PROYECTO DE INICIACIÓN A LA INVESTIGACIÓN DE INNOVACIÓN EN SECUNDARIA EN ANDALUCÍA

Estación Experimental del Zaidín

HIGH SCHOOL STUDENTS FOR AGRICULTURAL SCIENCE RESEARCH

VOL. IV MAY 2015

IN THIS VOLUME:
PROCEEDINGS OF THE 4TH CONGRESS PIIISA-CSIC (2015)
WINNING ARTICLE OF THE III EEZ SCIENCE AWARD (2014)

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CSIC CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

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High School Students for Agricultural Science Research

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PREFACE

This new edition of the journal "High School Students for Agricultural Science Research" includes four examples of the research activity carried out by secondary and primary school students in two different scenarios.

The first three articles correspond to the proceedings of the IVth PIIISA-Estación Experimental del Zaidín congress, which takes place the 5th of May 2015. This is a consolidated activity, organised in cooperation between the Education Delegacy of the Andalusian regional government, the Spanish National Research Council (CSIC) and the University of Granada.

In the present edition, three projects with a total of 18 students of the 3rd and 4th levels of secondary education, and 1st level of the high school diploma, from 11 bilingual schools of the province of Granada, have been developed at the Estación Experimental del Zaidín (CSIC, Granada, Spain). The main thematic focus of these proposal include genetic approaches to the determination of the spontaneous mutation rate of a soil bacterium and to the taxonomic identification of soil bacteria able to inhibit the growth of pathogens, as well as to determine the anti-inflammatory capacity of a component of the olive subproducts in diabetic patient.

Finally, the work developed through the 2013/2014 academic year, which deserved the allocation of the IIIth EEZ-CSIC award to the promotion of scientific careers was also included in this volume. This last work describes several observations as regard to the effects of mycorrhization in lettuce plants.

All manuscripts published here have been prepared in cooperation with researchers of the centre, or with professors of the educative institution, and reflect the multidisciplinary character of the Institute. Furthermore, they evidence what is even more representative, consisting in the capacity of work of these your researchers filled of enthusiasm. It is amazing the quick adaptation of these youths, eager of wearing the lab coats, handle those "sophisticated apparatuses", prepare de necessary reagents for an experiment, run to see the results, and rejoice or despair a little bit for what they have obtained.

From the standpoint of the researchers, it becomes rewarding witnessing such interest, realising that our work proposals are understood, and are able to generate the enthusiasm of these students, and that an idea is able to be developed in just a few sessions, no matters if the strategy has to be changed several times. If there is anyone that could possibly display such flexibility, they are these youngsters.

It is therefore extremely pleasant to see the result of the research presented by the authors in the form of a research article fulfilling all the criteria of a scientific publication, which also includes the personal appraisals of the students, thus enriching the perspective of this collaboration.

May these words of mine on behalf of the teachers and researchers involved in this project, to bring our immense gratitude to this new sap for keeping in constant motion the nutrients of this plant that is the science, allowing its development and the renewal of its tissues.

Juan de Dios Alché Ramírez

PREFACIO

Esta nueva edición de la revista "High School Students for Agricultural Science Research" engloba cuatro ejemplos de la actividad investigadora de estudiantes de secundaria y primaria en dos tipos de escenarios.

Los tres primeros artículos corresponden a los proceedings del IV congreso PIIISA-Estación Experimental del Zaidín, que tiene lugar el 5 de Mayo de 2015. Se trata de una actividad consolidada, organizada en colaboración entre la Delegación de Educación de la Junta de Andalucía de Granada, el Consejo Superior de Investigaciones Científicas (CSIC) y la Universidad de Granada.

En la edición de este año, tres proyectos, con un total de 18 estudiantes de 3º y 4º de ESO y 1º de Bachillerato, de 11 institutos bilingües de la provincia de Granada se han desarrollado en la Estación Experimental del Zaidín (CSIC, Granada, España). La temática de las propuestas incluye aproximaciones genéticas a la determinación de la tasa de mutación espontánea de una bacteria del suelo, y a la identificación taxonómica de bacterias del suelo capaces de inhibir el crecimiento de patógenos, así como la determinación de la capacidad antiinflamatoria de un componente de los subproductos de la aceituna en pacientes diabéticos.

Finalmente, se describe un trabajo desarrollado a lo largo del curso 2013/2014, y que mereció la concesión del III Premio EEZ-CSIC para el fomento de las vocaciones científicas. En este último trabajo se describen diversas observaciones sobre los efectos de la micorrización en plantas de lechuga.

Todos los manuscritos publicados han sido realizados en colaboración con investigadores del centro o con los profesores de los institutos implicados, y reflejan el carácter multidisciplinario del Instituto. Además evidencian lo que es más representativo aún, que es la capacidad de trabajo de estos jóvenes investigadores repletos de entusiasmo. Es asombrosa la rápida adaptación de estos jóvenes, ávidos por ponerse las batas, manejar los "sofisticados aparatos", preparar los reactivos necesarios para un experimento, ir corriendo a ver los resultados, y alegrarse o desesperarse un poco por lo que han obtenido.

Desde el punto de vista de los investigadores nos resulta gratificante ver ese interés, darnos cuenta de que los trabajos que proponemos se entienden y son capaces de entusiasmar a estos estudiantes y que en sólo unas sesiones, una idea es capaz de desarrollarse, no importa si hay que cambiar la estrategia unas cuantas veces. Si hay alguien que puede mostrar esa flexibilidad, son estos chicos.

Es por tanto tremendamente agradable ver el resultado de las investigaciones presentado por los autores en la forma de un artículo de investigación con todos los criterios de una publicación científica, que además contiene las valoraciones personales de los estudiantes, lo que enriquece la perspectiva de esta colaboración. Sirvan estas palabras mías en representación de los profesores e investigadores participantes en este proyecto, para aportar nuestro inmenso agradecimiento a esta savia nueva por mantener en continuo movimiento los nutrientes de esta planta que es la ciencia permitiendo su desarrollo y la renovación de sus tejidos.

Juan de Dios Alché Ramírez

Proceedings of the 4th Congress PIIISA-CSIC

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Granada. May. 5, 2015

MY ENEMY`S FOE IS MY FRIEND: DISCOVERING BACTERIAL BIOCONTROL AGENTS AGAINST PLANT PATHOGENS

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SUMMARY

The use of chemical compounds has been the usual way of combating plant diseases caused by pathogens or pests. However, beneficial microorganisms with the ability to challenge pathogenic fungi or bacteria constitute an interesting alternative (called biocontrol) that could help reduce the release of toxic chemicals in the environment. In this project, we have searched for soil bacteria with biocontrol capacities. We have isolated several bacterial strains that inhibit growth of certain pathogens. We have tested their effect on plant health, identifying two strains with plant growth promoting effect. By PCR amplification and sequencing of a fragment of their 16S rDNA, we have been able to make a preliminary classification of four of the strains. Three correspond to the genus *Pseudomonas* and one to *Enterobacter*.

INTRODUCTION

Plant infection and disease

Plant diseases play an important role in the reduction of agricultural and forestry resources. Alterations caused by pathogens can be related to synthesis or utilization of nutrients, or to mineral and water uptake, or to direct damage to plant tissues, resulting in reduced crop production. Damage can be due to very different organisms, including microorganisms: viruses, bacteria and fungi, and can affect the aerial parts of the plant or the base of the stem and the roots, depending on the infecting agent.

Fungal pathogens can develop their mycelia on the outside of the plant, with only a few hyphae penetrating in the plant tissue (e.g. *Oidium*; Figure 1A), or inside the parasitized plant. Penetration can be by directly breaking the epidermis or through wounds or natural openings such as stomata. Important fungal pathogens include:

- *Phytophtora*: it causes spots with an oily aspect, lesions in the intesections between branches and brown round patches on fruits and their peduncules. The base of the stem is also affected, causing external decay and size reduction. Finally, the root is attacked.
- *Fusarium*: it invades the vascular system through the roots, without the need for a wound being present. This causes yellowing of the basal leaves and progressive wilting.

- *Pythium*: this fungus infects at high temperatures, causing lesions in the roots and stem base.
- *Rhizoctonia*: it causes damage to stems, roots and fruits. In solanaceae, it produces softening of the base of the stem and spots in the fruits with progressive browning and rotting.

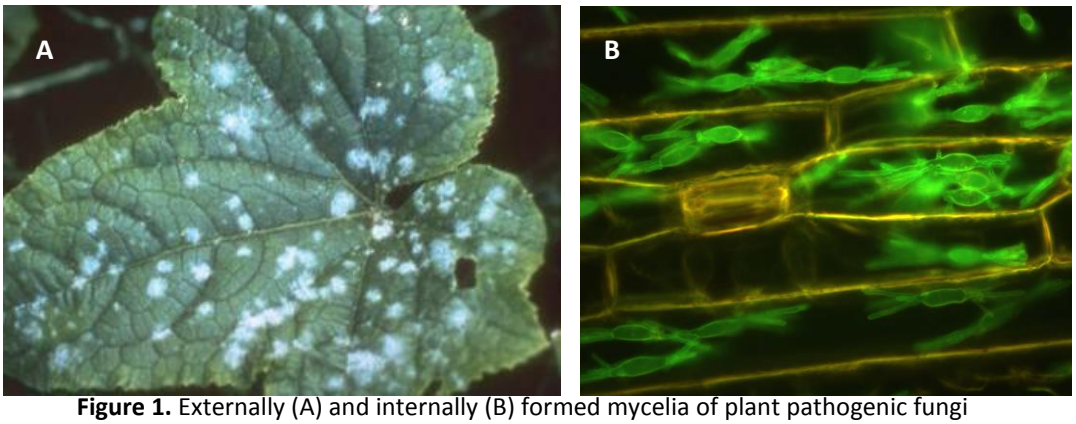

Figure 1. Externally (A) and internally (B) formed mycelia of plant pathogenic fungi

Bacterial pathogens usually attack the plant through natural openings or wounds. In this work we have focused on the following species:

- *Pseudomonas syringae*: it can infect a wide range of species, depending on the pathovar. Specific strains are assigned to one of the over 50 known pathovars based on their ability to infect different plant species [1]. *P. syringae* secretes virulence factors into the plant cell, and it can also produce ice nucleation, causing tissue damage (usually on aerial parts).
- *Ralstonia solanacearum:* it is a widely distributed pathogen found in tropical, subtropical, and some temperate regions of the world. The species has a very broad host range and infects hundreds of species in many plant families. This organism is the causal agent of brown rot of potato, bacterial wilt of tomato, tobacco, eggplant, and some ornamentals [2].
- *Dickeya dadantii* (formerly called *Erwinia carotovora*): It causes plant diseases such as necrosis, blight and "soft rot," which is a progressive tissue maceration. *D. dadantii* produces pectinases that are able to macerate and break down the plant cell wall material. This exposed part of the plant releases nutrients that can facilitate bacterial growth. Commonly infected plants include potato tubers, bulbs of vegetables, and ornamental crops [3].

Figure 2. Damage caused by *P. syringae* (left), *R. solanacearum* (center), and *D. dadantii* (right)

Biocontrol and plant growth promoting bacteria

For decades, the control of plant diseases has been done using chemical compounds. The abuse of these products can result in health risks for consumers and cause problems of environmental contamination. Their toxicity and persistence in the environment has led to the prohibition of several pesticides in many countries. Besides, there is an increasing demand in Europe for vegetables that have not been treated with chemicals. However, the production needs and the economic risks of a potential crop loss, makes it necessary to find alternative treatments, such as biocontrol: the use of microorganisms to combat plant pathogens or pests. Promising results have been obtained with plant growth promoting bacteria (PGPB), a group of different species that can increase plant development and crop production [ref]. The best known include species of *Rhizobium*, *Pseudomonas*, and *Azospirillum*. PGPB can be classified in two groups [4]:

- PGPB that influence plant nutrition. The mechanisms can be through their own metabolism (phosphate solubilization, hormone production or nithrogen fixation), altering the metabolism of the plant (hormones, enzimatic activities, stress resistance), increasing water and mineral uptake, or helping other beneficial microorganism for a combined action.
- PGPB with biocontrol activity. These promote plant growth by supressing phytopathogens, either directly (antibiotic production), or indirectly (competition for nutrients, iron sequestration, etc).

OBJECTIVES

In this work our objective was to isolate and identify potential PGPB with biocontrol activity against bacterial pathogens.

MATERIALS AND METHODS

Isolation and growth of soil bacteria

Soil samples obtained from the gardens of the EEZ were collected in 50 ml tubes. 15 ml of M9 medium were added to each tube and glass beads were added to mechanically separate the bacteria from soil particles. The tubes were then shaken for 2 minutes using a vortex. 100 µl of each sample were inoculated on Petri plates with M9+glucose (0.5%) as culture medium. For each sample, three plates were used and incubated for 24-48h at 30°C, 25°C, or room temperature. Cycloheximide was added to one of the plates to prevent the growth of fungi. For routine growth of bacterial cultures either in liquid or in 1.5% agar plates, rich medium (LB) was used.

Pathogen inhibition assays

To test the inhibitory effect of bacteria isolated from soil, three plant pathogens were chosen: *Ralstonia eutropha*, *Pseudomonas syringae* and *Dickeya dadantii*. Liquid cultures of each pathogen were inoculated as lawns on Petri plates with LB medium, and colonies of soil bacteria were picked onto each lawn. Alternatively, liquid cultures were used to spot 10 µl onto the lawn. The appearance of growth inhibition halos was recorded after 24h of incubation at 30°C.

