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

Estación Experimental del Zaidín

HIGH SCHOOL STUDENTS FOR AGRICULTURAL SCIENCE RESEARCH II

NEW ADVANCES IN THE INTERACTION OF PLANT AND THE ENVIRONMENT

PROCEEDINGS OF THE II CONGRESS PIIISA-ESTACIÓN EXPERIMENTAL DEL ZAIDÍN

Universidad de Granada
JUNTA DE ANDALUCIA Consejería de Educación
CSIC CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
GOBIERNO DE ESPAÑA MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD
FECYT FUNDACIÓN ESPAÑOLA PARA LA CIENCIA Y LA TECNOLOGÍA
Obra Social "la Caixa"

bPIISA

Universidad de Granada

JUNTA DE ANDALUCIA
Consejería de Educación

eez
Estación Experimental del Zaidín

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

GOBIERNO DE ESPAÑA
MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD

FECYT
FUNDACIÓN ESPAÑOLA PARA LA CIENCIA Y LA TECNOLOGÍA

Obra Social "la Caixa"

High School Students for Agricultural Science Research II. *New Advances in the Interaction of Plants and the Environment*

Proceedings of the II Congress PIIISA Program (Science research by young students in Science, Technology and Humanities)

2nd May, 2013
Estación Experimental del Zaidín. CSIC.

Scientific Editors

•Juan de Dios Alché
• Silvia Alguacil
•Elisabet Aranda
•Antonio Jesús Castro
•Francisco Javier Corpas
•Inmaculada García Romera
•Francisco Martínez-Abarca
•José Manuel Palma
• Mª Eugenia Ramos
• Ana Belén Robles

ISSN: 2340-9746

Edited in Granada, Spain by Estación Experimental del Zaidín. CSIC.

INDEX

PREFACE / PREFACIO

The Estación Experimental del Zaidín (EEZ) takes part in the new course of the PIIISA Programme. The success obtained last season was the challenge for all scientists in our centre this year. In 2012, we held the I Congress PIIISA – Estación Experimental del Zaidín, where six oral communications and their respective posters were displayed. Likewise, we published an ISBN referenced book which reported the proceedings of the congress. More than 70 people participated including students, teachers and researchers from the EEZ. Our activity was awarded the **First Prize** of the International Contest **Science in Motion** in the category of Didactic Materials in Science Outreach.

This year we propose another six projects, and new research groups are involved. About 38 students from 10 high schools and 17 scientists from three departments of the EEZ participate. The achievements from last year have been an incentive to propose more innovative projects to be boarded with audacity. We are confident the PIIISA at the EEZ will succeed again. Some of the results already obtained are the first notice in their respective fields and we envisage they will have some repercussion. As a consequence we will hold the II Congress PIIISA – EEZ and will edit the book *High School Students for Agricultural Science Research II. New Advances in the Interaction of Plants and the Environment.* This book is a real portrait of the PIIISA itself, so it combines Science and human attributes. Thus, each chapter is basically composed by two well differentiated parts: the first one is conjugated as a scientific article as displayed in any scientific journal; the second one, namely **My Own Ideas** and strictly written by the students, shows their feelings and their experiences in our laboratories at the EEZ.

To reach the target we have gone across a complex journey. All, students, relatives, teachers and researchers have put all our effort, and demand to our institutions to commit leading this kind of initiatives. The selection of students was hard, and this has improved some debility manifested last year. The participation of students from diverse villages from the province of Granada, that could have created some complexity, has been solved notoriously thanks to the will and devotion of students, parents and teachers.

Regarding the EEZ, this year some integrating projects in which different research groups have worked simultaneously have been achieved. There have been hard times. Our projects needed quite a lot more sessions than those proposed in the programme. That and the extra labour we are doing are the reasons why we have to applaud and acknowledge to all participants their involvement and their commitment.

All around PIIISA, with all details already indicated above and some other else, lead me to wonder about the future of this Programme and the role of the EEZ and of other CSIC participating centres. This subject is being accomplished right now in order to build the fundamentals to promote the innovative spirit and the virtues of this initiative in the coming years. We hope all of us, together our institutions, face this challenge which pursue the promotion of a higher scientific environment thus allowing our country being placed within the world scientific elite.

La Estación Experimental del Zaidín (EEZ) participa en la nueva edición del Programa PIIISA (Proyecto de Iniciación a la Investigación de Innovación en Secundaria en Andalucía). El éxito obtenido el año pasado suponía un reto para todos los investigadores de nuestro centro en la nueva edición. En 2012 celebramos el I Congress PIIISA – Estación Experimental del Zaidín, donde se presentaron seis comunicaciones orales con sus respectivos pósteres. Asimismo se publicó un libro con referencia ISBN donde se recogían las actas del congreso. En el mismo participaron más

de 70 personas entre estudiantes, familiares, profesores e investigadores de la EEZ. Nuestra actividad fue galardonada con el ***Primer Premio*** *del Certamen Internacional* ***Ciencia en Acción*** *en la modalidad de Materiales Didácticos de Ciencia y Trabajos de Divulgación Científica.*

En este curso contribuimos con otros seis proyectos, habiéndose incorporado nuevos grupos de investigación. Participan 38 alumnos de 10 institutos y 17 investigadores de tres departamentos de la EEZ. Los logros del año anterior han sido un incentivo para plantear en esta edición proyectos más innovadores que hay que abordar con cierta dosis de audacia. Confiamos en que este año el proyecto PIIISA vuelva a ser un éxito en nuestro centro. Algunos de los resultados obtenidos son una primicia en los respectivos campos y vislumbramos que tendrán gran repercusión. Y, por lo pronto, lo celebraremos en el II Congress PIIISA – EEZ y editaremos el libro High School Students for Agricultural Science Research II. New Advances in the Interaction of Plants and the Environment. Este libro es un retrato real del propio PIIISA, donde se combinan Ciencia y virtudes humanas. Así, cada capítulo se compone básicamente de dos partes: la primera se estructura como un artículo científico propio de revistas científicas; la segunda parte, denominada ***My Own Ideas*** *que ha sido escrito exclusivamente por los estudiantes, muestra sus inquietudes y su experiencia en nuestros laboratorios de la EEZ.*

Para llegar a la meta hemos tenido que recorrer un camino que no ha sido fácil. Todos, alumnos, padres, profesores de instituto e investigadores hemos volcado en los proyectos un esfuerzo enorme y demandamos que las instituciones que representamos abanderen este tipo de iniciativas. La selección de alumnos participantes fue exigente, lo que ha mejorado algunas debilidades de la edición anterior. La incorporación de alumnos de diversos pueblos de la provincia de Granada, que podría haber ocasionado una complicación, se ha saldado con solvencia gracias a la voluntad y dedicación de alumnos, padres y profesores.

Con respecto a la EEZ, este año se han realizado proyectos que integraban la labor simultánea de varios grupos de investigación. Ha habido momentos difíciles. Nuestros proyectos implicaban bastantes más sesiones que las establecidas obligatoriamente. Por eso, y porque todos estamos realizando una labor extra a la que estamos llamados a hacer, es por lo que hay que aplaudir y agradecer a todos los participantes su implicación y su compromiso.

Todo cuanto concierne al PIIISA, con los pormenores ya indicados y otros más, me lleva a plantearme varios interrogantes sobre el futuro de este Programa y del rol de la EEZ y de los otros centros del CSIC participantes en el mismo. Asunto sobre el que estamos trabajando para sentar las bases que permitan que en las próximas ediciones se mantenga el espíritu innovador, la calidad y las virtudes que posee esta iniciativa. Esperamos que todos nosotros, junto con nuestras instituciones afrontemos este reto que no persigue otra cosa que el fomento de un entorno con más cultura científica que lleve a nuestro país a ocupar el lugar que le corresponde en la élite científica mundial.

José Manuel Palma Martínez

This book presents the Second Edition of the PIIISA 2013 Program on Agricultural Science Research for High School Students. Based on our previous experience from last year, this Second Edition has been considerably improved. Additional sessions have been included and the selected students are more interested with the work they are expected to perform. On their side, the scientific personnel involved are also more conscious of the fact that they are not dealing with University

students, but with High School students. Bearing all these aspects in mind, the projects have been readapted to fit both the students and the researchers in charge, thus guaranteeing a higher rate of success than the previous year (close to 100%).

The projects reflect the students' astonishing capacities and maturity to process and perform the appropriate experiments in a short period of time. These time restrictions are common to all PIIISA projects and that is what makes them substantially different to any other research project. **Time** is the most difficult part to control and it is intrinsically divergent from research itself where "haste makes waste".

Throughout these pages you will find an atmosphere of 'scientific magic'; there will be plenty of time ahead to burst this bubble and for these brilliant students to get their heads stuck into pure, hard science.

The seed has been sown.

En este libro se presenta la segunda edición del "High School Students for Agricultural Science Research", correspondiente al Proyecto PIIISA2013 (Proyecto de Iniciación a la Investigación e Innovación en Secundaria en Andalucía). Como toda segunda edición presenta aspectos mejorados debidos a la experiencia del año anterior. En esta ocasión, ha habido más sesiones, el alumnado escogido ya era consciente del posible trabajo a llevar a cabo y los investigadores ya sospechábamos que los jóvenes con los que tratábamos no se correspondían a licenciados universitarios. Fruto de todo ello, los proyectos realizados se han adaptado en tiempo al tipo de investigadores que los presentan. De ello se deduce un mayor porcentaje de éxito que en la Edición anterior.

A pesar de ello (o como consecuencia de ello), de nuevo se respira en los proyectos la sorprendente capacidad del alumnado de secundaria (14-17 años) en adquirir y realizar los experimentos en un plazo breve de tiempo. Porque, esa brevedad es quizás el denominador común de todos los proyectos PIIISA y por otro lado diferenciador con cualquier otro Proyecto de Investigación. Es ese TIEMPO, quizás la parte más complicada de gestionar, ya que es antagónico con la investigación en sí, donde no siempre las "prisas" son buenas consejeras.

Estos proyectos no sólo versan sobre la investigación y su éxito sino sobre la capacidad de los mismos para entusiasmar a jóvenes investigadores por la Ciencia. Se ha vuelto a generar un ambiente "mágico" en la ciencia; más adelante, el tiempo determinará el que estos estudiantes sean capaces de poner sus brillantes mentes al servicio de la ciencia pura y dura

La semilla ya está plantada.

Francisco Martínez-Abarca Pastor

EFFECT OF THE STIGMA EXUDATE ON POLLEN PERFORMANCE IN *Lilium longiflorum*, AND SEQUENCE RECONSTRUCTION OF EXUDATE PROTEINS

Sergio Yeste Quiles[1], Charo López Barrientos[1], Elisa Zurita Ferrández[2], Yasmine Dris Ghali[3], Alejandro Zarco Sánchez[4], Luis Carlos Paredes Retamero[5], Aída López Amos[5], Krzysztof Zienkiewicz[6], María José Jiménez Quesada[6], Juan de Dios Alché Ramírez[6] and Antonio Jesús Castro López[6,*]

[1]*IES Alpujarra, C/ de Lora Tamayo s/n, 18400 Órgiva, Spain*
[2]*IES Miguel de Cervantes, Camino Real de los Neveros 1, 18008 Granada, Spain*
[3]*IES Luis Bueno Crespo, Avda. de las Palmeras s/n, 18100 Armilla, Spain*
[4]*IES Severo Ochoa, Avda. de las Alpujarras s/n, 18015 Granada, Spain*
[5]*IES Padre Manjón, C/ Gonzalo Gallas s/n, 18003 Granada, Spain*
[6]*Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, CSIC, C/ Profesor Albareda 1, 18008 Granada, Spain*
[*]*Corresponding author:* ***e-mail:*** *antoniojesus.castro@eez.csic.es*

SUMMARY

In plants with wet stigma, pollen grains land on a viscous secretion without any species selectivity. This secretion provides a water source for rapid hydration of the pollen grain and mediates pollen tube adhesion, nutrition and guidance. To date, the composition of the stigma secretome is poorly known. In this work, we studied the effects of the stigma exudate on pollen performance in *Lilium longiflorum* and we provided a comprehensive list of the exudate proteins. Data showed that the stigmatic secretion is a metabolically active site and shows a markedly catabolic profile. The addition of this secretion to the germination medium promotes both pollen germination and pollen tube growth in this species. The Eastern lily stigma secretome consists of at least 51 different proteins, mostly involved in sugar catabolism, defense against both biotic and abiotic stresses, and regulation of pollen tube growth and guidance through the female tissues.

INTRODUCTION

In Angiosperms, pollination begins when pollen comes into contact with the stigma surface and hydrates. In plants with a wet stigma, the surface cells release a viscous secretion in which pollen grains are embedded without any species selectivity [1]. This secretion mediates pollen capture and adhesion, and provides a water source for rapid hydration of pollen and a protective environment for fragile pollen tubes during the first steps of germination. Pollen tubes also uptake nutrients and other compounds from this fluid such as pectic wall precursors [2]. When non-compatible pollen grains reach this stage, further access is inhibited by blocking pollen tube growth.

The stigma exudate mainly consists of water, polysaccharides, lipids and proteins [3]. Other organic molecules such as phenolic compounds [4], amino acids [5], Ca^{2+} ions [6], and ROS and RNS [7] are also present in lower amounts. To date, the qualitative composition of the exudate protein pool and its involvement in pollen-stigma interaction is poorly known. In this context, the objectives of this work were: 1) to study the effect of the stigma exudate (SE) on

pollen performance in *Lilium longiflorum* (Eastern lily), and 2) to provide a comprehensive list of the proteins present in this secretion.

MATERIALS AND METHODS

Plant material

Flowering cuttings of Eastern Lily (*Lilium longiflorum* Thunb. cv. 'White Heaven') were purchased at a local market (Figure 1A). Flowers were emasculated just before anthesis and the drops of stigmatic secretion were collected from 20 flowers using a pipette. The protein content was estimated by the method of [3] using the Bio-Rad reagent (Bio-Rad, USA) and BSA as standard. Anthers were left to open on a filter paper and pollen was collected using a fine spatula.

Pollen viability assay

Eastern lily pollen viability was assayed using the FCR (fluorochromatic reaction) method described by [4].

In vitro pollen germination experiments

Pollen was incubated in a humid chamber for 30 min and then transferred to Petri dishes (0.01 g per dish) containing 1 ml of germination medium [10% w/v sucrose, 0.03% w/v $Ca(NO_3)_2$, 0.01% w/v KNO_3, 0.02% w/v $MgSO_4$ and 0.01% w/v boric acid]. Petri dishes were maintained at room temperature in the dark, and pollen grains were sampled after 3 h of culture. To determine the effect of the SE on pollen performance, the culture medium (0.5 ml) was supplemented with 0.5 ml of exudate and pollen was cultured as above. Experiments were carried out in triplicate.

Samples were observed with a Axioplan epifluorescence microscope (Nikon, Japan) under blue light irradiation and images were recorded with a ProgRes C3 camera (Jenoptik Laser, Germany) using the ProgRes CapturePro software (Jenoptik Laser). The germination rate (%) was calculated from 300 pollen grains randomly counted (100 grains per count × 3 independent experiments). Pollen tube length was measured from 75 germinated pollen grains (25 grains per count × 3 independent experiments) using the PowerPoint software (Microsoft, USA). The mean and standard deviation for each parameter were calculated and plotted using the Excel software (Microsoft).

SDS-PAGE

Exudate proteins (500 µl) were precipitated in 9 volumes of 20% w/v TCA and acetone for 6 h, and resuspended in 0.5 ml of sample buffer [6]. Protein samples (1, 2, 3, 4, 6, 8 and 10 µg) were separated by SDS-PAGE on 1 mm-thick slab gels using a 4% polyacrylamide stacking gel and a 5-15% resolving gel according to standard procedures [6].

Proteins were stained with a colloidal Coomassie Blue solution (0.12 % w/v Brilliant blue G250, 10% w/v ammonium sulfate, 10% v/v phosphoric acid and 20% v/v methanol). Images were recorded in a Pharos FX scanner (Bio-Rad).

Protein sequence analysis and reconstruction

Protein sequences obtained by MS/MS analysis were further anlyzed *in silico* using different bioinformatic tools including Pfam (clasification of proteins based on identified protein domains; //pfam.sanger.ac.uk/), FunCat (functional classification of proteins based on the Fun-CatDB functional catalogue; //mips.helmholtz-muenchen.de/proj/funcatDP), and GO component (describes subcellular locations of proteins; //www.ebi.ac.uk/QuickGO). Protein sequence reconstruction was performed using the COBALT tool (//www.ncbi.nlm. nih.gov/).

RESULTS

Effects of the stigma exudate on pollen performance in *L. longiflorum*

In Eastern lily, the stigma exudate was deposited as macroscopic drops on the stigma surface (Figure 1B). Pollen viability was first assessed using the FCR test (Figure 1C-D). Data showed that about 42% (SD±6.3%) of pollen grains were viable (i.e. fluorescent).

