymes with varying energetic needs and may hinder growth. Thus, we set out to decipher whether BSocial could predict a highly functional PGPM consortia starting from 8 PGPMs. PGPTs (siderophore production, phosphate solubilization, and IAA production) were tested for all 255 populations, resulting in three social consortia (X22, X93 and X149), of which X22 was above medium all functions tested [3]. Hence, BSocial analysis can rapidly (<5 days), cheaply and effectively narrow down PGPM consortia selection. Functional outcomes for phosphate solubilization and siderophore production were highly correlated ( $R=0.97$  and  $0.91$  respectively) with an increase in population richness. Overall functional stability was positively correlated with population richness, except for siderophore production. The latter possibly due to a diminished advantage for the siderophore producing species from high-energy requirements for siderophore production, and the lower chances of retrieving the Fe- siderophore complex in species-rich populations. Thus, functional redundancy outcompetes stability, since siderophore production increased with population richness. Further tests include PGPMs isolated from bottom-up approaches from cherry tomato or avocado rhizosphere, predicting 7-8 species-rich social consortia. Overall, the next generation of biofertilizers need to be specific to plant species, include both PGPMs and mycorrhiza [2], climate change resilient, and create social consortia which are stable and scalable [4] using the BSocial tool.

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## Bacterial volatiles as biofungicides. A new tool against phytopathogenic fungus *Botrytis cinerea*

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### Abstract

Volatile compounds are small, odorous compounds of low molecular mass. The antifungal role of these compounds has gained importance since they are effective in low concentrations, they diffuse through air-filled pores in soil, and they can act on pathogens without establishing actual physical contact with them [1]. A set of 70 bacteria was isolated from the inside of blackberries (*Rubus ulmifolius* Schott) and four strains were selected based on antifungal activity *in vitro* against phytopathogenic fungus *Botrytis cinerea*. The aim of this work is to evaluate the antifungal potential of the bacterial volatiles in tomato (*Solanum lycopersicum*) model plants.

Tomato seeds were pre-germinated and infected with phytopathogenic fungus while bacteria were grown in another plate, avoiding contact, to evaluate the antifungal potential. Volatile composition was evaluated by using head-space-solid phase microextraction technique. Simultaneously, genomic DNA was extracted, sequenced by Illumina technique, and analysed using KofamKOALA, dbCAN and antiSMASH tools. Finally, expression of five reporter genes related to activation of induced systemic resistance (ISR) and systemic acquired resistance (SAR) was evaluated using qPCR.

As results, the three bacteria reduced mortality in 67.5 – 90%. Volatile evaluation showed the production of volatile several known antifungal molecules such as acetoin, 2-Heptanone, 1-Butanol and, 2,4-Di-tert-butylphenol. Besides, genomic analysis has annotated genes involved in the biosynthesis of acetoin such as acetolactate synthase and alpha-acetolactate decarboxylase; butanol biosynthesis such as 3-hydroxybutyryl-CoA dehydrogenase; and terpene biosynthesis such as heptaprenyl diphosphate synthase.

Finally, the reported genes *pr1a*, *pi-1*, *pti4*, *etr4*, and *pal* for ISR and SAR induction in plants showed a decreased expression when plants were exposed to bacterial volatiles.

In conclusion, the volatile of three evaluated strains seems to have direct effect on *B. cinerea* development, proving that it may be a suitable option for the development of new biopesticides against this phytopathogen.

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# Impact of Arbuscular Mycorrhizal Fungi (AMF) on vegetative growth, yield, and fruit quality of tomato (cv. Moneymaker) growing under salinity

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## Abstract

Salinity is one of the most important climatic aspects affecting plant development, growth, and production [1]. Tomatoes can withstand moderate salinity, but excessive salinity impacts plant growth, seed germination, and fruit development [2]. Arbuscular Mycorrhizal Fungi (AMF) can increase mineral nutrition, photosynthetic activity, water status, and plant biomass under salinity [3]. This study wanted to examine in depth the influence of AMF on tomato plants grown under salt stress. *Solanum lycopersicum* L. (cv Moneymaker) plants were grown in 13L pots containing a 2:1 peat:sand mixture substrate, in a greenhouse at 26/15 °C day/night with a 14-h photoperiod. Half of the plants were inoculated (5 g per plant) with a mixture containing 100 spores per g of five mycorrhizal fungi: *Septoglomus deserticola*, *Funneliformis mosseae*, *Rhizophagus irregularis*, *Claroideogloium etunicatum* and *C. claroideum* (+M plants). Half of the plants remained uninoculated (-M plants). After mycorrhizal association was established in +M plants, both +M and -M tomatoes were subjected to either control (EC = 0 mScm<sup>-1</sup>), low salinity (EC = 60 mScm<sup>-1</sup>), or high salinity (EC = 120 mScm<sup>-1</sup>) conditions. At harvest, plants were separated into roots, leaves, stems, and fruits, and their fresh (FW) and dry weight (DW) were calculated. The water content in organs, yield, and fruit quality were also recorded. Results showed that low salinity negatively affected vegetative growth and yield in both +M and -M tomato plants, with the biomass of -M plants being one of the most affected parameters. In contrast, AMF counteracted the deleterious effect of high salinity on the water content in leaves and roots as well as on fruit yield. The ratio sugars to acids decreased in fruits only under moderate salinity regardless AMF inoculation. Salinity reduced lycopene levels in fruits of -M plants. In contrast, high salinity increased lycopene concentration in tomatoes of +M plants.

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# Post-fire temporal dynamics of soil properties and fungal communities in *Pinus pinaster* Ait. forests

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## Abstract

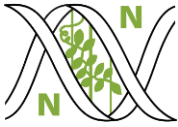
The rise in temperature and prolonged droughts make the Mediterranean region highly vulnerable to environmental disturbances, and fire is a primary agent of change [1]. Fungi play a pivotal role in the restoration of forest ecosystems under fire-induced stress, contributing to nutrient cycling, mediating plant responses and enhancing soil structure [2]. However, the post-fire dynamics of soil fungal communities and the key environmental factors influencing their recovery remain poorly understood. This study aimed to assess the impact of fire on soil fungal communities and physical-chemical properties, tracking their changes over time. Three independent fire events happened in *Pinus pinaster* Ait. forests across three locations in Segovia (Spain), in 2014. To assess the short- and mid-term effects of fire, soil samples were collected from both organic and mineral soil layers in burned and adjacent unburned areas in each site, one and nine years after fire. Soil properties were determined, the DNA extracted and the fungal ITS1 region sequenced by Illumina MiSeq. Data were analysed using linear mixed models, with fire and time as fixed factors and the site as a random factor. Results revealed that fire exerted varying effects on soil properties. Soil basification and a slight reduction in organic matter content were observed, both persisting throughout time. Additionally, significant variations in the concentration of bioavailable nutrients were noted, with an initial increase in calcium and potassium levels one year after the fire, followed by subsequent stabilization. In the short term, fire negatively impacted fungal richness, though a recovery trend was observed over time, particularly in the soil organic layer. These results contribute to a more comprehensive understanding of the immediate and transitional consequences of fire on soils. Nevertheless, further research is required in order to develop effective management strategies for the recovery and conservation of Mediterranean forest ecosystems.

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# Cabernet Sauvignon benefits more than Tempranillo from mycorrhizal association to enhance its resilience to elevated CO<sub>2</sub> and high temperatures

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## Abstract

Predictions confirm that climate change will have a negative impact on viticulture, particularly on grapevine physiology, yield and fruit quality [1]. The dynamic interactions between the vine rootstock and the soil microbiome could enhance the resilience of grapevines to climate change [2]. Our objective was to evaluate to what extent the association of Tempranillo (T) and Cabernet Sauvignon (CS) (both grafted onto R110 rootstock) with arbuscular mycorrhizal fungi (AMF) may counteract the deleterious effects of high temperatures (TE) and elevated atmospheric CO<sub>2</sub> (CE), acting either together (CETE) or separately (CATE, CETA), on vegetative vigor, physiology, productivity and fruit quality. At one year of age and once in the following two years, T and CS were inoculated with a mixture of five AMF or kept uninoculated. Plants were grown in temperature gradient greenhouses, either at ambient CO<sub>2</sub> (CA) and air temperature (TA) (CATA) or at elevated CO<sub>2</sub> (700 ppm) combined or not with elevated air temperature (ambient temperature+4°C). Shoot dry matter production and rootstock thickening after one year were determined, as were gas exchange and leaf organic solutes two weeks after veraison, and leaf area, production and must quality at harvest. The results showed that AMF increased yield and leaf proteins under CATA conditions. Yield was also improved by AMF under elevated CO<sub>2</sub> or high temperature acting separately (CETA, CATE), but not when these factors acted together (CETE). The beneficial effect of AMF on yield was more pronounced in CS than in T. In addition, in CS, AMF favored the accumulation of anthocyanins and the maintenance of must acidity under CETE conditions. There were other benefits of AMF on CS under high temperature alone (CATE): shoot dry matter, rootstock thickening, leaf conductance, photosynthetic rates and leaf soluble sugar concentrations were higher in mycorrhized plants.

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# New biotreatments against saline rising on Ribatejo (Portugal) farmlands using Iberian Peninsula salt flats-isolated, halophile strains

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## Abstract

Saline soils are a major problem affecting several kinds of crops across the globe [1]. Underlying soil salinity affects 20% of irrigated land that produces one-third of the world's food [2] being the Mediterranean countries the most affected in the EU. Soil salinization happens through the accumulation of excess ions that will inhibit plant function and growth by disturbing the nutrients and water uptake by the plant [3]. Rising temperatures and altered precipitation patterns from climate changes intensify soil salinization, threatening agricultural productivity and food security [4]. In environments characterized by stress and custom biodiversity, there is a microbiome that has evolved to cope with osmotic stress and these microorganisms have the potential to assist plants to deal with it as well [5]. The development of a new bioformulation with the adapted microbiota could be a game-changer, enabling plants to thrive in these stressful conditions. This study aims to create a halophile bacteria-based bioformulation to enhance plant growth and salt tolerance in saline environments. Among the candidate strains selected for their plant growth-promoting and salt-stress-relieving traits, 80% of the candidates enhanced photosystem II quantum yield under stress in tomato plant (*Solanum lycopersicum*). The strains *Rosellomorea vietnamensis* 123, *Kushneria marisflavi* AA, *Idiomarina loihiensis* EA and *Virgibacillus salarius* GC enhanced general growth under such conditions. Besides *Idiomarina loihiensis* EA or *Bacillus swazeyi* 119 were able to increase the shoot length and root length in maize plant (*Zea mays*), respectively; meanwhile the strains *Halomonas sulfidaeris* DE, *Rosellomorea aquimaris* 104 and *Halobacillus faecis* 109 increased both parameters under stressing conditions. To improve the resolution, consortiums including different strategies can be proposed. These results show a promising potential to improve crop resilience and growth in saline environments leading to a higher agricultural productivity.

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## Context-dependent symbiosis between a halophytic grass and its fungal endophyte

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### Abstract

*Festuca rubra* subsp. *pruinosa* (= *Festuca pruinosa*) is a perennial grass found in rocky sea cliffs along the Atlantic coasts of Europe. In sea cliffs, plants grow in rock fissures where soil is absent, and the exposure to sea spray and marine winds is intense. Therefore, nutrient limitation and osmotic stress due to salinity and desiccation are characteristic stressors of this habitat.

*Festuca pruinosa* can form symbiotic associations with *Epichloë festucae*, an endophytic fungus that colonizes systemically the intercellular space of leaves and stems, and is vertically transmitted to seeds. In natural populations of *Festuca pruinosa*, about 70% of the plants are symbiotic with *Epichloë festucae* [1]. Symbioses of grasses with *Epichloë* endophytes are often defined as defensive mutualisms because host plants may acquire antiherbivore resistance through toxic alkaloids synthesized by *Epichloë* endophytes [2]. However, large herbivores are not present in sea cliffs, and the defensive mutualism hypothesis seems to be inadequate in this otherwise inhospitable habitat.

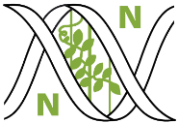
We present the results of an experiment whose main objective was to investigate whether the fungal symbiont could contribute to the habitat adaptation of the plants through improved salinity tolerance. For this purpose, symbiotic and non-symbiotic *Festuca pruinosa* plants were subjected to salinity (400 mM NaCl) and control (tap) watering treatments. The results show that the symbiosis is beneficial when plants are exposed to salinity, but not when salinity is absent. Harboring the fungus without salinity seems to be too costly to the plant. This fact can help to understand why the prevalence of *Epichloë festucae* is lower than 100% in most populations studied. In addition, information on the physiological plant response for adaptation to salinity was obtained, showing that it is a true halophyte, growing better under salinity than in its absence.

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## Multi-stress effects on PGPM Social Behaviour

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### Abstract

The application of microbial consortia in biotechnological areas, such as bioremediation or biofertilisation, has proven to be much more efficient than the use of individual microorganisms due to their greater stability and functional efficiency [1]. The main difficulty in the study of microbial consortia lies in the complexity of their interactions and the high testing capacity needed to explore population from the complete combinatorial of their individual. Nowadays, there are models/protocols that allow the study of the complexity that lies in microbial interactions of >2 species. One example is the BSocial tool (<http://m4m.ugr.es/BSocial.html>) which assigns social behaviour (positive, neutral or negative) to each strain based on its contribution to overall population growth (parameters: number of generations or growth rate) of all combinatorial populations, through which a “social consortium” is defined with positive and neutral assigned species [2]. In addition to the intrinsic complexity of these interactions, it is to be expected that those may be influenced by external factors leading to changes in population dynamics, and thus may influence social behaviour after stress. Biofertilizers require efficacy under vast and constant environmental fluctuations. In order to determine whether the social behaviour of a strain is susceptible to environmental changes, the social behaviour of 8 PGPMs (*Azospirillum*, *Bradyrhizobium*, *Ensifer*, *Pseudomonas spp.*, *Mesorhizobium*) were analysed against multi-stress conditions of temperature, salinity and drought. During standard conditions, two social consortia were established. After 27 stress conditions, we found that *Bradyrhizobium valentinum* LmjM3 and *Mesorhizobium olivaresii* CPS13 maintained their social status mainly under temperature and salinity stresses, and that *Pseudomonas* species, with initial negative social behaviours, maintained their behaviour after stress. Hence, with some exceptions, social behaviour remains unchanged immediately after stress conditions if environmental conditions are restored.