Gram stain

Selected bacteria were grown in liquid LB medium. 2 µl of each culture were placed on a microscope slide and fixed with heat. The samples were stained as follows:

- Stained with crystal violet 1', and rinsed with distilled water.
- Incubated with lugol 1', and rinsed.
- Discolored with acetone for 20" and rinsed.
- Stained with safranin 30", rinsed and allowed to dry.

Slides were then observed under the microscope. Those stained red are Gram + and those stained purple are Gram -.

Plant growth assays

Selected bacteria were grown in liquid LB medium. 10 µl of each culture were diluted in 4 ml of M9 salts and this suspensions were used to inoculate corn seeds by incubating 4 seeds/suspension for 1 h at room temperature. Inoculated seeds were planted in microcosms containing commercial peat. Two seeds were inoculated per microcosm and duplicates were made for each bacterial strain. A control without added culture was prepared in the same way. Plants were kept in a growth chamber with cycles of 16 h light (22°C) / 8 h darkness (16°C), and watered as demanded. After 8 days plants were collected, removing soil from the roots by shaking and immersion, and different parameters were recorded: root and shoot length and total weight. Results are averages and standard deviations of the two independent microcosms (4 plants per treatment).

Polymerase Chain Reaction (PCR)

To identify the selected microorganisms, we performed PCR amplification of part of the 16S ribosomal DNA. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Universal 16S primers containing conserved sequences were used for amplification, with colonies of the different bacteria as starting material for template. The PCR conditions were as follows:

Initial denaturing	95°C	5'	
| Denaturing	95°C	30''	|
| Annealing	48°C	30''	| × 30 cycles
| Extension	72°C	1' 30''	|
Final extension	72°C	5'	

Electrophoresis

Electrophoresis is a technique for separating molecules according to their mobility in an electric field, and it was used to check the results from PCR amplification. 5 µl from the PCR reaction were run in a 0.8% agarose gel in TAE buffer. The gel was stained with ethidium bromide and visualized under UV.

Sequence analysis

Sequencing of 16S rDNA was done at the IPBLN sequencing facility. Sequences were analyzed and compared with the SILVA databases using SINA Alignment Service (http://www.arb-silva.de/aligner/), with the default parameters.

RESULTS AND DISCUSSION

Isolation of soil bacteria

We collected six samples of soil around plant roots obtained from different locations in the gardens of the Estación Experimental del Zaidín. 15 ml of M9 medium were added to each sample and bacteria were detached from the soil by shaking the mix with glass beads for 2 minutes. The suspension was allowed to settle and 100 µl were spread on M9 agar plates with glucose as carbon source, in triplicate. One set of the plates contained cycloheximide, which is an inhibitor of protein synthesis in eukaryotes, to prevent fungal growth. The plates were incubated overnight at 30°C, 25°C or room temperature. Examples of the plates corresponding to 30°C and 25°C are shown in Figure 1. We note that there were significant differences betwwen samples in terms of the number of culturable bacteria recovered. Also, at higher temperatures the colonies grow more easily and the plates containing cycloheximide favor the reproduction of bacterial colonies.

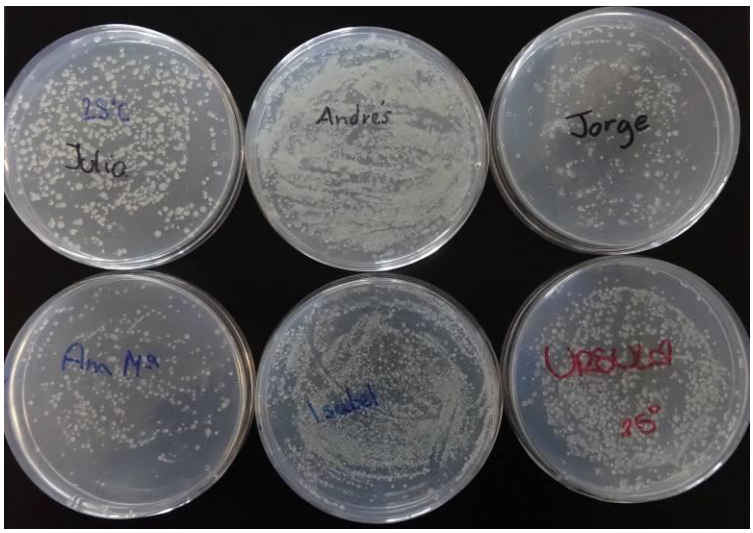

Figure 1.
Isolation of soil bacteria from de EEZ gardens. Images correspond to representative examples of plates after 24 h of growth at 30°C or 25°C.

Selection of strains with inhibitory activity against plant pathogens

To identify bacteria with potential biocontrol activity, 18 colonies were collected from one plate of each soil sample with sterile toothpicks and placed on Petri dishes where we had previously inoculated different plant pathogens covering the whole surface: *Pseudomonas syringae*, *Ralstonia eutropha* and *Dickeya dadantii* (formerly *Erwinia*). Plates were incubated at 30°C overnight and the appearance of a clear halo around the colonies was analyzed. This would indicate inhibition of growth of the underlying pathogen culture by certain bacteria. Nine colonies showed promising results: three in the *P. syringae* plate (that we named **P1**, **P2** and **P3**); five in the *R. eutropha* plate (named **R1** to **R5**); and one in the *D. dadantii* plate (named **D**). These 9 strains were tested again to confirm the results. In this case, the strains were grown in liquid LB medium and 10 µl were spotted on three different plates (one for each class). Once the spots were dry, a suspension of each pathogen was overlaid on the corresponding plate, and plates were incubated overnight. The results are shown in Figure 2. All of the selected strains produced clear inhibition halos, although none of them was very large.

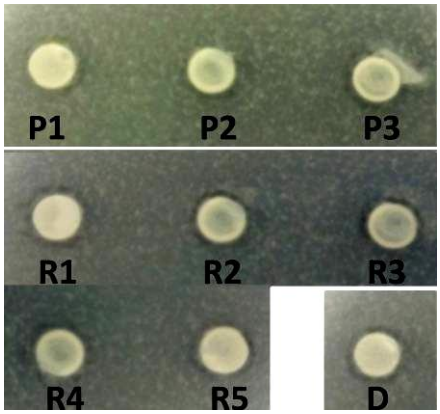

Figure 2.
Growth inhibition of *Pseudomonas syringae*, *Ralstonia eutropha* and *Dickeya dadantii* on plates by selected biocontrol strains.

The inhibitory efficiency was measured by calculating the area of the halo and substracting the area occupied by the patch of soil bacteria. Results are indicated in Table 1. The best inhibitors were strain P3 against *P. syringae* and strains R1 and R4 against *R. eutropha*. The only selected strain against *D. dadantii* was among the most efficient of the nine.

Table 1. Size of growth inhibition halos (cm^2)

P1: 0.34	R1: 0.51	R4: 0.51
P2: 0.51	R2: 0.34	R5: 0.45
P3: 0.63	R3: 0.34	D: 0.63

Influence of potential biocontrol agents on plant growth

Besides being able to block the growth of plant pathogens, a biocontral agent must have no negative influence on the plant in order to be useful for field appliactions. Some bacteria with biocontrol properties have even been identified as plant growth promoting rhizobacteria (PGPR), implying that their association with the root stimulates plant health and growth (ref). The 9 strains selected as potential biocontrol agents were tested to determine if their presence had any positive or negative effect on plants, or if they were neutral to the plant. For this purpose, corn seeds were inoculated with the 9 selected bacteria, as described in Materials and Methods, with a set of uninoculated plants as controls. Seeds were then sown in microcosms with commercial peat as substratum and kept in a controlled growth chamber for 8 days. Plants were monitored every day, watered when necessary and photographed at day 6 (Figure 3). At this time, some differences between specific treatments and the control plants were already evident.

After 8 days, plants were removed from the microcosms (Figure 4) and roots were washed to discard attached soil. To determine the effect of the different bacteria on plant health, the whole plants were weighted, and the length of shoots and primary roots were recorded.

The results from these analyses are presented in Figures 5 (shoot length), 6 (root length) and 7 (fresh weight), and summarized in Table 2.

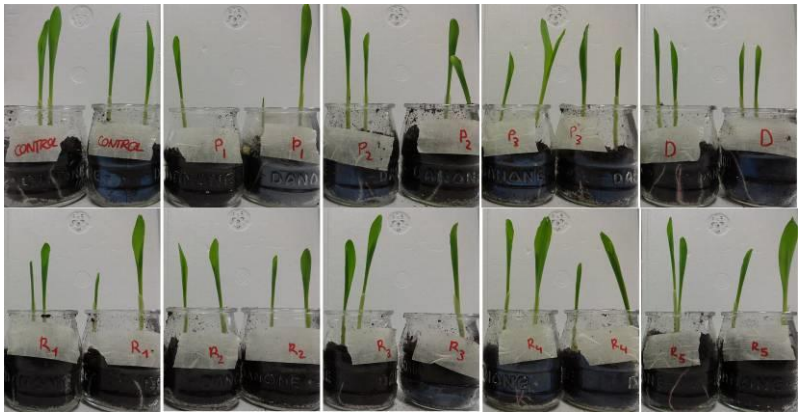

Figure 3. Corn plants 6 days after inoculation with the 9 soil bacteria, compared to the non-inoculated control.

Figure 4. Plants after being removed from the microcosms, 8 days post-inoculation. Shown are the control and three treatments as examples.

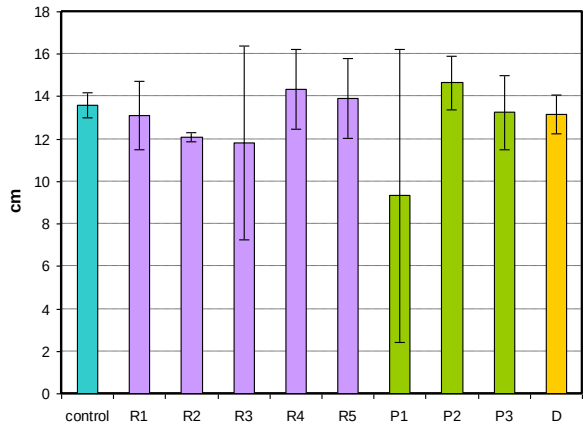

Figure 5. Effect of selected bacteria on shoot length length

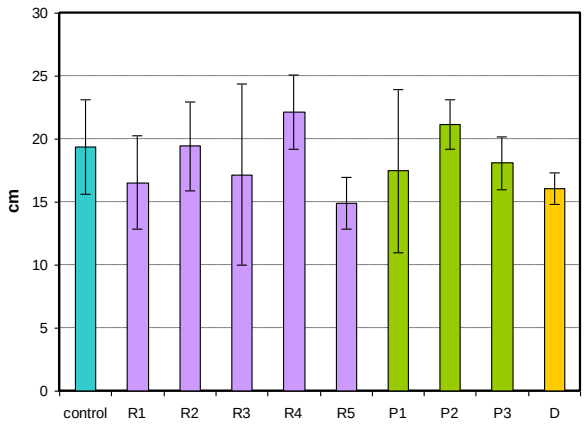

Figure 6. Effect of selected bacteria on root

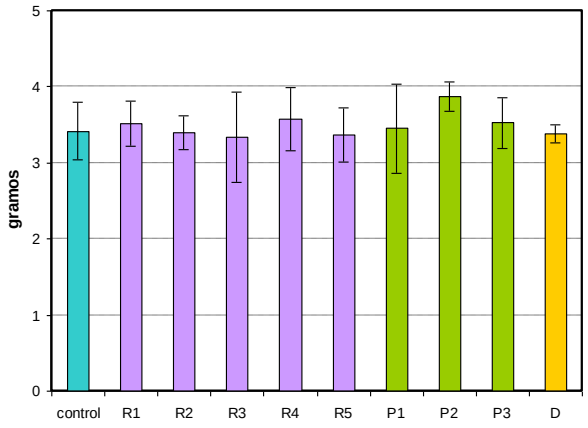

Figure 7. Effect of selected bacteria on plant weight

	increase/decrease (% with respect to control)			
strain	**shoot length**	**root length**	**fresh weight**	**global**
R1	-3.49	-14.47	2.71	
R2	-11.03	0.39	-0.59	
R3	-13.05	-11.24	-2.41	
R4	5.51	14.34	4.68	
R5	2.39	-22.87	-1.39	
P1	-31.43	-9.69	1.02	
P2	7.72	9.30	13.31	
P3	-2.57	-6.46	3.22	
D	-3.31	-16.80	-1.02	

Table 2. % increase or decrease in each parameter with respect to the control without treatment is indicated. A global evaluation of each strain is shown using a color intensity code (red=negative effect; blue=positive effect; grey=neutral effect).

Based on these results, of the five potential biocontrol agents against *R. solanacearum*, R4 had a clearly positive effect on plant growth and R3 was clearly detrimental to the plants. The other three were negative for one parameter and neutral for the rest. In the case of the inhibitors of *P. syringae*, P2 had plant growth promoting effects, P3 was relatively neutral for the plants, and P1 had a negative influence on root and shoot length. Finally, the only strain isolated with inhibitory effect against *D. daddantii* had a negative impact on root length. These data indicate that R4 and P2 could be promising candidates as biocontrol PGPB.