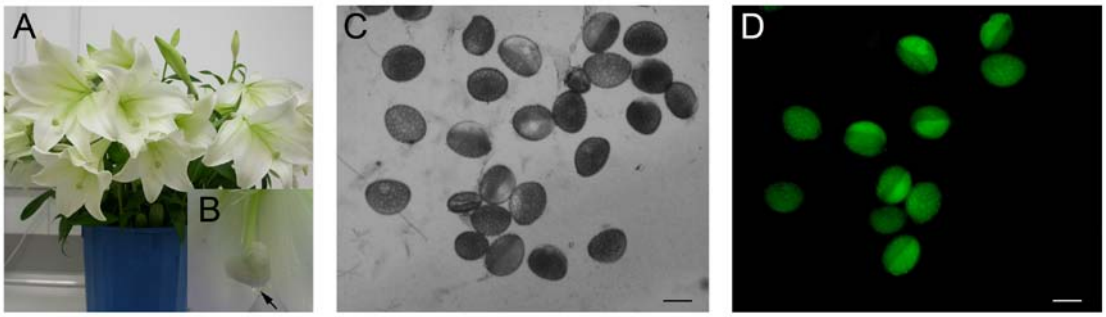


Figure 1. (A) Eastern lily (*L. longiflorum*) flower cuttings. (B) Macroscopic drops of exudate (arrow) are formed on the stigmatic surface. (C-D) Assessment of pollen viability. Photomicrograph of Eastern lily pollen grains under the transmitted white (C) and UV (D) light, respectively. Bars= 50 µm.

To determine the effect of the stigma exudate on pollen performance, pollen was germinated in a culture medium supplemented with this secretion. We observed that the percentage of germinated pollen grains increased two-fold in exudate-containing experiments compared with non-treated controls (Figure 2A). In addition, pollen tubes also grow to a higher rate in the presence of the exudate (Figure 2B).

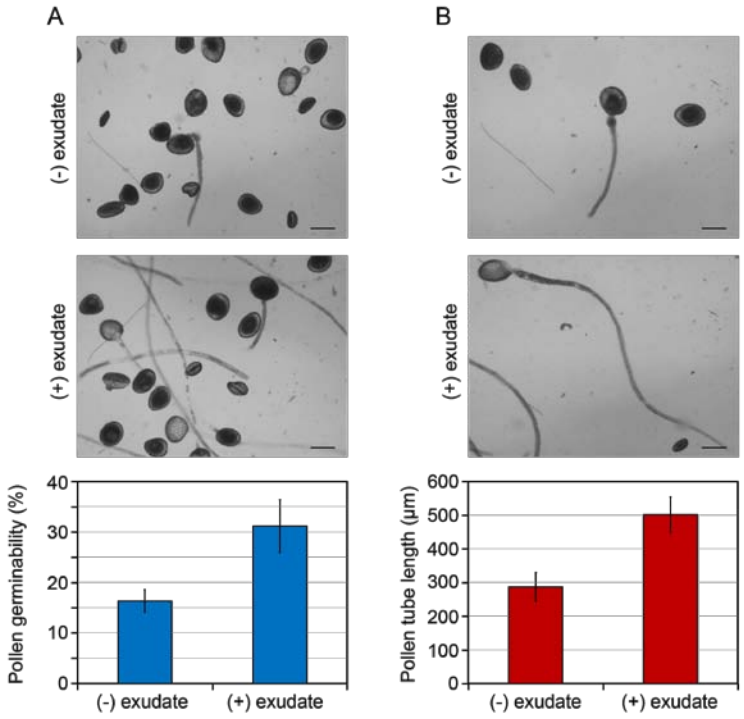


Figure 2. Effect of the stigma exudate on the lily pollen germinability (A) and the pollen tube growth (B) rates. Bars= 50 µm.

Further, we analyzed the 1-D protein profile of the Eastern lily stigma secretome by SDS-PAGE. After staining, up to 21 protein bands were visible on polyacrylamide gels (Figure 3).

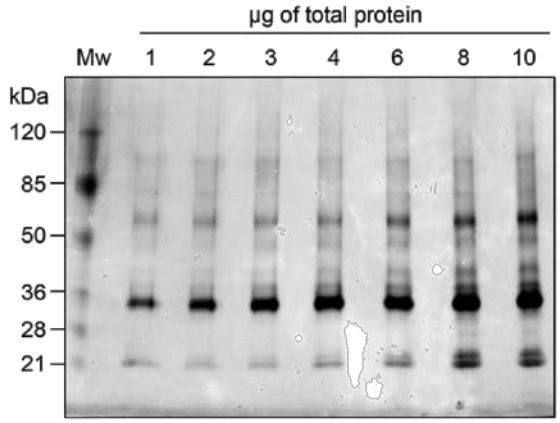


Figure 3. 1-D protein profile of the *L. longiflorum* stigma secretome. Protein markers are displayed on the left.

Protein bands were analyzed by mass spectrometry (data not shown), resulting in a comprehensive list of 396 putative exudate proteins. In order to overcome protein redundancy, we carried out partial reconstruction of protein sequences using a multiple alignment tool (i.e. COBALT). Following this strategy, we found that the stigma secretome consisted of at least 51 proteins, half of which were polymorphic (i.e. two or more isoforms). For instance, the amino acid sequence of a GDSL-like lipase enzyme was partially reconstructed from two putative GDSL-like lipases (gi numbers: 71143481 and 164519779) identified in the exudate. This sequence showed microheterogeneities in at least six different amino acid positions (Figure 4).

```
  1 ------------------------------------------------------------------------------
                                        L
 81 ------------------------GVNYASAAAGIR--------BLDFTGQVTNYK-----QVVDLL------------

161 ------------------------------------------------------------------------------
       L     L           T DG
241 ---BINVANQIFNNK---LVDDFNSNSPDAK----------------------BVTNAGCCGVGR---------------

321 ------------------------------------------
```

Figure 4. Partial reconstruction of the amino acid sequence of a GDSL-like lipase enzyme present in the stigma exudate of *L. longiflorum*.

The predicted subcellular localizations of exudate proteins are shown in Figure 5. There were no data available in GO database for more than half of proteins identified. Only 5.5% of proteins showed a predicted extracellular location, although 20% of proteins were expected to localize in the apoplastic space or the cell wall (Figure 5). This is a site for accumulation of secreted proteins that are further released during development. Unexpectedly, about 20% of matched proteins were predicted to be either intracellular or associated to the plasma membrane.

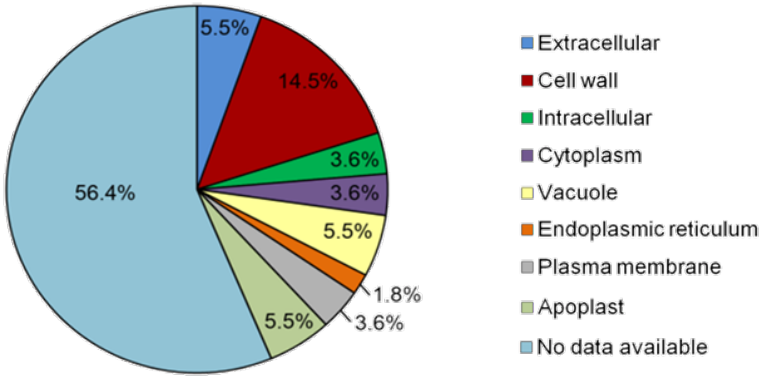


Figure 5. Cellular distribution of *L. longiflorum* SE proteins according to Gene Ontology (GO) classification [11].

Lily stigma proteins were classified into 58 protein families (data not shown) [12]. We also found that the stigma secretome might be involved in 11 different functions. Major functional categories included carbohydrate metabolism, energy, regulation of protein activity and fate, and response to biotic and abiotic stresses (Figure 6). Other important functions were cell wall organization, cell signaling, cell adhesion and cellular transport (Figure 6).

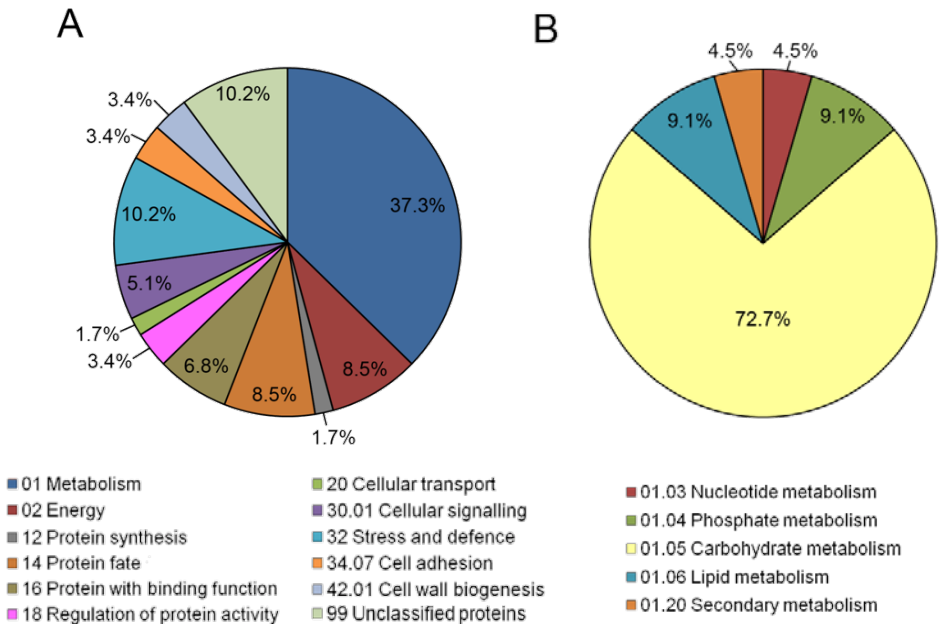


Figure 6. Distribution of functional classes of the Eastern lily stigma exudate proteins according to FunCat classification [13]. (A) Main functional categories. (B) Secondary functional categories referred to metabolism.

CONCLUSIONS

1. The stigmatic secretion promotes pollen germination and pollen tube growth in *L. longiflorum*.
2. The lily stigma secretome consists of at least 51 different proteins, of which about 50% are polymorphic.

3. The lily stigma exudate is a metabolically active site and shows a markedly catabolic profile. Based on its protein profile, this secretion is likely involved in pollen tube nutrition and adhesion, and regulates pollen tube growth and guidance through the female tissues.

ACKNOWLEDGEMENTS

This work was supported by ERDF-cofinanced projects AGL2008-00517 and BFU2011-22779 (Spanish Ministry of Science and Innovation, MICINN), and P2010-CVI5767, P2010-AGR6274 and P2011-CVI7487 (Junta de Andalucía). We also thank Prof. María Isabel Rodríguez-García for scientific discussion and Concepción Martínez-Sierra for expert technical assistance.

REFERENCES

[1] Sanchez AM, Bosch M, Bots M, Nieuwland J, Feron R, Mariani C (2004) Pistil factors controlling pollination. The Plant Cell 16: S98-S106.
[2] Labarca C, Loewus F (1972) The nutritional role of pistil exudate in pollen tube wall formation in *Lilium longiflorum*: utilization of injected stigmatic exudate. Plant Physiology 50: 7-14.
[3] Suárez C, Castro AJ, Rapoport HF, Rodríguez-García MI (2012) Morphological, histological and ultrastructural changes in the olive pistil during flowering. Sexual Plant Reproduction 25: 133-146.
[4] Martin FW (1969) Compounds from the stigmas often species. American Journal of Botany 56: 1023-1027.
[5] Konar RN, Linskens HF (1966) Physiology and biochemistry of the stigmatic fluid of *Petunia hybrida*. Planta 71: 372-387.
[6] Zienkiewicz K, Rejón JD, Suárez C, Castro AJ, Alché JD, Rodríguez-García MI (2011) Whole-organ analysis of calcium behavior in the developing pistil of olive (*Olea europaea* L.) as a tool for the determination of key events in sexual plant reproduction. BMC Plant Biology 11: 150.
[7] Zafra A, Rodríguez-García MI, Alché JD (2010) Cellular localization of ROS and NO in olive reproductive tissues during flower development. BMC Plant Biology 10: 36.
[8] Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.
[9] Heslop-Harrison J, Heslop-Harrison Y (1970) The evaluation of pollen viability by enzymatically induced fluorescence: intracellular hydrolysis of fluorescein diacetate. Stain Technology 45: 115-120.
[10] Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
[11] The Gene Ontology Consortium (2010) http://www.geneontology.org.
[12] Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, Khanna A, Marshall M, Moxon S, Sonnhammer ELL, Studholme DJ, Yeats C, Eddy SR (2004) The Pfam protein families database. Nucleic Acids Research 32: D138-D141.
[13] Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Güldener U, Mannhaupt G, Münsterkötter M, Mewes HW (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Research 32: 5539-5545.

MY OWN IDEAS

Charo López Barrientos, IES Alpujarra, Órgiva

In this project we have studied some aspects of plant reproduction. Specifically, we have study the role of the stigma exudate on pollen performance in *Lilium longiflorum*. The exudate of this plant contains all the necessary nutrients that allow pollen germination as well as the fertilization of ovules. Its biological function is closely related with the adhesion and hydration of the pollen, and accumulates storage substances that contribute to nourish the pollen tube. The main goal of this proposal has been the reconstruction and identification of proteins from the stigma exudate from peptide sequences obtained from previous proteomic analysis in the laboratory, using bioinformatics tools for sequence alignment and searching for homology in databases. I met PIIISA through our teachers in our high school. They informed us about the existence of several ongoing research projects in several scientific areas that will allow our active contribution. I chose this project because it provoked me a great interest and curiosity. The variety of knowledge acquired during this work has helped me to understand and increase my expertise on scholar subjects that were either unknown or partially known for me.

En este proyecto hemos estudiado la reproducción de las plantas. En el desarrollo del proyecto nos hemos centrado en estudiar el papel del exudado del estigma en la función biológica del polen, concretamente en Lilium longiflorum. El exudado de las plantas contiene nutrientes necesarios para que se produzca la germinación del polen y, a su vez, la fecundación del óvulo. Su función biológica está relacionada con la adhesión e hidratación del polen, y acumula sustancias de reserva que contribuye a la nutrición del tubo polínico. El objetivo principal de este proyecto era la reconstrucción e identificación de proteínas del exudado a partir de las secuencias peptídicas obtenidas en un análisis proteómico previo realizado en el laboratorio, utilizando para ello herramientas bioinformáticas de alineamiento de secuencias y búsqueda de homología en las bases de datos. Conocí PIIISA a través de los profesores de nuestro instituto, quienes nos informaron de una serie de proyectos de investigación en diferentes campos científicos que se iban a llevar a efecto y en los que podíamos participar activamente. Elegí éste porque me causó gran interés y curiosidad. Los conocimientos adquiridos en el desarrollo del mismo me han ayudado a comprender o a ampliar conocimientos de mis materias escolares de las que sólo tenía una vaga referencia.

Yasmine Dris Ghali, IES Luis Bueno Crespo, Armilla

This experience has made me to learn that beings to which we give a minor importance, such as plants, are very complex and very interesting, and very important when we talk about life in the universe. To begin with, I would like to make you a summary of our project. We have tested the efficiency of the pollen germination in vitro using two different experimental models: an artificial culture medium and the stigma secretion in which pollen naturally germinates. With this experiment, we could

observe how pollen germinates in these two media. Moreover, with this project we could answer two questions: a) are their differences in terms of germination between the exudate and the artificial media? and b) are their differences in the pollen tube length in these two experimental models? The answer is "yes". In conclusion, this project is better than it seemed to me, and is a very good experience, because I learned lots of things and put them into practice not only studying, but you do what you like and you meet people with the same interests.

Con esta experiencia he aprendido que seres a los que le damos poca importancia, como las plantas, son muy complejos y muy interesantes, y muy importantes a la hora de hablar de vida en el universo. En este proyecto hemos estudiado la eficiencia de la germinación in vitro del polen según el medio en el que se encuentre: exudado del estigma de la propia planta o un medio de cultivo creado artificialmente en el laboratorio, intentado imitar las mismas propiedades del exudado de la planta. Con este experimento, pudimos ver si era o no viable la germinación del polen en otro medio de reproducción que no fuese el mismo medio de su propia reproducción natural. Y lo que es más, con este proyecto pudimos contestar dos preguntas: ¿hay diferencias entre la germinación en un medio con y sin exudado?, y ¿hay diferencias en la longitud del tubo polínico entre un medio y otro? Y sí las hay, tal y como pudimos constatar. En conclusión, este proyecto es mejor de lo que parecía ser, es una experiencia muy buena, ya que haces aquello que te gusta y lo pones en práctica y conoces a gente con los que compartes cosas en común.

Aída López Amos, IES Padre Manjón, Granada

Through this project I have learned that for human survival it is necessary the existence of cells which are considered as insignificant as pollen grains. Without them, it would be impossible the existence of life because they produce plants. Although pollen is often seen annoying and useless it is something much more complex and without it we could not survive. In this project, we have focused on pollen germination in vitro under different experimental conditions, including a synthetic germination medium that mimics the stigma exudate and the stigma exudate itself. Thanks to these experiments, we have demonstrated that pollen grains germinate better and grow faster in the stigma exudate than in the artificial medium. In conclusion, this project has made me to think on the importance of research to have a good knowledge of nature, which is very important for us. I found it rewarding to carry out these experiments as I like natural sciences very much and I would like to deal with this field in the future, in addition to the personal and educational experience that this project represented to me.