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# The olive holobiont: the role of plant-microbiome crosstalk in tolerance to verticillium wilt of olive

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## Abstract

Recently, some studies are focusing on assessing the relationship between a phenotypic trait of interest in a cultivable plant variety and its microbiome from a holistic point of view. In our case, in regard to verticillium wilt of olive (VWO), a disease caused by the soil-borne fungus *Verticillium dahliae*, we wanted to focus on the microbiome of the root system of this plant. In fact, in a recent study [1] we were able to confirm that olive trees with very different endophytic microbiota show differences in the expression levels of key genes in response to both abiotic and biotic stresses. Going a step further, thanks to the recent reclassification in the degree of tolerance/susceptibility of a large number of olive cultivars to VWO [2], it is of great interest to study the belowground microbial communities (bacteria and fungi) of olive trees with an extreme phenotype (extremely susceptible and very tolerant to VWO). By confronting these opposed profiles, it is possible to look for a relationship between the innate disease evasion abilities of a given genotype and the microbiota it recruits. In addition, we can deepen our understanding of plant-microbiome communication and highlight genes and microorganisms that may be the key to future disease management strategies.

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## Exploring the mechanism of microbiota inheritance and the interference of heat stress using tomato plant as model

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### Abstract

The interactions between plants and microorganisms causes a continuous adaptation, being very evident for pathogenesis processes or in the case of nitrogen fixation by *Rhizobium* in legume nodules. However, more subtle cases have gone unnoticed, as with the inherited microbiota. This mechanism is expected to play a crucial role in the pre-adaptation of the plant populations to stressed suffered by parental plants. This work aims to unveil some bases on the processes of microbiota inheritance, also including the interference of heat stress. For this study we used tomato plant (*Solanum lycopersicum*) variety Micro-Tom as a model [1] under controlled conditions of a growth chamber, facilitating the manipulation of different temperatures and the evaluation the effects on fertilization and reproduction, and using natural soil as main source of microorganisms. The conditions implemented basically banish the horizontal transference and reduce the microbial inheritance processes to vertical transmission. Here, we evaluated the bacterial populations of the flowers and next-generation seeds by metagenomics (16S rRNA metabarcoding) and culturomics, under regular and heat conditions [2]. These approaches showed that the predominance of the classes Bacilli, Clostridia and Erysipelotrichia (Firmicutes) under both conditions, detecting however lower populations and biodiversity under heat conditions. Among the species isolated in flowers and next-generation seeds, strains form the families *Bacillaceae* and *Rhodanobacteraceae* supposed promising models to study such phenomena in detail. Furthermore, we simultaneously characterized the metabolomic profile of tomato flowers in order to identify structural components and metabolites involved in the inheritance mechanism and their conditioners [3]. The evaluation of the models, comparative metagenomic-metabolomic datasets, and several generation treatments will give insights about inheritance mechanisms, eventually allowing the optimization of microbe transmission and plant resistance in microbiota-mediated breeding approaches.

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# Microbial strains from the Atacama Desert: a source of biocontrol agents for agriculture

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## Abstract

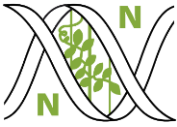
The Atacama Desert, one of the most arid and extreme places on the Earth, harbors a unique microbial biodiversity adapted to extreme environmental conditions [1]. Numerous endemic species are described to this region, yet the biotechnological prospects of their microbiome remain largely unexplored [2]. During this study, microbial diversity inhabiting the rhizosphere and internal tissues of *Astragalus paposanus*, an endemic plant of Chile's Atacama Desert, has been investigated, assessing their potential for secondary metabolite production. Employing a suite of culture-dependent, genomic, and metabolomic methodologies, the isolates were identified and characterized. Moreover, their capacity to synthesize secondary metabolites of biotechnological relevance was evaluated. Our findings unveiled a remarkable diversity of secondary metabolites produced by those isolates, especially compounds with potential antimicrobial and antifungal properties. Notably, several isolates demonstrated inhibitory effect against a spectrum of plant pathogens including *Aspergillus*, *Alternaria*, *Botrytis*, or *Fusarium* amongst others. Furthermore, our study identified ecological variations in the production of secondary metabolites among strains inhabiting distinct microhabitats. This research underscores the untapped biotechnological potential of the Atacama Desert's microbiota. The identified metabolites offer a reservoir of bioactive compounds for various biotechnological applications, particularly in agriculture, while the gifted strains identified serve as promising candidates for the development of biocontrol products.

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## Funding

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# ***Trichoderma* and arbuscular mycorrhizae induction of drought tolerance in wheat**

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## **Abstract**

Wheat is one of the most important crops worldwide, but its production is affected by biotic and abiotic stresses. Drought represents the main limiting stress for wheat development and production, and its intensity and severity are increasing due to the climate change. The application of beneficial microorganisms in agricultural systems has been proposed as a promising strategy for contributing to drought tolerance of crops [1, 2], of high importance in rainfed wheat [3]. To investigate the main mechanisms by which fungi promote drought tolerance in wheat plants, we conducted two greenhouse experiments. In the first study, we determined wheat responses to drought stress in the presence of *Trichoderma simmonsii* T137 and *T. asperellum* T25. And later, we explored the effect of T137 strain in combination with the mycorrhizal fungus *Rhizoglyphus irregularis*. As a result, *Trichoderma*-treated plants showed better drought tolerance than those untreated, as detected by physiological, biochemical, and molecular data, with slight differences between T137- and T25-treated plants. Additionally, the presence of *T. simmonsii* and *R. irregularis* induced the antioxidant enzymatic activities in wheat plants. Results show the potential of the combined application of beneficial microorganisms to increase crop tolerance to drought.

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## **Funding**

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# Microbial Technologies in Combatting Climate Change: Study of Drought-Resistant Legumes in the Iberian Peninsula

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## Abstract

Facing the challenges of climate change in the arid regions of the Iberian Peninsula requires innovative approaches to sustain agriculture, particularly for essential crops like beans and soybeans. This study delves into the native microbiota associated with drought-resistant legumes such as *Medicago sativa* (Alfalfa) and *Bituminaria bituminosa* (Pitch trefoil), which flourish under these conditions. These legumes benefit from specialized bacterial communities that bolster their drought resilience. Our extensive fieldwork, which involved collecting soil, roots, and seeds from over 60 locations, led to the isolation of a diverse array of more than 750 bacterial strains. Among the well-known plant growth promoting bacteria identified, genera such as *Bacillus* (*Bacillaceae*) and *Pseudomonas* (*Pseudomonadaceae*) were prevalent, along with a significant detection of *Stenotrophomonas maltophilia* (*Xanthomonadaceae*) in over 90% of seed samples. Additionally, the presence of *Glutamicibacter* (*Micrococcaceae*) was associated with higher germination rates, a correlation that corroborates findings from previous studies. This bacterium emerged as a particularly promising candidate due to its performance in biochemical tests for ACC deaminase activity, biofilm production and auxin production, highlighting its potential role in enhancing plant drought resilience. Extending our research, we are assessing nutrient dynamics, including the ability of the microbiota to fixate nitrogen and solubilize essential nutrients such as phosphorus, potassium, and sulfur. These processes are crucial for plant health and growth. We are also examining siderophore production, which plays a key role in microbial iron acquisition. In this way, our study purposes to evaluate how well these bacteria can provide nutrients to plants, thereby supporting the development of sustainable farming strategies that influence microbial interactions. Metagenomic analysis and metabarcoding were also included to provide a descriptive analysis of the microbiota. By examining these relationships, we are laying the groundwork for potentially utilizing microbial technologies to mitigate the adverse impacts of climate change on legume cultivation.

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## Efficient endosymbionts of common bean under drought conditions with reduced capacity to produce nitrous oxide

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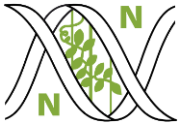
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### Abstract

Rhizobia-legume symbiosis allows biological nitrogen fixation, representing an environmentally friendly alternative for crops. It supplies nitrogen to the plant in an assimilable form, reducing the need for mineral fertilizers associated with leaching and nitrous oxide (N<sub>2</sub>O) emissions. Although nutrient limitation is the main factor affecting plant growth, there are other environmental stresses that have a negative impact on plant development, such as drought. Therefore, the aim of this study is to search for new efficient endosymbionts with low N<sub>2</sub>O emission capacity that improve drought tolerance of common bean symbiotic nitrogen fixation. In this study, *Burkholderia alba* and *Rhizobium leucaenae* strains were isolated from soil samples collected from the Misión Biológica de Galicia (MBG-CSIC) using the “plant-trap” method. The capacity of these isolates to emit or reduce N<sub>2</sub>O was investigated under free-living conditions. The symbiotic efficiency of *B. alba* and *R. leucaenae* was evaluated by inoculating seeds of the Matterhorn common bean variety with both strains separately or co-inoculated. *Rhizobium etli* CFN42 was used as positive control. After plants growth under standard or moderate drought conditions (seven days without watering), we analyzed the following physiological parameters: shoots dry weight, number of leaves, trifolia, number of flowers, N content, nodules weight and number, leghemoglobin, plant water content and stomatal conductance. Our results show that *B. alba* and *R. leucaenae* do not emit N<sub>2</sub>O. Moreover, based on the parameters measured, we found that plants inoculated with the *B.alba* + *R. leucaenae* consortium increased plant development, nodule biomass and nitrogen fixation when compared to those inoculated with *R. etli* under both standard or drought conditions. Therefore, we propose this consortium as a suitable biofertilizer for common bean plants, since, in addition to being efficient N fixers under drought stress, they are not a source of N<sub>2</sub>O emission.

### Funding

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## Bacterial inoculation as a biotool for wetland habitat restoration

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### Abstract

The State Strategy for Green Infrastructure, Connectivity, and Ecological Restoration proposes the recovery of wetland habitats due to their significance in production, climate regulation, water and carbon storage, water quality enhancement, and protection against extreme weather phenomena. However, wetlands suffer severe conservation issues due to human activity, thus increasing the restored wetland area is sought as part of the ecological transition [1]. The main actions developed in wetland restoration include terrain manipulation and revegetation with native species, yet the success in the survival and establishment of target species has been minimal. In this regard, the novelty of our project and its general objective are the design and development of a protocol to optimize the effectiveness of revegetation efforts in restoring degraded wetlands of community interest through bacterial inoculants. To achieve this, the first objective is to develop a vegetative propagation protocol that includes the use of bacterial consortia with Plant Growth-Promoting (PGP) properties to increase the production of halophyte plant material for wetland revegetation [2]. The second objective is to study the capacity of these bacterial inoculants in improving plant resilience to environmental stresses and post-transplantation in the natural area [3]. Although the project is in a very early stage, preliminary results suggest that inoculations positively affect the rooting of the plants' underground part and enhance tolerance to saline and thermal stress.

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# Effects of plant growth-promoting microorganisms on wheat growth under hydric stress

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## Abstract

Drought represents one of the main limiting factors of agricultural productivity in the current climate change scenario. In the case of wheat (*Triticum aestivum* L.), one of the most important cereal species for global food security, hydric stress causes different morphological, physiological and biochemical effects that influence its performance and significantly reduce yield [1, 2].

Likewise, the plant undergoes changes in gene expression and hormonal balance that influence, among other aspects, the development of the root system, stomatal movement, osmolyte accumulation and detoxification of prooxidant molecules, which together improve water uptake or reduce water loss, alleviate experienced damage and improve stress tolerance [3]. In addition, some microorganisms, such as plant growth-promoting microorganisms (PGPMs), can play an important role in increasing plant tolerance against water stress. In addition to other PGP activities, such microorganisms help plants cope with drought through various mechanisms, such as the secretion of plant hormones, the accumulation of osmolytes or 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity [4, 5].

In this context, four bacterial strains of the genera *Azotobacter*, *Pseudomonas*, *Peribacillus* and *Priestia* and a fifth strain of the yeast genus *Pichia* with different PGP activities were independently inoculated into wheat plants exposed to drought by water shortage to assess their ability to alleviate hydric stress by measuring changes in plant growth parameters, biochemical markers of abiotic stress and expression profiles of genes related to plant response to water stress.

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## Funding

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# Maize crops under rising temperatures: plant growth promoting rhizobacteria as a mitigation strategy

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## Abstract

The increase in the temperature of the earth's surface is already impacting species, ecosystems functioning and agricultural production. The reported effects of increasing temperature on plants include decreased growth and yields, oxidative stress, cell damage, changes in cellular metabolism and cell membrane integrity [1]. Currently, there are few solutions to increase the tolerance of plants to temperature, being plant breeding one of the most used [2], but new alternatives are coming into play. Plant growth promoting bacteria (PGPB) inoculation is emerging as a promising methodology to cope with temperature stress. However, there is a lack of knowledge on how bacteria can increase plant tolerance to high temperature. This study aims to bring novel information to enlarge this knowledge. For that maize plants were grown at two temperatures (26 and 36 °C) and different inoculation conditions. Temperature and inoculation effects were estimated through morphometric, biochemical, physiological and lipidomic parameters. Results showed that seed germination was negatively affected by temperature and was increased by bacterial inoculation. Bacteria increased shoot and root dry weight at 26 and 36 °C. High temperature induced osmolyte levels, antioxidant enzyme activity and membrane damage, yet bacteria were able to ameliorate these effects, especially *Pantoea* sp. Lipidomics analysis evidenced significant differences induced both by temperature and inoculation. At 36 °C inoculation with *Pantoea* sp. reversed the effect of high temperature and brought the lipid profile closer to plants exposed to control temperature. This effect is an important feature showing the impact that inoculation with PGPB strains may have on plants, conferring protection to high temperature and increasing plant tolerance. Thus, our study underlines the effectiveness of PRPB inoculation as a strategy to increase crop establishment under unfavourable conditions, contributing to sustainable production and food security in climate change scenarios that include increases in temperature between 1.8 and 4.4 °C by 2100 [3].