Taxonomic classification of strains P2, P3, R4 and D

As a first step in the classification of the chosen bacterial strains, the nine candidates selected from the inhibition assays were stained using the Gram stain. All were Gram-negative, except R1, which was classified as Gram-positive.

Next we decided to do a preliminary taxonomic classification based on partial sequencing of the 16S ribosomal DNA, which is considered the most reliable method used routinely for identification and classification of bacterial species. For this study, we chose only those isolates that had a positive or neutral effect on plant growth (P2, P3, R4). We included also the only isolate with inhibitory effect against *D. daddantii* (D), although this strain might not be a good candidate for its use in crop protection. Using a small amount of culture of each strain as template, we did a PCR amplification with universal oligonucleotides designed to amplify a fragment of 16S rDNA, as detailed in Materials and Methods. After confirming by electrophoresis that the amplifications were successful (Figure 8), the PCR products were sequenced using the same oligonucleotides.

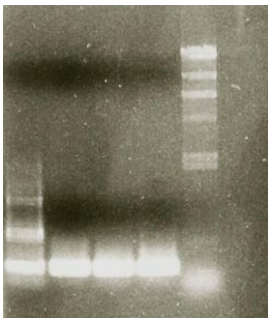

Figure 8. Electrophoresis of PCR products resulting from amplification with 16S rDNA oligonucleotides. Lanes: 1: strain P2; 2: strain P3; 3: strain R4; 4: strain D; 5: Molecular weight marker.

The obtained sequences (shown below in the 5'-3' orientation, after removing the poor quality tail fragments and confirming the base identities in both strands) were used for comparison with SILVA rDNA database [5,6].

>P2

CCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAATAACCT
CAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG
TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACTTGGG
AACTGCATTCTAAACTGGCAGGCTAGAGTCTGGTAGAGG

>P3

TGCCGCGTGTGTAAGTAGGTCTTCGGATTGTAAAGACTTTGGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATT
TTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTG
CATCCAAAACTGGCAAGCTAGAGTATGGTAG

>R4

CGGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTAACCTAATAC
GTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAA
GCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCT
GGGAACTGCATTCAAAACTGTCGAGCTAGAGTATGGTAGAGGG

>D1

TTAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGC
AGTTAGCGAATACCTGACTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAAT

ACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCC
CCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAGGGTAGAGGGG

The results of the database analysis are presented in Table 3. The four best hits are included, based on the following criteria:

- Alignment quality: the value is indicative of the reliability of the result, based on the length of the aligned sequence and the number of indeterminations.

- Aligned: it corresponds to the % of the query sequence that can be aligned with one database sequence. If it is less than 100% it indicates that there are gaps or that some bases in either end of the sequence are not included in the alignment.

- Identity: it indicates the % of identical bases between the query sequence and one database sequence. Although the other three parameters must be taken into account, this is the main one. It is considered that the optimum to identify a species is >99.5% (in any case no less than 99%).

strain	organism name	origin	alignment quality	aligned	identity
P2	*Enterobacter ludwigii*	urine	99.88	100	99.93
	uncultured bacterium	soil	99.91	100	99.50
	Enterobacter sp. JDG192	roots of *Gynostemma pentaphyllum*	99.68	98.83	98.81
	Enterobacter cloacae CHS 79	urine	99.87	99.41	98.68
P3	*Pseudomonas sp*. ps1-4	soil	100	100	100
	Pseudomonas sp. PG-2010-9	soil	100	100	100
	Pseudomonas fluorescens NBRC12568	water	99.91	100	99.79
	Pseudomonas sp. W15Feb9B	not indicated	99.92	100	99.67
R4	*Pseudomonas thivervalensis*	root tissues of *Salvia miltiorrhiza*	99.89	100	99.93
	Pseudomonas sp. SAM1	soil of cauliflower field	99.67	100	99.93
	Pseudomonas sp. PB2	soil contaminated with PAH	99.56	100	99.85
	Pseudomonas sp. SJ7b	mentha rhizospheric soil	99.91	100	99.73
D	*Pseudomonas plecoglossicida*	clean room	98.87	100	99.14
	Pseudomonas sp. JCM 5481	not indicated	99.84	100	99.04
	Pseudomonas rhizosphaerae OW2	soil	99.00	100	98.94
	Pseudomonas oryzihabitans	clean room	98.67	100	98.95

Based on these results, it seems that the four isolates studied are different organisms. We can confidently adscribe strain P2 to the genus *Enterobacter*, closely related to *E. ludwigii*, a species isolated from urine, although relatives have been identified in soil and plant roots. P3 and R4 are unequivocally classified as *Pseudomonas*, and the closest neighbors are in general strains isolated from soil or plant roots. Different *Pseudomonas* species are known to have biocontrol or PGPB properties, and are frequently associated to plants, therefore it is not surprising that we have isolated strains of this genus. Strain D is also most likely within the genus *Pseudomonas*, but the similarity with species included in the rRNA database is less clear than that of P3 or R4. Thus, it is possible that D corresponds to a new species, although more evidence will be required to verify this.

CONCLUSIONS

- We have been able to isolate 9 bacterial strains that inhibit growth of different plant pathogens: three against *Pseudomonas syringae*, five against *Ralstonia solanacearum*, and one against *Dickeya dadantii*. The inhibitory effect on plates was not very large.
- Some of the isolated strains have clear negative effects on plants and therefore will not be useful as biocontrol agents.
- Isolates P2 (*Enterobacter* sp.) and R4 (*Pseudomonas* sp.) have a positive effect on plant parameters and could act as plant growth promoting bacteria (PGPB) with biocontrol properties against *Pseudomonas syringae* and *Ralstonia solanacearum*, respectively.

ACKNOWLEDGEMENTS

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MY OWN IDEAS

Ana Ramírez

Sobre mis primeras impresiones quería destacar que me ha parecido muy interesante, desde un principio me hacía una idea de en qué consistía pero a la hora de la verdad me ha sorprendido gratamente, tanto las instalaciones como las personas que allí he conocido. Investigar y trabajar en un laboratorio ha sido mi sueño de siempre y ver que por unos días ese sueño se hace realidad me encanta. Tanto el ámbito ecológico como el cutivo de colonias de bacterias me parece una manera fácil y amena de acercarnos a ese mundo que es la ciencia.

Isabel Peinado

Mi primera impresión sobre el proyecto fue muy buena. Me encantó el trabajo que realizamos con las placas y las bacterias, y además creo que todos hemos congeniado muy bien y vamos a disfrutar mucho de este proyecto.
Este proyecto no fue uno de los que elegí, pero por el momento me está gustando bastante

Julia Salas

Mi primera impresión sobre el proyecto PIIISA fue genial. Nada más plantearnos en nuestro centro esta actividad me llené de entusiasmo al pensar que podriamos trabajar en un laboratorio con científicos y demás y también pensé que podría servirme en el futuro a la hora de elegir una vocación. Al llegar allí el primer día mi impresión no fue nada distante de la anterior, me encantó. Aquello era un sitio ideal en el que trabajar el día de mañana y puede que despertase una curiosidad en mi sobre botánica.

Andrés Moreno

Me he llevado una buena impresión del proyecto en sí, las instalaciones, la gente y lo que vamos a estudiar, ya que siempre me a gustado la biología este proyecto me interesa. Hay buen ambiente, espero que siga así y me lleve una buena experiencia aprendiendo nuevas cosas. Solo le veo un inconveniente, es la exposición en inglés. No me importa explicar lo que vamos a hacer, pero el inglés no se me da bien y menos el hablado aunque me esforzaré para poder hacerlo medianamente bien.

ANTIINFLAMATORY EFFECTS OF FLOURS PURIFIED FROM OLIVE SEEDS IN DIABETIC PATIENTS.

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HIGHLIGHTS

- Olive seed flours are new materials prone to be used as animal and human foods.
- In vitro assays consisting in blood culture and induction of inflammatory processes are useful methods to determine the effects of challenging foods and molecules.
- Defatted olive seed flour displays an interesting anti-inflammatory effect on diabetic patients tested this way.

SUMMARY

Flours from olive seeds represent a protein-rich novel material with potential uses for animal and even human consumption. We have tested here the effects of the addition of olive seed flour, defatted by using supercritical fluid technology, to whole blood cultures where a inflammatory response has been triggered by means of chemical inductors. Two types of patients were assayed: healthy patients and diabetics, as the later group has been described to develop subclinical inflammatory reactions. Three key markers were analysed in plasma samples after culture by using Western blotting approaches: presence of iNOS and IL-1β, and finally the plasma SDS-PAGE profiles relatives to protein-bound 3-nitrotyrosine, as a marker of inflammation and NO production. The three markers were enhanced in inflammation-induced samples in comparison with controls. Challenge with olive flour together with the induction resulted in significantly lower enhancement for diabetic patients, but not for the healthy group. Finally, the olive flour itself did have no inflammatory effect in healthy patients, but minor, although significant inflammatory effect in diabetics. The described effects are promising for the development of new therapeutically valuable tools. However, much progress is needed in order to dissect the precise causes of the described effects.

INTRODUCTION (AND OBJECTIVE)

Olive oil plays a well-established, beneficial role in promoting good health. Oil retains the compounds that the fruit develops in response to environmental stress, especially phenolic compounds. Moreover, virgin olive oil is an integral ingredient of a Mediterranean diet. Its nutritional and medical benefits are widely known and approved. Indeed, virgin olive oil has been used as a folk remedy for combating diseases due to its hypotensive, cardio protective, antimicrobial, anti-hyperglycaemic, anticancer, and anti-inflammatory pharmacological properties (Michas et al., 2014; Parkinson and Keast, 2014).

Olive seeds are not widely used for feeding purposes, as the whole olive fruit is generally pressed for the generation of olive oils. However, recent investigations are beginning to undercover the high potential of this by-product, which can be isolated using devoted machinery in oil factories (almazaras) and industries for the generation of table olives. Preliminary studies have been able to characterize the presence in this material of seed storage proteins (SSPs) of the 11S type, similar to legumins (Alche et al., 2006), as well as enzymes like peroxidases, and superoxide dismutases among others. Preliminary data also exist as regard to digestibility of these proteins, lack of relevant allergenicity and absence of gluten in this material, which therefore could be potentially used for animal and even human consumption.

A subclinical inflammatory reaction has been shown to precede the onset of type 2 (non-insulin-dependent) diabetes (Kaur, 2014). An olive-oil-rich diet is not only a good alternative in the treatment of diabetes. It may also help to prevent or delay the inflammatory reaction of the disease. The effects of extra virgin olive oils and functional olive oils on the human immune system in response to inflammation induction are being tested within the frame of a NUTRAOLEUM research project by the SPR-EEZ-CSIC group. Here, we wanted to analyse the effects of olive seed flours de-fatted by means of supercritical fluids on this same test system.

MATERIALS AND METHODS

Generation of defatted flours from olive seeds

Specialized flours were obtained from olive seeds isolated from olive fruits by the company Elayo (Castillo de Locubín, Jaén, Spain). The flours were obtained by cold pressing of isolated seeds in order to remove most oil, and then were further de-fatted by using supercritical fluid technology (SCF) by the company Fluysur (Castillo de Locubín, Jaén, Spain).

Patients

Informed consent was obtained from each participant. The study comprised two healthy subjects and two diabetic patients. The subjects were unrelated, recruited and diagnosed at the coverage area of basic area “Pedro Martínez” (A.G.S. NorthEast Granada, Spain). Venous blood was collected from the cubital vein in 4-ml lithium-heparin tubes.

Whole blood culture, inflammation induction and challenge with FSC defatted flour

1ml samples of whole blood were incubated in 24-well plates at 37°C, 5% CO_2 in humidified air with and without FSC defatted flour from olive seeds (25 µg/mL), and each one of the following inflammation inductors: none (negative control), 1µg/ml Phorbol 12 myristate 13 acetate + ionomicine (PMA+IO), 1µg/mL phytohaemaglutinin (PHA) and 1µg/mL *Escherichia coli* lipopolysaccharide (LPS). Plasma samples were obtained from each combination assayed by collecting well content after 24h of culture stimulation and centrifugation at 3000rpm for 15 min. Supernatants were then harvested and frozen at -20°C until use.

Western blot detection of inflammation markers

Samples of plasma containing 50–75 µg of protein were separated by SDS-PAGE (9–14% acrylamide) and transferred to PVDF membranes. Nonspecific binding was blocked by preincubation of the PVDF membranes in PBS containing 5% BSA for 1 h. The membranes were then incubated overnight at 4°C with appropriate antibodies. Antibodies against nitrotyrosine and the inducible form of nitric oxide synthase (iNOS) (130 kDa) were purchased from Abcam (Cambridge, UK). Antibody against IL-1β (17 kDa) was purchased from BIOMOL International (Plymouth Meeting, PA). Bound primary antibody was detected with the use of a peroxidase-conjugated secondary antibody (Dako Cytomation, Glostrup, Denmark) and with the use of an enhanced chemiluminiscence kit (Biorad, Hercules, CA, USA). The density of the specific bands was quantitated with an imaging densitometer.

Statistical analysis

Results are shown as means ± SE. Data for Western blots are presented as percentages from control values. Data were analysed using one-way ANOVA. Post hoc comparisons were carried out by the Newman-Keuls test. A value of $P < 0.05$ was regarded as significant. All calculations were performed by using the SPSS 14.0 statistical software (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Detection of iNOS and IL-1β in the plasma samples.