Gracias a este proyecto he aprendido que para la supervivencia humana es necesario de la existencia de células que consideramos tan insignificantes como es el polen. Sin ellas sería imposible la existencia de la vida ya que gracias a él se producen las plantas. Pese a que el polen pueda parecer molesto e inservible es algo mucho más complejo y sin el cual no podríamos vivir. En este proyecto nos hemos centrado en el polen,

tratando la germinación in vitro desde varios puntos (según el medio en el que se encuentre y según el tipo de polen). Hemos trabajado con dos medios principalmente: exudado del estigma de la propia planta y un medio de germinación creado artificialmente en el laboratorio que imita el exudado de la planta. Gracias a ello comprobamos si el polen era viable o no en su germinación tratándolo desde un medio que no fuera el suyo y con ello comprobar si había diferencias en el tubo según el medio en el que se trataba. Como conclusión, este proyecto me ha hecho reflexionar sobre la importancia de la investigación para el conocimiento de la naturaleza y su comportamiento que nos condiciona infinitamente. Me ha resultado gratificante a la hora de realizarlo porque es algo que me gusta y a lo que me querría dedicar en un futuro, además de la experiencia a nivel social y educativo que supone.

Elisa Zurita Ferrández, IES Miguel de Cervantes, Granada

En este proyecto se investigaron las proteínas del exudado del estigma del lirio. Hemos investigado también sobre la germinación de los granos de polen y si el polen germina y crece más y mejor en presencia del exudado del estigma o con un medio de cultivo creado artificialmente. Para ello, hemos utilizado microscopía de fluorescencia entre otras técnicas. También hemos visto las proteínas del exudado en un gel. ¿Hay diferencias entre la germinación en un medio u otro? La respuesta a esta pregunta es que sí las hay. Con el exudado del estigma de la propia planta, el polen germina más y los tubos polínicos crecen más largos y a mayor velocidad que los tubos que crecen en el medio artificial. Ha sido una experiencia interesante y esto me ha llevado a preguntarme sobre qué sería de los granos de polen a diferentes temperaturas, a condiciones de vida extremas, o si en otra planta crecerían de otra manera o no.

Alejandro Zarco Sánchez, IES Severo Ochoa, Granada

Mi experiencia en el proyecto PIIISA ha sido muy satisfactoria ya que este ha sobrepasado todas mis expectativas sobre él. Me embarqué en este proyecto debido a que la idea de saber más sobre las plantas y su reproducción me atraía bastante, después del trabajo realizado durante estos meses las conclusiones obtenidas han sido bastante fructíferas, ya que la mayoría de las diferentes hipótesis realizadas durante el proyecto se han visto verificadas por las experiencias realizadas para demostrarlas. El tema que más se ha tratado durante el proyecto ha sido la viabilidad del polen según el medio en el que lo hiciéramos germinar (el propio exudado de su planta, o un medio creado en el laboratorio recreando esas condiciones). Después de diversas experiencias, se ha observado que la viabilidad del polen aumenta en el exudado de la planta. Mi experiencia en PIIISA ha sido muy gratificante ya que me ha hecho crecer intelectualmente y me ha hecho barajar otras posibilidades sobre mi vida académica en un futuro.

Luis Carlos Paredes Retamero, IES Padre Manjón, Granada

With this project we have learned a lot about plant reproduction, pollen, and the biological role of the stigma exudate. We worked with Lilium longiflorum pollen and exudate using microscopy, electrophoresis and bioinformatic techniques and tools. We germinated pollen and checked whether these are or are not viable and we have also checked how fast pollen tubes grow with or without exudate. In summary, I liked a lot this project and we learned enough to encourage all who want to join this PIIISA program because it is a unique experience.

SHEEP AND SHEPHERDS: GREAT ALLIES FOR THE MEDITERRANEAN MOUNTAINS

Gala Marañón García[1], Sara Castillo de Leyva[2], Francisco Javier Roldán de la Rosa[3], Lorena López Muñoz[4], Luz Divina Muñoz Travé[4], María Arana Fernández[2],Paula López[1], Francisco Mario Cabeza Arcas[5],Mauro Tognetti Barbieri[5], Ana Belén Robles Cruz[5], María Eugenia Ramos Font[5]

[1]I.E.S. Generalife. Huerta del Rasillo s/n, 18004 Granada, Spain
[2]IES Fray Luis de Granada, Huerta del Rasillo s/n, 18004 Granada, Spain.
[3]IES-Padre Manjón,Gonzalo Gallas s/n, 18003 Granada, Spain
[4]IES-Zaidín Vergeles, Primavera 26-28, 18008 Granada, Spain
[5]Grupo de Pastos y Sistemas Silvopastorales Mediterráneos, Estación Experimental del Zaidín,CSIC, Profesor Albareda 1, 18008 Granada, Spain
**Corresponding author: eugenia.ramos@eez.csic.es*

HIGHLIGHTS

Sheep may help to revegetate natural areas while grazing

SUMMARY

In this work we try to determine whether sheep grazing can be a method for revegetation purposes, by means of the dispersal ability of herbivores. Seed recovery, seed germination and seedling establishment in greenhouse conditions are evaluated for three legume species of the Mediterranean mountains (*Anthyllis cytisoides* L., *Astragalus edulis* Bunge and *Genista versicolor* Boiss.). Seed recovery was high for *G. versicolor* (33%) and moderate or low-moderate for *A. edulis* (14%) and *A. cytisoides* (7.4%). Most seeds were recovered during the first 72 h and continue appearing even during the fourth and the fifth day. On the one hand, for *A. cytisoides* and *G. versicolor* gut passage increased germination and seedling establishment with respect to control seeds. This is due to the softening of the coats by means of digestive liquids. On the other hand, *A. edulis* obtained similar germination and seedling establishment percentage for ingested and control seeds. We hypothesized that this species have two kinds of seeds: soft coated seeds, which would be destroyed after gut passage, and very hard coated seeds, whose coats would not be softened by digestive processes. We may conclude that sheep can help to revegetate Mediterranean mountains with the studied species as they can disperse large quantities of seeds through several days, allowing seeds to colonize distant areas far from the mother plants, germination is boosted in all but one species, and seedling emergence is not inhibited by manure.

INTRODUCTION

Mediterranean landscapes cannot be understood without the presence of herbivores. In fact, herbivores play an essential role in determining the vegetation structure and the flora in Mediterranean environments (Emanuelsson, 2009; González Rebollar, 2009). Throughout the natural history of the earth, vegetation has developed a variety of adaptations which allowed the different species to protect themselves against herbivory (e.g. thorns, chemical substances) and/or to obtain benefits from a mutualistic relationship with the animals (e.g. developing features which favored zoochory) (Willson and Traveset, 2000; Rigueiro et al. 2011).

Endozoochory (i.e. the dispersal of seeds after passage through the vertebrate gut) may constitute a mutualistic relationship between plants and animals in which plants trade food for displacement (Herrera 2002). On the one hand, destruction of seeds occur due to mastication and to the action of digestive liquids; on the other hand, species may colonize far areas from the mother plants, germination maybe enhanced by the softening of the coats during gut passage and faecal material may facilitate the establishment due to nutrients release (Traveset and Verdú, 2002; Moussie, 2004). However, the determination of whether endozoochory is advantageous for a plant requires an analysis of all the stages involved: from seed ingestion to seedling establishment.

Shrubs are crucial fodder sources for wild herbivores and livestock in Mediterranean environments (Le Houérou, 1980). Shrubby species, and mostly legumes, provide an important fraction of the protein requirements of the animals, especially in the months were grass is not available (i.e. end of summer and winter). Legumes shrubs and herbs also play a pivotal ecological role in Mediterranean mountains, as increasing soil fertility and soil protection against erosion. Many of them are adapted to fire and are pioneer species, thus they are excellent colonizers after disturbances.

The role of livestock in Mediterranean mountains is a controversial issue for the management and protection of natural resources, and it is full of questions, prejudices and half-truths, therefore, any effort focused on a better understanding of the above mentioned role can be crucial for managers. In this work we try to determine whether sheep grazing can be a method for revegetation purposes, by means of the dispersal ability of herbivores. For this aim, three legume species (shrub and herbaceous) were studied and three main questions are posed: 1) What is the percentage of seed recovery after seed ingestion for each species? 2) What is the temporal pattern of seed recovery? 3) How does gut passage affect to seed germination? 4) How does dung affect to seedling establishment and survival, in greenhouse conditions?

MATERIALS AND METHODS

Ripe fruits (pods) of *Anthyllis cytisoides* L., *Astragalus edulis* Bunge and *Genista versicolor* Boiss. were manually collected (Table 1) . After collection, seeds were manually removed from the fruits (except for *A. cytisoides,* as we used pods) and stored at 4º C in glass jars containing silica gel. Three types of experiments were carried out: 1) seed recovery after gut passage, 2) seed germination after gut passage, 3) seedling establishment from dung.

Table 1. Data from seed collection.

Species	Locality	Date
Anthyllis cytisoides	Guadix (Granada)	23/06/2009
Astragalus edulis	Sierra Filabres (Gérgal, Almería)	10/05/2010
Genista betica	Puerto de la Ragua (Granada)	11/08/2011

Seed recovery after gut passage

On October 2012, seeds were provided to adult sheep (*Ovis aries*, Segureña race) kept in individual pens. Seeds of each species were offered to three different sheep, thus, nine sheep were used for the experiment. The number of seeds eaten by each sheep is listed in Table 2.

To facilitate the intake, seeds were soaked in water and then, intermingled with oats, their usual diet. Animals had *ad libitum* access to water, alfalfa hay and straw. Faeces were collected at 24, 48, 72, 96 and 120 h after ingestion, dried at room temperature, weighed and stored in laboratory until de beginning of the experiment.

Table 2. Data of ingested seeds.

Species	Seed mass (g)	Seed size (mm)	Mass of ingested seeds/sheep (g)	# of ingested seeds/sheep
Anthyllis cytisoides	0,0026	3-4 x 2	100	35315
Astragalus edulis	0,0114	4 x 4	33	2763
Genista betica	0,0045	1,7-2,5 x 2-2,7	12,5	2661

Faeces from the three sheep which ingested the same species and for the same day were pooled together to constitute a single composite sample for each sampling date, i.e., for each species we obtained one sample per sampling date. For every composite sample we took 10 subsamples of 5 g each. Each subsample was gently sieved under running water to concentrate the sample and facilitate seed retrieval. The number of seeds per subsample was annotated.

The percentage of seeds recovered (PSR) at each interval for each species was estimated by the following expression:

$$PSR = \frac{m\sum s_i}{5 \cdot S} \cdot 100$$

where m is the total mass of faeces by time interval, s_i is the number of seeds found per subsample, S is the number of seeds ingested by sheep, 5 is the weight of each subsample (g).

Seed germination after gut passage

From the dung of the previous experiment we collected the necessary number of seeds for the germination experiment. We tested the three sampling periods with the highest seed recovery (24, 48 and 72 h after ingestion), as on the other days we could not get enough seeds due to low recovery (see Results). For this experiment we set four different treatments per species: 1) Control, intact seeds that were not ingested; 2) 24 h, seeds recovered 24 h after ingestion; 3) 48 h, seeds recovered 48 h after ingestion; 4) 72 h, seeds recovered 72 h after ingestion. *A. cytisoides* has an indehiscent monosperm fruit, therefore, we used the fruits instead of seeds (even after gut passage). Additionally, seeds that were scarified with sandpaper (*A. cytisoides*) or a scalpel (*G. versicolor* and *A. edulis)* were sown for germination to determine seed viability. Also, at the end of the trial, those seed that did not germinate were scarified and placed again in the growth chamber during one month. For each treatment (including scarified seeds) and species six replicates (Petri dish) of 25 seeds were set.

All seeds were disinfected by immersion in a 1% sodium hypochlorite solution for 10 min, and thoroughly rinsed with sterile distilled water. Seeds were placed in sterile, plastic Petri dishes (10 cm diameter) containing filter paper disks. Dishes were initially moistened with 3 ml sterile water and sealed with a strip of Parafilm ®, being thereafter moistened as needed. Petri dishes were placed in a growth chamber with a photoperiod of 16 h day/ 8 h night, at 22/16º C. Germination, identified as visible radicle protrusion, was daily recorded during the first 21 days, and, thereafter, at 2-days interval until the end of the experiment (60 days from the seeding date). Germinated seeds were removed from the dishes.

Seedling establishment in greenhouse conditions

The potential of germination of seeds encased in dung and of seedling establishment was evaluated in a greenhouse experiment. Seeds were distributed in two treatments: 1) Control, seeds placed on the soil surface; 2) Faeces, seeds recovered after 24, 48, and 72 h were placed on the soil surface inside manually crumbled pellets, simulating crumbling of pellets under natural conditions (i.e. from rainfall or from animals trampling the pellets). For this treatment, using a needle, we made a hole 3 mm deep in the pellet, inserted the seed, and sealed the opening of the hole by gently pressing the pellet. After that we crumbled it. The proportion of 24h, 48h and 72 h seeds in the pellets corresponded to the recovery proportion for each species.

Seeds or crumbled pellets were placed in 1 L pots containing a mixture 8:1 of turf and vermiculite. For each species and treatment there were 12 pots with 16 seeds or pellets, depending on the treatment. The experiment started on February 2013 in a greenhouse (23º C and natural day/night cycle). Pots were watered by imbibition and manually sprinkled as needed (around two-day interval) with tap water. As visualization of the moment of radical protrusion was not possible in seeds from the Faeces treatment, emergence was used as the criterion for analysis, considering a seedling to be emerged by the appearance of cotyledons. Each emerged seedling was allowed to grow for 35 days.

Data analysis

Seed germination after gut passage was analyzed using a one-way ANOVA for each species, and a Tukey test for *post-hoc* comparisons.

For seedling emergence in greenhouse conditions the U-Mann Whitney test was used.

To determine the overall probability of recruitment we multiplied the obtained probabilities for each stage, i.e., proportion of seed recovery, proportion of germination, and proportion of emergence. We made a diagram (Fig. 6) considering of each stage and the above mentioned overall probability.

RESULTS AND DISCUSSION

Seed recovery after gut passage

Seed recovery was high for *G. versicolor* (33%) and moderate or low-moderate for *A. edulis* (14%) and *A. cytisoides* (7.4%) (Fig. 1). Many authors (Pakeman et al. 2002; Razanamandranto et al. 2004; Moussie et al. 2005) have hypothesized about the main factors affecting seed recovery. Seed size and, specially, hardseedness are the most relevant seed features, i.e., the smaller and the harder is a seed, the higher is the seed recovery percentage. According to Table 1 to the seed features of the studied species, we may confirm that seed size is not as important as hardseedness seems to be, and therefore, presumably, *G. versicolor*'s seeds should be harder than *A. cytisoides.*

Most seeds were recovered during the first 72 h and continue appearing even during the fourth and the fifth day. Free-ranging sheep usually graze in open grasslands where they can walk up to 25 km per day (transhumant livestock). Therefore, seeds ingested during grazing can be spread over a large area.

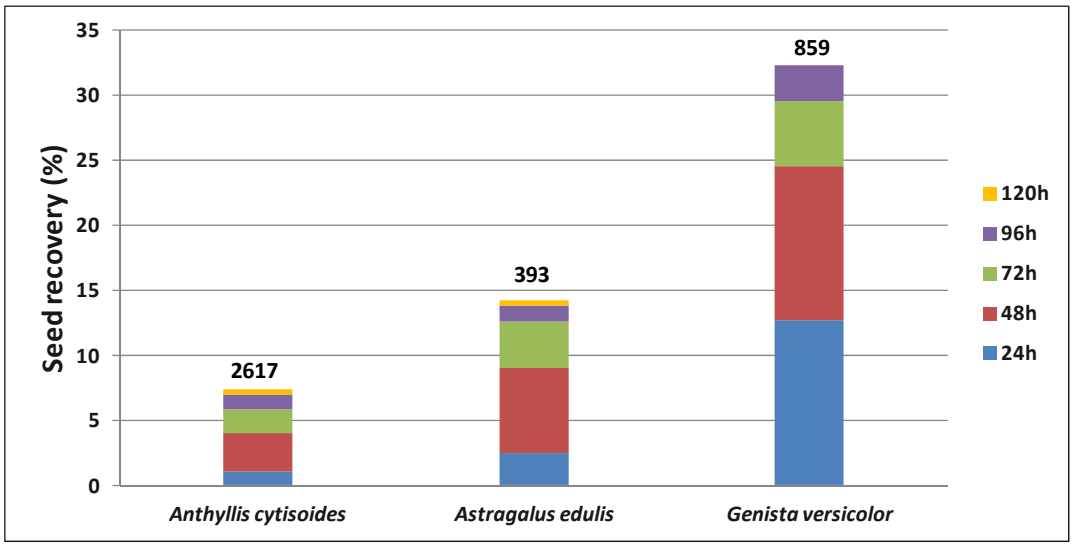


Figure 1. Daily percentage of seed recovery of three legume species after seed consumption by sheep. Numbers above bars are the estimate of total seeds recovered for one sheep.

Seed germination after gut passage

Depending on the species, gut passage favored or did not affect seed germination, compared to control seeds. Legume seeds are characterized by a physical dormancy imposed by hard coats (Baskin and Baskin, 1998), therefore, any factor softening the coats without damaging the embryo may enhance germination (Ramos et al. 2006). Ruminal liquid contains proteolytic and cellulolytic enzymes which, together with the acidic medium of the abomasum and duodenum are responsible of seed coat erosion (Prins and van der Vostenbosch 1975; Gardener et al. 1993). For *A. cytisoides* gut passage boosted germination from eight-fold up to twelve times with respect to control seeds (Fig. 2). Moreover, 48h and 72h treatments showed higher germination percentages than 24h (F-value = 56.391; d.f. = 3; p-value <0.0001). This could be due to the greater exposure of 48h and 72h to digestive liquids, and therefore, greater seed coat softening. *G. versicolor* also seemed to benefited from gut passage, as germination percentage increased around two-fold in relation to control seeds, however, the differences among between control and ingested seeds were not statistically significant (F-value = 2.487; d.f. = 3; p-value = 0,09). *A. edulis* obtained similar germination percentages for all treatments (F-value = 0.840; d.f. = 3; p-value = 0.48). We hypothesized that this species have two kinds of seeds: soft coated seeds and very hard coated seeds. When seeds go through sheep's gut, soft seeds would be destroyed whereas only the hardest ones would be retrieved, and these are so hard that digestive processes will not be able to soften the coats. This hypothesis was confirmed by the data obtained from scarification of ingested seed after the study (100% of ingested seeds were viable).