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# Impact of mercury contamination on soil fertility and bacterial communities: a study in the Almadén Mining District, Spain

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## Abstract

Approximately one third of the world's soil surface is degraded, a proportion expected to increase due to the rapid global changes currently occurring because of natural or anthropogenic causes [1]. Mercury (Hg) is one of the most dangerous environmental pollutants, and its presence highly contributes to soil degradation [2]. Given the critical importance of preserving soil multifunctionality, the restoration of degraded soils requires improving and maintaining their physicochemical and biological properties [3]. The main objective of this study was to evaluate the impact of Hg contamination on soils, including its effects on soil physical-chemical properties and bacterial communities.

Soil samples were collected from nine locations in the Almadén Mining District (Ciudad Real, Spain), comprising both uncontaminated control areas and sites with medium and high Hg contamination. Parameters such as soil pH, relative humidity, organic matter content, electrical conductivity and bioavailable nutrients were measured. Additionally, soil DNA was extracted, and the 16S rRNA gene was sequenced by Illumina MiSeq. Data were analysed using generalized linear models with Hg contamination as a fixed factor and location as a random factor. Bioinformatics analysis of bacterial sequences was performed using DADA2 and the SILVA database with the R software.

The presence of Hg did not affect the electrical conductivity or the bioavailability of macro- and micronutrients in the soils. However, a trend toward increased pH and organic matter content was observed in highly contaminated soils. The results will elucidate whether Hg-contaminated soils exhibit alterations in the diversity and/or structure of soil bacterial communities compared to non-contaminated soils, and whether dominant bacterial taxa indicative of Hg-contaminated soils can be identified.

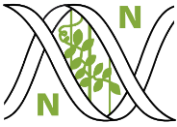
Getting insights into the changes induced in soil by heavy metal contamination will facilitate the identification of biomarkers useful for soil quality assessment, which can in turn inform the design of remediation and restoration strategies.

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## Impact of drought stress on the mobilome of *Medicago truncatula*

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### Abstract

The constant increase in the world population requires new approaches to enhance food production. The development of crops that are more tolerant to stress conditions could lead to higher yields. Understanding the mechanisms of adaptation to plant stress is a key step in achieving this goal. Recently, extrachromosomal circular DNA (eccDNA) has emerged as a new mechanism that regulates genome plasticity in eukaryotes. EccDNA are circular molecules of DNA, composed of coding or non-coding sequences. They can be formed by homologous recombination between adjacent repeats, such as amplified genes or tandem repeats, or they can result from linear extrachromosomal forms of active transposable elements. The presence of eccDNA has been described in multiple organisms, including yeast, plants, insects, and cancer cells [1]. In plants, this field is still little explored, with few works describing the presence of eccDNA as a potential mechanism to adapt to biotic or abiotic stresses [2]. The production of eccDNA has been shown to depend on plant tissue [3], so the response to stress could be triggered in some cells but not in others.

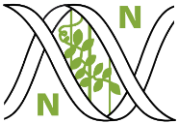
The aim of this work is to test whether eccDNA can be related to drought stress responses in the model legume *Medicago truncatula*, as part of a tolerance mechanism. We are currently optimizing the methodology for the isolation, purification, and enrichment of eccDNA molecules in *M. truncatula* samples for subsequent mobilome sequencing. This work would represent the first mobilome characterization of a legume plant.

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### Funding

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## ***Bacillus amyloliquefaciens* and its metabolites increase intensive olive orchard production under water-limiting conditions**

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### **Abstract**

The ability of *Bacillus amyloliquefaciens* QV15 and its metabolites to stimulate olive tree adaptive metabolism to water limitation was evaluated on intensive orchard production in two consecutive years. Two groups of plants were marked (100% irrigation- 75% irrigation) and treatments were delivered every 2 weeks, from April through October (flowering to harvest). After summer, photosynthesis, stress metabolic markers were determined; early harvest took place in November 2020 and November 2021. With full irrigation, root delivered QV15 induced a moderate increase in olive yield (4%), while its metabolites, delivered to leaves, achieved a higher increase (14%) as compared to controls. Water limitation induced a 25% yield decrease; under these conditions, QV15 induced a 20% yield increase, overcoming controls with full irrigation. The second year, QV15 was combined with its metabolites resulting in a synergistic 40 % increase as compared to the strain (9%) or its metabolites (22%), under water restriction.

These results indicate the potential of this strain as active matter of a biofertilizer to address a decrease in water demand in intensive production. This is especially relevant under the current global climate change, and more specifically to Spanish intensive production in Southwest Spain where water limitation for irrigation is already taking place.

### **Funding**

CDTi.

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## Combination of PGPR and biochar improves olive tree resilience to drought

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### Abstract

Water scarcity is one of the most important problems in Mediterranean agriculture, as it leads to a reduction in crop yields in a scenario of exponential population growth [1]. The olive tree, a dominant crop in the Mediterranean and the world's largest producer, is severely affected by reduced rainfall, resulting in lower production and serious economic consequences [2]. It is therefore crucial to look for alternatives that help the plant to maintain yield under drought conditions. The aim of this work was to evaluate the effects of inoculation with a Plant Growth-Promoting Rhizobacteria (PGPR) strain, the addition of biochar and their combination in helping olive plants to cope with water stress, in order to understand the underlying mechanisms.

Four-month-old olive plants were grown in pots in a phytotron. A total of eight treatments including the corresponding controls were evaluated (control, *Bacillus* strain, biochar and combination of strain-biochar under drought conditions and the same treatments under optimal growth conditions with three replicates per treatment). The parameters assessed were fresh and dry weight, chlorophyll content, biochemical stress markers and gene expression related to abiotic stress conditions. The data was statistically analysed with ANOVA.

The best treatment was the strain-biochar combination, showing the synergistic action of both components under water stress. Our results suggest that this combined treatment may mitigate plant stress by modulating various tolerance and defence mechanisms. This study presents a promising strategy for improving olive tree resilience to water stress, which is crucial for sustainable agricultural practices.

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### Funding

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### Acknowledgements

The authors would like to thank Viveros Criado (Castro del Río, Córdoba, Spain) for providing the olive plants for the experiments carried out in this work.



## ***Bacillus G7* increases tomato yield under drought conditions, enhancing CO<sub>2</sub> fixation, lowering photosynthetic pigments and oxidative stress**

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### **Abstract**

Water shortage is an increasing problem which directly affects crop production food. Drought impacts physiological state of plants, inducing water stress and compromising their development and production. Is urgent to find new alternatives to afford and improve crop production meanwhile water use is reduced.

As plants are sessile organisms, they have developed an inducible adaptation metabolism in response to biotic and abiotic stress. Among other elements which activate adaptive metabolism, PGPR bacteria have demonstrated their ability to enhance plant adaptation to adverse conditions [1].

Tomatoes are one of the most cultivated plants in Europe. It's an intensive crop requiring great water input. Thus, this study focuses on tomato plants treated with G7 under water stress conditions, evaluating the physiological effects (photosynthesis, oxidative stress, osmolytes) and production in open-field experimental plots, comparing plants under water stress with those without [2].

When plants have abundant water, PGPR does not show any improvement in growth and development. However, under drought stress, G7-treated plants exhibited a significant increase in CO<sub>2</sub> fixation and transpiration (E). Additionally, a great decrease in photosynthetic pigments content was observed, resulting in reduced oxidative stress due to lower energy input into the system and better energy utilization, reflected also in the production compared to the control. These results indicate increased efficiency in energy capture and utilization, thus better adaptation to water stress induced by G7. This strain presents a promising candidate for the development of biofertilizers to decrease water use in intensive agriculture [2].

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## Bacterial symbionts from *Ips typographus* beetles have the potential to protect *Durum Wheat* crop from *Fusarium* head blight

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### Abstract

Durum wheat (*Triticum turgidum* subsp. *durum*) is a key crop in the Mediterranean region, which is responsible for half of global production. An estimated 21,5% of wheat yield is lost due to diseases, with fungi accounting for 70-80% of those losses. Among them, Fusarium head blight (FHB), caused by *Fusarium graminearum*, is one of the most damaging wheat diseases [1]. The overuse of chemical pesticides to protect crops has not only led to environmental and public health concerns, but also decreased effectiveness due to resistance development in phytopathogens, which demands alternative strategies [2].

Recent research revealed that insect-associated bacteria harbour a wider diversity of antagonistic strains to a broader spectrum of pathogens than those of soils or plants [3]. In a previous study, we characterized bacterial associates of the spruce bark beetle (*Ips typographus*), which showed strong capacity to antagonize entomopathogenic fungi [4].

Three isolates from this collection - *Bacillus halotolerans* (C3L22), *Pseudomonas yamanorum* (LC49), and *Streptomyces albidoflavus* (MMI15B) – exhibited remarkable *in vitro* antagonism against the wheat root-lesion nematode (*Pratylenchus penetrans*) and to different phytopathogenic fungal strains, including a *F. graminearum* strain. Furthermore, when C3L22, LC49 and MMI15B were inoculated on wheat seedlings not adverse effects were observed on early plant development but enhanced plants capacity to resist FHB infection at the spike developing stage. This enhanced resistance is likely attributed to the priming of different systemic defence mechanisms, as suggested by the differential gene expression (qPCR) of markers for resistance mediated by salicylate (SAR), jasmonate, ethylene (ISR), and phenylpropanoid.

Bacterial isolates from *I. typographus* have a strong antagonistic effect to a broad range of phytopathogens. Notably, isolates C3L22, LC49, and MMI15B demonstrated potential to be used as biocontrol agents for wheat crops. Finally, our study provides evidence supporting that insects might be a potential niche for obtaining biocontrol agents.

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### Funding

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The authors thank Dr. Carla Varanda from the MED, University of Évora, for providing the collection of fungal phytopathogens used in this work.



## Enhanced plant growth under salt stress by using sugarcane molasses as eco-friendly fertilizer

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### Abstract

Since the beginning of the industrial era, food production has mainly been promoted by the use of chemical fertilizers. However, nowadays, society is looking forward to more sustainable foods, highlighting the use of sustainable fertilizers as is the case of the use of the so-called by-products of ecological industries. In this respect, the generated by-products following the processing of sugarcane and sugar beet molasses have been proposed as by-products that can promote plant growth [1]. However, the effects of these compounds in the amelioration of salinity stress are not well known. For that purpose, the effect of the liquid residue of the ethanolic fermentation of the molasses after a maturation step performed in anoxic conditions was evaluated as fertilizer in pea plants (*Pisum sativum*) Lincoln variety. The plants were grown in a hydroponic solution [2] supplemented with 0.5% agar for 28 days. A control condition plant set (absence of the molasse-related by-product) and a supplemented one (1 % v/v of the by-product) were addressed under 0 mM and 25mM of NaCl in a controlled growth chamber. At the end of the experiment, the height and the weight of the aerial fraction were significantly increased in the plants supplemented with the by-product, compared to those of the non-supplemented plants, independently of the salinity stress. Strikingly, biomass production was incremented in the supplemented set by 27% and 39% under 0 and 25 mM of NaCl compared to the two control sets, respectively. Besides, the photosynthetic status and the N and C contents of the plants were increased, when the molasse-related by-product was added to the hydroponic solutions, independently of the NaCl concentration applied. Therefore, these results stand out the beneficial effects of the use of environmentally friendly by-products generated by the alcoholic industries.

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## Litter quality influences dehesa soil microbiology

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### Abstract

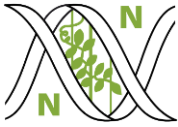
Here we report on how litter quality influences dehesa soils microbiology [1,2]. To this end, we conducted an incubation experiment under controlled laboratory conditions, in which different types of litter were added to soils with different microbiomes, resulting in 10 combinations. Soils and litter were taken from natural grasslands: abandoned (AB), grazed plot (RO), legume-rich sown pasture (LEG) and control (continuous grazing) plot (CT). The experiment was sampled three times: at the beginning (T1), at 9 (T2) and at 18 months (T3). Our results shown that soil respiration and microbial biomass were higher at T1. In addition, nutritional stress increased with time  $T1 < T2 < T3$ . At T1, higher microbial respiration was observed in rotational soils (RO-RO and RO-CT) than in the reference ones (CT-CT), while no differences were found for the other soil-litter combinations. Bacterial  $\alpha$ -diversity and, in general, the relative abundance of the 10 most abundant *phyla* was also higher at T1 compared to T3. Among fungi, there was no difference in  $\alpha$ -diversity between T1 and T3, however, the relative abundance of Glomeromycota decreased at T3 compared to T1. Moreover, the combinations with AB soil (AB-AB and AB-CT) showed a larger relative abundance of Rozellomycota than CT-CT. In terms of  $\beta$ -diversity, population structures of fungi and bacteria were found to be different between T1 and T3. When we examined functionality, we noticed differences in  $\beta$ -Glucosidase and  $\beta$ -D-Celobiohydrolase activities ( $T1 > T3$ ) and in phosphatase, which was higher at T2. Furthermore,  $\beta$ -Glucosidase was higher in RO-RO and RO-CT than in CT-CT and Phosphatase was higher in RO-CT than in CT-CT, while it declined in AB soils (AB-AB and AB-CT). These data suggest that nutrient recycling is favoured in rotational grazed soils, whereas the opposite may occur when they are abandoned.

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### Funding

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## Influence of native arbuscular mycorrhizal fungi on the response of olive plantlets to drought

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### Abstract

Olive (*Olea europaea* L.) trees, the most typical fruit crops of the Mediterranean basin, will be affected by climate change due to decreasing water availability in terms of rainfall, irrigation resources and progressive salinization, and extreme climate events. This impact will lead to a reduction in suitable crop areas, lower yields and poor-quality food products. Although the olive tree is considered a drought-tolerant species, enhancing its drought tolerance is necessary to maintain and even improve productivity in the current context of climate change. Arbuscular mycorrhizal (AM) fungi, which form beneficial symbiosis with most land plants, are known to increase plant fitness under drought stress. While olive trees are highly dependent on AM fungi under arid and semi-arid conditions, the effect of the AM symbiosis depends on the plant cultivars and AM fungal species involved. The aim of this work was to select the most efficient AM fungal species to increase the fitness of two olive varieties (Arbequina and Picual) under drought stress. To that end, rooted cuttings of these two olive varieties were inoculated with seven AM fungi isolated previously from the rhizosphere of mature field-grown olive trees collected in a major olive plantation area in the province of Jaén and one isolated from an arid ecosystem. Five-months post-inoculation, half of the Arbequina and Picual olive plantlets from each treatment were subjected to drought stress for 2 months, while the other half were well-watered. It was found that olive exhibits a high mycorrhizal dependency. All the AM fungi improved the development of both varieties, with *Funneliformis geosporum* being the most efficient in enhancing the growth of well-watered plants. Under drought conditions, all fungi equally promoted the growth of Picual; however, *Septoglomus constrictum* was the most efficient in improving the growth of Arbequina. The underlying mechanisms of the observed effects will be discussed.