Figure 1 displays an example of the detection of both iNOS (Figure 1A) and IL-1β (Figure 1B) in plasma samples corresponding to basal and induced conditions. iNOS is detected as a single band of c.a. 130 kDa, whereas IL-1β appears also as a single band of lower Mw (17 kDa). Such intensities are in good agreement with the molecular weights described for these molecules. The intensity of bands is markedly higher in induced samples when compared to the basal ones for both markers. Therefore, the intensity of bands seems to be a good indicative for the presence of the molecule, and this parameter was further used for semi-quantitative purposes.

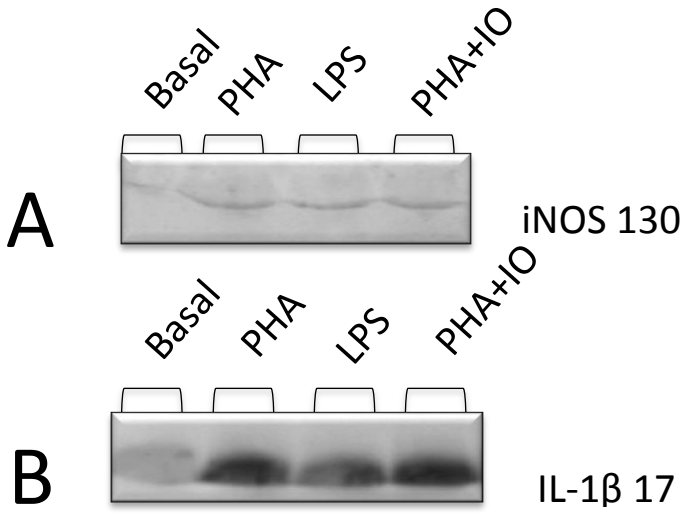

Figure 1. Example of Western blotting detection of iNOS (A) and IL-1 β (B) in basal and induced plasma samples.

Production of iNOS and IL-1β in healthy patients

The presence of both iNOS and IL-1β (Figure 2A, 2B, respectively) in the plasma of healthy patients was significantly increased by the inductors PHA, LPS and PMA+IO, as compared with basal samples (obtained from non-cultivated, non-induced blood) or control samples (obtained from cultivated, non-induced blood). The addition of flour simultaneously to the inductors did not significantly reduce the levels of iNOS, nor of IL-1 β in the patients analysed. We also included an additional control, consisting in the addition of the flour alone at two different concentrations. The flour didn't induce itself a significant increase in the production of iNOS or IL-1β in the healthy patients.

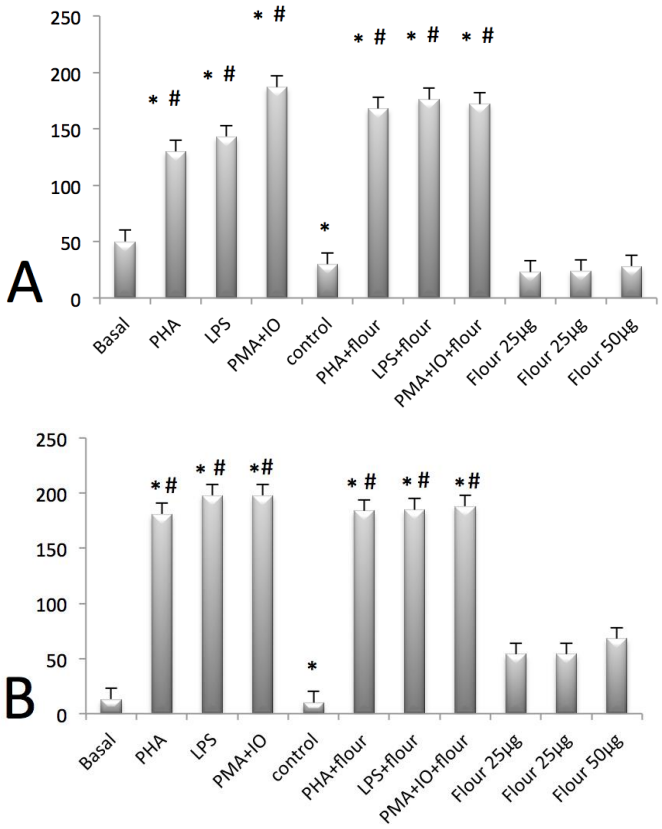

Figure 2. Quantification of iNOS (Figure 2A) and IL-1β (Figure 2B) in plasma samples of healthy patients after different treatments of induction. Basal and control samples (obtained from non-cultivated, non-induced blood, and from cultivated, non-induced blood, respectively) were included. *Statistically significant comparisons ($p<0,05$) between the control and the induced samples with and without flours. #Statistically significant comparisons ($p<0,05$) between induced samples with and without flours.

Production of iNOS and IL-1β in diabetic patients

The presence of both iNOS and IL-1β (Figure 3A, 3B, respectively) in the plasma of diabetic patients was significantly increased by the inductors PHA, LPS and PMA+IO, as compared with basal samples (obtained from non-cultivated, non-induced blood) or control samples (obtained from cultivated, non-induced blood). Interestingly, and also oppositely to what was observed in healthy patients, the addition of flour simultaneously to the inductors did significantly reduce the levels of iNOS and IL-1 β in the patients analysed. For diabetic patients we also

included the additional control also used with healthy patients, consisting in the addition of the flour alone at two different concentrations. In this case, the flour did induce itself a significant production of iNOS or IL-1β in the diabetic patients.

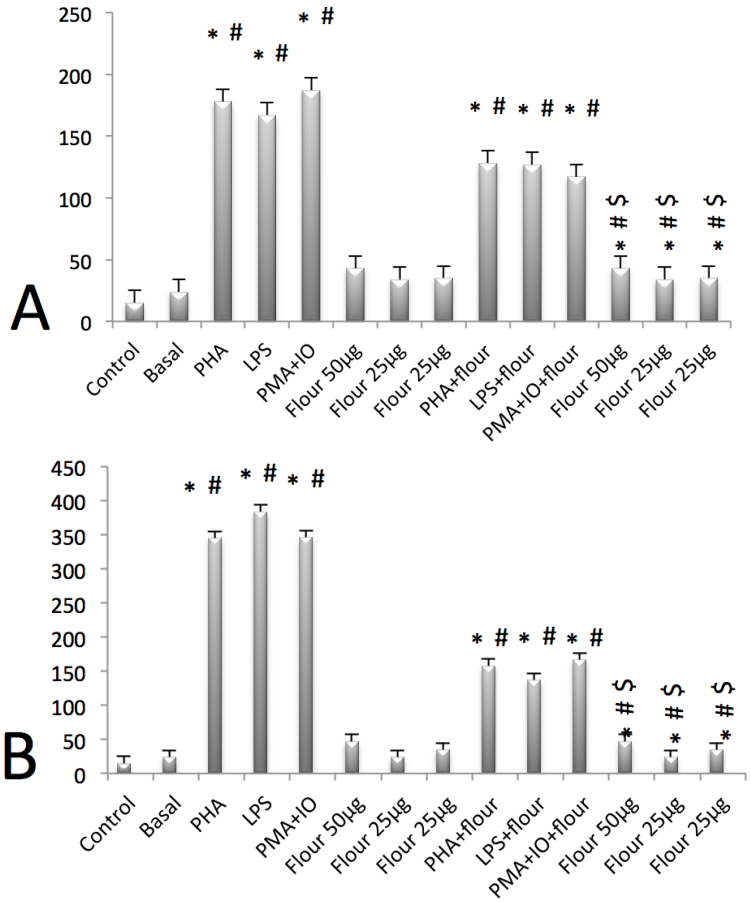

Figure 3. Quantification of iNOS (Figure 3A) and IL-1β (Figure 3B) in plasma samples of diabetic patients after different treatments of induction. Basal and control samples (obtained from non-cultivated, non-induced blood, and from cultivated, non-induced blood, respectively) were included. *Statistically significant comparisons ($p<0,05$) between the control and the induced samples with and without flours. #Statistically significant comparisons ($p<0,05$) between induced samples with and without flours. $ Statistically significant comparisons ($p<0,05$) between the control and non-induced samples with flours.

Analysis of protein-bound 3-nitrotyrosine profiles in plasma samples

Superoxide and NO are known to bind to form peroxynitrite. Peroxynitrite and/or peroxynitrite-derived intermediates can nitrate free or protein-bound tyrosine residues to form 3-nitrotyrosine. The detection of protein-bound 3-nitrotyrosine is often used as a marker of inflammation and NO production (Winyard et al, 2011). Figure 4 shows the SDS-PAGE profiles of nitrated proteins present in plasma samples. Detected immunoreactive bands in healthy patients indicate a significant increase of endogenous tyrosine nitration after the incubation with the inductors, compared with that shown in control and basal patients'

plasma. Treatment with the olive seed flour in different concentrations partly suppressed this increase. However, diabetic patients showed a higher decrease in the nitrated protein after the incubation with olive seed flour than shown in the three different inductors compared with the control group.

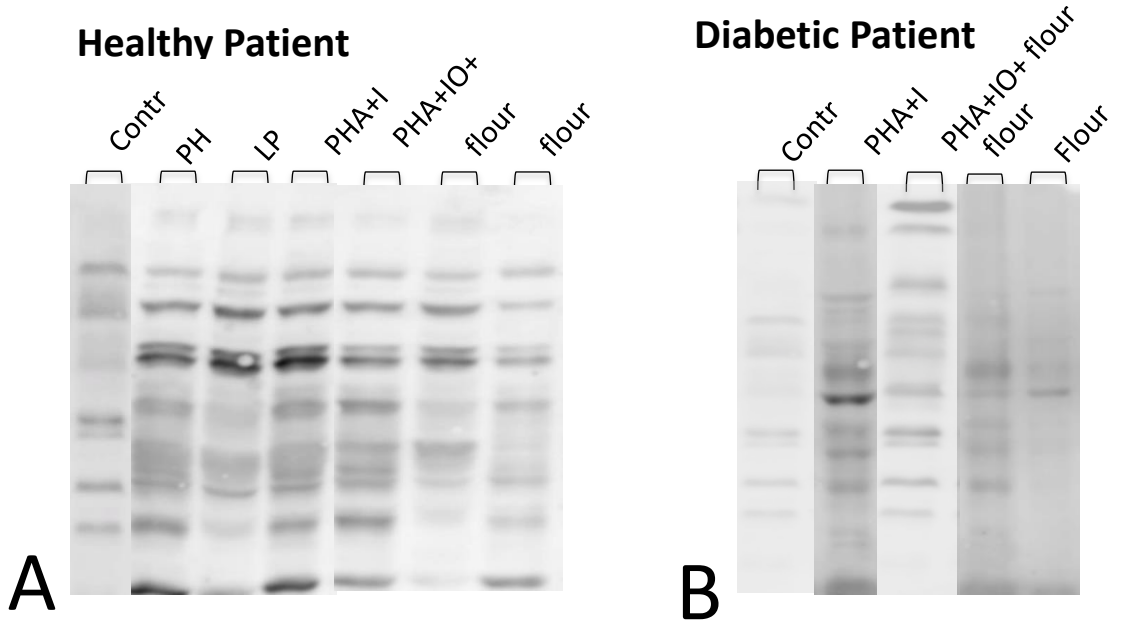

Figure 4. Western blotting showing protein-bound 3-nitrotyrosine profiles of nitrated proteins present in plasma samples corresponding to a healthy patient (A) and to a diabetic one (B).

The results obtained here provide further evidence as regard to the tight relationships between oxidative metabolism and the development of certain diseases, as it is the case of diabetes. Diabetic patients display a differential behaviour in comparison to healthy patients concerning the three markers used here. Moreover, the significant induction of the markers in presence of the flour alone, which doesn't produce such effect in healthy patients, may reflect these differences as well.

The results obtained here are difficult to assign to a particular explanation. First, scarce numbers of experiments were carried out, and in a very limited number of patients only. Therefore the statistical significance of the observations has to be taken carefully. Second, three markers, although very informative, constitute little information when compared with the plethora of molecules regulating inflammation response in humans. Finally, previous studies carried out by the BRP-EEZ-CSIC group have identified already the presence of numerous protein forms in the olive seed (Krebesova, 2014). Many other non-protein components are yet to be described and identified in this material. Thus, the identification of the precise molecules responsible for the described effects is at present a challenging task. However, we have to focus into the relevance of the present work, as it represents a novel approach to the analysis of potential benefits of a by-product generated for olive industries, with plenty of potential. This might be the basis for future areas of research, perhaps directed to the design of new therapeutical approaches for these patients.

CONCLUSIONS

- Plasma samples from diabetic patients in whole blood cultures are hyper-responsive to inflammation induction by PHA, LPS and PMA+IO, as demonstrated by the increased levels of IL-1β and iNOS markers in plasma, and 3-nitrotyrosine production.

- The addition of extracts from olive seed flour produced unequivocal signs of reduced inflammatory response, mainly in diabetic patients. This is likely mediated through modifications of the complex subclinical inflammatory reaction, and is witnessed here as reduced levels and/or biological activity of IL-1β, iNOS and presence of 3-nitrotyrosine.

- The specific components of the olive seed flour ultimately responsible for these anti-inflammatory effects are yet to be characterised. Such characterization will help to design new therapeutical approaches for these patients.

ACKNOWLEDGEMENTS
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MY OWN IDEAS

Sandra Carmona

Al principio del proyecto estaba nerviosa porque aunque había leído en lo que íbamos a trabajar no tenía muy claro cómo iba a salir ya que yo no había utilizado instrumentos tan complejos.