The percentage of germination for scarified seeds (viability) was 94%, 100% and 87% for *A. cytisoides*, *A. edulis* and *G. versicolor*.

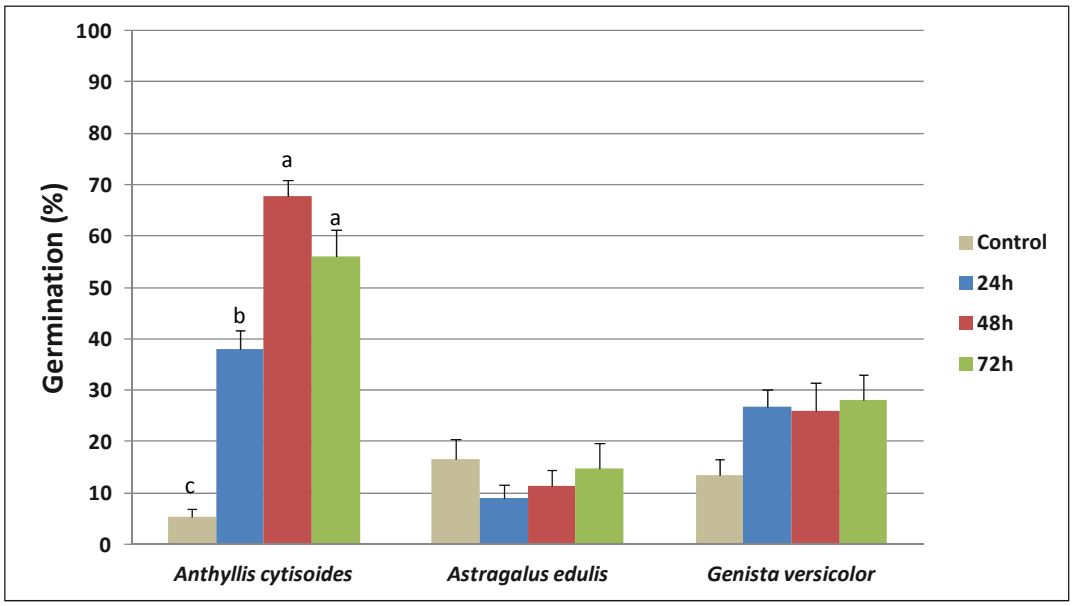


Figure 2. Percentage of seed germination of three legume species recovered from sheep faeces (24, 48 and 72 h after seed ingestion). Different letters above bars inside are significant differences among treatments within one species according Tukey test (α=0.05).

Gut passage may modify not only final germination percentage, but also the germination pattern. Figure 3 shows germination rate for each species, i.e., the accumulated proportion of seeds that germinate in each sampling date in relation to the total germination for each treatment. In *A. cytisoides* similar curves were obtained for the scarified treatment and for all the gut passage treatments (24, 48 and 72 h), and they indicate that scarification and gut passage increased the speed of germination compared to control seeds (Fig. 3). This will confirm that gut passage soften seed coats up to the point of being similar to scarified seeds. On the contrary, in *A. edulis* curves differed among treatments and gut passage seemed to slow down the germination rate compared to control seeds. As mentioned above, *A. edulis* may have soft seeds and hard seeds, and after gut passage only the hardest would be recovered and therefore, germination is slower than control seeds. *G. versicolor* have an intermediate behaviour: during the first third period of the assay control and gut passage treatments had similar curves, whereas from that date on gut passage boosted germination rate.

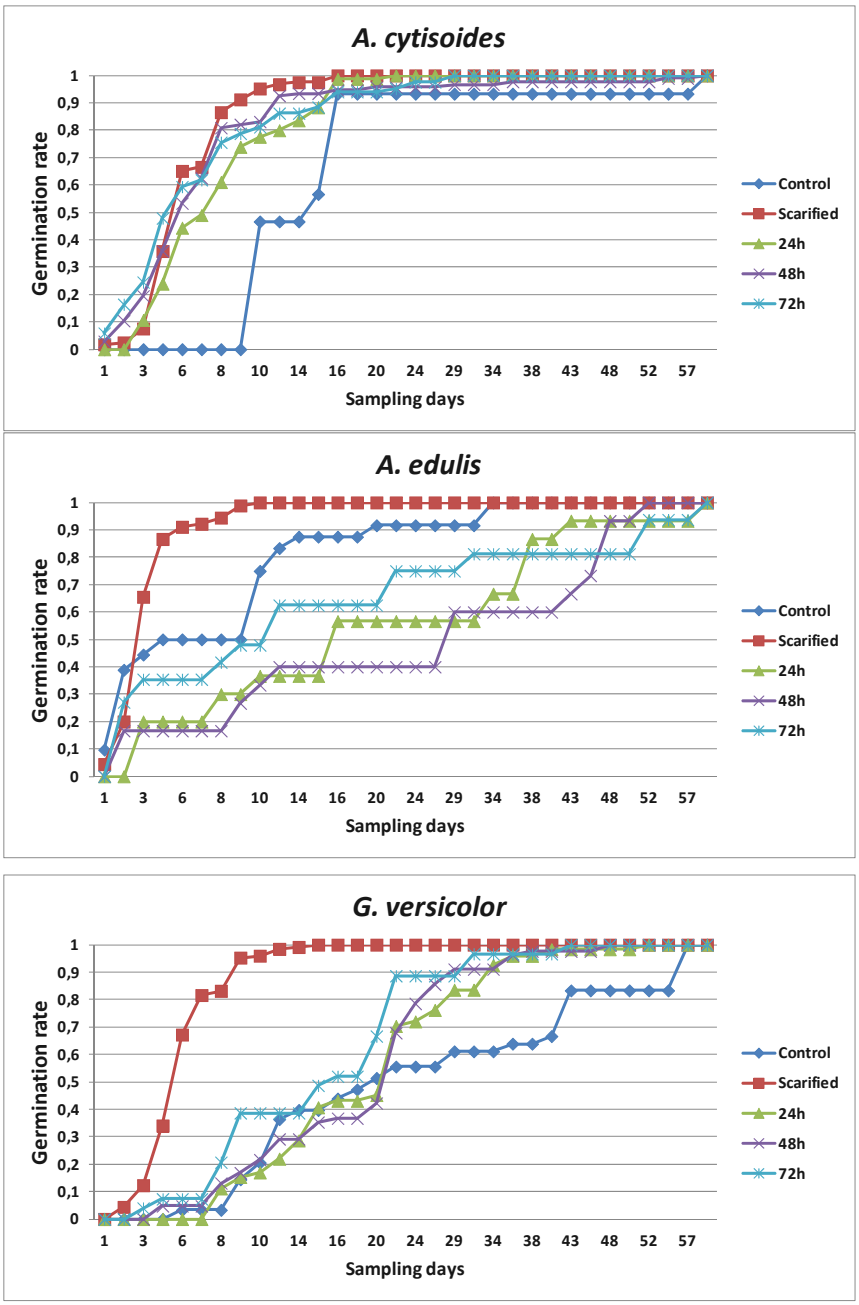


Figure 3. Curves of germination rates (accumulated proportion of seeds germinating in each sampling date in relation to the number of germinated seeds) of three legume species seeds recovered from sheep faeces (24, 48 and 72h after seed ingestion).

Seed establishment in greenhouse conditions

In order to test the effect of faeces in seedling establishment, seeds inside dung pellets were compared with control seeds. Results were consistent with those obtained for germination in Petri dishes (see Figure 4), although establishment values were lower than the latter probably due to suboptimal conditions for germination in the greenhouse. Emergence was greater in the faeces treatment than in the control treatment for *A. cytisoides* (*U*-Mann Whitney = 9; d.f. = 1; *p*-value < 0.01) and for *G. versicolor* (*U*-Mann Whitney = 26; d.f. = 1; *p*-value < 0.01), whereas there were no differences between treatments for *A. edulis* (Fig 4).

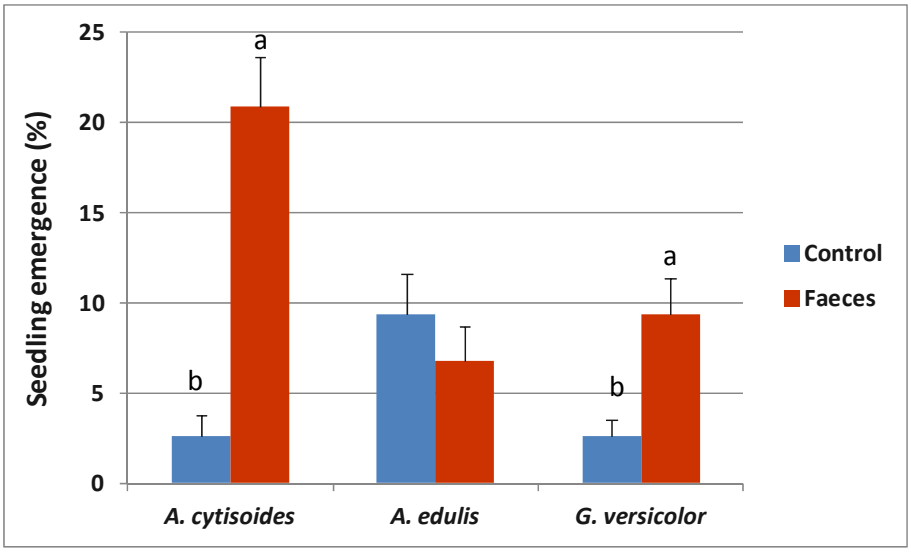


Figure 4. Percentage of seedling establishment of three legume species from intact seeds (control) and from seeds inserted in dung pellets (faeces). Different letters above bars inside are significant differences between treatments within one species, according to U-Mann Whitney test.

The curves of emergence rates (Fig. 5) responded similarly to the curves of germination rates (Fig. 3). In *A. cytisoides*, seeds from pellets emerged faster than those from the control treatment; whereas *A. edulis* responded in the opposite way. *G. versicolor* had similar curves for both treatments, no clear differences could be detected (Fig. 5).

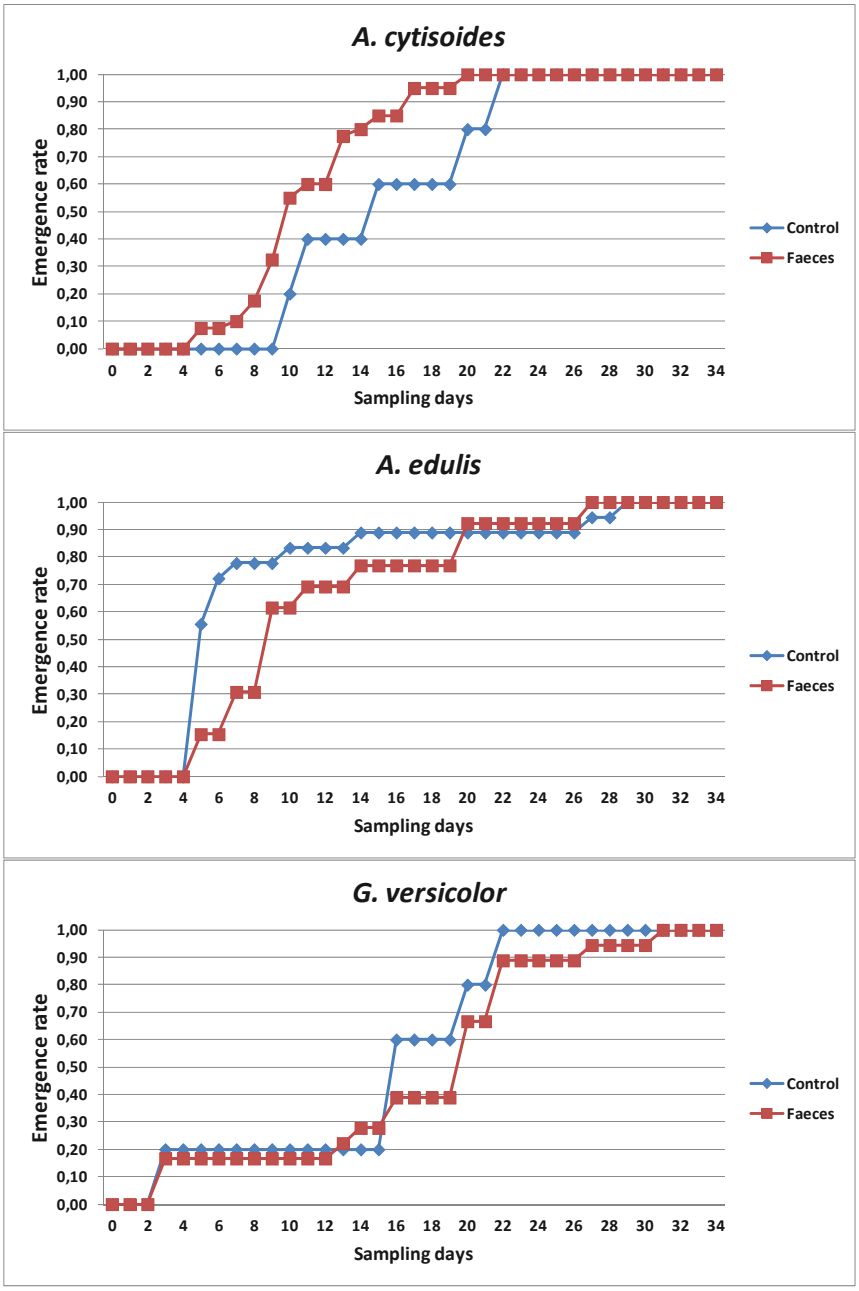


Figure 5. Curves of emergence rates (proportion of seeds emerging in each sampling date in relation to the number of germinated seeds) of three legume seeds recovered from sheep faeces (24, 48 and 72h after seed ingestion).

Seedling survival at the end of the experiments was high and similar for all treatments and species, ranging from 78% *(G. versicolor* faeces) up to 100% (*A. cytisoides* control). This will demonstrate a positive or neutral effect of faecal materials on seedling emergence.

When pooling all the stages analyzed, the overall probability of recruitment, i.e. the product of all the probabilities of the stages considered, shows that seed consumption may increase the overall probability of recruitment of *A. cytisoides (seven times)* and of *G.*

versicolor (two fold) whereas for *A. edulis* seed consumption decreases that probability (fourteen times) (Figure 6).

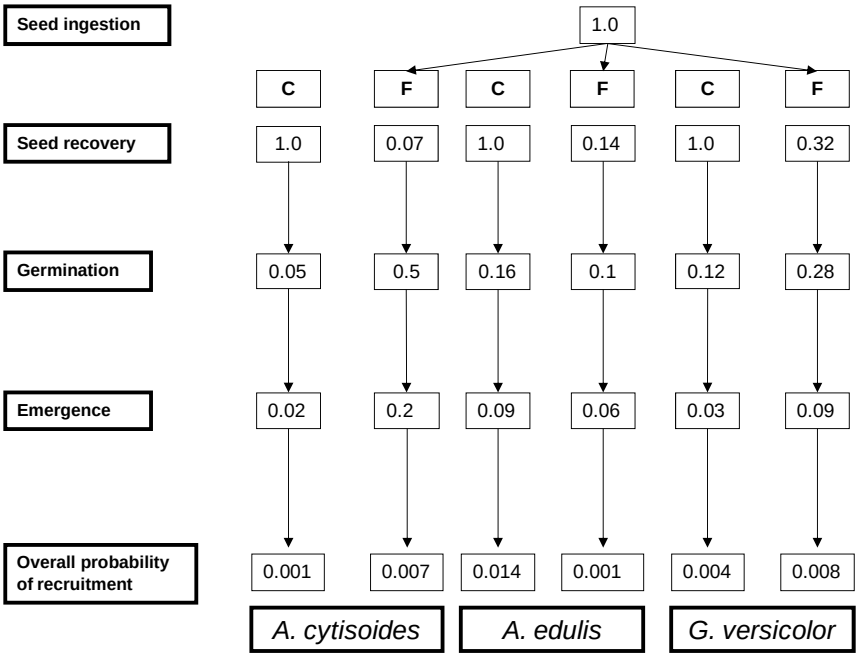


Figure 6. Diagram showing all the stages from seed ingestion to emergence for three legume species. Transition probabilities are shown for every stage. C= Control (non-ingested seeds); F = faeces (ingested seeds contained in crumbled pellets).

CONCLUSIONS

We may conclude that sheep can help to revegetate Mediterranean mountains with the studied species as they can disperse large quantities of seeds through several days, allowing seeds to colonize distant areas from the mother plants, germination is boosted in all but one species, and seedling emergence is not inhibited by manure.