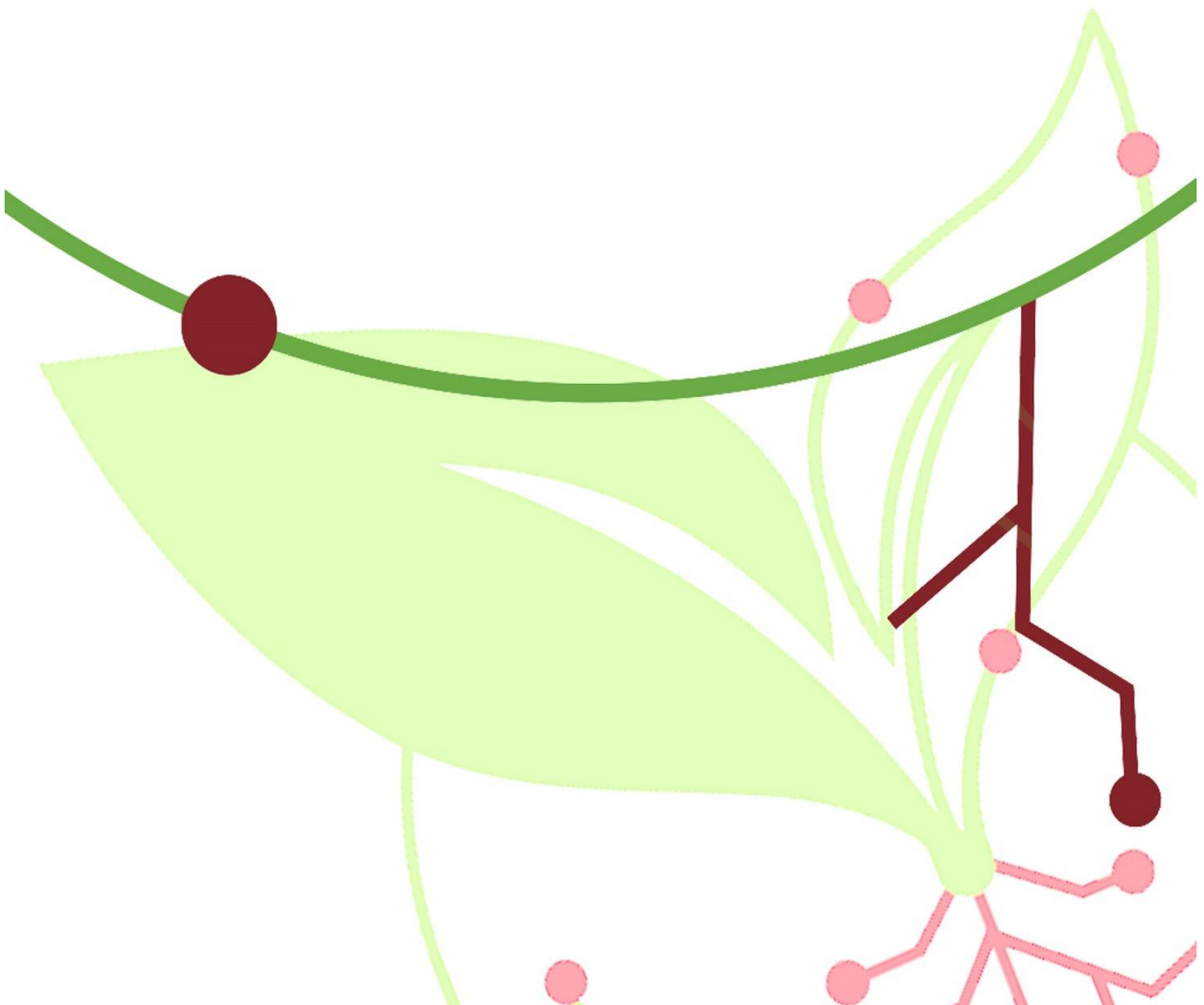
### Funding

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## Session 4

# Molecular biology of plant-microorganism interactions





## Regulation of *Sinorhizobium meliloti* nitrogen fixation genes by antisense RNAs

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### Abstract

Massive sequencing of strand-specific cDNA libraries (RNAseq) has revealed the great complexity of prokaryotic transcriptomes, unveiling a huge number of small-sized non-protein-coding transcripts (sRNAs) [1]. Most sRNAs regulate extensive post-transcriptional networks that support almost every adaptive response of bacteria to environmental changes. Therefore, it is increasingly evident that no microbial process can be fully understood without considering the regulation of gene expression by sRNAs [2].

In this work, unconventional RNAseq protocols such as Differential RNAseq (dRNAseq), Cappable seq or Term seq were used to determine the ends of transcripts encoded in the genome of the legume symbiont *Sinorhizobium meliloti*. Simultaneously, new conventional RNAseq experiments carried out in bacteroids have allowed us to evaluate changes in the coding and non-coding transcriptome of *S. meliloti*, and to identify sRNAs with possible symbiotic function. Here, I present a preliminary characterization of the promoter activity and the effect of overexpression on their target mRNAs of several antisense RNAs potentially involved in the regulation of nitrogen fixation during symbiosis between *S. meliloti* and *M. sativa*.

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### Funding

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## Mechanosensing and motility in plant-colonizing *Pseudomonas* species

Alonso-Caballero, A.<sup>1\*</sup>, Montoya, M.<sup>1,2</sup>, Carrera, L.<sup>1</sup>, Duran, D.<sup>1</sup>, Martin, M.<sup>1</sup>, Rivilla, R.<sup>1</sup>

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### Abstract

*Pseudomonas* is a gram-negative genus that includes growth-promoting and plant-pathogenic species [1]. Plant colonization requires bacteria cells to approach, recognize, and attach to the host's target tissues. These cell behaviors largely depend on the type IV pili (T4P), which are extracellular micrometer-long appendages that participate in bacterial motility, mechanosensing, adhesion, and biofilm development. Hundreds of proteins compose the pilus; however, the tip-end PilY1 protein stands out for its key role in T4P functions. PilY1 protein comprises three major domains, each harboring features linked to its activities in T4P mechanosensing, adhesion, and dynamics. Due to its location, PilY1 is exposed to mechanical stimuli that can alter its domains' conformations and functions [2]. Hence, understanding how mechanical forces modulate PilY1 structural-based functions can provide insights into the molecular basis governing the cell behaviors that drive plant colonization. Herein, we show (1) how mechanical forces alter PilY1's adhesive and pilus-dynamics regulatory domains at the single-molecule level and (2) how PilY1 mutagenesis impacts *Pseudomonas* cell behaviors. Our results show that PilY1's adhesive domain exhibits low mechanical stability and force-sensing capability, which could aid the bacterium in distinguishing abiotic from biotic—target—substrates. By contrast, the PilY1 pilus regulatory domain is mechanically stable, and this stability is increased in the presence of calcium, a cognate ligand of PilY1. This observation provides a mechanistic rationale to support the antagonistic effect of calcium binding on pilus dynamics previously described [3]. Cell-scale assays prove that PilY1 elimination severely affects *Pseudomonas* motility and biofilm formation, which provides us with a benchmark for testing molecular strategies targeting PilY1 in the future. A better understanding of how mechanical forces influence bacteria behaviors at the nano and microscale could aid in developing agrobiotechnological tools to harness *Pseudomonas* plant colonization *ad hoc*—assisting plant-growth-promoting bacteria while tackling pathogenic species.

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### Funding

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### Acknowledgments

The authors thank Dr. Fernando Rojo (CNB-CSIC, Madrid) for providing the *Pseudomonas* strains used in this work.



# Metal-binding protein RLV\_3444 is a component of a symbiotically relevant zinc ABC transporter system in *Rhizobium leguminosarum*

Soldek, J.N.<sup>1\*</sup>, Ballesteros, M.<sup>1</sup>, Palacios, J.M.<sup>1,2</sup>, Albareda, M.<sup>1,2</sup>

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## Abstract

Nitrogen-fixing *Rhizobium*-legume symbiosis constitutes an alternative to the use of nitrogen fertilisers. To establish the symbiosis, both partners need to adjust their behaviour through sophisticated plant- and bacteria-dependent mechanisms which lead to the formation of nodules where rhizobia are differentiated into bacteroids, the symbiotic nitrogen-fixing form. Proteomic analysis of bacteroids induced by *Rhizobium leguminosarum* bv. *viciae* from pea and lentil nodules revealed that the expression of more than 100 proteins depends on the legume host [1], suggesting that the host induces responses dependent on the rhizobial strain. Among these proteins, a metal binding protein (RLV\_3444), a component of the ABC transporter system RLV\_3442-3444, was identified as being overexpressed in pea vs. lentil bacteroids suggesting that the provision of metal(s) to the bacteroid might be more restrictive in the *Rhizobium*-pea symbiosis. This work aims to study the functional role of the RLV\_3442-3444 metal transporter system in *Rhizobium*-legume symbiosis. Results have shown that in pea bacteroids induced by a mutant strain affected in the transporter system, the zinc concentration decreased compared to the wild-type. Functional analysis of the RLV\_3442-3444 system revealed that RLV\_3444 may replace the role of ZnuA, the metal-binding component of the well-known ZnuABC high-affinity zinc transporter system, under both free-living and symbiotic conditions. The growth defective phenotype of the RLV\_3444/ZnuA double mutant under zinc-depleted conditions was reverted by supplementation with zinc, but not with manganese or iron, or by expression of wild-type copies of the respective genes. Transcriptional fusions to the reporter gene *gusA* have shown that the expression of the system is regulated by zinc via the Zur transcriptional regulator.

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## Genetic factors involved in the production of a Mixed-linkage $\beta$ -glucan (MLG) in rhizobia

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### Abstract

Different agronomical relevant rhizobia, including *Ensifer meliloti* and *Rhizobium etli*, are able to produce a linear Mixed-Linkage  $\beta$ -Glucan (MLG) activated by the second messenger c-di-GMP [1,2]. This exopolysaccharide (EPS) is important for bacterial aggregation and biofilm formation and is required for efficient attachment to the roots of the host plants. MLG also adds to the growing list of promising biopolymers with potential biotechnological applications.

We previously described the *bgsBA* operon required for MLG production, where *bgsA* encodes an inner membrane glycosyltransferase and *bgsB* a companion HlyD-like periplasmic protein [1,2]. By analogy to other related EPS like cellulose, it is hypothesized that additional genes and proteins must participate in MLG biosynthesis. We have followed various genetic approaches to uncover other functions involved in the synthesis and secretion of MLG by *E. meliloti*. Among the genes essential for MLG production is *tolC* which encodes an external membrane porin involved in secretion of different biopolymers and tolerance to toxic compounds and abiotic stresses [3].

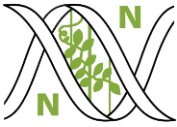
In order to maximize MLG production by rhizobia, we have constructed mutants in other c-di-GMP-regulated EPS that may metabolically compete with MLG. Production of MLG was quantified in *E. meliloti* strains unable to produce EPS I, EPS II and the recently described APS (arabinose-containing polysaccharide), as well as in a cellulose mutant of *R. etli*. While blocking the biosynthesis of EPS I, EPS II and APS do not seem to have a significant impact on MLG production by *E. meliloti*, a mutant in the cellulose operon greatly increase MLG production in *R. etli*. The results suggest that c-di-GMP activated MLG production is greatly dependent on the bacterial genetic background.

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## Understanding the molecular basis of *Sinorhizobium fredii* HH103-soybean compatibility conferred by bacterial secreted proteins

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### Abstract

To establish N-fixing symbiosis, rhizobia bacteria must bypass plant immunity [1]. Like plant pathogenic bacteria, many rhizobial strains suppress plant defence by delivering effector proteins into the host cell via Type III Secretion System (T3SS) [2]. Conversely, some rhizobia T3SS effectors can also directly activate the plant symbiotic program [3]. Unravelling how plant proteins recognize and respond to T3SS effectors is key to unlocking novel insights into symbiotic compatibility.

*Sinorhizobium fredii* HH103 is symbiotically compatible with the agronomically-important soybean Williams 82 cultivar (W82). However, using a subset of 55 soybean genetically diverse lines, we have demonstrated that HH103 cannot establish symbiosis with all soybean cultivars. For example, the soybean JL2 cultivar inoculated with HH103 did not form any nodules. We hypothesized that HH103 gain and/or loss of nodulation might be driven by evolution processes through the recognition of T3SS effectors by the host. We constructed loss-of-function mutants in the T3SS ( $\Delta T3SS$ ) and the T3SS secreted NopM effector ( $\Delta nopM$ ) of HH103. We observed both effector-dependent gain or loss of nodulation phenotypes in both W82 and JL2, supporting that T3SS effectors are involved in HH103-soybean compatibility.

It is unknown whether these phenotypes are result of the activation of the plant symbiotic program or the suppression of plant defence. To determine the role of NopM in the plant cell, we performed *in vitro* Size Exclusion Chromatography and Small-angle X-ray scattering (SAXS), and *in vivo* bacterial two-hybrid assays and observed the formation of homomeric proteins. Additionally, unravelling the NopM targets will provide novel insights into the molecular mechanisms driving rhizobia-host compatibility. Thus, we are currently performing Genome Wide Association Studies (GWAS) to identify soybean *loci* involved in HH103-soybean compatibility, and IP-MS to identify NopM targets in W82.