Conforme fuimos avanzando tanto en días como en el proyecto en sí se nos presentaron algunas dificultades que supimos afrontar gracias a los coordinadores. Estas dificultades han hecho que cambiemos un poco nuestro objetivo pero hasta ese aspecto ha sido positivo porque nos ha ayudado a aprender que aunque algunas cosas salgan mal siempre tendremos oportunidad de sacarle su lado positivo.

Otra apreciación que me ha gustado ha sido la confianza que hemos tomado en nosotros mismos desenvolviéndonos en el laboratorio con más soltura debido al paso de los días y la experiencia.

Me alegra haber escogido este proyecto tanto como por la amabilidad de los coordinadores y sus ayudantes como por haber aprendido algo más de la ciencia que ha hecho que me guste más.

Another finding that I liked was the confidence we had in ourselves knowing develop ourselves in the laboratory with more ease due to the passage of time and experience.

I'm glad I chose this project as well as the friendliness of the coordinators and assistants like to have learned something of science that has made me like it more.

At the beginning of the project I was nervous because although I had read about what we were going to work I was not sure how it would go because I had not used such complex instruments.

As we were moving both days in the project itself we presented some difficulties that we knew face thanks to the coordinators. These difficulties have to change a bit our goal but until that point has been positive because it has helped us learn that although some things going wrong always have a chance to get its positive side.

Cristina Pedrosa

First I want to thank all the researchers who have been with us, and to my Secondary School. This have given me a new view about science, as if we were scientists, because we've been doing a lot of procedures, which, thanks to the help of the people who were helping us in our laboratory, we have been able to learn and enjoy something that we don´t know if will be repeated throughout our lives.

This project has been a new, gratifying and interesting experience because I had never used these kind of methods, for example with the gels and all of the machines that we have used. It has been very rewarding to meet people who really like science and therefore are dedicated to advance further into it. The best thing is that we have been put in to practise things we learned in class but it goes further than I can study at the school, I have learnt things much more interesting that those ones I have learnt in the classroom, discovering that things were even more interesting than it seems. It is very important to get into science in a more experimental way because it is something that will always be there the rest of our lives. Sometimes we needed to sacrifice our spare time in order to do the investigation, however it was worthy because this is a unique

experience that not many young people even adults have the opportunity to live. I´m very happy to have been involved in this great experience. I hope that PIIISA continue a lots of years and that the practice of teaching young people what really is science never get lose.

Menyao Pan

Gracias al proyecto PIIISA he tenido la oportunidad de realizar por primera vez una investigación en laboratorio y ha sido una excelente experiencia. Al ser novata y no conocer mucho sobre proteínas, no sabía qué hacer al comienzo pero tanto el investigador como los compañeros nos hemos ayudado mutuamente y conseguimos realizar a cabo exitosamente los procesos. Pese a los errores y las situaciones adversas logramos unos resultados decentes, aunque mejorables; pero como se suele decir, de los errores se aprende y efectivamente durante la segunda fase del proyecto obtuvimos mejores resultados.

Aparte de la práctica con utensilios propios de laboratorio (un aspecto muy interesante y divertido para todos los que nos guste la ciencia) hemos aprendido cosas sobre biología molecular y utilidades que antes desconocíamos de una planta tan común en Andalucía como el olivo.

Estas sesiones me han hecho conocer más aspectos de la investigación científica y me han ayudado a disfrutar aun más de la ciencia, aclarando mi vocación en esto. Ha sido una experiencia increíble y si tuviera la oportunidad de volver a hacerlo, lo repetiría.

Thanks to the PIIISA project I had the chance of making some research in a laboratory and it has been a sterling experience. Being a rookie and not knowing much about proteins I didn't know what to do at the beginning but both the researcher and mates we have helped each other and we got to carry out the processes successfully. Despite the mistakes and adverse situations we have achieved proper results -improvable, though. But as say, you learn from mistakes and in fact during the second phase of the project we obtained better results.

Apart from practicing with laboratory ware (a very interesting and fun aspect for everyone who likes science like us) we became acquainted with molecular biology and uses we didn't know before about a so common plant in Andalusia such as the olive tree.

These sessions helped me to get to know more things related to scientific researching and thanks to this, currently, I enjoy science even more, having my vocation about this clearer. It's been an amazing experience and if I ever had the oportunity of doing it again, I would.

José Miguel Benavente

For me it has been an awesome different experience. It is interesting to do this type of things because you can learn a lot of them. The idea to know that an olive's molecule can help to eradicate or weaken an ill is so curious.

When I was younger I wanted to be a scientist, thinking that I could do an important experiment for the world. Maybe this project is the first step to initiate me in this exciting career.

I want to thank the help that the people of my group gave to me (staff and students).

Para mí ha sido una experiencia totalmente diferente. Es interesante hacer este tipo de cosas porque puedes aprender mucho de ellas. La idea de saber que una molécula de una aceituna pueda debilitar o acabar con una enfermedad es muy curiosa.
Cuando era más joven quería ser científico, pensando que podría hacer un experimento importante para el mundo. Quizás este proyecto puede ser solo el primer paso para meterme en esa apasionante profesión.
Quisiera agradecer la ayuda que mi grupo me ha dado (coordinadores y estudiantes).

VARIATIONS IN THE SPONTANEOUS MUTATION RATE OF A SOIL BACTERIUM. EFFECT OF GENE LOCATION

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All students contributed equally to this work

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HIGHLIGHTS

We were able to determine the mutation frequency for *Sinorhizobium meliloti* bacteria and surprisingly, we found that it significantly differs depending where the reporter gene is located.

SUMMARY

Rates of spontaneous mutation vary hugely within and between organisms. This rate has been established for only a group of bacterial model and pathogens. *Sac*B gene - which encodes levansucrase- production in *E. coli* and other Gram negative bacteria is lethal in the presence of 5% sucrose in agar medium. This property can be used to determine the mutation frequency by studying the Sacarose sensitive phenotype.

Our bacteria the study is a soil Gram- bacterium named *Sinorhizobium meliloti* that belongs to a group collectively referred to as rhizobia that together with leguminous plants contributes the largest input of combined nitrogen into terrestrial ecosystems. It is a genetically tractable model species for investigating rhizobial biology.

In a previous study, we inserted the Mob-Sac cassette including the gene *Sac*B in different locations of the Chromosome of *S. meliloti* strain RMO17 generating derivatives with a resistant to Kanamycin and Sucrose Sensitive phenotype. Based on these strains we were able to determine the mutation frequency for *Sinorhizobium meliloti* bacteria and surprisingly, we found that this frequency significantly differs depending where the *SacB* gene is located.

INTRODUCTION

Rates of spontaneous mutation vary hugely within and between organisms. This rate has been established for only a group of bacterial model and pathogens. In addition, both the kinds of mutations and the processes that generate them are diverse and only partially discovered [1].

The measure the genomic mutation rate must satisfy the following criteria: (i) the experimental mutational target gene should be large enough to sufficiently sample the genome (a gene or operon 1-5 kb it should be enough for the majority of bacteria. (ii) mutants should grow at same rate as their progenitors. [1].

The isolation of the structural gene *sacB* from *Bacillus subtilis* have made it possible to design a means to positively select mutations in Gram negative bacteria [2]. The *sacB* gene encodes levansucrase (sucrose:2,6-P-D-fructan6-3-Dfructosyl-transferase; EC2.4.1.10). The production of levansucrase in *E. coli* and other Gram negative bacteria is lethal in the presence of 5% sucrose in agar medium, causing lysis within 1 h or inhibition of growth [2]. This property has been used to develop suicide vectors which inserted in bacterial genomes generates strains with Sacarose sensitive phenotype (Figure 1) [3].

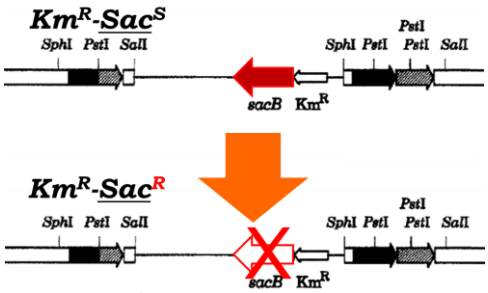

Figure 1. Scheme of *the Mob-Sac system designed to generate bacterial strains with sacarose sensitive phenotype* [3]. *The Mob-Sac cassete is integrated into the chromosome via homologous recombination. Only cells disrupted in the 1.4 kb SacB gene are able to grow on TY agar plates containing 5% sucrose and Kanamycin 200 ug/ml.*

Our bacteria the study is a soil Gram- bacterium named *Sinorhizobium meliloti* that belongs to a group collectively referred to as rhizobia that together with leguminous plants contributes the largest input of combined nitrogen into terrestrial ecosystems (4). It is a genetically tractable model species for investigating rhizobial biology.

In a previous study, we inserted the Mob-Sac cassette including the gene *SacB* in different locations of the Chromosome of *S. meliloti* strain RMO17 generating derivatives with a resistant to Kanamycin and Sucrose Sensitive phenotype [5].

In this work, we aimed to obtain a mutation frequency for the soil bacteria *S. meliloti* based in the *SacB* gene cassette. Surprisingly, we found that this mutation frequency significantly differs depending on the gene location. Further studies will confirm these results.

MATERIALS AND METHODS.

Bacterial strains. The bacterial strains used in this work, their source and relevant characteristics are listed on table 1.

Table 1. Bacterial strains used in this study.

***S. meliloti* strains**	**Relevant characteristics***	**Reference or source**
RMO17	Sac^R/Km^S ; wild type	(6)
RMO17(3G)	Sac^S/Km^R; MobSacB vector (11,764 nt)	(5)
RMO17(1F)	Sac^S/Km^R; MobSacB vector (2,952,963 nt)	(5)
RMO17(1H)	Sac^S/Km^R; MobSacB vector (1,858,431 nt)	(5)

*In parenthesis is indicated the insertion site in the choromosome of the 11.3 Kb of the *MobSacB* cassete.

Media and growth conditions. Triptone yeast (TY) solid media were used for maintenance and grow at 28ºC *S. meliloti* strains. Eventually parallel plates were cultured at home in a warm and dry folder. Liquid media cultures were grown in agitation chamber at 28ºC.

-Optical Density measurements. Different bacterial cultures were grown in agitation chamber for 3-4 days and generally the Optical density at 600 nm of a dilution of 1/5 of the culture was measured on a spectrophotometer (Pharmacia Biotech Company).

-Bacterial Plate dilutions and spread. In order to establish a general scheme to count total and mutant bacteria present in the different cultures a general protocol was determined. Serial dilutions of TY media containing the bacterial cultures were performed in a series of 100 ul in a 1 ml (vortexing carefully) in such way that: dilution (0) is equivalent to a bacterial culture reaching to OD_{600nm} of 0.5. 100 ul of dilutions (0) and (-1) were spread on plates and used to obtain an accounting number of colonies of SacR mutants (Approx 10-1000 per plate); On the other hand, 100 ul of dilutions (-5) and (-6) were used for accounting the total number of cells in the culture.

RESULTS

Evaluation of the spontaneous arising of SacB Sinorhizobium meliloti mutants.

As a preliminary result, we determine the amount of bacteria and dilutions required to obtain tractable numbers of colonies per (see example in Figure 2). In this regard, we estimate that a single colony contains between 0.8 to 15 x 10^6 bacteria, and liquid cultures at 0.5 OD600 present 3 to 9 x 10^8 bact /ml. The presence of *SacB* mutants in those cultures (plates containing Sacarose at 5%) varies between 0.7 to 20.0 x 10^3 mutants/ml (see further).

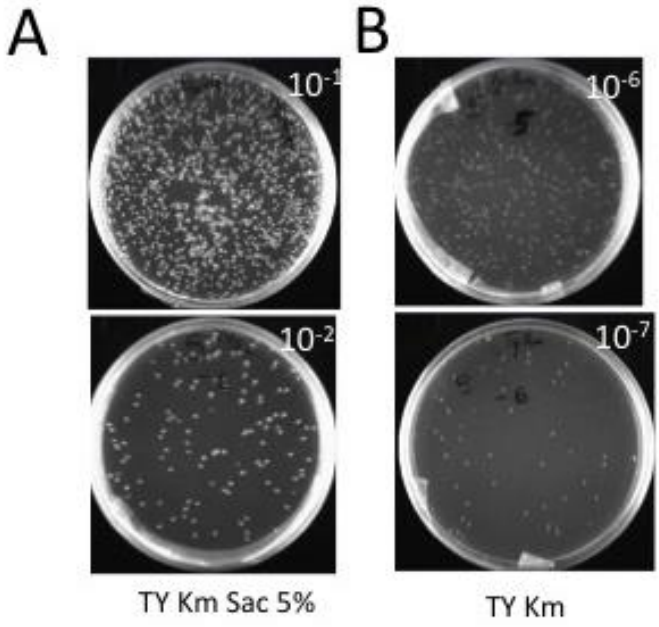

Figure 2. Example of accounting plates performed. *An example of dilutions and bacterial growth on TY media containing 5% sacarose and 200 ug/ml of Kanamycin (A) or in TY media with 200 ug/ml of Kanamycin (B). Counting single colonies allow calculating the number of bacteria and bacterial mutants present in every liquid culture.*

Estimation of mutation frequency on S. meliloti bacteria based on Sac B gene.