ACKNOWLEDGEMENTS

We thank the staff of the Patronato Rodríguez Penalva (Diputación of Granada) for lending us the experimental animals and the facilities for carrying out the seed ingestion stage. This study has being funded by Consejería de Agricultura, Pesca y Medioambiente (Junta de Andalucía, Dirección General para la Gestión del Medio Natural, through a FEDER project. Ganadería extensiva y biodiversidad

REFERENCES

Baskin C.C. and Baskin J.M. (1998). Seeds. Ecology, biogeography and evolution of dormancy and germination. Academic Press, San Diego.

Emanuelsson U. (2009). The Rural Landscapes of Europe. How man has shaped European nature. The Swedish Research Council Formas, Värnamo, Suecia.

Gardener C.J., Mc Ivor J.G., Jansen A. (1993). Survival of seeds of tropical grassland species subjected to bovine ingestion. Journal of Applied Ecology 30: 75-85.

González Rebollar J.L. (2009). Allí donde los desconocidos se saludan. Ambienta, 88. http://www.revistaambienta.es/WebAmbienta/marm/Dinamicas/secciones/articulos/Gonzalez.htm

Herrera C.M. (2002). Seed dispersal by vertebrates. In : Plant-animal interactions: an evolutionary approach (Herrera C.M. and Pellmyr O., eds.). pp. 705-727, Blackwell Science, Oxford, Reino Unido.

Moussie M. (2004). Seed dispersal by large herbivores: implications for restoration of plant diversity. PhD Thesis. University of Groningen, Groningen.

Mouissie A.M., van der Veen C.E.J., Veen G.F.C. and van Diggelen R. (2005). Ecological correlates of seed survival after ingestion by Fallow Deer. Functional Ecology 19: 284-290.

Pakeman R.J., Digneffe G. and Small J. L. (2002.) Ecological correlates of endozoochory by herbivores. Functional Ecology 16: 296-304.

Prins R.A and van der Vorstenbosc C.J.A.H.V. (1975). Interrelationships between rumen micro-organisms. In: Binnerts W. T. (eds.), Physiology of digestion. Veenman H. and Zonen B.V., Wageningen.

Ramos, M.E.; Robles, A.B.; Castro, J. (2006). Efficiency of endozoochorous seed dispersal in six dry-fruited species (Cistaceae): from seed ingestion to early seedling establishment. Plant Ecology 185: 97-106

Razanamandranto S., Tigabu M., Neya S. and Odén P.C. (2004). Effects of gut treatment on recovery and germinability of bovine and ovine ingested seeds of four woody species from Sudanian savanna in West Africa. Flora 199: 389-397.

Rigueiro-Rodríguez A., Rois-Díaz M., Mosquera-Losada M.R. (2011). Integrating silvopastoralism and biodiversity conservation. In: Biodiversity, Biofuels, Agroforestry and Conservation Agriculture Vol 5. 359-373 (Lichtfouse E.,ed.), Springer Science+Business Media B.V., London.

Traveset A., Verdú M. (2002). A Meta-analysis of the Effect of Gut Treatment on Seed Germination. In: Seed Dispersal and Frugivory: Ecology, Evolution and Conservation (Levey D.J., Silva W. R., eds.). pp 339-350. CAB International, Wallingford, Oxford.

Willson M.F., Traveset A. (2000). The ecology of seed dispersal. In: The Ecology of Regeneration in Plant Communities. 2nd ed (Fenner M., ed.), pp 85-110. CAB International. Wallingford, Oxford.

MY OWN IDEAS

Gala Marañón García, IES Generalife, Granada

This Project gave us the chance to introduce ourselves in the world of scientific investigation by the hands of people who knew how to teach us the researcher's skills and their vocation for science.

We had the opportunity to work in a laboratory, to use specialized material, methods and resources and to fully experience the life of a researcher. We learned to be patient and meticulous with the experiments and not to give up easily when the results are not as expected.

We found out that with very simple materials (seeds and excrements) we could see how clever and awesome nature can be. In this case, the dispersion of seeds through excrements and the importance of the digestive system of animals to help them to prepare for germination.

It would be very interesting to carry on with this project, experimenting with seeds and excrements from other kinds of plants and animals (maybe birds or other kinds of mammals and plants with agronomic interest).

This project would be useful in the future to study further the dispersion of specific species of plants, which could be useful to the regeneration of damaged ecosystems or to understand the distribution patterns of other plants or animals.

Este proyecto nos ha dado la oportunidad de introducirnos en el mundo de la investigación en manos de personas que han sabido enseñarnos las cualidades de los investigadores y mostrarnos su vocación por la ciencia.

Tuvimos la oportunidad de trabajar en un laboratorio, así como de usar material y medios especializados y poder introducirnos de lleno en la vida de un investigador. Hemos aprendido a tener paciencia con los experimentos, ser meticulosos y no desanimarnos si no obtenemos los resultados que esperábamos.

Hemos descubierto que con sencillos materiales (semillas y heces) podemos darnos cuenta de lo impresionante que puede llegar a ser la naturaleza, en este caso, en la dispersión de semillas por medio de heces animales.

Sería interesante continuar este proyecto con experimentos usando semillas y defecaciones de otro tipo de animales, aves o mamíferos.

Este proyecto puede servirnos en el futuro para profundizar en el estudio de la dispersión de especies concretas de plantas, lo que puede ser útil para la regeneración de ecosistemas degradados o entender los patrones de distribución de plantas y animales.

Sara Castillo de Leyva, IES Fray Luis de Granada, Granada

In my opinion this project is a good way to show young people how the world of investigation is. Also here I have learned a lot of things, like what the scientific procedure is, how we use the different apparatus (that I didn't even know of their

existence before) and how important they are for the development, and the hours that this needs, which are a lot.

Here you can be an researcher for some days, and its funny and interesting to work with people that have scientific studies and work on this. It also makes you open your mind to new possibilities for the future.

The research in wich I'm working I think is stirring, because it is related to nature and the environment, that is an important thing. I think there should be more investigations like this (that studies how we can revegetate our mountains with shrubs with the help of sheeps) because we have to take care of our world, and if we don't do things like that, we will finally destroy it completely.

In conclusion I'm glad I've participated on this proyect and one of the things that I have learned is how important the investigation is (and has been), and how important is to do it well too.

En mi opinión este proyecto es una buena manera de enseñar a la gente joven cómo es el mundo de la investigación. También aquí he aprendido muchas cosas, como los procedimientos científicos, como usamos los diferentes aparatos (que no sabía de su existencia antes) y lo importantes que son para el desarrollo, y las horas que esto necesita, que son muchas.

Aquí tú puedes ser un investigador por algunos días, y es divertido e interesante trabajar con gente que tiene estudios científicos y trabaja en esto. También te hace abrir tu mente para nuevas posibilidades en el futuro.

La investigación en la cual estoy trabajando, yo creo que es emocionante, porque está relacionada con la naturaleza y el medio ambiente, que es importante. Yo pienso que debería haber más investigaciones como ésta (que estudia cómo nosotros podemos revegetar nuestras montañas con árboles y diferentes plantas con la ayuda de ovejas) porque tenemos que cuidar nuestro mundo, y si no hacemos cosas como ésta, destruiremos el planeta finalmente.

En resumen estoy orgullosa de haber participado en este proyecto y una de las cosas que he aprendido es lo importante que es investigar y esto, es hacer bien las cosas.

Francisco Javier Roldán de la Rosa, IES Padre Manjón, Granada

In my opinion, this project is very important for the nature. Nowadays, there is a decrease in forest areas, and that's so bad for our planet. I've discovered that sheep can reforest all the land because their droppings, where there are the seeds of the plants that they eat. I've learnt a lot with this, and it's a very important fact for our future lives. Now, I know how to use different laboratory instruments, new kind of plants… It was very interesting. However, I think that it has been so short. It would have been great if we had be more time with our teachers and with all the

experiments. It's a pity that something practical like this don't have so much time for us, I believe it's more important to investigate these kinds of projects that go to school, because we learn a lot making different things. This is something that I've never done, but now I'm so happy to participate with all my partners, and in the future, I wish make more projects like this. It's a way to have fun, but learning.

En mi opinión, este proyecto es muy importante para la naturaleza. Actualmente, existe una disminución de la superficie forestal, y eso es muy malo para nuestro planeta. He descubierto que las ovejas pueden reforestar la tierra debido a sus excrementos donde se encuentran las semillas de plantas que ellas mismas se comen. He aprendido mucho con esto, y es un factor muy importante para nuestras vidas futuras. Ahora conozco cómo usar diferentes aparatos del laboratorio, nuevos tipos de plantas… Fue muy interesante. Sin embargo, pienso que ha sido corto. Hubiera estado genial si hubiéramos estado más tiempo con nuestros profesores y los experimentos. Es una pena que algo práctico como esto no tenga mucho tiempo para nosotros, creo que es más importante investigar este tipo de proyectos que ir al colegio, porque aprendemos muchas cosas diferentes. Esto es algo que no había hecho, pero ahora estoy muy contento por participar con todos mis compañeros, y en el futuro deseo hacer más proyectos como este. Es una forma de divertirse, pero aprendiendo.

Lorena López Muñoz, IES Zaidín-Vergeles, Granada

The main aim of our project is to know if sheep can help to reforest our mountains. This project is very interesting because it helps us to join even more in the world of science. We can see how a laboratory is, we can meet scientists, and we can check if our hypothesis is confirmed or not.

It is very interesting because our project has to be done both in the laboratory and in the countryside. It is very important to be in the environment where sheep live and where they spread the seeds they have eaten. There we can find the answer to our main question: Can sheep help us to reforest our mountains?

Next year I would like to participate in PIIISA project again because it is an unique and unforgettable experience that let us to learn how scientists work in a laboratory and the day to day of experiments. Of course we can make new friends too.

To be in contact with our coordinators is marvelous and we are very thank to them because they have made possible our project. In my opinion, next year there must be more projects with less people in every group in order to take the most of them.

El objetivo principal de nuestro proyecto es conocer si la oveja es capaz de reforestar nuestros montes. Este proyecto es muy interesante porque nos ayuda a implicarnos en el mundo de la ciencia. Podemos ver cómo es un laboratorio, conocer a científicos y comprobar si nuestras hipótesis se confirman o no.

Es muy interesante porque nuestro proyecto tiene que hacerse en el laboratorio y en el campo. Es muy importante estar en el ambiente donde viven las ovejas y donde diseminan las semillas que hay comido. Ahí es donde podemos encontrar la respuesta a

nuestra principal pregunta: Pueden las ovejas ayudarnos a reforestar nuestras montañas?

El año que viene me gustaría volver a participar en un proyecto PIIISA de nuevo porque es una experiencia única e inolvidable que nos permite conocer cómo trabajan los científicos y el día a día de los experimentos. Por supuesto, podemos hacer también nuevos amigos.

Ha sido maravilloso estar en contacto con nuestros coordinadores y estamos muy agradecidos a ellos porque han hecho posible este proyecto. En mi opinión, el año que viene debería haber más proyectos con menos gente en cada grupo para que sea más provechoso.

Luz Divina Muñoz Travé, IES Zaidín-Vergeles, Granada

Our project is called: Can sheep help to revegetate our forests?

To answer this question we had to do many tests, graphics and others with our coordinators, by which we know today about a laboratory, tools, and applications.

I think the PIIISA project is a great opportunity that is given to students to move in a way that is not ours and learn from it.

It is interesting for us to participate in it because apart from learning at the laboratories, we learn to value things that previously we ignored as the importance of the work of our coordinators and their peers, and we also learn that thanks to them many things can be remediate, as the revegetation of the mountains, in our case.

I think it would be good for the project and for ourselves that we would have had more sessions with our coordinators and that we would have put more seed species.

Finally thank you for allowing us to live this wonderful experience, and I hope to continue it next year.

Nuestro proyecto se llama: ¿pueden las ovejas ayudar a repoblar nuestros montes?

Para responder a esta pregunta hemos tenido que hacer muchas pruebas, gráficos y demás junto a nuestros coordinadores; gracias a los cuales sabemos hoy sobre un laboratorio, sus herramientas, y usos.

Creo que el proyecto PIIISA es una gran oportunidad que se nos da a los estudiantes para movernos en un medio que no es el nuestro y así aprender de ello.

Es interesante para nosotros hacerlo porque aparte de aprender en los laboratorios, aprendemos a valorar cosas que antes ignorábamos como la importancia del trabajo que nuestros coordinadores y sus compañeros realizan, y que gracias a ellos se remedian y saben muchas cosas que nos incumben como la revegetación de los montes en este caso.

Pienso que sería positivo para el proyecto y para nosotros mismos que pudieran hacerse más sesiones con nuestros coordinadores y también que hubiésemos puesto más especies de semillas.

Por último doy las gracias por haber permitido que vivamos esta bonita experiencia, y espero continuarla el año que viene.

María Arana Fernández, IES Fray Luis de Granada, Granada

I found that the project is very interesting and clever, and very practical because sheep grazing can help our mountains and forest can facilitate the work of farmers.

The first thing we did was to separate the seeds from the droppings and count, select and classify them.

One of the things I liked most was looking through the magnifying glass to differentiate types of seeds closely. Also I would like to go out to see the animals and the plants and see the vegetation from Granada.

El proyecto me ha parecido muy interesante, inteligente y muy práctico, porque al comprobar si las ovejas pueden ayudar a revegetar nuestros montes y bosques facilita el trabajo de los agricultores y ganaderos.

Lo primero que hicimos fue separar las semillas de los excrementos y contarlas y seleccionarlas, clasificarlas. Una de las cosas que más me ha gustado ha sido mirar al través de la lupa para diferenciar el tipo de semillas muy de cerca. También me gustaría ir al campo para ver las ovejas y ver las plantas que salen de las semillas, la diferente vegetación de Granada.

Paula López, IES Generalife, Granada

This project has taught me how to make things differently. What we tried to do was search through sheep feces seeds of various types of species. We have used instruments in order not get dirty and did everything carefully. We tidy everything that we used and we cleaned all the dirty instruments. What I liked most is that sessions have been very entertaining and what I disliked is that there have been very few.

Este proyecto me ha enseñado a ver las cosas de otra manera. Lo que se trataba de hacer era buscar entre heces de oveja varios tipos de especies. Hemos usado instrumentos para no mancharnos y hacerlo todo con cuidado. Ordenábamos todo lo que poníamos en medio y limpiábamos lo ensuciado. Lo que más me ha gustado es lo ameno que han sido las sesiones y lo que menos me ha gustado esque ha habido muy pocas.

CHARACTERIZATION AND POTENTIAL USES OF "PROTEIN ISOLATES" FROM OLIVE AND ARGAN SEEDS

Andrea Rueda[1], Irene Martín-Aznarte[1], Ada Fernández-Márquez[1], Sara Al-lach[1], Adoración Zafra[2], Agnieszka Zienkiewicz[2], Alfonso Clemente[3], Antonio Jesús Castro[2], Juan de Dios Alché[2*]

[1]IES Generalife, Huerta del Rasillo s/n, 18004, Granada, Spain.
[2]Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain
[3]Institute in formation for Animal Feeding, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain

*[*]Corresponding author: **e-mail:** juandedios.alche@eez.csic.es*

INTRODUCTION AND OBJECTIVES

Flours obtained from different plant seeds are currently available either for human and animal consumption and can be obtained in a commercial basis. These include wheat, maize, soya, cotton, chickpea, sunflower, pea, bean, and others flours. Intakes of many of these products have been recommended due to their content in high quality proteins. Some of these proteins function as store, and have been thus named Seed Storage Proteins (SSPs). Among them, those called 11S proteins are some of the most abundant. They are so-called legumins as well, as they have been deeply studied in several legumes. Legumins belong to the SSP family of globulins, which are soluble in buffered solutions.

The olive tree is an important crop in Spain and many other Mediterranean countries. The argan tree is on the other hand, an endemic wild species from Morocco, currently endangered of extinction because of over-exploitation because of its highly appreciated oil and the use of its fruits for animal feeding. Whereas the olive oil is mainly obtained under industrial parameters from the mesocarp (pulp), the argan oil is produced manually from seeds by local women, or just occasionally under semi-industrial conditions.

SSPs of the 11S-type have been biochemically characterized in the olive seed [1, 2]. However, just a preliminary determination of the presence of the counterpart proteins in the argan seed has been reported up to date [3]. The aim of the study was to attempt the preparation of aqueous solutions -socalled "protein isolates"-, enriched in highly nutritious proteins (mainly 11S Seed Storage Proteins of the legumin type, present in these seeds). For this purpose, two different alternatives previously described in the literature were used. These protein isolates may have further use for animal or even human nutrition.

MATERIALS AND METHODS

Material

Seeds from olive and argan were manually separated from the endocarp by using a cutting tool. Once we obtained 40 g. of each material, a cooking grinder was used to obtain the flours.

Partial removal of lipids from the flours & pre-treatment.

The flours were shaken with 100 ml of hexane (modified from [4]) for 20 min and the upper phase (hexane) was removed. This process was repeated 3 times. Remaining hexane was left

to evaporate at room temperature (RT). Four washes were then made by shaking the flours with 100 ml of water for 20 min. each. A final wash with 20% (v/v) ethanol was made. To end up, flours were left to dry at RT.

Protein extraction

Two slightly different methods were tested [4]. For the first method, 20 g. of defatted flour were stirred in 200 ml of 0.2 % (w/v) NaOH, pH 12.0 for 1h and then centrifuged at 8.000 g for 15 min. The supernatant was stored at 4ºC and the pellet used for a new re-extraction with the same conditions above described. A total of 3 extractions were performed. The method 2 was similar, although a solution of 0.25% (w/v) Na_2SO_3 pH 10.5 was used instead.