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## ***Fusarium oxysporum*, demon or angel? Endophytes to control Fusarium wilt**

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### **Abstract**

*Fusarium oxysporum* is the fifth most important fungal pathogen which determines severe damages in relevant crops (banana, tomato, common bean) worldwide [1]. In addition to the strict restrictions imposed by the European Union in the use of agrochemicals, the harmful effects of these products on soil, water resources and biodiversity limit the options of the farmers to control the pathogen. "Suppressive soils" have been used as an appropriate source of non-pathogenic strains of *F. oxysporum* with beneficial effects on plants [2]. Following this strategy to identify beneficial strains, we explored healthy common bean crops in the region of El Barco de Ávila (Ávila, Spain). We have isolated and characterized several endophytic strains of *F. oxysporum* from these plants. The pathogenicity assays performed with combinations of endophytes and pathogens have shown a drastic reduction of the disease symptoms in common bean and tomato plants. The study of the colonization pattern in common bean plants demonstrated the ability of the endophytes to colonize the root system, the root crown and hypocotyls, although to a lesser extent of the pathogenic strain. These results suggested that the ability to control the disease is not a consequence of spatial competition with the pathogenic strains. We performed an expression analysis by means of qPCR to determine the plant resistance response in common bean plants inoculated with the endophyte and compared the results with those obtained from plants inoculated with the highly virulent strain FOP-SP1. We detected a significant induction of the expression of *ERF1* (a marker for ethylene-mediated response) at 3 and 21 dpi in plants inoculated with the endophytic strain ABE-22, in contrast with the results previously described for the endophytic strain Fo47 in tomato plants [3]. Our results suggest that the endophyte-mediated resistance (EMR) depends on the strain of endophyte and/or the plant species.

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# Phosphotransferase System (PTS<sup>Ntr</sup>) links to metabolism and motility in *Rhizobium leguminosarum*

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## Abstract

Careful coordination of intracellular carbon and nitrogen levels is crucial not only for optimal cellular growth but also for maintaining nitrogen fixation and symbiosis with the plant host. One of the key metabolic regulators in rhizobia and other  $\alpha$ -proteobacteria is the Phosphotransferase System (PTS<sup>Ntr</sup>), which acts at the post-translational level by protein-protein interactions. This system comprises an initial sensor protein PtsP, a phosphotransfer protein NPr, and two output regulatory proteins, PtsN and ManX. Depending on their phosphorylation states, PtsN and ManX regulate downstream cellular functions. We have previously shown how unphosphorylated ManX activates the TCA cycle, while phosphorylated PtsN activates ABC transporters [1, 2].

We are now studying the molecular mechanisms and potential effectors of PTS<sup>Ntr</sup> responsible for carbon storage regulation and its link to motility and chemotaxis systems. We hypothesise that under nitrogen-poor conditions, excess carbon is allocated into different polymers, such as the internal polymers polyhydroxybutyrate (PHB) and glycogen or the surface polymer EPS, and these dynamic changes are likely to be mediated through PTS<sup>Ntr</sup>. We have quantified intracellular and extracellular polymer production and assessed the activity of TCA cycle enzymes, showing how PTS<sup>Ntr</sup> acts on TCA cycle dehydrogenase enzymes, resulting in increased carbon flux from the TCA cycle into storage. We have also examined the complexities of control of motility, showing links to the regulation exerted by PTS<sup>Ntr</sup> [3]. Surprisingly, chemicals from the TCA cycle cause the bacteria to completely stop moving. Understanding how soil bacteria respond to nutrients and influence their symbiotic behaviour is of crucial importance for their survival and adaptation both in soil and when interacting with their plant hosts.

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## Characterising the *Sinorhizobium fredii* USDA257 Type VI Secretion System

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### Abstract

For agriculture, the symbiosis carried out by rhizobia with legumes stands out as crucial for both economic and environmental reasons. In this process, the bacteria colonize the roots of the plants, inducing the formation of plant organs called nodules. Within these structures, rhizobia fix the environmental nitrogen into ammonia reducing the demand for this essential element required for plant growth. Various bacterial secretion systems (TXSS, Type X Secretion System) are involved in the establishment of this symbiosis, with the T3SS being the most extensively studied [1]. The T6SS is a nanoweapon present in 25% of gram-negative bacteria commonly used against other gram-negative bacteria, though some of them use it to manipulate eukaryotic cells [2]. Interestingly, although T6SS is widely distributed among rhizobia, whether it has a specific role in symbiosis with legumes remains unknown. *Sinorhizobium fredii* USDA 257 is a fast-growing rhizobium with the capacity to nodulate a great variety of legume plants [3]. This strain harbors a single T6SS cluster, containing the genes encoding all the structural components of the system and two genes encoding potential effectors that could target the cell wall of the plants. We have demonstrated that the system is active and can be induced in poor culture media. Additionally, we have seen by fluorescence microscopy that the T6SS is active in nodules. Competition assays between USDA257 and different preys have shown that USDA257 cannot kill any of them using its T6SS in the tested conditions. However, we are currently testing the role of this machinery in *in planta* competition assays during the nodulation process.

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# The *Bradyrhizobium diazoefficiens* ClpAP<sub>1</sub>S<sub>1</sub> proteolytic system is involved in the abiotic stress response and in the symbiotic interaction with soybeans

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## Abstract

Rhizobia-legume symbiotic interaction implies major physiological and metabolic changes of the bacteria from the free-living state in the soil to the symbiotic form in the oxygen-limited environment of plant root nodules. Moreover, environmental stresses are limiting factors for an effective nitrogen-fixing symbiosis. Clp-type chaperone-proteases are conserved energy-dependent proteolytic systems that degrade unfolded or misfolded proteins, as well as specific substrates [1]. They consist of an AAA+ ATPase-type chaperone, that recognizes and denatures the substrate, and a protease cylinder. Specific adapters also modulate their proteolytic activity. Clp-type proteins are involved in the bacteria response to several abiotic stresses, though a limited knowledge is available in the context of rhizobia in both free-living and symbiotic states [2].

Here, we investigated the role of the ClpAP<sub>1</sub>S<sub>1</sub> chaperone-protease system of the soybean endosymbiont *Bradyrhizobium diazoefficiens*, which is involved in the proteolytic control of FixK<sub>2</sub>, a key regulator for the microaerobic metabolism of this bacterium [3]. For this purpose, strains deficient in the chaperone ClpA and its adaptor ClpS<sub>1</sub> were characterized, as well as a ClpP<sub>1</sub> protease overexpressing strain. Our results indicated that while the ClpA chaperone is required for tolerance to heat shock and acid pH, *clpS<sub>1</sub>* mutation increases the resistance to salt stress, and the entire ClpAP<sub>1</sub>S<sub>1</sub> system is involved in the tolerance to alkaline pH. Inoculation of soybean plants with single or mixed inoculum (parental:mutant) at different ratios showed that both *clpA*- and *clpS<sub>1</sub>*-mutants are affected in the infection and nodulation processes as well as in nodule occupancy. Taken together, these data suggest that both ClpA and ClpS<sub>1</sub> proteins may play a role at some stage of nodule colonization, which is currently being investigated.

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# Volatile-mediated growth promotion and induction of biochemical alterations in *Arabidopsis thaliana* by *Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8

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## Abstract

*Rhizobium* sp. and *Flavobacterium* sp. are important soil bacteria that display several plant growth promotion abilities. While mechanisms such as the establishment of nodules for nitrogen fixation found in rhizobia require an intricate and specific molecular cross-talk between the host legume and the bacteria, other mechanisms such as production of indol acetic acid are somewhat unspecific and open the door for wider application beyond legume plants. Here we report a novel mechanism of growth promotion induced by *Rhizobium* sp., which is not specific to legumes, as shown by growth promotion of the non-legume *Arabidopsis thaliana*, and which was shared with a *Flavobacterium* strain, also isolated from a legume. This mechanism consists of the release of volatile organic compounds that promote plant growth and although it has been reported before for other genera of bacteria, this ability integrates an extensive list of benefits of rhizobia, highlights the possibilities of its use with non-legumes and raises questions regarding plant-bacteria communication. We also examined several biochemical endpoints of the plant in response to the bacterial volatile organic compounds (BVOCs) released, having found responses in chlorophyll content, protein, electron transfer system, lipid peroxidation and superoxide dismutase. This is also, to our knowledge, the first report applying two-dimensional gas-chromatography to the study of plant growth-promoting bacteria (PGPB), while also comparing the results obtained by one-dimensional gas-chromatography. We were able to discriminate distinct VOC profiles (with a wide range of compounds belonging to several different chemical families) between the two PGPB bacteria and to identify BVOCs with reported bioactivity.

## Funding

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## Role of HkmA, GcbA, HdmA and HdmB in the regulation of motility in *Pseudomonas ogarae* F113

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### Abstract

*Pseudomonas ogarae* F113 is a gamma-proteobacteria with high ecological and agronomic relevance due to its role as plant growth-promoting rhizobacteria (PGPR). Its ability as PGPR is largely determined by its ability to compete in rhizosphere colonization mainly by changing from a sessile to a motile phase [1]. This transition is regulated by the presence or absence of the second messenger cyclic diguanylate monophosphate (c-diGMP), whose production is carried out by diguanylate cyclases (DGC) and it is degraded by phosphodiesterases, both regulated by the AmrZ/FleQ node [2]. This repressor/regulatory node triggers a regulatory cascade in the presence of environmental stimulus that results in the synthesis and regulation of flagellar apparatus in F113. Within this framework we found three related proteins: HkmA, HdmA and HdmB. HkmA is a possible histidine kinase that acts as an environmental sensor. Mutation in *hkmA* is associated with a hypermotile phenotype, reduced biofilm formation and altered extracellular matrix components due to the alteration of the environmental signal transduction pathway. On the other hand, HdmA and HdmB are two proteins with an HDOD domain, which is also found in SadB, another protein present in the motility regulatory pathway, that shows interaction with c-diGMP [3]. Mutations in *hdmA* and *hdmB* result in reduced motility, but the ability to form biofilm is not altered. HdmA seems to be necessary for FliC synthesis, which encodes flagellin, but HdmB does not.

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# Deciphering the hidden potentials of bacterial communities isolated from root nodules of native legumes growing in Tunisian arid regions

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## Abstract

In an Era where climate change imposes difficult challenges for agricultural sustainability, there's a growing urgency for the development of novel strategies to guarantee both plant productivity and soil fertility. In this context, particular attention was given to plant growth promoting bacteria (PGPB) associated with native legumes in arid habitats due to their natural adaptability to extreme environments. Despite the large number of identified species, the potential application of this microbial community in food legumes wasn't thoroughly explored in current research endeavours. In this context, we proceeded for the isolation and characterization of root nodules endophytes from 15 wild legumes, originating from Tunisian drylands. In a collection of 210 strains, phylogenetic analysis revealed the presence of a large diversity of rhizobial and non-rhizobial isolates belonging to  $\alpha$ -,  $\beta$ -, and Gamma-proteobacteria, Firmicutes and Actinobacteria, among which the strain IRAMC:0171 was introduced as a new species in the *Mesorhizobium* genus. Furthermore, genomic characterization of the Saharan isolate *Sinorhizobium meliloti* IRAM:0087 showed the presence of some unique genetic features, conferring to the strain an outstanding capacity to tolerate a variety of abiotic stressors such as heat, drought, salinity, and heavy metals. Interestingly, the same isolate was able to nodulate *Vachellia tortilis* (Forssk.) Gallaso & Banfi subsp *tortilis*, a plant-tree well adapted to harsh environments. Moreover, the evaluation of the impacts of heat and salt stress on *Pisum-Rhizobium* and *chickpea-Mesorhizobium* symbiosis respectively, showed not only a decrease in the growth and plant nodulation of both legume species, but also a substantial change in the composition of phenolic compounds in root exudates, which negatively affected the early molecular signalling in legume-rhizobium symbiosis. Nevertheless, these detrimental impacts were successfully mitigated by the co-inoculation with non-rhizobial endophytic consortium from our collection, proving the promising potential of nodules microbiome from wild legumes in boosting the survival and productivity of food legumes in drylands.

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## Characterization of a *Sinorhizobium meliloti* protein that controls phenotypes associated with bacterial life on surfaces

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### Abstract

Bacterial colonization of plant roots is an important stage for the establishment of different plant-bacteria interactions. As a proxy to decipher *Sinorhizobium meliloti* mechanisms of root colonization, we investigate the molecular bases of surface motility in the alfalfa symbiont. During these studies, we found that *S. meliloti* emits volatile compounds and one of them, 2-tridecanone (2-TDC), affects surface-associated bacterial behaviors and hampers plant–bacteria interactions by interfering with microbial colonization of plant tissues [1]. In *S. meliloti*, 2-TDC promotes a mode of surface translocation, which is largely flagellum-independent [1]. To gain further insights into the mechanism of action of 2-TDC and unveil genes putatively involved in plant colonization, *S. meliloti* transposants impaired in 2-TDC-induced surface translocation were isolated. One of the mutants is affected in a gene, *mkiG*, that encodes a protein with domains characteristic to proteins involved in cyclic diguanilate (c-di-GMP) metabolism [2]. The mutant shows swimming motility similar to the wild type strain but is impaired in surface translocation and unable to respond to volatile 2-TDC. In addition, the mutant exhibits increased biofilm formation, strong Congo red staining, and increased levels of c-di-GMP, but it establishes symbiosis with alfalfa plants as effectively as the wild type strain. The characterization of mutants with different deleted versions of *mkiG*, complementation experiments, and isolation of mutations that suppress the *mkiG*'s mutant phenotypes, demonstrate that c-di-GMP homeostasis is fundamental in the mechanism of action of 2-TDC and indicate that MkiG is a bifunctional protein, which is crucial in the control of phenotypes associated with bacterial life on surfaces.

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# Analysis of the c-di-GMP dependent intracellular proteome of *Rhizobium etli*

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## Abstract

The second messenger cyclic diguanylate (cdG) is a bacterial lifestyle switch molecule, well known for its role in biofilm formation and the regulation of the production of many biofilm matrix components. We have previously reported that elevated c-di-GMP levels in *R. etli* promote the export of numerous cytoplasmic proteins to the cell exterior, in addition to proteins involved in adhesion and biofilm formation [1]. Here we present a study of the changes induced by artificially elevated cdG levels on the intracellular proteome of this bacterium. The cell-associated cdG-dependent proteome was determined by i-TRAQ labelling coupled to quantitative MS-based proteomics. A total of 171 cell-associated proteins displayed Differential Abundance (DA) in the high cdG strain, of these 122 were increased and 49 were reduced. Similar to the extracellular proteome, the majority of intracellular cdG regulated proteins are predicted to have a cytoplasmic localization. However, there were striking differences between the cell-associated and the extracellular cdG-dependent proteomes of *R. etli*. With only some exceptions, cdG regulated proteins in the cell fraction were generally unrelated to DA proteins found in the supernatants. Our data support that cdG affects the expression and/or stability of many proteins involved in very diverse processes and functions, from proteins involved in adhesion, exopolysaccharide production or plasmid transfer, to housekeeping and secondary metabolisms. Interestingly, nearly a dozen regulatory proteins displayed DA under elevated c-di-GMP levels.