In order to estimate a mutation frequency for every strain (table 1) liquid cultures were grown at 28ºC for three days at TY Km media. After Optical density measures, a Dilution 0 was made at 0.5 OD600 for every culture. A serial of dilutions of the culture (1 ml each) were performed and

100 ul of the corresponding dilution were plated either on TY km Sac5% media (dilutions -1 and -2) or on TY km media (dilutions -6 and -7). After 4 days growth colonies were count in order to determine the frequency of spontaneous mutant for every strain (table 2).

Table 2: Counting of total and mutant bacterial colonies present in three replica cultures for every RMO17 derivative strain.

			Colony counting					
				Km/SacR			TY/Km	
Cultures	Nomenclature	O.D. 600nm	vol (ul) (1st Plate)	dil(-1)-Sac5%	dil(-2)-Sac5%	dil(-2)-Sac10%	dil(-6)	dil(-7)
3G	1	0,405	120	70	12		587	30
3G	2	0,36						
3G	3	0,49	102	87	30		563	59
3G	4	0,445	113	ND	ND		519	65
1F	5	0,42	119	ND	142	134	418	63
1F	6	0,41	122	ND	221		299	36
1F	7	0,4	125	ND	102		274	29
1F	8	0,38						
1H	9	0,345	147	ND	145		571	70
1H	10	0,355	142	ND	43		247	83
1H	11	0,435	116	ND	204		523	58
1H	12	0,325						

**Four diferent liquid cultures were performed for every* SacB *insertion derivative (3G, 1F and 1H). Only cultures reaching to OD600 close to 0.5 were used for further serial dilution plate analysis (red, blue and green coloured data for 3G, 1F and 1H derivatives respectively). In general data of at least two plates were used to generate the ratio of the three cultures per bacteria.*

Counting of colonies allow determining the spontaneous mutation frequency of the *SacB* gene for every isolate by dividing the number bacteria able to growth in Sac 5% by total bacteria present in the culture. Numbers of colonies obtained allow to estimate a mutation of spontaneous frequency of 2.75 x 10^{-6} (margins: 1.19 to 5.3 x 10^{-6}) when the cassette *sacB* is close to the Replication Origin (RMO17 derivative 3G) and 43.19 10^{-6} (margins: 21.3 to 73.9 x 10^{-6}); 22.60 10^{-6} (margins: 5.2 to 39.0 x 10^{-6}) for the two other locations 1F and 1H respectively. These differences are statistically significant and are not due to the previous presence of Sac^R bacteria individuals in the cultures since a drop assay indicates the absence of single colonies Sac^R in the primary inocula (data not shown).

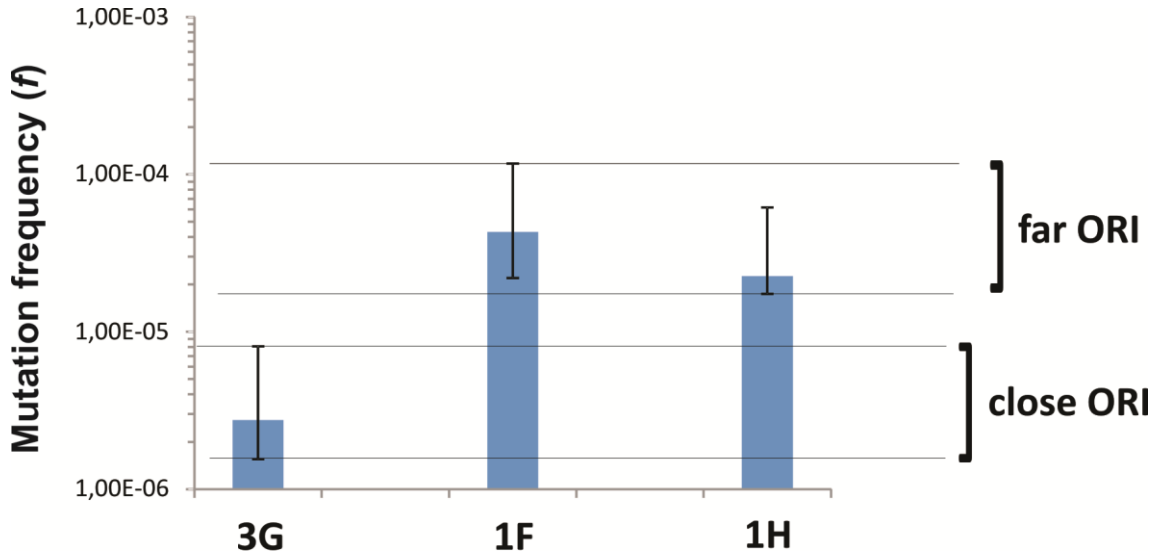

Figure 3. Mutation frequency estimated for *S. meliloti*. *Three different vales of Mutation frequency were obtained from data of table 2. Error bars are margin indication based on three replicas per bacteria. Significant differences can be accounted between 3G isolate and the other two 1F and 1H.*

Discussion

Because mutation is the ultimate source of all variation, the evolutionary process of any organism will be incomplete until a detailed account has been made of the rate of spontaneous alterations of their genomes [7]. One easy manner to calculate this rate is based on mutation of reporter constructs in a particular species [1]. In this work we follow to get this number based on using a fraction of cultures without mutants. Then, knowing the fraction of mutants (f) we can obtain finally a mutation rate (μ) based on the known Genome size. In the case of other Gram negative bacteria, *f* values varies between 1.7 x 10^{-6} to 3.5 10^{-6} [1]. In our experiments, we obtain similar values for one of the locations (close to the replication Origin, 3G derivative). However, in all studies performed no comparison has been made among different locations. In our work a difference of 8 to 15 fold less was found when the reporter gene was localized close to the Replication Origin. Causes of this difference can be result of different types of gene disruption occurred in different locations. Further studies confirm this result, which could be an indication of different speed of variability of the genes depending where are localized. If that is the case this fact could have evolutionary implications in the genome localization of the bacterial genes depending of their conservation degree.

CONCLUSIONS

1) Levan sucrose gene (*sac*B) is a good target for mutation rate estimation in *Sinorhizobium meliloti*.

2) Mutations in *sac*B gene are irreversible.

3) The estimated mutation frequency depends on gene location being 8 -15 fold lower when gene is located close to replication Origin of the chromosome.

ACKNOWLEDGEMENTS

This work was performed in the Microbiology and Symbiotic Systems department in the Estación Experimental del Zaidín – Consejo Superior de Investigaciones Científicas. It was supported by research projects MICINN Consolider-Ingenio 2010. CSD2009-00006; BIO-2011-24401 and BIO2014-51953-P; Secondary Schools Institutes and Centers: Francisco Ayala, Zaidín Vergeles, Miguel de Cervantes and CDP Virgen de Gracia from Granada Capital and Illiberis (Atarfe). Also we appreciate the coordination in the project of teachers D Angel

Venegas, Ermila, Fernández Palomino, Mª Antonia Padial Santín, Esperanza Vaquera and particularly to Antonio Quesada Ramos for his help in developing our blog: http://dondemutanlosgenes.blogspot.com.es/.

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MY OWN IDEAS

Mª Elena Leal Raya

The original objective was to evaluate certain mutations of *Sinorhizobium meliloti*, the mutation speed and test if the genes location at genome could modify something. These mutations determine the survival when there's sucrose.

We started working with the cultures 1H and 3G phenotypes but then, we added culture 1F. We found an answer to the questions about the generation number, the division time... We did it using sensitivity and resistance tests, dilutions, colonies count and mathematical operations. We had some weird results as when we observed that the 3G derivative colonies' size was smaller than 1H's, we even had to repeat the experiment because we received new information about the dilutions' concentration. We also had to confirm some data and take into account the margin of error.

At the end, we achieved to have the mutation frequency and the variation derived from the location in genome.

I've really enjoyed a lot this project since it has been a great and unique opportunity to see how scientist investigation works. It has been exciting being in a laboratory next to people who work there. This experience has allowed me reaffirm my tastes and without any doubt it's going to influence me when I had to decide what I want to study and where I want to work. I've learnt a lot so I want to thank all the people who have made possible PIIISA.

Mario Iglesias Alarcón

I started this Project thinking that it was going to be another regular and educative activity, but the first day, Francisco Abarca made clear that it was serious. Serious doesn't mean boring, of course. We started with some math, but later we began the work in the laboratory: plaques, pipettes and a lot of patience. It wasn't easy, I found difficult some calculations and I lost some meetings because of illnesses but, finally, we did it, we completed the table that summarize all the work, all our work was there. I felt proud, however, I thought that I did less work than the others. It has been a fantastic experience, something that has made me feel special, unique and lucky, because not everyone has the chance to do something like that.

Laura Pardo García

Participating in this project has been one of the most significant experiences in my life. To carry out activities related to everything that I like has been a real pleasure.

For me, everything about PIIISA projects was totally unknown. When entering a new Institute I have known closely the opportunity to work on a project like this.

I have to say that at the beginning of the investigation, most of the information was so strange for me that it started to scare me a little. The months went by and we became more familiar with the objective of the research.

In addition to many other things, we have learned different techniques of Microbiology for counts of bacteria, which consist of the preparation of culture media, picking colonies and preparing bacterial solutions.

It is a great satisfaction to have completed the investigation with consistent results. For my part, I have tried to put all my effort into the good development of the project. I´m proud to say that I am one of the few people that at my age, have collaborated on scientific research

After all the work done, is it more difficult to convince who hears you that what we say is true and that we also have to prove it. In fact our research has not finished, it has only just started.

Victor Fernández Trespando

In the research project "Where mutated genes?" organized by the EEZ, we were searching the rate of mutation of genes in *Sinorhizobium meliloti* bacteria by using sensitive and resistant to sucrose test. The first days were spent learning to calculate approximately the number of bacteria

present in a single colony by plate dilution. The final experiment consisted to prove in which of the three locations of the Sucrose sensitive cassette the genes mutated faster.

From my point of view the project has exceeded all my expectations. At the begining, as I consider it's normal, I was a little bit lost, as it was a matter (Biology, …) of who did not know much. But with time and practice I managed to understand everything better.
I leave very happy with the project and I believe I have learned quite a bit about Microbiology.

Clara Benavente

In this project, I met a small part of the microbiology, which is an important field in Biology, because microorganisms play important roles in our lives. At first I did not understand the concepts and how to work in a laboratory, but gradually, with the help of fellow project and researcher Francisco Martinez-Abarca I began to understand the calculations and at the end we have investigated mutations in the bacteria *Sinorhizobium meliloti*. The results were very accurate what turned out to be a surprise. We learned that repetition and not commit any fault (in silly things p. Ej) is important as it can lead to faulty assumptions.

Although it was a bit difficult, because I had to study the secondary course, we were able to complete the project on time.

I have learned to do a research project and this is an advantage that not everyone can have. It will be important throughout my life, because it is possible that my future studies are directed towards the world of research. I feel very fortunate.

Antonio J. Padilla Molina

In this project, we have look for a possible clue showing that a determinate gene mutation rate (in this case the gene is *SacB*) depends on where the gene is located in the genome. Our conclusion has been affirmative to this question. I think the real mean is that a particular gene mutation rate depends on where is located and the growth speed of the host cells.
I have learnt a lot in this project, from counting bacteria to some logistic questions that I wouldn't be able to think if I have not participate in this project. I think these months have opened mi mind in respect with some scientist thoughts.
I wouldn't change anything of the project. It has been great and I feel like so lucky. Now I see the biology from a different perspective and so much more important than before. I think PIIISA is a good project because of the ideas it gives to the students. I believe that some projects can completely change their student mind. I feel I'm not the same. After PIIISA I am even thinking of studying a Biology grade, that I would never have consider before. I will recommend some classmates' this is a great experience but it is not easy. Not everyone would be that happy on studying outdoors.

-WINNING ARTICLE OF THE III EEZ SCIENCE AWARD-

"HELPING FROM THE ROOTS"

SYMBIOSIS BETWEEN LIVE BEINGS. OBSERVING THE EFFECTS OF MYCORRHIZATION IN LETTUCE PLANTS.

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Key words : lettuce, mycorrhizas, symbiosis, natural balance in soil

SUMMARY

The fifth grade began the 2013/2014 school year studying the kingdoms of live beings. As part of this study, the fifth grade class at Alquería visited the EEZ-CSIC, to complete and enrich the research project started in the classroom. During this field trip, one of the concepts that aroused the interest of the students was symbiosis. After returning from the visit we decided to investigate more about this concept. We contacted the biologist Dr. Alberto Bago, who visited our school and told us about his research. Through him, we learned of the existence fungi that live in symbiosis with plant roots and benefit them in their growth.

With this knowledge we decided to observe and measure the effect of symbiosis on the growth of lettuce plants, specifically romaine lettuce, through different variables such as the number of leaves, stem thickness, and the fresh and dry weight of the plant. After completion of the experiment, we demonstrated that the lettuce plants which had contact with mycorrhizal fungi had higher values in the measured variables as compared to the group of lettuce plants that had no added mycorrhiza. On the other hand we observed that growth in both groups was negatively influenced by variables that we could not control, such as high temperatures that occurred during some weeks of our experiment and the presence of pests that affected the development of the plants. Another variable that affected our experiment was the supply of water to plants. We found it difficult to learn the right balance for watering plants, so we lost some plants in both groups, mainly due to stalk rot. At times we provided excess moisture to the soil, hoping the plants wouldn't lack water. However, we can conclude that due to the higher measured variable values in the group of mycorrhizal lettuce we achieved our goal of observing the effect of the symbiosis between fungi and lettuce. This successful partnership was confirmed in the laboratory, which showed us that there was mutually beneficial symbiosis between the lettuce and the fungus in the mycorrhizal lettuce.