Protein quantification

For the measurement of the quantity of protein present in the samples, a commercial kit based in the colorimetric method of Bradford [5], was used.

Isoelectric point (Ip) titration of the proteins present in the flours

The supernatants corresponding to the first protein extraction of each flour were used for Ip titration. For this purpose, aliquots were prepared and each one was adjusted to a pH in the range 7.0 to 2.0 (in 0.5 unit intervals) by adding 0.5 M HCl 0,5M. Aliquots were centrifuged at 8.000 g for 30 min at 4ºC and both, the supernatants and the pellets were stored a 4ºC. The Bradford method was used to quantify the proteins of the supernatants.

SDS-PAGE

The proteins of the supernantants and pellets of the 11 alicuots obtained from the method of extraction 1 (that with higher yield of proteins) were separated electrophoretically by a using a Criterion™ System (Bio-Rad Laboratories) in a 4-20% gradient SDS-PAGE gel. The samples were denatured and ran under reducing conditions, and the gels stained with silver [6]. Parallel sets of gels electrophoresis were transferred onto PVDF membranes (Bio-Rad) aimed to perform immunobloting. A specific antibody to olive 11S SSPs, developed in rabbit, was used. An Alexa-488 conjugated anti-rabitt IgG secondary antibody (Agrisera) was used as the secondary antibody. To reveal the presence of the labelled antibody over the membranes, we used a Pharos FX fluorescence scanner system (Bio-Rad). Finally, the intensities of the bands obtained were quantified using the Quantity One (Bio-Rad) software.

Light and electron microscopy

Mature seeds from olive/argan were processed for its visualization at light (LM) and transmission electron microscopy (TEM) [1]. Thin (1 μm) and ultrathin (70 nm) sections were stained and contrasted with toluidine blue and uranyl acetate/lead citrate, respectively. Finally, they were visualized into a light microscope (Zeiss Axioplan) and a TEM (JEOL TEM-1011).

RESULTS AND DISCUSSION

Both the olive and the argan stones are formed by a woody endocarp with the seeds localized inside (usually one in the case of olive and up to three in the case of argan) (Figs. 1A and 2A). These can be isolated after breaching and separation of the endocarps (Figs. 1B and 2B). As the result of almonds milling, a lipid-rich paste (Figs. 1C and 2C) was obtained for each material, which was treated by using organic solvents, followed by washing and drying procedures in order to generate a de-fatted flour (Figs. 1D and 2D). These flours were considered the original material to further proceed with the generation of protein isolates.

Figure 1. A) Woody endocarps of olive. B) Seeds recovered after endocarp breakdown were stored at 4ºC. C) After grinding of seeds, an oily paste was obtained. D) Most lipids from the oily paste were removed using hexane, and partially defatted flour was obtained as the result.

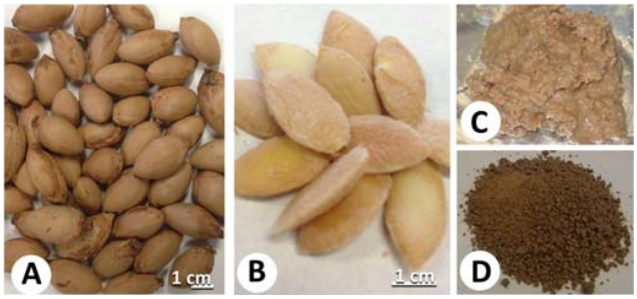


Figure 2. A) Woody endocarps of argan. B) Seeds recovered after endocarp breakdown were stored at 4ºC. C) After grinding of seeds, an oily paste was obtained. D) Most lipids from the oily paste were removed using hexane, and partially defatted flour was obtained as the result.

This process can be considered simple and effective, and has been designed in order to prevent the formation of lipid-protein complexes, which may deteriorate the quality of the final product [7]. However, the use of alternative methods in order to potentially improve yields further cannot be discarded. These procedures may include the use of physical procedures (e.g. pressing) to obtain olive seed oil and therefore generate partially defatted flours. Another alternative procedure widely described in the literature is the use of Soxhlet for this same purpose [7-9].

The preparation of protein isolates succeeding flour defatting in leguminous plants, is currently performed by one of the following procedures: i) isoelectric precipitation of proteins, and further separation of proteins from the remaining soluble molecules by centrifugation, ii) protein concentration by ultracentrifugation, or iii) protein extraction by using sodium chloride and precipitation by micelation [7, 9-10]. The present work uses the first one of these procedures. In this case, two types of isolates have been prepared from each material, including the use or not of sodium sulfite in the extraction medium. The extraction by using NaOH is economically preferable, although the use of sulfite also represents several advantages, like the use of milder conditions for the extraction (pH 10,5 instead of pH 12), as well as the fact of sulfite being able to inhibit the oxidation of polyphenols present in the sample, avoiding putative secondary reactions. As regard to the protein yield, the amount of proteins obtained is substantially higher after using NaOH (Fig. 3). Successive extractions

(extractions rounds 2 and 3) from the defatted flour do not substantially improve the protein yield obtained from the first extraction (Figs. 3A and 4A).

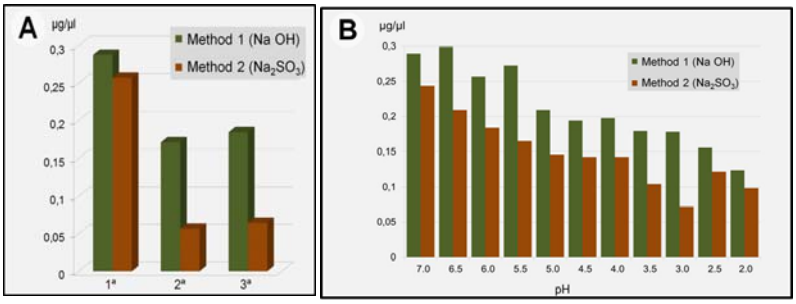


Figure 3. Bradford quantification of the protein extracts obtained from defatted olive flour prior and after isoelectric precipitation. A) Successive re-extractions of flours by using two different solutions (NaOH and Na_2SO_3). Quantification of the proteins in the supernatants revealed a clear drop in the quantity of proteins extracted over the successive re-extractions. It was also observed that the method 1 works better than method 2. B). Proteins in the supernatants resulting from isoelectric titration of the extracts. The results revealed an exponential decrease of soluble proteins from pH=7.0 to pH=2.0. Optimal pH for the precipitation of the proteins was 4.0-4.5.

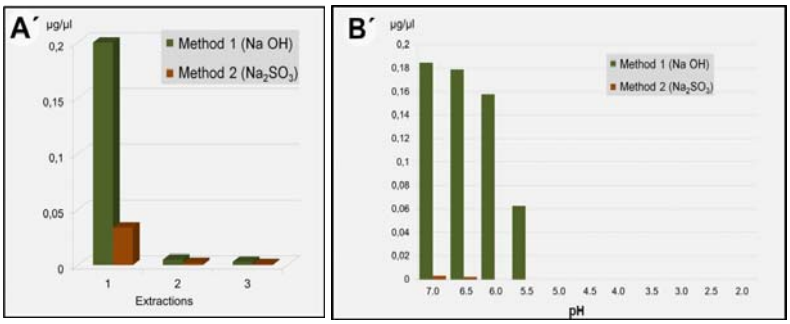


Figure 4. Bradford quantification of the protein extracts obtained from defatted argan flour prior and after isoelectric precipitation. A) Successive re-extractions of flours by using two different solutions (NaOH and Na_2SO_3). Quantification of the proteins in the supernatants revealed a clear drop in the quantity of proteins extracted over the successive re-extractions. It was also observed that the method 1 works better than method 2. B) Proteins in the supernatants resulting from isoelectric titration of the extracts. The results revealed an exponential decrease of soluble proteins from pH=7.0 to pH=2.0. Optimal pH for the precipitation of the proteins was 5.0.

Along the corresponding titrations, the solubility of the proteins present in the extracts decreased exponentially from pH 7.0 to reach a minimum between pHs 4 and 4.5 for the olive, and 5.0 in the case of argan (Figs. 3B and 4B). Titrations to lower pHs (4.0 to 2.0), although also produced lower readings of the protein amount, according to the method of Bradford,

produced a change in the graph tendencies, likely caused by some kind of interference in the spectrophotometric readings, thus limiting the accuracy of the Bradford method at these pHs. Such measurements should be therefore contrasted by using alternative methods for protein quantitative measurement (e.g. Lowry) or by determining the presence of nitrogen in the supernatants, referred to the total nitrogen extracted [8]. As observed in Figs. 3 and 4, protein extraction ability is higher after using the method 1 (NaOH) than after using method 2 (Na_2SO_3). The average Ip of the proteins present in the olive extract is therefore close to 4.0-4.5, and 5.0 for the corresponding argan extracts. These figures are compatible with previous studies carried out using several sources of defatted flours like those from oilseed and chickpea [8, 11].

The SDS-PAGE analysis of the protein profiles present in the supernatants and precipitates obtained at different pHs throughout titration (Figs. 5 and 6), also confirmed Bradford quantification of proteins displayed previously. This is particularly evident in olive extracts, particularly at pH 4.0 (Fig. 5B) and for argan extracts at pH 5.0 (Fig. 6)

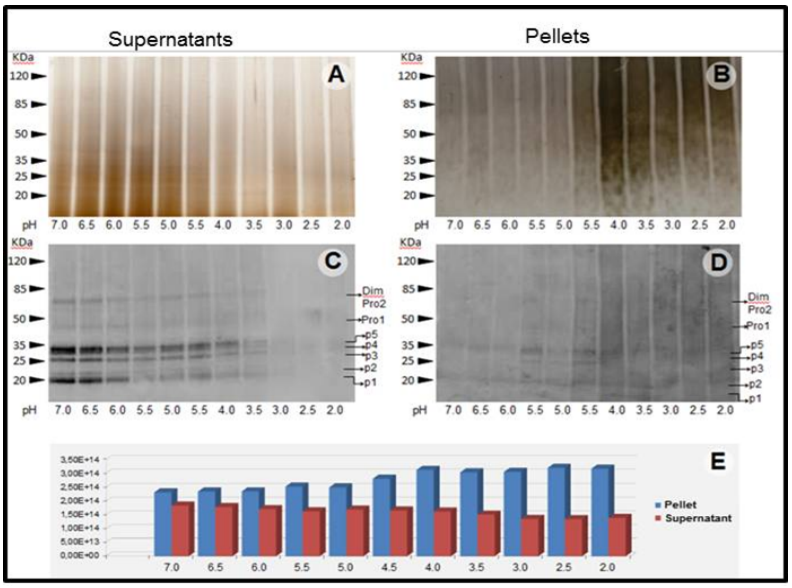


Figure 5. SDS-PAGE Protein profiles and 11S-protein Immunoblots corresponding to the olive seed protein isolates. A, B) Silver-stained gels corresponding to the different fractions after Ip precipitation confirmed Bradford quantification, as they showed an increase in the protein species present in the precipitates, occurring at pH=4.0. C,D) We used a anti-legumin 11-S antibody in immunoblotting experiments. As the result, 7 bands were observed in the olive (2 protein precursors and 5 individual proteins) (Alché et al. 2006) E) Quantification of the bands obtained in C, D.

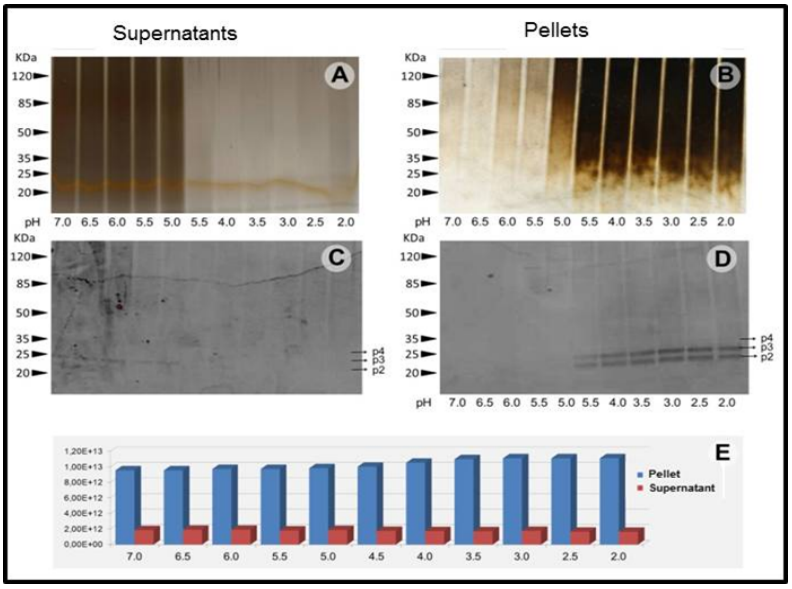


Figure 6. SDS-PAGE Protein profiles and 11S-protein Immunoblots corresponding to the argan seed protein isolates. A, B) Silver-stained gels corresponding to the different fractions after Ip precipitation confirmed Bradford quantification, as they showed an increase in the protein species present in the precipitates, occurring at pH=5.0. C, D) We used a anti-legumin 11-S antibody in immunoblotting experiments. As the result, 3 major bands were observed (individual proteins) E) Quantification of the bands obtained in C, D.

The immunoblotting experiments carried out allowed confirming that the peptides present in the profiles for the different pHs correspond to seed storage proteins of the 11S-type, similar to legumins (Fig. 5C, D, E and 6C, D, E) as the result of their cross-reaction with the antibody and their characteristic pattern of band sizes after using denaturing, reducing conditions for SDS-PAGE [1-2, 12]. This characteristic pattern is also well conserved among different byproducts resulting from olive processing for the industrial production of olive oil [13-14], and is also shared at least partially among different plant species [3].

Finally, analysis of the cellular and subcellular structure of the seed tissues allowed identifying these places accumulating lipid and protein accumulation, which corresponded to lipid and protein bodies, respectively (Figs. 7, 8). These structures showed a subcellular distribution highly organized and repetitive. Such distribution displayed a number of modifications as the result of the physiological changes that seeds experiment during processes like germination [15].

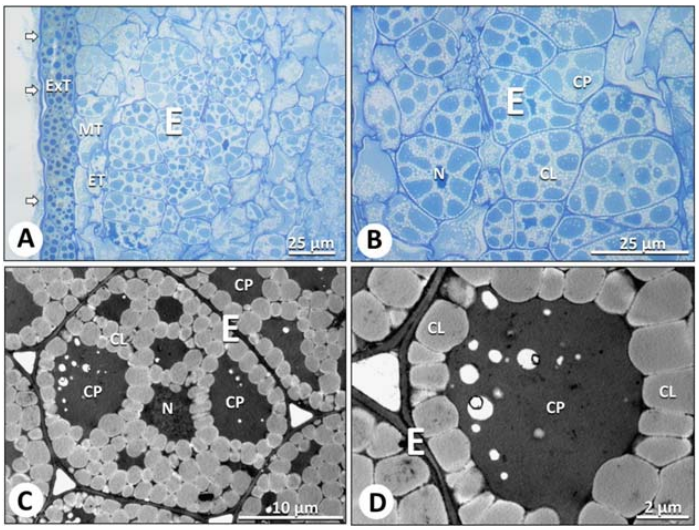


Figure 7. LM and TEM analysis of olive seeds histology. A, B) LM analysis of semi-thin sections displaying the teguments, composed of the cuticule (arrows), and the testa, stratified in exo-, meso-, and endotesta, and surrounding the endosperm cells (E). The endosperm cells show protein bodies (CP). In the case of the olive, lipid bodies (CL) surround the protein bodies. The results are confirmed by TEM analysis on ultra-thin sections, which display different electron density for CL and CP.

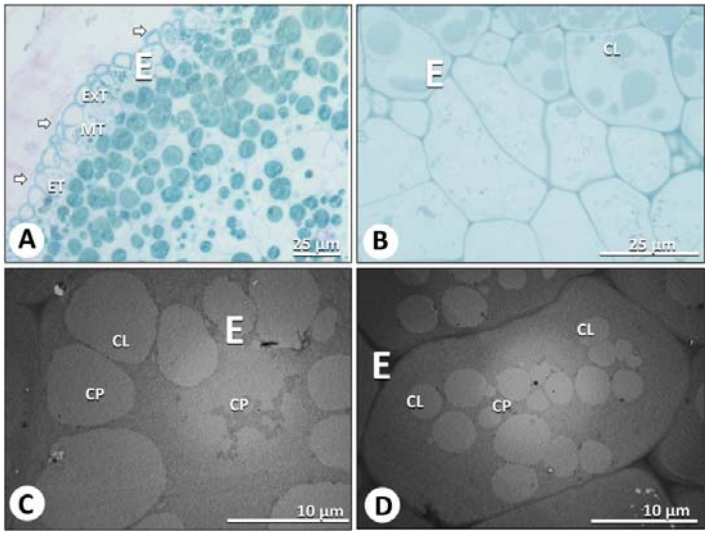


Figure 8. LM and TEM analysis of argan seeds histology. A, B) LM analysis of semi-thin sections displaying the teguments, composed of the cuticule (arrows), and the testa, stratified in exo-, meso-, and endotesta, and surrounding the endosperm cells (E). The endosperm cells show protein bodies (CP). The organization is slightly different from olive, with a much larger proportion of oil bodies compared to protein bodies, particularly in the inner layers of cells corresponding to the endosperm. The results are confirmed by TEM analysis on ultra-thin sections, which display different electron density for CL and CP.