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## The *hex1* gene role in *Trichoderma simmonsii* biology

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### Abstract

*Trichoderma* spp. are soilborne fungi used as biological control agents. Some strains are effective colonizers of plant roots while activating plant defences against biotic and abiotic stresses [1]. The *hex1* gene is conserved in filamentous Ascomycete fungi and encodes the hexagonal peroxisome protein 1, a major component of the Woronin Bodies. These are vesicles specialized in sealing septal pores in Ascomycetes to regulate cytoplasmic traffic, preventing cytoplasmic bleeding in case of mechanical injuries [2]. Several studies have shown that *hex1* is involved in pathogenicity of fungal phytopathogens [3,4]. We have focused on the characterization of *hex1* in *Trichoderma simmonsii* T137, a strain isolated from healthy wheat crop plants and previously selected due to its ability to protect wheat plants against drought [5]. We have generated *hex1* knock-out transformants of strain T137 and evaluated their tolerance to abiotic stresses, biocontrol potential against several phytopathogens, ability to colonize wheat roots, and the effect on wheat tolerance to water stress. Results indicate that *hex1* is involved in the biocontrol activity of *T. simmonsii*, but not in wheat root colonization and protection of this plant against water stress. Further studies should be addressed to determine other possible roles of *hex1* on the biology of this fungus and its interactions with plants.

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## Reassessing the role of the stringent response in the free-living and symbiotic lifestyles of *Sinorhizobium meliloti*

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### Abstract

The stringent response (SR) is a bacterial regulatory mechanism that aids adaptation to stressful conditions by controlling a broad range of processes such as DNA replication, transcription, protein synthesis or lipid metabolism [1]. The key components of SR are phosphorylated nucleotides [(p)ppGpp] also known as alarmones, whose levels are modulated by members of the RSH (RelA/SpoT) protein superfamily. In addition to its function in managing stress, SR is important during normal cellular growth and essential for a specialized type of bacterial motility [2]. In a forward genetic approach aimed at deciphering the molecular mechanisms governing surface motility in *Sinorhizobium meliloti* GR4, a *relA::Tn5* transposant (GRS3009) was isolated. This mutant showed swimming motility similar to that of the wild-type strain, but impaired surface translocation and response to 2-tridecanone, a volatile compound that triggers surface spreading in *S. meliloti* [3]. Analyses of the GRS3009 volatilome also revealed reduced volatile production. Interestingly, our *relA::Tn5* transposant is able to induce nitrogen-fixing nodules in alfalfa albeit exhibiting slower nodulation kinetics than the wild-type strain. This behaviour contrasts with the symbiotic phenotype described for *S. meliloti* Rm1021-derived *relA* mutants, which fail to induce nodulation on alfalfa [4]. To further decipher the role of SR in the control of surface motility and volatile production in free-living *S. meliloti* as well as its impact on the establishment of symbiosis, a GR4-derived *relA* deletion mutant and complementing strains are currently being characterized.

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## New discovered functions of the TtsI transcriptional regulator of *Sinorhizobium fredii* HH103

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### Abstract

*Sinorhizobium fredii* HH103 is a nitrogen-fixing symbiont of dozens of legumes, including soybean [1]. The set of bacterial genes whose expression is affected by the presence of appropriate flavonoids (the *nod* regulon) is subjected to a complex regulatory network in which NodD1 acts as the main positive regulator, and NodD2, NolR, SyrM, and TtsI participate as secondary regulators with either positive or negative roles depending on the specific regulated gene [1,2]. In this work we summarize our recent findings about the role of TtsI, the positive regulator of the symbiotic type 3 secretion system (T3SS) that is present in this strain. On the one hand and combining different kind of studies of gene expression (RNAseq, qPCR, and  $\beta$ -galactosidase analyses), we have recently shown that TtsI has a role (direct or indirect) as a repressor of several genes belonging to the *nod* regulon, including *syrM* and *ttsI* itself [2]. On the other hand, we have found that not only *ttsI* but also the T3SS apparatus are required for the genistein-induced surface motility exhibited by *S. fredii* HH103 [3]. In fact, TtsI is required for the genistein-induced expression of the flagellar gene *flgJ* through a previously unidentified *tts* box [4]. Thus, our work shows a connection between the *nod* regulon and bacterial motility and highlights previously unknown function of TtsI, which might affect not only to secretion of effectors and bacterial motility but also to other processes that remain to be elucidated.

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## Studying *Sinorhizobium fredii* HH103 trans-sRNAs putatively involved in symbiosis with legumes

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### Abstract

*Sinorhizobium fredii* HH103 is a broad-host range rhizobium capable of establishing a symbiotic relationship with many legumes. This relationship involves complex molecular communication, with various molecular signals from both parties. On the bacterial side, these signals include lipochitooligosaccharides (LCOs), also known as Nod Factors (NF), effector proteins secreted by the Type III secretion system (T3SS), and different surface polysaccharides.

Previously, we have investigated the regulation of the production of these signals at the transcriptomic level [1-4], but the role of small RNAs has been overlooked. In addition, the genome sequencing of HH103 left different gaps in plasmid d, which is the one carrying most of the symbiotic bacterial genes. To address these issues, we have carried a new genome sequencing by using PacBio technology. Concurrently, we have employed a specialized RNA-seq technique called Cappable-Seq to identify transcription start sites (TSSs) in our bacteria, complemented by Term-Seq to identify transcription terminations with the final goal of characterise the repertoire of trans sRNAs of HH103.

By integrating this data with strand-specific transcriptomic analyses that were conducted using total RNA extracted from different treatments (free-living bacteria grown either in the absence or presence of flavonoids, bacteroids of different host legumes), we compared differently expressed TSSs in bacteroids from indeterminate (*Glycyrrhiza uralensis*) and determinate nodules (*Glycine max*). The aim was to identify trans sRNAs potentially involved in the symbiotic relationships with these two kinds of legumes. We identified five candidates, three of them were differentially expressed in *G. max* bacteroids, one in *G. uralensis* bacteroids, and the remaining one was up-regulated in both types of bacteroids. Our current goal is to elucidate the putative symbiotic roles of these small RNAs by deleting the regions containing these small RNAs and conducting nodulation assays with both legumes.

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### Funding

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## Deepening in genomic features from *Trichoderma* species widely used as biocontrol agents

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### Abstract

*Trichoderma* is a genus of filamentous fungi with more than 450 species, some of them are of interest in agriculture as direct biocontrol agents (BCAs) of phytopathogens. Some strains able to colonize rhizosphere and/or showing endophytism, are exploited as indirect BCAs due to the activation of local and systemic defences in the plant, and also as biostimulants by promoting plant growth and the adaptation to adverse environmental conditions [1]. To get more knowledge about *Trichoderma*-plant interactions, we have analysed the *T. atroviride* T11 and *T. asperellum* T25 genomes. These strains have demonstrated high biocontrol properties in field assays. In addition, strain T25 increased defence against pathogenic fungus in tomato plants and the wheat tolerance to drought. DNA was sequenced using a GridION platform (Oxford Nanopore Technologies). The assembled draft genomes (depth > 100X) followed the JGI MycoCosm [2] pipeline for genome annotation by using genome annotations from closely related *Trichoderma* species present in the MycoCosm repository coupled to *de novo* predictors; and the KEGG database (Kyoto Encyclopedia of Genes and Genomes), among others, was used to perform a functional enrichment. Our analyses identified high levels of CWDE-encoding genes in the T11 strain; and secreted CAZyme families such as Glycoside hydrolases, Polysaccharide lyases, Carbohydrate esterase and Carbohydrate-binding, previously involved in *Trichoderma*-root interactions [3], were highly represented in these strains.

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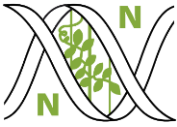
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### Funding

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## Analysis of the *Pseudomonas ogarae* F113 secretome reveals two new type vi secretion systems effectors

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### Abstract

*Pseudomonas ogarae* F113 is a model rhizobacterium considered a relevant biocontrol agent because of its ability to produce a diverse set of antibacterial and antifungals compounds such as DAPG. The genome sequence of *P. ogarae* F113 encodes three copies of Type Six Secretion Systems (T6SSs; F1-, F2- and F3-), a bacterial nanomachine involved in interbacterial competition and rhizosphere colonization. In addition to the structural elements, another five orphan VrgG protein clusters unrelated to a specific T6SS cluster are present. An in-silico analysis of F113 genomic sequence revealed genes encoding eight T6SSs effectors, some of them associated with their corresponding immunity protein. All this Effector-Immunity pairs are associated either with T6SSs structural operons or with orphan VgrG proteins. We have characterised the secretome of the wild-type strain and mutants affected in each of the T6SS structural clusters. This analysis showed that under the conditions tested, only T6SS F-1 was functioning and allowed us to identify two potentially new effectors not detected in our previous in silico analysis (*tfe9* and *tfe10*). The preliminary results revealed that Tfe9 seems to have an immunity protein and unknown function, additionally Tfe10 is orthologous to *Salmonella typhimurium* Tae4 (type VI amidase effector) without an immunity protein. Our results suggest that these effectors are necessary for the antibacterial activity of *P. ogarae* F113 against *E. coli*.

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# Nitrogenase is a major substrate of a microaerobically induced host-dependent small heat shock protein expressed by *Rhizobium leguminosarum* in pea nodules

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## Abstract

During the establishment of the symbiosis rhizobia are exposed to hostile physical and chemical microenvironments to which it must adapt to obtain effective symbiosis. Proteomic studies of bacteroids induced by *Rhizobium leguminosarum* bv. *viciae* (Rlv) UPM791 in pea and lentil plants identified more than 50 host-dependent expression proteins including stress-response proteins [1]. Among them, small Heat-shock proteins (sHsp) were identified. sHsp act as chaperones stabilizing other partially denatured proteins in response to different types of stress. One of them, RLV\_1399, was observed to be expressed at high levels in pea bacteroids. In this work, a relevant role of RLV\_1399 protein in the symbiosis of Rlv UPM791 with pea plants has been demonstrated. In addition, *rlv\_1399* gene has been shown to be transcribed from a FnrN-dependent promoter activated under microaerobic free-living conditions, consistently with the presence of two anaeroboxes in its regulatory region. Overexpression of RLV\_1399 improves microaerobic cells tolerance to the presence of hydrogen peroxide or cationic nodule-specific cysteine-rich (NCR) peptides, suggesting that this protein might protect rhizobia against oxidative stress and antimicrobial peptides present in the nodule. Results from proteomic analysis of pull-down experiments indicate that this sHsp might be a general binding protein stabilizing denatured proteins under symbiotic conditions, playing an important role in the adaptation of the bacteria against specific stress conditions inside their host. Furthermore, the identification of nitrogenase structural subunits as major sHsp interactors suggests that this protein might be required to obtain optimal levels of nitrogen fixation in symbiosis with pea plants.

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## Characterization of a novel effector from *Sinorhizobium fredii* HH103

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### Abstract

*Sinorhizobium fredii* HH103 is a nitrogen fixing bacterium able to nodulate a broad range of legume plants, although its natural host is soybean (*Glycine max*). Nod factors, surface polysaccharides and secretion systems, like the Type III Secretion System (T3SS), are the bacterial mechanisms involved in the establishment of this symbiosis [1]. Like several other Gram-negative plant-pathogenic and symbiotic bacteria, *S. fredii* HH103 utilizes a T3SS to deliver protein effectors (T3E) into their host cells. In rhizobia, these proteins are involved in the host-range determination, plant defences suppression and the nodulation efficiency. Moreover, the nodulation genes and the T3SS genes are co-regulated in these bacteria. Flavonoids and NodD induce the expression of nodulation genes and the *ttsI*, by attaching to the *nod* boxes located in the promoter region of these genes. *TtsI* is the main regulator of the T3SS genes and it attaches to the *tts* boxes located upstream the structural T3SS and T3E genes [2].

In this work we studied a novel effector, Sfe1. This effector was initially identified through *in silico* analysis and it is homologous to TAL effectors (TALE, transcription activation like effector), which have only been previously identified in phytopathogenic bacteria. Expression assays have performed in free-living bacteria to determine whether the *sfe1* gene is regulated by the T3SS key regulator, *TtsI*, and by other important symbiotic regulators, such as *SyrM*. In addition, we have demonstrated by microscopy analysis that this gene is expressed in soybean nodules. Moreover, secretion assays have conducted to determine whether Sfe1 is secreted by the T3SS machinery.

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# Root System Architecture Remodelling Triggered by *Pseudomonas* sp. CDVBN10: Molecular Insights into Growth Promotion of *Brassica napus*

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## Abstract

Since roots explore soil to acquire water and nutrients, root system architecture (RSA) optimization, by changing primary root (PR) length, lateral root (LR) structure and abundance, and root hair (RH) development, have been proposed as breeding targets for improve resource utilization, stress resilience and crop yield in modern agricultural paradigms [1,2]. Plant growth-promoting rhizobacteria (PGPR) have a considerable influence on RSA and plant growth through the production of phytohormones, volatile organic compounds, and secondary metabolites, thereby augmenting nutrient exchange surfaces and other rhizospheric effects [3]. Among these, *Pseudomonas* sp. CDVBN10 strain, initially isolated as a *Brassica napus* root endophyte, has been described as a promising PGPR for this crop [4,5]. In this work, we analysed the RSA changes induced by CDVBN10 strain in *B. napus* seedling. Additionally, we performed a differential gene expression analysis (DEA) to elucidate the molecular cross-talk during the interaction. Our results elucidate a distinctive RSA phenotype triggered by CDVBN10, characterized by enhanced LR and RH elongation and proliferation, alongside a reduction in PR length. Transcriptomic profiling revealed significant upregulation of auxin-related pathways and downregulation of genes associated with flavonoid biosynthesis and photomorphogenesis. Moreover, quantification of endogenous and exogenous indole-3-acetic acid (IAA) levels in roots revealed an increase in exogenous IAA concentration concomitant with a reduction in endogenous IAA levels, suggesting a potential mechanism of redistribution of auxin and flavonoids within roots. Notably, the observed overexpression of the transcription factor HY5 in inoculated plants leads us to hypothesize that HY5 may play a central role in mediating PGPR-induced RSA alterations, potentially maintaining a balance between auxins and plant secondary metabolism.