INTRODUCTION

At the beginning of the project the year 5 students had a very basic level of knowledge regarding the living things with which we intended to begin our experiment. Thus in the first phase, we decided to learn about lettuce and mycorrhizal fungi. This research process was conducted in small collaborative work groups, which subsequently shared their information with the whole group. The information produced by the working groups includes the following:

1. The Plants.

The origin of lettuce plants is not very clear, and while some authors believe that it originated in India, others also think that the Egyptians were the first to cultivate it. The cultivation of lettuce dates back 2,500 years. It is probable that in ancient times, the Greeks and Romans already knew about it. The first known lettuces were open leaf. Closed head lettuces were known about in Europe in the sixteenth century. Today, lettuce still remains a favourite crop because it is one of the most common foods in our daily diet because it provides vitamins A and C. Lettuce can be cooked, but it is more often consumed fresh and raw. It is a fresh food and commonly used in salads.

Lettuce is an annual plant that can be grown throughout the year. Its botanical name is Lactuca sativa. The root is short and never exceeds a depth of 25 cm. It's pivotal and branches.
The leaves are arranged in a rosette; in some cases they retain this shape throughout their development (Romaine varieties), and others close into a head later. The edges of the leaves can be straight, wavy or serrated. The stem is cylindrical and branched.

2. Cultivation.

Romaine lettuce does not form a true closed head; the leaves are oblong, with complete borders and firm ribs. As the roots of lettuce are short, they do not need much space, or depth for growth. The lettuce grows in a variety of soils, but prefers well-drained soil.

Romaine lettuce needs warm temperatures to develop, but hot temperatures impede proper growth. Their ideal growing temperature ranges from 15 to 20 degrees during the day and less than 10 degrees overnight. It is essential that the lettuce is cultivated with a difference in temperature between day and night. Romaine lettuce can be better cultivatedin low temperatures than high; if the temperatures are higher than 25 or 30 degrees, its leaves can taste bitter.

Lettuce withstands drought, or lack of water, for a short period of time. Excess moisture is also a problem. Relative humidity suitable for lettuce is 60 to 80% and outdoor cultivation is recommended. Irrigation is important to keep the soil moist but should not be excessive. Sunlight is essential for normal plant development.

3. Mycorrhizas.

Scientists have observed filaments of fungi in the roots of the oldest plant fossil dating from 420 million years ago. Certain plants associate with organisms to get nutrients, and some fungi help plants get the nutrients needed for growth. Plants develop their roots in the soil and grow surrounded by a large number of living creatures, many of them microscopic, including fungi.

Some fungi associated with plants live together with them in a very intimate way that is known as mycorrhiza (Greek for fungus-root). The fungi help the plant "friend" not only to take the soil nutrients and water they need to grow and develop, but they also help them fight their "enemies" and confront negative environmental situations, such as lack of water.

Mushrooms and truffles, which grow near forest trees, are common types of fungi, and the mycorrhizas that most people are familiar witth. In other plants, such as vegetables, mycorrhization occurs with microscopic fungi. Therefore, mycorrhizas are a type of fungi that form symbioses with the roots of terrestrial plants. As previously mentioned, a mycorrhizal fungus obtains plant nutrients (carbohydrates) through the plant photosynthesis, since fungi cannot photosynthesize. Meanwhile the plant grows, because the fungus is introduced into the root surface and root biomass. The fungus helps in the absorption of minerals such as nitrogen (N), phosphorus (P) and potassium (K), which areb transferred to the plant through its hyphae, or filaments.

"Filaments" are how normal fungi grow. The filaments are thin, resistant strands forming a "spider

web" around the root and spreading over the ground. Virtually all plants grow with mycorrhizal fungi in a symbiosis that allows the plant to occupy more land with its roots with fungal filaments and therefore have better nutrition. The fungus takes advantage of the photosynthesis of the plant and the plant receives the nutrients from the fungus that it could not otherwise get. It also helps plants survive adverse environmental conditions such as drought.

4. Symbiosis.

According to the dictionary, symbiosis is the mutually beneficial association of different species of living organisms. The word symbiosis comes from Greek and can be translated as "livelihood". It is a biological association between individuals of different species. This word is used when both organisms derive benefit from this partnership.
Endosymbiosis occurs when one of the bodies is introduced into cells from another or space between one cell and another.
Ectosymbiosis occurs if one of the bodies is placed on the body of another.

5. Substrate

Substrate is essential for plant life as it is their food source and where the roots develop.
There are two different substrate types: those formed of earth and peat-based. Peat is an organic, natural product formed by the decomposition of plant residues. Peat-based substrate has few nutrients and needs to be fertilized during the first few weeks. It has good aeration and moisture retention.
The peat substrate used for our experiment also contained white perlite beads. Perlite retains moisture and fertilizes. Perlite is an inert, light, porous substance made of expanded volcanic rock. Perlite:

- Facilitates aeration.
- Cushions the soil.
- Helps drainage, when there is excess water and water circulation through the substrate.
- Facilitates growth and early plant root development.

6. Ecological Importance

Mycorrhizas associated in symbiosis with plants "fertilize without pollution" to help plants obtain nutrients when they can make or get their own food. This partnership between plants and fungi reduces the use of fertilizers and chemicals that can be harmful to the environment and our health, if used in excess. Mycorrhizas are also used in the recovery of areas with little vegetation and damaged soils as they help plants to grow in harsh environments. Additionally, they are also important in the recovery of threatened or endangered flora and species, including some plants endemic to the Sierra Nevada in Granada, Spain.

OBJECTIVES

The purpose of our experiment and work is to observe and measure the effects of this symbiosis in the growth of lettuce plants. The ground where mycorrhization occurs, in which plants live in symbiosis with fungi, will be a richer, healthier and more bio-diverse soil.

MATERIALS

1. Plants

30 **Romaine lettuce** seedlings: Lactuca sativa, long leaf variety.

2. Micro-organisms

Arbuscular Mycorrhiza in gel was provided by MYCOVITRO, a technological company associated with CSIC (Consejo Superior de Investigaciones científicas) whose technology comes from the Estación Experimental del Zaidín (Experimental Station, Zaidín, Granada, Spain.)

Figure 1: Romaine lettuce seedlings

Figure 2: mycorrhiza in gel

3. Pots

30 **pots.** Volume 1.5; dm 3; Height: 12.5 cm; base diameter: 11cm; diameter of the opening 13.5 cm. The pots were new and had not been previously used for another plant.

- **Substrate**: Black peat with perlite.

- **Fertilizer:** fertilizer DMPP by COMPO: 3 gr per pot. This fertilizer adds nitrogen (N), phosphorus (P), and potassium (K).
  Fertilization Date: 14 March, one week after the initial planting.

Figure 3. Substrate preparation

Figure 4. Calculating Volume

Figure 5. Pot Measurement

Figure 6. Watering the substrate

Figure 7. Preparation and Fertilization

METHODS

The plants were organized into two groups of 15 seedlings each.
The plants in the **control group (C.G.)** were numbered from 1 to 15, and called C.G. 1, C.G. 2, etc. and they were not exposed to the fungi.
The plants in the **experimental group (E.G.)** were numbered from 1 to 15 and labelled E.G. 1, E.G. 2, etc. These lettuce plants were exposed to mycorrhizal fungi.

Figure 8. Labelling the experimental and control group pots.

The variables measured were as follows:

- Length of the longest leaf.
- Number of developed leaves.
- Stem thickness.
- Fresh weight and Dry weight: shoot and root.

Environmental Conditions measured daily include: Temperature: maximum and minimum. Relative Humidity: maximum y minimum. Hours of sunlight.

The following tables show data from the measured variables. Collection methods included first, direct collection of the maximum and minimum temperature with a thermometer and also consultation on weather websites: http://www.eltiempo.es/ y http://www.aemet.es/to record the relative humidity and hours of sunshine, noting the times of the sunrise and sunset, to later calculate the hours of daily sunshine.

	Maximum T. (°C)	Minimum T. (°C)
Week 1	20	6
Week 2	22	13
Week 3	19	6
Week 4	19	6
Week 5	25	10
Week 6	26	10
Week 7	22	7
Week 8	24	10
Week 9	28	10

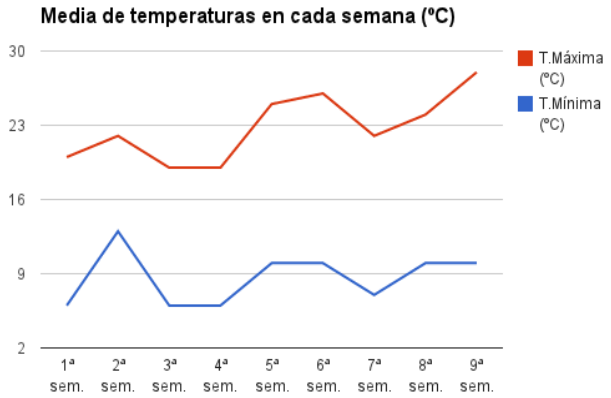

Figure 9. Weekly Temperature Measurements. Red: Maximum T. Blue: Minimum T

	Minimum Relative Humidity	Maximum Relative Humidity
Week 1	40%	84%
Week 2	38%	74%
Week 3	37%	90%
Week 4	40%	90%
Week 5	36%	85%
Week 6	32%	75%
Week 7	40%	86%
Week 8	30%	74%
Week 9	33%	76%

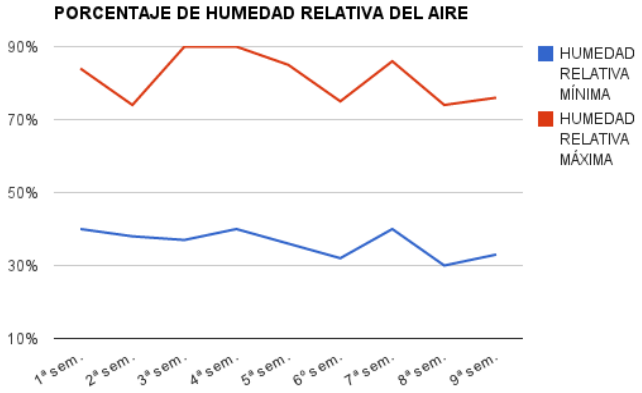

Figure 10. Percentage of Relative Humidity in the Air.
Blue: Minimum Relative Humidity – Red Maximum Relative Humidity.

DAILY HOURS OF SUNLIGHT (WEEKLY AVERAGE)	
Week 1	11h 43min
Week 2	12h 02min
Week 3	12h 19min
Week 4	12h 35min
Week 5	12h 50min
Week 6	13h 08min
Week 7	13h 24min
Week 8	13h 40min
Week 9	13h 52min

Figure 11. Daily hours of sunlight

Maximum temperatures were above 20 degrees, nearing 30°C for a few weeks. Meanwhile, the minimum temperatures were always over 5°C, reaching 10°C or higher for 5 weeks. Taking into account optimum temperatures for lettuce development, our plants experienced high temperatures in their development, both at the maximums and minimums. The minimum relative humidity was around 35% -40%. The maximum ambient humidity was above 70% -90%. As our experiment was conducted in late winter/ early spring, our developing plants were exposed to more than 12 hours of **sunlight daily**.

Date of transplantation and first measurements: 7 March 2014

Figure 12. Transplantation and first measurements.

Mycorrhization date for the E.G.: 7 March 2014
Quantity of mycorrhizas: 5 ml to 15 plants in the Experimental Group (E.G.)

Figure 13. Addition of mycorrhizal fungi

Irrigation:

Two systems of irrigation were used to water the plants in our project:

a. Cotton Ropes

In our project we used cotton ropes, stemming from bottles, and reaching the plant pots. The water rose through these ropes and reached the substrate in the pots, based on the principle of capillarity. Then the hairs from the roots of lettuce absorbed water and transported it to the leaves.

Figure 14. Preparing the bottles and cotton rope for irrigation

b. Irrigation from a plate.

Another form of irrigation involves filling a plastic plate beneath the pots with water. Water rises through capillarity to the small hairs on the roots that carry water to the leaves.

Figure 15. Measurement and recording of water taken up by the plants

Our experiment taught us about a property of water: **capillarity**. Capillarity is when water is able to rise through small tubes, via its the ability to stick to the duct walls. The smaller the conduit, the more water will rise.

Figure 16. Cotton rope irrigation

All of the water to offered to the plants was controlled. We recorded the amount of water that we originally gave them in an Excel table (google-drive).The total amount of water supplied to each plant during the two-month experiment is summarized in the following table.

Control Group	Water supplied		Experimental group	Water supplied
C.G.1 (20/3/14)	0,870 L		E.G. 1	4,470 L
C.G.2	4,620 L		E.G.2 (26/3/14)	1,270 L
C.G.3	4,320 L		E.G.3 (21/4/14)	2,670 L
C.G.4 (23/4/14)	2,420 L		E.G. 4	4,420 L
C.G.5	4,620 L		E.G.5	4,920 L
C.G.6	4,470 L		E.G.6	4,920 L
C.G.7	2,820 L		E.G.7	4,470 L
C.G.8 (28/3/14)	0,870 L		E.G.8 (27/3/14)	2,420 L
C.G.9	4,770 L		E.G.9	3,220 L
C.G.10	0,870 L		E.G. 10	4,770 L
C.G.11	4,470 L		E.G.11	3,470 L
C.G.12	4,470 L		E.G. 12 (11/4/14)	2,120 L
C.G.13 (3/4/14)	0,870 L		E.G. 13	3,370 L
C.G.14 (21/4/14)	2,420 L		E.G.14	4,920 L
C.G.15	4,420 L		E.G.15 (26/3/14)	0,870 L

Figure 17. TABLE: Amount of Irrigation Water. The brackets indicate the date on which the plant was lost and therefore stop receiving water.