The results obtained here represent the first aproach to the generation of protein isolates from olive and argan seeds. These results should be complemented with the analysis of other chemical components present in the isolates (total protein by using other methods, fiber, residual fats, sugars…), antinutritional factors, aflatoxins, tripsin and other protease inhibitors, lectins etc. Aditional analyses of the digestibility of both flours and protein isolates should also be implemented, and compared.

CONCLUSIONS

1. This is the first approach to obtain protein isolates from flours prepared from olive and argan seeds.
2. Efficiency of the extraction with NaOH is much higher than that of Na_2SO_3 for both species.
3. After the first extraction, the quantity of proteins decreases drastically in the successive re-extractions.
4. Most proteins precipitate at pH= 4.0 in olive and pH=5.0 in argan.
5. The use of the antibody anti-legumin 11-S corroborates the presence of this protein in both species, with a different molecular composition for each one.
6. Structural studies reveal the presence of lipid bodies surrounding protein bodies in the olive, with a higher proportion of lipid bodies in the argan.

ACKNOWLEDGEMENTS

This work was supported by ERDF partially co-financed funds: project MCINN BFU2011-22779 and "Proyectos de Excelencia": (JA) P2010-AGR6274, P2010-CVI5767 and P2011-CVI-7487. We also would like to thank C. Martínez Sierra for expert technical assistance, Prof. Mª Isabel Rodríguez-García for scientific discussion and the Confocal and Transmission Electron Microscopy (CTEM) core facilities of the EEZ-CSIC for providing excellent scientific environment. We also thank "almazara El Picón S.L." (Albanchez de Mágina, Jaén, Spain) and Elayotecnia S.L. (Castillo de Locubín, Jaén) for providing part of the material used in this work.

REFERENCES

[1] Alché JD, Jimenez-Lopez JC, Wang W, Castro AJ, Rodríguez-García MI (2006) Biochemical characterization and cellular localization of 11S type storage proteins in olive (Olea europaea L.). Journal of Agricultural and Food Chemistry 54: 5562-5570

[2] Wang W, Alché JD, Rodríguez-García MI (2007) Characterization of olive seed storage proteins. Acta Physiologiae Plantarum 29: 439-444

[3] Allach M, M´rani M, Sabouni I, Alché JD (2009) Preliminary characterization of SSPs (Seed Storage Proteins) in Argania spinosa L. Proceedings 3rd International SMBBM Congress, IUBMB Special Meeting & 6th FASBMB Congress, Marrakesh, 358-362

[4] Sánchez-Vioque, R, Clemente A, Vioque J, Bautista J, Millán F (1998) Polar lipids of defatted chickpea (Cicer arietinum L.) flour and protein isolates. Food Chemistry 63: 357-361

[5] Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254

[6] Rabilloud T, Brodard V, Peltre G, Righetti PG, Ettori C (1992) Modified silver-staining for immobilized pH gradients. Electrophoresis 113: 262-266

[7] Clemente A (1998) Estudio de la Calidad Proteica de la Semilla de Garbanzo (Cicer Arietinum L.) Var Athenas: Obtención de Aislados e Hidrolizados Proteicos. Ph. D. Thesis Universidad de Sevilla. Departamento de Bioquímica, Bromatología, Toxicología y Medicina Legal.
[8] Sánchez-Vioque R, Clemente R, Vioque J, Bautista J, Millán F (1999) Protein isolates from chickpea (Cicer arietinum L.): chemical composition, functional properties and protein characterization. Food Chemistry 64: 237-243
[9] Robles MC, Mora ER (2012) Influencia del método de obtención en las características fisicoquímicas y estructurales de aislados de soja. IX Congreso de Ciencias de los Alimentos y V Foro de Ciencia y Tecnología de Alimentos. XXV Aniversario de la Carrera de Ingeniería en Alimentos en el Instituto de Ciencias Agrícolas, Universidad de Guanajuato, México, pp. 716-721
[10] Vioque J, Sánchez-Vioque R, Pedroche J, Yust M, Millán F (2001) Obtención y aplicaciones de concentrados y aislados proteicos. Grasas y Aceites 52: 127-131
[11] Gonçalves N, Vioque J, Clemente A, Sánchez-Vioque R, Bautista J, Millán F (1997) Obtención y caracterización de aislados proteicos de colza. Grasas y Aceites 48: 282-289
[12] Castro AJ, Jiménez-López JC, Rodríguez-García MI, Alché JD (2007) Proteínas de almacenamiento tipo 11S en semillas de olivo (Olea europaea L.). Caracterización mediante técnicas de proteómica. Comunicaciones del Simposium Científico –Técnico EXPOLIVA 2007. Fundación del olivar, Jaén. ISBN 978-84-934503-3-5, pp. 1-6
[13] Jiménez-López JC, Alché JD, Wang W, Rodríguez-García MI (2007) El contenido proteico mayoritario del alpeorujo corresponde a proteínas de almacenamiento de semillas tipo 11S. En: El Aceite de Oliva. Actas del Simposium Científico-Técnico EXPOLIVA 2005. Fundación del olivar, Jaén. ISBN 978-84-934503-0-4, pp-365-376
[14] Ben Ali S (2011) Caracterización de proteínas de almacenamiento en la semilla del olivo y en subproductos de la extracción del aceite. "Master of Science en Olivicultura y Elaiotecnia". Universidad de Córdoba and CIHEAM.
[15] Zienkiewicz A, Jiménez-López JC, Zienkiewicz K, Alché JD, Rodríguez-García MI (2011) Development of the cotyledon cells during olive (Olea europaea L.) in vitro seed germination and seedling growth. Protoplasma 248: 1-15

MY OWN IDEAS

Andrea Rueda Arranz, IES Generalife, Granada

- The part of this investigation that I liked the most was when we were doing the gels of proteins because it was very enjoyable.
  (La parte de la investigación que más me gustó fue cuando hicimos los geles de proteínas porque fue muy entretenido).
- We would like to check digestion in animals like mice or chickens.
  (Nos gustaría probar la digestión en animales como ratón o conejo).
- The results of our investigation might be used to develop animal or human foods.
  (Los resultados de nuestra investigación podrían usarse para desarrollar alimentos animales o humanos).
- My experience at the laboratory has been very good with a view to the future. My opinion is that we have learned many laboratory techniques and has seemed very interesting to me.
  (Mi experiencia en el laboratorio me ha parecido muy buena con vista a mi futuro. Mi opinión es que hemos aprendido muchas técnicas de laboratorio que me han parecido muy interesantes).

Sara Al-lach Cazalla, IES Generalife, Granada

- At first we didn´t know exactly how the project we were involved in was, maybe because we didn´t understand the title of the project. But all these thoughts changed the first day when we really knew the details of the project. Little by little we started to work in the lab and at the end of each session we were looking forward to the next one. The experiments I liked the most were the titration and the proteins acrylamide gels. It was fun to change the pH looking for the Isolectric point.
  (Al principio no sabíamos exactamente como era el proyecto en el que íbamos a trabajar, quizá porque no entendíamos el título del proyecto. Pero todas esas dudas cambiaron el primer día cuando conocimos los detalles del proyecto. Poco a poco empezamos a trabajar en el laboratorio y al final de cada sesión estábamos ansiosas de que llegase la siguiente. Los experimentos que más nos gustaron fueron la titulación y los geles de acrilamida para separar las proteínas. Fue divertido cambiar el pH con el objetivo de conocer el punto isoeléctrico).
- I think that learning these techniques was very interesting for us because now, we know more about the laboratory work and we have helped to carry out an investigation. All of us have felt very comfortable at the CSIC with all the researchers.
  (Creo que haber conocido todas estas técnicas fue muy interesante para nosotros porque ahora sabemos más sobre el trabajo de laboratorio y hemos ayudado a llevar a cabo un proyecto de investigación. Destacar sobre todo que nos hemos sentido muy cómodas con los investigadores del CSIC).

Ada Fernandez Marquez, IES Generalife, Granada

- The truth is I don't quite know what to display in this small space that has been entrusted to me in this project, but I guess the first thing is to say thank you.
  It has been a new experience and it quite shaped me because I have seen and lived how is working in a real laboratory, in a true important center, and with professionals dedicating themselves to what they like. The thing that I have enjoyed the most has been the wonderful opportunity that I have had, experimenting pure research, thinking of different ways to get those results you crave, to can "become dirty" in a laboratory and apply everything that in class seems so boring and useless.
  It has been, in short, something I needed, because if we apply what we learn and we see that all the hours and hours of studying, the ones that we spend listening to a teacher in class and at home doing homework and trying to understand what seems so confusing and hard in a book, then why so hot head? I have loved that many of my classmates have been enjoying this experience too because enjoying experiences like this is the only way to realize if you really like something, if you would be willing working on this for almost the rest of your life, to see that what you studying worth, that's what really matters and with this project I have been able to verify many of these things. I may not want to do research in a lab like this because biology is not my forte but this opportunity has shown me that, behind a subject, there is much more, there are very cool professions, very friendly professionals who explain what you do and try to help and very nice and rewarding projects.

  La verdad es que no sé muy bien qué exponer en este pequeño espacio que me ha sido confiado dentro de este proyecto; pero supongo que, lo primero es dar las gracias.
  Creo que ha sido una experiencia nueva y que me ha marcado bastante pues he podido ver y vivir cómo es trabajar en un laboratorio de verdad, en un centro de verdad, importante, y con unos profesionales dedicándose a lo que les gusta. Con lo que de verdad me quedo es con la oportunidad tan genial que he tenido de poder experimentar la investigación en estado puro, el pensar en diferentes métodos para obtener aquellos resultados que ansiamos, el poder "pringarme" en un laboratorio y en aplicar todo lo que en clase parece tan aburrido e inútil.
  Ha sido, en resumen, algo que me hacía falta, porque si no aplicamos lo que aprendemos y no vemos que todas las horas y horas de estudio, las que pasamos escuchando a una profesora en clase y las que dedicamos en casa a hacer deberes y entender lo que parece tan enrevesado plasmado en un libro, entonces ¿para qué calentarse tanto la cabeza? Me hubiera encantado que muchos de mis compañeros de clase hubieran podido disfrutar de esta experiencia como lo he hecho yo porque proyectos como este son la única manera de poder determinar si de verdad te gusta algo, si estarías dispuesto a trabajar en eso durante prácticamente el resto de tu vida, ver que lo que estudias vale la pena, eso es lo que realmente importa y con este proyecto yo he podido comprobar muchas de estas cosas. Vale que no quiera dedicarme a la investigación en un laboratorio como este porque la biología no es mi fuerte pero esta oportunidad me ha demostrado que, detrás de una asignatura, hay mucho más, hay profesiones muy chulas, profesionales muy simpáticos que te explican lo que hacen y te intentan ayudar y proyectos muy bonitos y gratificantes).

Irene Martín Aznarte, IES Generalife, Granada

- We have never thought to experience the science so close, not as simple spectators. We have really worked and feel part of the project as true researchers. We are lucky because we felt we were helped by researchers at the same time we helped them to carry out the project. We were part of a team and the researchers treated us as equals. The laboratory tools that we have never heard about, but after a brief explanation they were in our hands. We have broadening our knowledge and discovered new techniques in biology that were difficult to understand for us, but after a while they became easy to learn. The obligation of presenting the project in English has improved our level. This experience has made me to think about the possibility of working in a research laboratory in the future. A world that was very far for us became closer because we started to learn in a different way. Sometimes we needed to scarify our spare time in order to do the investigation, however it was worthy because this is a unique experience than not many young people even adults have the opportunity to live.

Nunca creímos que íbamos a vivir esta experiencia desde tan cerca no mirando sino actuando y sintiéndonos parte del proyecto como unos científicos más. Hemos tenido suerte porque pensábamos que íbamos a ser unos simples espectadores más. Nos sentíamos ayudadas y sentíamos que ayudábamos, que colaborábamos, que éramos parte de un equipo, que nos trataban como iguales. Las herramientas que no sabíamos ni que existían antes de pisar el CSIC de pronto estaban al alcance de nuestras manos tras explicárnoslo. Hemos ampliado conocimientos y descubierto nuevos avances de la biología que nos parecían imposibles de entender a nuestro nivel pero que no fue difícil de comprender. A fuerza de tener que presentar los proyectos en inglés, hemos mejorado el idioma. Me han dado ganas de querer trabajar también en un futuro en un laboratorio de investigación. Un mundo que teníamos muy lejano se volvió de pronto mucho más cercano porque aprendíamos de otra manera. Aunque a veces requiriera sacrificar nuestro tiempo para poder investigar mereció la pena porque esta experiencia la tienen pocas personas de nuestra edad o incluso mayores la oportunidad de vivirla).

WHAT DOES A FUNGUS LIKE YOU DO WITH A SEED LIKE THIS?

Leticia Castellano Sánchez[1], Ana Rodríguez Ronchel[2], Lourdes Almagro Martín[2], Isabel García Martín[3], Yolanda Torres Montero[3], Antonio Montero Ruiz[4], Carmelo Ruiz[5], Francisco J. Corpas[5], José M. Palma[5], Inmaculada García-Romera[6], Elisabet Aranda[6]

[1]IES-Generalife, Huerta del Rasillo 1, Granada, Spain; [2]IES-Miguel de Cervantes, Camino Real de los Neveros 1, Granada, Spain; [3]IES-Alpujarra, Lora Tamayo s/n, Órgiva, Granada, Spain; [4]IES-Fray Luis de Granada, Huerta del Rasillo s/n, Granada, Spain; [5]Departamento de Bioquímica Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Granada, Spain; [6]Departamento de Microbiología del Suelo y Sistemas Simbióticos, CSIC, Granada, Spain

HIGHLIGHTS

●It has been proved that, under certain circumstances, the effect on plant growth is due to the fungi volatiles with no physical contact between both organisms.

●The parasitic and saprophytic features of the studied fungi are not universal, so using Arabidopsis, different growth patterns from those reported previously in other plant species were found.

●The interaction of the fungi activates the metabolism of reactive oxygen species in plants.

INTRODUCTION AND OBJECTIVE

The association between living organisms from different species is a phenomenon not unusual in nature. Thus, complex networks of mutualistic and antagonistic interactions occur among organisms in nature, which strongly impact on their own survival and on the stability of the whole population. Fungi, in particular, can take part in natural as well as in man-managed ecosystems due to their ubiquitous occurrence and the range of interactions they establish with plants, animals and other microbes. The different mutualistic and antagonistic fungal interactions are of particular interest for their ecological role, or because they can be exploited by man to improve plant health and/or productivity in sustainable agriculture and forestry. The living organism association needs the communication between both parts of the network mainly based on a package of molecular signals ("words") that allow the identification of partners, their interaction, and the specific responses that may favour the attraction or promote repulsion.

It is known that biotic and abiotic stresses enhance the production of reactive oxygen species (ROS) in plants. The main toxicity of ROS stems from their ability to cause rapid and deleterious oxidation of most cell components (Apel and Hirt 2004). Plants possess several enzymatic and non-enzymatic mechanisms that protect cells against excessive oxidative damage (Mittler, 2002). Among enzymatic antioxidants, catalase (CAT) plays an important role in the ROS removal by scavenging hydrogen peroxide.

Our study was designed to obtain more detail about the interaction between some soil and wood fungi *Cladosporium variabile*, *Fusarium lateritium*, *Coriolopsis rigida* and *Auricularia auricula-judae* in the growth of the model plant *Arabidopsis thaliana*. We studied the action of these fungi, which show different patterns in the association with plants (either as pathogenic

or saprophytic), both in *in vitro* seed germination and under greenhouse conditions. We also examined some of the possible mechanisms involved in such interactions. In addition, we tried to determine whether these fungi are capable of inducing oxidative stress in plants and, consequently, to study the defence responses mediated by the enzymatic systems.

MATERIAL AND METHODS

Fungi and plants

The fungi used were: *Cladosporium variabile* (CBS-KNAW Cult No.), *Fusarium lateritium* (BAFC cult. No. 2317), *Coriolopsis rigida* LPSC strain 232 (CECT 20449) and *Auricularia auricula-judae* (DSZM Cult No. 11326). Stock cultures were kept at 4 ºC on 2% malt extract agar (MEA).

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia were surface sterilized for 5 min in 70% (v/v) ethanol containing 0.1% (w/v) SDS and then placed for 20 min in sterile water containing 20% (w/v) Na-hypochlorite and 0.1% (w/v) SDS and washed four times in sterile water. The seeds were sown for 2 d at 4 ºC in the dark for vernalization on a basal growth medium composed of 4.32 g/L commercial Murashige and Skoog (MS) medium (Sigma-Aldrich) with a pH of 5.5, containing 1% (w/v) sucrose and 0.8% (w/v) phyto-agar.

In vitro experiments

Two experimental designs were followed. In the first design, diffusible antifungal activity was studied using full Petri dishes (90 mm) with MS medium. Petri dishes containing Arabidopsis seeds were inoculated with the fungi by transferring a 5-mm plug cut out from the margin of a 5-day-old colony on the bottom of the plate. The control plates had only the Arabidopsis plants growing in them.