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## Role of T6SS of two different *Rhizobium* spp. in symbiosis with beans and peas

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### Abstract

The T6SS is a bacterial nanomachine mainly involved in bacterial competition but also in host-bacteria interactions. Genes encoding components of T6SS are present in more than 60% of symbiont rhizobia, but the relevance of the system is not well understood in this group of bacteria. Our bacterial T6SS models are from *Rhizobium etli* Mim1 (ReMim1) and from *Rhizobium ruizarguesonis* UPM1134 (UPM1134) that efficiently nodulate beans and peas, respectively. In the first one, the T6SS is active in free-living cells and in bacteroids, and a positive effect on symbiosis was shown [1]. It was analyzed whether any non-structural gene of the T6SS cluster could be responsible for the positive effect on symbiosis. ReMim1 T6SS cluster encodes three modules with potential effectors: 1) Re78-79, a new effector-immunity protein pair important in bacterial competition for bean nodulation; 2) Re81-82-83 homologues to Adaptor-Dnase-Immunity proteins from *Agrobacterium*; and 3) Re84-89 encode six proteins, mostly T6SS immunity and truncated proteins. Mutations in genes of the three modules do not affect the symbiosis with bean. This suggests that other effector genes may exist elsewhere in the genome, or that T6SS structural components could be recognized by the legume [2]. In this work, we present transcriptomic data of bean roots inoculated with the wt strain or a mutant in the T6SS structural gene, that shows the T6SS of ReMim1 is able to modulate the host immune response in the early phase of symbiosis. In the second symbiotic model, UPM1134 has an inactive T6SS both in free living and in pea bacteroids. We are currently studying whether an active T6SS in UPM1134 negatively affects pea symbiosis. These data suggest that T6SS is not only a machinery that favours bacterial competition but is also involved in the *Rhizobium*-legume compatibility depending on the species involved.

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## Understanding the molecular basis of *Sinorhizobium fredii* HH103-soybean compatibility conferred by bacterial secreted proteins

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### Abstract

To establish N-fixing symbiosis, rhizobia bacteria must bypass plant immunity [1]. Like plant pathogenic bacteria, many rhizobial strains suppress plant defence by delivering effector proteins into the host cell via Type III Secretion System (T3SS) [2]. Conversely, some rhizobia T3SS effectors can also directly activate the plant symbiotic program [3]. Unravelling how plant proteins recognize and respond to T3SS effectors is key to unlocking novel insights into symbiotic compatibility.

*Sinorhizobium fredii* HH103 is symbiotically compatible with the agronomically-important soybean Williams 82 cultivar (W82). However, using a subset of 55 soybean genetically diverse lines, we have demonstrated that HH103 cannot establish symbiosis with all soybean cultivars. For example, the soybean JL2 cultivar inoculated with HH103 did not form any nodules. We hypothesized that HH103 gain and/or loss of nodulation might be driven by evolution processes through the recognition of T3SS effectors by the host. We constructed loss-of-function mutants in the T3SS ( $\Delta T3SS$ ) and the T3SS secreted NopM effector ( $\Delta nopM$ ) of HH103. We observed both effector-dependent gain or loss of nodulation phenotypes in both W82 and JL2, supporting that T3SS effectors are involved in HH103-soybean compatibility.

It is unknown whether these phenotypes are result of the activation of the plant symbiotic program or the suppression of plant defence. To determine the role of NopM in the plant cell, we performed *in vitro* Size Exclusion Chromatography and Small-angle X-ray scattering (SAXS), and *in vivo* bacterial two-hybrid assays and observed the formation of homomeric proteins. Additionally, unravelling the NopM targets will provide novel insights into the molecular mechanisms driving rhizobia-host compatibility. Thus, we are currently performing Genome Wide Association Studies (GWAS) to identify soybean *loci* involved in HH103-soybean compatibility, and IP-MS to identify NopM targets in W82.

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### Funding

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## Impact of iron availability on arbuscular mycorrhiza development and function

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### Abstract

Iron (Fe) is an essential micronutrient for the correct development and survival of all organisms and influences the outcome of many cross-kingdom interactions. Despite being abundant in most soils, it is poorly available to plants. To cope with Fe deficiency, plants have evolved a suite of adaptive strategies aimed at increasing its acquisition. A widespread strategy engaged by plants to overcome nutrient deficiencies is the formation of a mutualistic symbiotic interaction, referred to as arbuscular mycorrhiza (AM), with certain soil-borne fungi belonging to the subphylum Glomeromycotina within the phylum Mucoromycota. Arbuscular mycorrhizal fungi are obligate biotrophs that colonize the root cortex and develop an external mycelium that overgrows the rhizosphere. This hyphal network represents, therefore, an adaptation strategy to increase the supply of mineral nutrients to the plant. Additionally, AM symbiosis enhances tolerance to pathogens by priming the plant's immune system, a phenomenon known as mycorrhiza-induced resistance (MIR). The aim of this work was to analyse the effect of AM on Fe homeostasis and the impact of Fe deficiency on AM establishment and function. Using the *Solanum lycopersicum*-*Rhizophagus irregularis* mycorrhizal system, we observed a decrease in the translocation of Fe from roots to shoots in mycorrhizal plants. This suggests that the AM fungus *R. irregularis* acts as an iron sink. Iron deficiency resulted in a reduction in AM colonization and symbiotic phosphate transport. Furthermore, Fe deficiency increased plant tolerance to *Botrytis cinerea* infection in non-mycorrhizal plants. We are currently analysing the impact of Fe nutrition on MIR to *B. cinerea*. The significance of Fe homeostasis in AM is being confirmed by using the Fe-inefficient mutants *fer* and *chloronerva* of tomato.

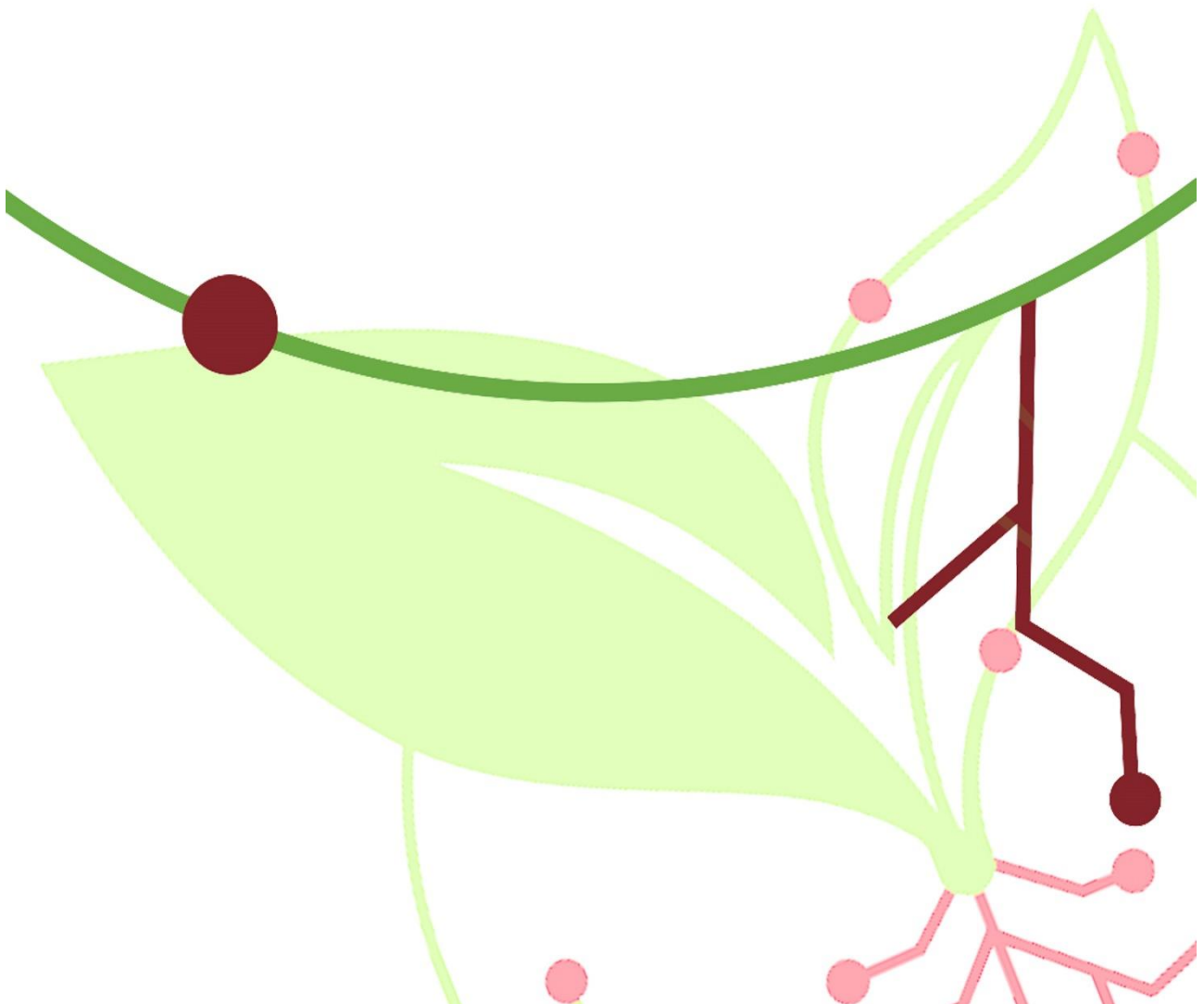
### Funding

This work was supported by grant PID2021-1255210B-I00 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe", by the "European Union".



## Session 5

# Symbiotic nitrogen fixation





## Analysis of extracellular membrane vesicles from *Rhizobium tropici* CIAT 899 in the presence and absence of the inducing- flavonoid apigenin

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### Abstract

Rhizobia are a group of soil proteobacteria capable of establishing a symbiotic interaction with legumes. These bacteria can fix atmospheric nitrogen into ammonia within specific plant root organs called nodules. The rhizobia-legume interaction begins with flavonoids exuded by the plant roots, initiating a complex molecular dialogue. In response, signaling molecules called Nod factors (NFs) are secreted by the bacteria. These NFs are sensed by specific plant receptors, leading to the colonization of the root by rhizobia and the development of nodules, where rhizobia can fix atmospheric nitrogen [1].

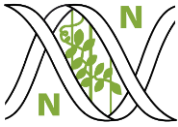
The legume-rhizobia symbiosis is well-characterized to date. However, a new player has recently emerged that could be involved in the symbiotic process: the extracellular membrane vesicles (EMVs) released from rhizobia [2, 3]. EMVs are nanospherical lipidic structures released from the cell envelope into the external environment, playing crucial roles in various biological processes. For example, these nanocompartments serve as transport vessels and can elicit responses in host target cells. EMVs from certain plant pathogenic bacteria carry molecules that may trigger plant immune responses [4]. Regarding symbiosis, it has been demonstrated that EMVs produced by some rhizobia carry NFs in the presence of an inducible flavonoid [2]. Here, we isolate and characterize the EMVs released from *Rhizobium tropici* CIAT 899 in the presence and absence of the flavonoid apigenin. Our results indicate that the presence of apigenin increases the formation of CIAT 899 membrane vesicles. Furthermore, we investigate whether this increase in vesicle formation is due to the effect of the apigenin flavonoid or as a consequence of NFs synthesis, that could be could potentially disrupt the stability of the external lipid membrane.

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### Funding

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## Unrestricted intimate relationship: proteomic analysis of extracellular membrane vesicles from bacteroids

Ayala-García, P.<sup>1</sup>, Herrero-Gómez, I.<sup>1</sup>, Jiménez-Guerrero, I.<sup>1</sup>, Müsken, M.<sup>2</sup>, Van Ham, M.<sup>3</sup>, Ollero, F.J.<sup>1</sup>, Borrero de Acuña, J.M.<sup>1</sup>, Pérez-Montaño, F.<sup>1\*</sup>.

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### Abstract

Bacteria and eukaryotes secrete extracellular membrane vesicles (MVs) into the extracellular milieu to preserve and transport elevated concentrations of a given cargo across long distances. MVs encapsulate metabolites, DNA, RNA, and proteins, whose abundance and composition fluctuate depending on environmental cues. Importantly, MVs are involved in eukaryote-to-prokaryote communication owing to their ability to navigate different ecological niches and exchange cargoes between the two domains. Amongst the different bacterium-host relationships, rhizobium-legume symbiosis is one of the closest known to nature. A crucial developmental stage of symbiosis is the formation of N<sub>2</sub>-fixing bacteroids, which are endocytosed rhizobia confined by the plant-derived membrane inside of the root cells. The unrestricted interface between the two membranes is the symbiosome space. To date many molecular aspects of symbiosis have been extensively studied, but the possibility of interdomain molecule trafficking by MVs in the symbiosome space was not questioned so far. Here we unveil intensive MV trafficking within the symbiosome interface of several rhizobium-legume systems by developing a robust MV isolation procedure. We analyse the bacterial MVs-encased proteomes encountered in the symbiosome space of several bacterium-host partnership uncovering both conserved and differential traits of every symbiotic system. This study opens the gates for designing MV-based biotechnological tools suitable for sustainable agriculture.

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### Funding

EMERGIA20\_00048 from the Junta de Andalucía, Consejería de transformación económica, industria, conocimiento y universidades; the ProyExcel\_00450 from the Junta de Andalucía, Consejería de Universidad, Investigación e Innovación; and the PID2021-122395OA-I00 from the Agencia Estatal de Investigación of the Spanish Ministry of Science and Innovation.