Tying each head of lettuce: 21 Abril 2014

Figure 18. Tying head of lettuce

Final Harvest Date: 7 May 2014

Figure 19. We took the plants out of their pots, removed the substrate and washed the roots.

RESULTS AND DISCUSSION

a. Stem thickness.

Stem thickness	C.G. (cm)	E.G. (cm)
7/03/2014	0.3	0.3
11/04/2014	1	1.2
7/05/2014	1.1	1.4

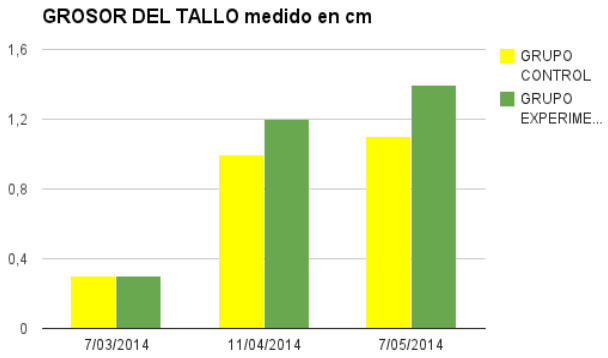

Figure 20. Stem thickness measured in cm. Yellow: Control Group – Green Experimental Group. Observation and data recording dates

In the first measurement the average stem thickness is equal in both groups.
In the middle of the experiment the average measurements of the mycorrhizal lettuce group exceed the control group by 0.2 cm.
In our last measurement, the average stem thickness in the experimental group exceeded the average measurements of the stem thickness in the control group by 0.3 cm.

b. Length of the largest leaf.

Leaf Length	C.G.	E.G.
7/03/2014	12,42	11,9
11/04/2014	22,5	22,2
7/05/2014	26,4	27,1

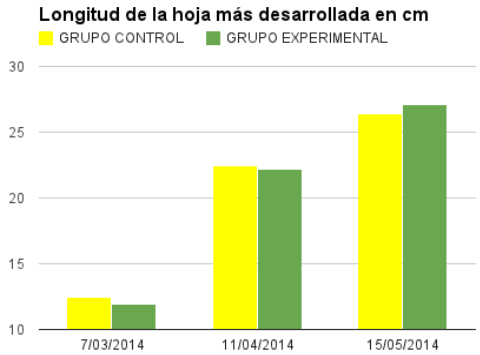

Figure 21. Length of the largest leaf measured in cm. Yellow: control group. Green: experimental group Observation and measurement dates

The leaf length in the control group at the first measurement (prior to adding fungus to the plants) was 0.52 cm longer than the average of the lengths in the experimental group.
In the second measurement this difference was reduced to 0.30 cm, though the lettuce in the control group without mycorrhizas still exceeded the experimental group in size.
In the last data collection, the average leaf length measurements in the experimental group of mycorrhized lettuce exceeded the average of the measurements of the group without mycorrhizas by 0.70 cm.
Therefore the increase in the control group was 13.98 cm, while the increase in the mycorrhizal group was 15.20 cm.

c. Number of healthy leaves.

Number of leaves	C.G.	E.G.
7/03/2014	5,4	5,1
24/03/2014	6	7,2
4/04/2014	10,2	11,5
11/04/2014	13	16
7/05/2014	26,4	33,6

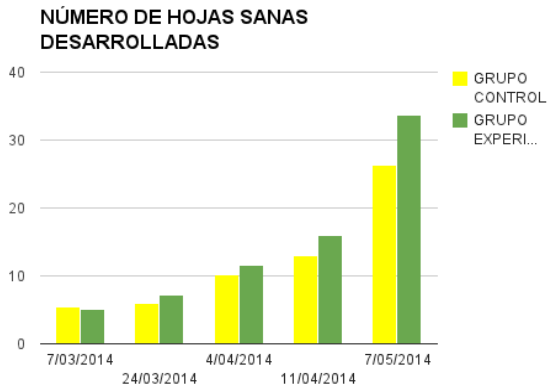

Figure 22. Number of Healthy Leaves. Yellow: Control Group – Green Experimental Group
Observation and data recording dates

When we first recorded data, the lettuce plants in both groups had almost exactly the same number of leaves. In the second count, the average number of leaves in the experimental group exceeded the number in the control group by 1.2. On the third count the difference in number of leaves increased slightly to 1.3. In the fourth count there was a difference 3 leaves more in the experimental group. Finally in the last data collection, the average number of developed leaves in mycorrhized group was 7.2 more than in the lettuce without mycorrhiza.

d. Fresh Weight.

When the lettuce plants were picked (7 May 2014) we separated the aerial parts of the plant from the root and we weighed them separately.

Fresh weight (g)	Roots	Aerial parts
C.G.	6,8	99,8
E.G.	11,3	109,3

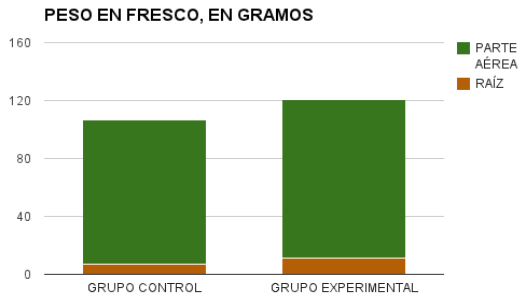

Figure 23. Fresh Weight, in grams. Green: Aerial part. Orange: Root.
Control Group – Experimental Group

The average weight of the lettuce roots in mycorrrhizal group was 4.5 gr. more than the average weight of the roots without mycorrhizas.
Additionally, the weight of the aerial part of the lettuce plants in the mycorrhizal group was 9.5 gr. more than the control group.

e. Dry weight.

After harvesting and weighing the fresh plants, the plants were dried in the sun, wrapped in absorbent paper for a week. Once dried, the root and aerial parts were weighed separately to measure the amount of organic matter without water.

Dry Weight (g)	Roots	Aerial part
G.C.	0,01	0,06
G.E.	0,06	0,1

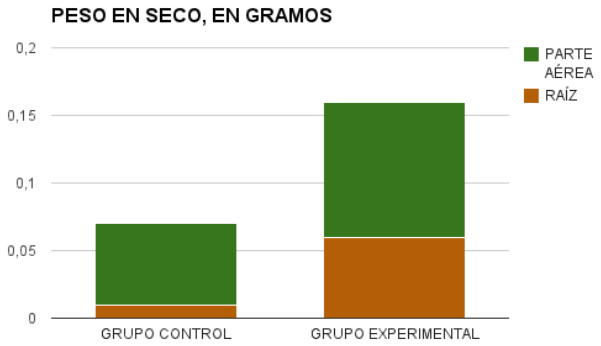

Figure 24. Dry Weight in grams. Green: Aerial part. Orange: Roots.
Control Group - Experimental Group

The average weight of the roots of mycorrhizal lettuce was 0.05 gr. greater than the lettuce in the control group. The dry weight of the aerial part of mycorrhizal group also exceeded the dry weight of the control group lettuce by 0.04 gr.

LABORATORY OBSERVATION OF THE MYCORRHIZATION.

In the final phase of our project, we contacted the company MYCOVITRO and requested their cooperation to check if our lettuces had been mycorrhizal and at what rate.
Thus, on the 27 May we visited this lab and we found that there had been a 16% mycorrhization of our lettuce plants. This data supports the data obtained in our measurements, as our results showed the experimental group to have higher measures than the control group in all variables measured.
Thanks to the collaboration of MYCOVITRO, who prepared the roots of our lettuce with a stain, we observed mycorrhization microscopically.
In the microscopic observation it was clear that the mycorrhiza introduced into the roots of our lettuce was fundamentally arbuscular. This means there were many arbuscules, indicating that symbiosis worked well and both the plant and the fungus benefitted from the association. Spores were also observed, which means that the fungus was reproducing to keep living in that soil and continue colonizing new roots. This indicates that we were helping enriching the soil, fostering biodiversity and making healthier substrate.

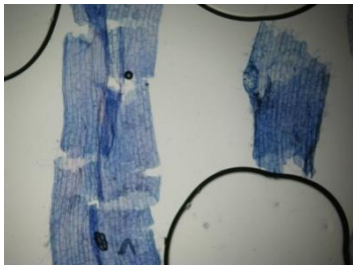

Figure 25. Root with no mycorrhizal association in the C.G.

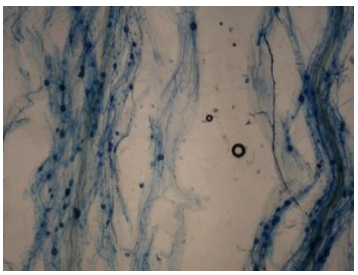

Figure 26. Mycorrhizal root with spores from the E.G.

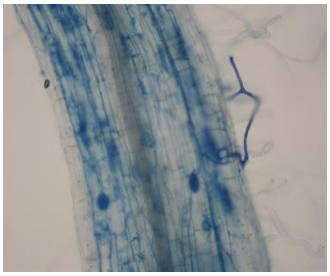

Figure 27. Roots with early stage arbuscules and spores.

DIFFICULTIES ARISING DURING THE EXPERIMENT.

Stem rot.

One of the main difficulties we encountered in our project was stem rot in various plants. Five plants from each group were afflicted due to excess water. It proved difficult to find balance of water required for proper plant development.

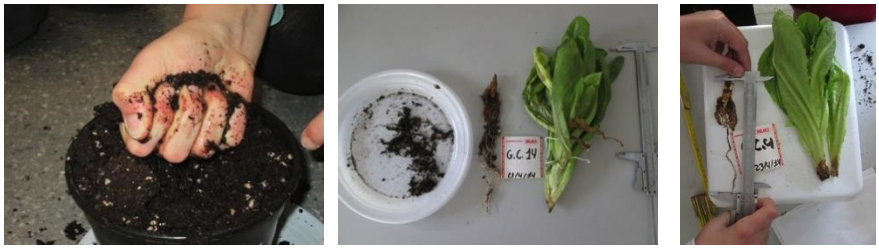

Figure 28. Stem rot

Appearance of Pests.

Another difficulty encountered during the course of the experiment was the emergence of aphids on all plants. Furthermore, although less problematic than the aphids, snails appeared, and the leaves were pecked by birds and caterpillars.

Figure 29. Appearance of Pests.

Finding solutions: biological control.

Due the appearance of the aphids in the last phase of our project, we researched the predators of these insects and we introduced these predators to attempt control the pests. Ladybugs and lacewings were introduced to the plants. However, because the plague was rampant and the incorporation of this auxiliary fauna occurred in the last stage of the project, we were unable to test the effects of these predators on aphids.

Figure 30. Finding solutions: biological control

Defective development of lettuce heads.

The stems of the lettuce, in both groups, grew too big, impeding correct development. We believe this may have been due to the excessive temperatures, as both the maximum and minimum were higher than those recommended for proper plant growth.

Supplementary images: experimental follow-up of the project

Measurement of the length of the healthiest, biggest leaf

Measuring stem thickness

Counting number of healthy developed leaves

Weighing and recording the weights of fresh aerial lettuce parts and roots

Measuring and recording the dry weights of the roots and aerial plant parts

Microscopic observation in Mycovitro lab

CONCLUSIONS

Upon the completion of our project we reached the following conclusions:

- The lettuce associated with mycorrhizas obtained higher average measurements in measured variables.
- We were able to prove that symbiosis between the fungus and root really occurred as shown by microscopic observation on our visit to the laboratory. This is consistent with the differences in the measures in favour of the experimental group of mycorrhizal lettuce.
- The average measurements were very different in each group.
- The percentage of mycorrhizal colonization according to the laboratory was around 16%. This data, which indicates a low level of mycorrhization, justifies the small differences found between the two groups.
- We found it difficult to determine the right amount of water to give the plants. In several plants excess water caused stem rot. On the other hand, if we watered too little or infrequently, some leaves dried up and fell off the plant.
- When the first aphids appeared, we waited too long to introduce a biological control using predators, such as ladybugs. The aphids weakened the plants.
- We believe that the high temperatures, with 30ºC maximums, during several weeks of the experiment led to an incorrect development of the lettuce head as the stem grew excessively.
- As a result of this experience, we propose continued studies about mycorrhization. One proposal includes using a different vegetable and planting it in the school garden as opposed to pots.
- Another future project could study the interaction of auxiliary fauna: using ladybugs and lacewing insects to combat aphids to determine the benefit to the plants.
- After this experience, taking into account everything we have researched and experienced, we have concluded that mycorrhizas are beneficial partners in the development of plants and their natural protection.
- The use of mycorrhiza favours the natural balance of the environment and the growth of natural fresh vegetables. It generates richer, healthier soils with greater biodiversity.
- If by using mycorrhiza, the use of chemical substances to promote plant growth can be reduced, this will benefit the health of people and the environment.

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- **EEZ-CSIC**, for spreading the world of Science and Scientific Research, motivating us at the start of this research project.
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