## Harnessing Soybean Genetic Diversity and Rhizobia Effectors to enhance Nitrogen-fixing symbioses

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### Abstract

To meet the EU's demand for sustainable soybean proteins, efforts are underway to boost domestic production and reduce reliance on Nitrate fertilizers [1]. Rhizobia present a sustainable solution to enhance soybean yield by fostering symbiotic N-fixing relationships. Many rhizobia employ a Type III Secretion System (T3SS) to deliver effector molecules into host plants, where they either activate symbiotic signaling or suppress plant defence responses, promoting nodulation. However, certain plant genotypes harbour specific Resistance (*R*) Proteins capable of detecting rhizobial effectors, triggering immunity and limiting colonization. Understanding these dynamics is key to optimizing N-fixing interactions with soybeans. NopI effector from rhizobia *Sinorhizobium fredii* HH103 promotes nodulation in commercial soybean cultivar W82 (*Glycine max*) [2] but restricts nodulation in wild soybean cv. JL2 (*Glycine soja*). Thus, we hypothesized that NopI may promote symbiotic signaling or suppress defence in some varieties (like W82) while being recognized by *R* proteins in others (like JL2). To determine soybean genes involved in NopI-dependent nodulation (and enhanced nodulation overall) we are employing Genome-Wide Association Studies (GWAS). This analysis harnesses soybean genetic diversity, integrating phenotype and genotype data to identify candidate genetic loci associated with a phenotype. We have curated a unique soybean collection (over 700 SNP-genotyped cultivars) representing global diversity [3, 4]. This collection remains unexplored in nodulation and includes cultivars highly relevant to European agriculture. After refining our focus to 200 genotypes based on population structure and phylogenetic relationships, nodulation assays with a subset revealed significant nodule number variation, making this population highly promising for further analyses. Additionally, we are performing complementary approaches to identify soybean proteins targeted by NopI, including immunoprecipitation coupled with Mass Spectrometry (IP-MS) and yeast-2-hybrid analyses. Together with these analyses, we aim to unravel the interplay between rhizobia effectors and soybean, crucial for optimising N-fixing interactions for sustainable soybean production.

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### Funding

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## Several UMAMIT plant amino acid transporters are required for efficient symbiotic nitrogen fixation in *Medicago truncatula*

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### Abstract

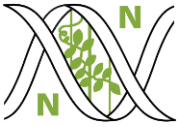
Legumes are able to establish symbiotic interactions with soil bacteria, collectively known as rhizobia, to acquire nitrogen, essential for their growth. This interaction culminates in the formation of a new nitrogen-fixing organ, the root nodule, within which the bacteria fix atmospheric nitrogen into ammonia and transfer it to the plant in exchange of carbon sources. Rhizobia infection and nodule organogenesis are tightly regulated by the plant and controlled by main plant transcription regulators [1]. Likewise, for an efficient colonization by nitrogen-fixing bacteria, the plant must restructure its metabolism to provide the bacteria with organic substrates for its multiplication and colonization. Particularly, the bi-directional amino acids transport between the plant and the bacteria is essential for an effective symbiosis [2]. In this sense, we explore the role of a new plant protein family involved in amino acid bi-directional transport called UMAMIT (Usually Multiple Acids Move In and out Transporters) [3]. Previous works suggest that amino acid transport mediated by transporters of this family could be a key point to maintain an adequate infection and nodular development process [3]. In this work, we carried out an expression analysis of the 88 UMAMITs described in *M. truncatula* using several public RNA-seq data sets. The results show several candidates whose expression is correlated with nodulation with a likely redundant function. We physiologically characterized a CRISPR/Cas9 triple mutant, targeting *MtUMAMIT14*, -17 and -36. Our results indicate that these genes are required for the establishment of an effective nitrogen fixation, with the triple mutant presenting lower biomass and N deficiency symptoms.

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## Metabolome and hormone profiling of hemoglobin *glb1-1* and *glb2-1* mutants in *Lotus japonicus*

Esquinas-Ariza, R.M.<sup>1\*</sup>, Matamoros, M.A.<sup>1</sup>, Román, Á.<sup>1</sup>, Pérez-Rontomé, C.<sup>1</sup>, Zamarreño, Á.<sup>2</sup>, Sandal, N.<sup>3</sup>, Perez de Souza, L.<sup>4</sup>, García-Mina, J.M.<sup>2</sup>, Fernie, A.<sup>4</sup>, Becana, M.<sup>1</sup>

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### Abstract

Legumes contain two main types of hemoglobins: symbiotic (leghemoglobins) and non-symbiotic (phytoglobins, Glbs). The latter are categorized in three classes based on phylogenetic and biochemical properties. *Lotus japonicus* has two class 1 Glbs, two class 3 Glbs, and two rare hemoglobins (Glb2-1 and Glb2-2) with intermediate features of leghemoglobins and class 2 Glbs [1]. At least one function of Glb1-1 is to contribute to nitric oxide homeostasis [2] whereas the function of Glb2-1 is completely unknown. To gain insight into the functions of both hemoglobins (to simplify, Glb1 and Glb2) under (non)symbiotic conditions, we generated homozygous single mutants *glb1* and *glb2* as well as the double mutant *glb1/2*. The three mutants showed a delayed growth phenotype, but an additive effect was not observed in *glb1/2* compared with the single mutants. Primary metabolites and phytohormones were profiled in non-nodulated plants (NIT; 5 mM NH<sub>4</sub>NO<sub>3</sub>) and nodulated plants (NOD; 0.25 mM NH<sub>4</sub>NO<sub>3</sub>) at 7 weeks from germination. Leaves, roots, and nodules, especially of *glb1/2*, showed altered levels of amino acids, fatty acids, and sugars. In leaves, abscisic acid (ABA) decreased in *glb2* and *glb1/2* in NIT but remained unchanged in NOD; salicylic acid (SA) increased in *glb1* of NIT and NOD; and jasmonic acid (JA) and JA-Ile decreased in *glb2* and *glb1/2* of NIT and in *glb2* of NOD. In roots of NIT, ABA and the cytokinins isopentenyladenine and its riboside decreased. In nodules, indole acetic acid (IAA) and JA decreased in the three mutants and SA increased in *glb1*. Thus, phytohormone profiles depend on the mutant, plant organ, and N source. Based on these results, in the next step we will tackle regulatory mechanism(s) in leaves and nodules, with the focus being on the relation of Glb1-1 with SA and of Glb2-1 with JA.

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### Funding

R.M.E-A. is the recipient of a FPI fellowship from MCIN (PRE2021-099765). This work was funded by grants from MCIN/AEI/10.13039/501100011033 (PID2020-113985GB-I00) and Gobierno de Aragón (group A09\_23R).



## Unveiling new metallo-proteins in *Medicago truncatula* nodules

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<sup>1</sup> Centro de Biotecnología y Genómica de Plantas (UPM-INIA/CSIC), <sup>2</sup> Laboratory of Plant Biophysics and Biochemistry, Biology Centre, Czech Academy of Sciences, <sup>3</sup> Faculty of Sciences, University of South Bohemia, <sup>4</sup> Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria, y de Biosistemas, Universidad Politécnica de Madrid

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### Abstract

Symbiotic nitrogen fixation carried out in legume root nodules relies in a steady supply of metals. In just 5% of the total plant biomass, we can find up to a third of the total plant metal content [1]. This makes nodules a great model system to identify new plant metalloproteins. To satisfy nodule requirements, transporters must transfer metals to cytosolic shuttling proteins (metallochaperones) to be delivered to the final accepting metalloproteins. As “free” metals are toxic [2], this must be carried out through protein-protein interactions, *i.e.* as a “bucket-brigade”. Therefore, metalation relies in the compatibility of docking interfaces and not simply relative metal binding affinities. We hypothesize that by identifying the proteins interacting with metal transporters, we will be able to determine new metallochaperones and new downstream metalloproteins. As a proof-of-concept, by characterizing a nodule-specific *Medicago truncatula* Cu<sup>+</sup>-chaperone [3], NCC1, and determining its interactors, we have identified novel nodule-specific Cu<sup>+</sup>-binding proteins. Similarly, by using known nodule iron transporters as baits, we have selected a subgroup of uncharacterized iron-binding proteins with a likely role in iron transfer.

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## Structure and spectral properties of LjGlb2-1, an unusual hemoglobin of the legume *Lotus japonicus*

Becana, M.<sup>1\*</sup>, Villar, I.<sup>1</sup>, Minguillon, S.<sup>1</sup>, Esquinas-Ariza, R.M.<sup>1</sup>, Pérez-Rontomé, C.<sup>1</sup>, Martínez-Júlvez, M.<sup>2</sup>

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### Abstract

Plant hemoglobins are classified into symbiotic, including leghemoglobins (Lbs) of legume nodules, and phytohemoglobins (Glbs), ubiquitous in tissues. The *L. japonicus* genome encodes 3 Lbs, 4 typical Glbs, and 2 additional hemoglobins that we termed Glb2-1 and Glb2-2. These two share properties of Lbs and class 2 Glbs. Thus, Lbs show pentacoordination (C5) in ferric (3+) and ferrous (2+) forms and class 2 Glbs show hexacoordination (C6) in both heme oxidation forms, whereas Glb2-1 is C6 in 3+ and C5 in 2+. Also, like Lbs, Glb2-1 is expressed mainly in nodules, but, unlike Lbs, Glb2-1 and Glb2-2 contain one Cys residue. We have determined the 3D structure of Glb2-1. The protein (152 aa) was expressed in recombinant form with a Strep-tag (12 aa) at the N-terminus and was purified by streptavidin-affinity chromatography. This was used to successfully produce high-quality crystals that diffracted at 1.6 Å in the B13 (XALOC) beamline of the ALBA synchrotron. The asymmetric unit contains two coordinated Glb2-1 molecules, each with one heme group and one cyanide anion, and also 183 water molecules. Site-directed mutagenesis was conducted and the UV-visible spectra of the proteins C76A, H75V and Y42F were examined. C76A shows similar spectra to the wild-type protein, but unlike it, is unable to retain O<sub>2</sub>. Thus, the single Cys of Glb2-1 is involved in the stability of O<sub>2</sub> binding. However, the most interesting findings are that H75V remains C6 in 3+ but becomes C6 in 2+, and that Y42F is C5 in both 3+ and 2+ forms. This indicates that Y42, and not H75, is responsible for the C6 state of ferric Glb2-1. Furthermore, this C6 is due to the OH of tyrosine because when it is replaced by phenylalanine, coordination becomes C5. Currently, we are modeling the homologous hemoglobin Glb2-2 (154 aa) using Glb2-1 as a template.

### Funding

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## Expression of leghemoglobins and phytoglobins in response to nutritional stress in *Lotus japonicus*

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### Abstract

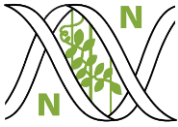
The genome of *Lotus japonicus* encodes nine hemoglobins: three leghemoglobins (Lb1, Lb2, Lb3) that are expressed in nodules, four nonsymbiotic hemoglobins or phytoglobins (Glb1-1, Glb1-2, Glb3-1, Glb3-2) that are widespread in plant tissues, and two unusual hemoglobins (Glb2-1, Glb2-2) that display intermediate features of the two other groups. To gain insight into hemoglobin functions, here we have focused on the transcriptional response of *Lb* and *Glb* genes in leaves and nodules of plants subjected to macronutrient deficiency (-N, -Ca, -K, -P, -S). First, non-nodulated plants (2.5 mM NH<sub>4</sub>NO<sub>3</sub> for 3 weeks) and nodulated plants (0 N for 4 weeks) were grown in plates to set up the deficiency conditions by ICP analysis of leaves. We also measured growth parameters of leaves and nodules. Second, we determined *Glb* and *Lb* expression profiles. The most relevant results are as follows (data refer to mRNA levels). *Leaves of non-nodulated plants*: large increases of Glb1-1 and Glb3-1 in -K; decreases of Glb1-1 and Glb1-2 in -Ca; disappearance of Glb1-1 in -N and large decrease in -P; large decrease of Glb1-2 in -N; and decrease of Glb3-2 in -S. *Leaves of nodulated plants*: no effects except an increase of Glb1-1 in -K and increases of Glb3-2 in -Ca and -K. *Nodules*: only minor effects. Thus, nodulated plants seem to be more tolerant to deficiencies than are non-nodulated plants in terms of hemoglobin expression profiles. The next step of our study will be focused on regulatory mechanisms of Glb1-1 mRNA and protein in leaves of non-nodulated plants under K, Ca, P and N deficiencies. Special attention will be paid to our hypothesis that at least some of these changes are mediated by nitric oxide (NO). For this purpose, we will perform immunoblots and will measure parameters of nitrate metabolism such as nitrate and nitrite reductases (mRNAs and activities) and nitrite and NO contents.

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## New regulators involved in the ammonium-mediated inhibition of nitrogen fixation in *Medicago truncatula*

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### Abstract

The culture of legume crops promotes the entry of N into agrosystems thanks to the symbiotic nitrogen fixation process. However, when N availability is high in soils, the process of nodule establishment and functioning is rapidly shut down. Understanding how legumes sense and signal their N supply status to regulate nodulation is key for developing more sustainable agriculture using lower inputs of chemical fertilizer and applying good practices for organic N amendments. In recent years, the involvement of transcription factors belonging to the nodule inception (NIN) family (a.k.a NIN-like proteins, NLPs) in nitrate signaling has been demonstrated in several plant species [<sup>1, 2</sup>]. On the other hand, the plant hormone ethylene has been related to this nitrate response. However, the role of ammonium, one of the major sources of inorganic N and organic amendments are applied to soils, has been less studied. Ammonium is taken up by the roots via high- and low-affinity transport systems (HATS and LATS, respectively). The HATS Ammonium Transporters (AMT) family has not been described in detail in legumes, and the response of its members when ammonium is applied to N-fixing legume plants is unknown. Our first goal is to identify members of this gene family in the model legume *Medicago truncatula* Gaertn. cv. Jemalong A17, using a reciprocal BLAST approach against the latest genome version (v5). Once identified, we will use existing RNA-seq expression data sets to investigate whether specific ammonium transporters are regulated in a rhizobium-dependent manner. Finally, we will explore the link between ammonium signaling and the activation of ethylene biosynthesis [3] in N-fixing plants. To do so, we will compare the responses of N-fixing plants of wild-type *M. truncatula* A17 and the ethylene-insensitive mutant *sickle* (*skl*) to ammonium application at the phosphoproteomic level.

### References

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