In the second design, antifungal volatile activity was studied using 2-half divided Petri dishes (90 mm). In this case, the fungi were inoculated onto one half of the divided plate containing MEA with a 5-mm plug cut from the margin of an actively growing culture. Arabidopsis was placed onto the other half of the plates containing MS medium. For controls, the MEA´s compartment was left empty. The plates were incubated in vertical position.

The Petri dishes containing the *Arabidopsis* seeds and the fungus were then grown at 16 h light, 22 ºC/8 h dark, 18 ºC (long-day conditions) under a light intensity of 100 $\mu Em^{-2}s^{-1}$. Determinations were screened from a five-replicate experiment.

The growth the plants and the fungi were measured every 4 days, as compared to the control, over a period of 14 days.

In vivo experiments

The experiments were carried out in 0.15 L pots containing a mixture of peat:vermiculite, in a proportion 2:1 (v:v), which was steam-sterilized three times. A total of 7 barley seeds contaminated with each fungus were mixed with the substrate on the pots. For barley seeds fungal contamination, the content of 4 agar plates containing the fungus homogenized in 80 mL sterile water (55%; w/v) were inoculated. The fungi were grown in the barley support during one week. Uncontaminated barley seeds were added to the controls.

Arabidopsis seeds, previously sterilized, were planted on the surface. *Arabidopsis thaliana* plants were grown in a greenhouse under controlled conditions, watered and fed with a nutrient solution with 10 mL per week (Hewitt, 1952). Plants were harvested after 4 weeks and dry matter weight was determined. The experiments were conducted in a completely randomized design, with four replicates for each treatment.

Enzyme determination

For Arabidopsis crude extracts, plant tissues were frozen in liquid N_2 and ground in a mortar with a pestle. The powder was suspended in a homogenizing medium containing 50mMTris-HCl, pH 7.8, 0.1 mM EDTA, 0.1% (v/v) Triton, 10% (v/v) glycerol and 5 mM DTT (dithiothreitol), the last one, extemporarily added. Homogenates were centrifuged at 17,000 *g* for 30 min, and the supernatants were used for the assays. Catalase (CAT EC 1.11.1.6) activity was determined according to the method of Aebi (1984), following the H_2O_2 consumption at 240 nm (ε = 38,52 10^{-3} $M^{-1}cm^{-1}$). Total proteins were determined according to the Bradford method (1976) with bovine serum as standard.

Chromatographic analysis

The extraction of volatiles was carried out by head space (HS), using a SPME apparatus and 65 µm polydimethylsiloxane–divinylbenzene (PDMS/DVB) PDMS fibre (Supelco, Bellefonte, USA).

Samples were prepared directly in 10 mL vials. For this, 2.5 mL of MEA was sterilized in the vial and then a 5-mm plug cut from the margin of a 5-day-old colony of *F. lateritium* was placed on the bottom of the MEA. The control vials had only the MEA. The vials were closed using a cotton stopper and they were incubated during 5 days. Immediately after cultivation they were analyzed by gas chromatography-mass spectrometry (HS-SPME-GC-MS).

RESULTS

The germination of Arabidopsis seeds and the growth of plantlets were modified by the presence of most of the fungi tested in our experiments. As Fig. 1 shows, fungi *C. variabile* and *F. lateritium* grown in the presence of Arabidopsis seeds during 14 days decreased the plant fresh weight. Arabidopsis plants were not affected by the presence of the fungus *C. rigida*. However, *A. auricula-judae* was able to increase the growth of Arabidopsis after 14 days of cultivation.

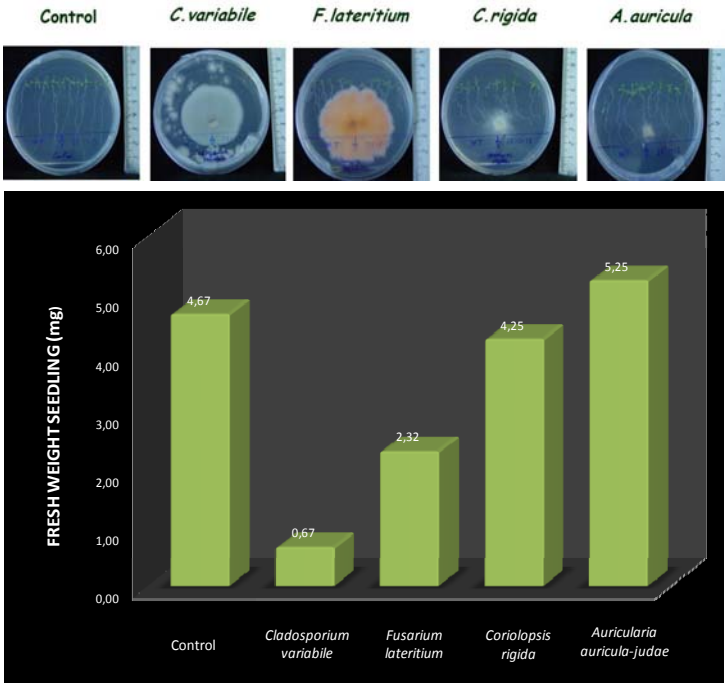


Figure 1. (Top) *In vitro* experiment performed in full Petri dishes, with Arabidopsis (Control) and the fungi *Cladosporium variabile*, *Fusarium lateritium*, *Coriolopsis rigida* and *Auricularia auricula-judae* at 14 days of cultivation. (Bottom) Arabidopsis fresh weight determined after harvesting (mg per plant).

Some of the results obtained in the experiment with divided Petri plates were different to those made in full Petri plates (Fig. 2). In fact, we found that Arabidopsis weight was higher in the presence of *C. variabile* and *A. auricula-judae* than in the control. However, incubation with *F. lateritium* and C*. rigida* decreased the Arabidopsis growth. It is worth to point out that the fungus *C. variabile* showed opposite results in the experiments with the full or divided Petri dishes. In fact *C. variabile* was able to reduce the growth of Arabidopsis in normal Petri dishes experiments but improved the growth in divided Petri dishes. It seems that *C. variabile* produces diffusible exudates that could affect the growth of our test plants; however, *F. lateritium* decreased the development of the plant either by diffusible or volatile exudates. On the other hand, *C. rigida* improved the growth of the test plants by diffusible exudates while the volatile exudates produced by this fungus decreased the growth of Arabidopsis. *A. auricula-judae* was able to improve the growth of plant either by its diffusible or volatile exudates.

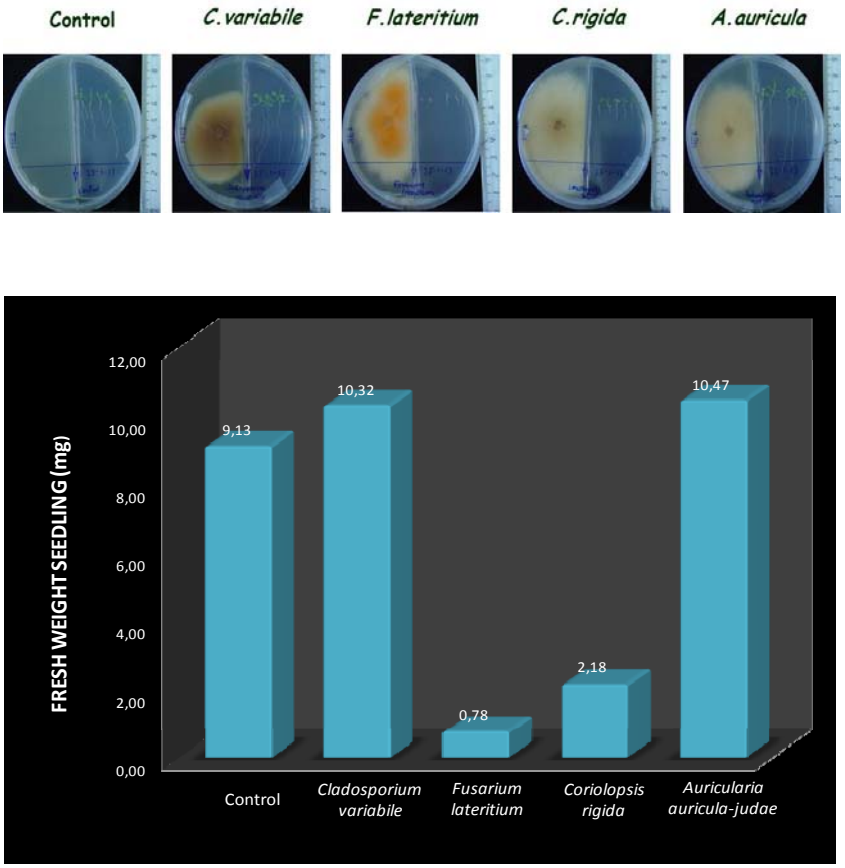


Figure 2. (Top) *In vitro* experiment performed in divided Petri plates, with Arabidopsis (Control) and with the fungi *Cladosporium variabile*, *Fusarium lateritium*, *Coriolopsis rigida* and *Auricularia auricula-judae* at 14 days of cultivation. (Bottom) Arabidopsis fresh weight determined after harvesting (mg per plant).

The *in-vivo* experiments carried out in the greenhouse showed similar results to the *in vitro* assays previously described (Fig. 3). After one month, the fungus *C. rigida* and *A. auricula-judae* were able to promote the Arabidopsis growth. In contrast, the growth impact of *C.*

variabile and *F. lateritium* on the development of Arabidopsis was negative. This study clearly confirms the result obtained in the *in vitro* experiment.

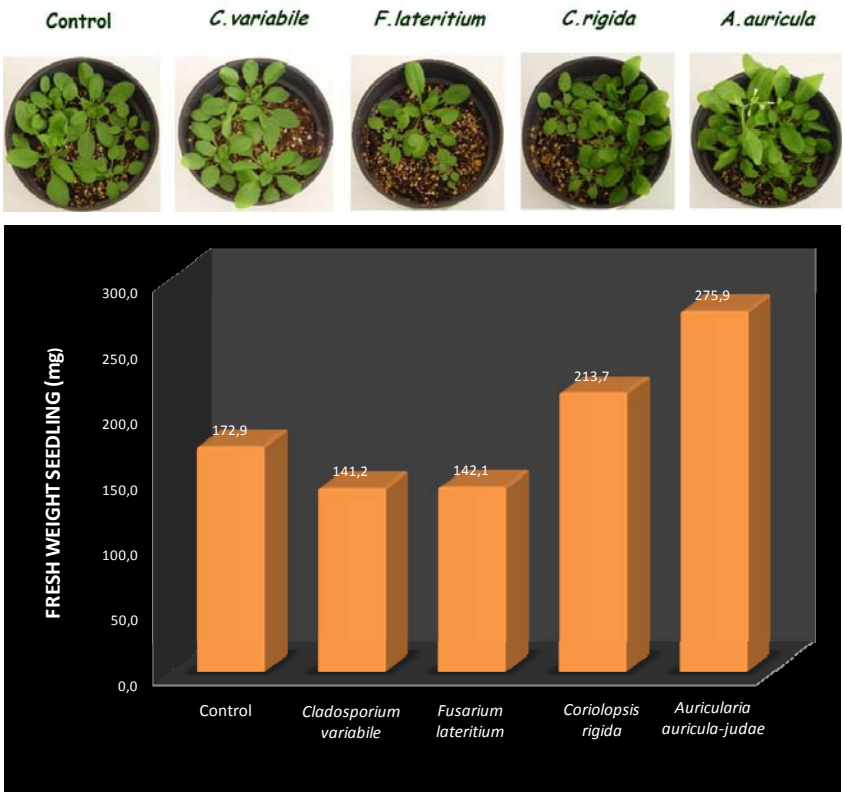
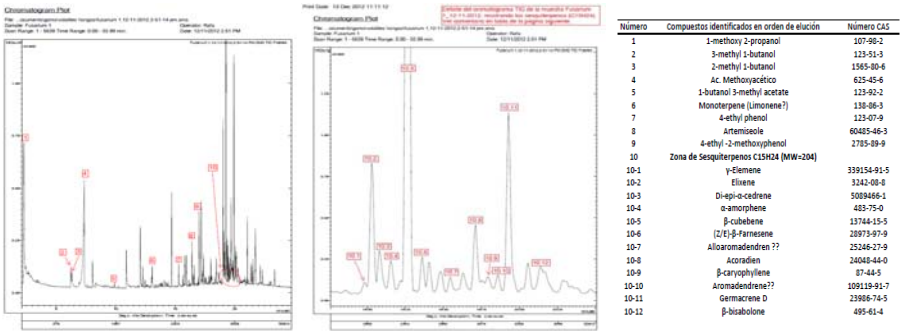


Figure 3. (Top) *In vivo* experiment with Arabidopsis (Control) growth in the presence of the fungi *Cladosporium variabile*, *Fusarium lateritium*, *Coriolopsis rigida* and *Auricularia auricula-judae* at 14 days of cultivation. (Bottom) Arabidopsis fresh weight determined after harvesting (mg per plant).

More detailed studies will be required to explain the mechanisms of these fungi-plant interactions. In a first approach, we studied by HS-SPME-GC-MS the composition of the volatile exudates produced by the fungus *F. lateritium* (Fig. 4). The main compounds detected were terpenoids (monoterpenoids, sesquisterpenes) and alcohols. As it is well known, terpenoids are the most widespread, chemically interesting groups of secondary metabolites (Wang et al., 2005). Many sesquisterpenes have biological activities, and in particular, some sequisterpenoids are produced by several species of *Fusarium*. Mycotoxins produced by *Fusarium*, belonging from this group, have been studied for the variety of their metabolic effects and phytotoxic activities, including the ability to cause necroses, chlorosis, growth inhibition, wilting, and inhibition of seed germination (Eudes et al., 1998). We can conclude that the growth inhibitory effect detected in Arabidopsis cultivated with *Fusarium* could be due to the mycotoxin produced by this fungus.



Número	Compuestos identificados en orden de elución	Número CAS
1	1-methoxy 2-propanol	107-98-2
2	3-methyl 1-butanol	123-51-3
3	2-methyl 1-butanol	1565-80-6
4	Ac. Methoxyacético	625-45-6
5	1-butanol 3-methyl acetate	123-92-2
6	Monoterpene (Limonene?)	138-86-3
7	4-ethyl phenol	123-07-9
8	Artemisiole	60435-46-3
9	4-ethyl -2-methoxyphenol	2785-89-9
10	**Zona de Sesquiterpenos C15H24 (MW=204)**	
10-1	γ-Elemene	339154-91-5
10-2	Elixene	3242-08-8
10-3	Di-epi-α-cedrene	5089466-1
10-4	α-amorphene	483-75-0
10-5	β-cubebene	13744-15-5
10-6	(Z/E)-β-Farnesene	28973-97-9
10-7	Alloaromadendren ??	25246-27-9
10-8	Acoradien	24048-44-0
10-9	β-caryophyllene	87-44-5
10-10	Aromadendrene??	109119-91-7
10-11	Germacrene D	23986-74-5
10-12	β-bisabolone	495-61-4

Figure 4. TIC chromatogram of HS-SPME of *Fusarium lateritium* (a), TIC chromatogram detail of the sesquisterpen area (b). The main compounds detected (c).

The analysis of a ROS parameter, catalase, also showed important differences among the different treatments (Fig. 5). Thus, an increase of about 25-30% was obtained in plants grown in the presence of *C. variabile, C. rigida* and *A. auricula-judae*.

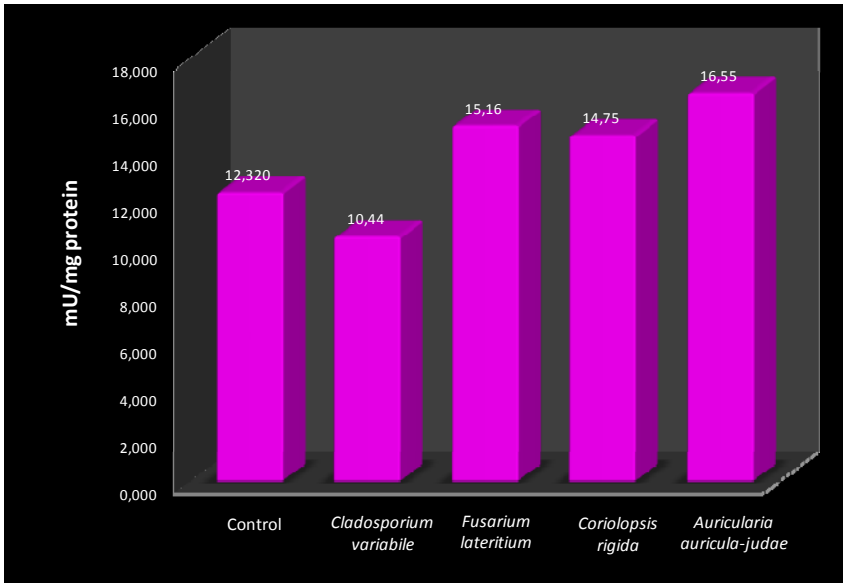


Figure 5. Catalase activity in leaves from Arabidopsis plants grown in the presence of the fungi *Cladosporium variabile*, *Fusarium lateritium*, *Coriolopsis rigida* and *Auricularia auricula-judae.*

CONCLUSIONS

1. The fungi *C. variabile* and *F. lateritium* cultured in Petri plates are able to inhibit the growth of Arabidopsis. The mechanisms implicated in this effect probably are related

with the diffusible exudates in the case of the *C. variabile* and both diffusible and volatile exudates for the *F. lateritium*.

2. Arabidopsis growth was improved by the fungi *C. rigida* and *A. auricula-judae*. The promotion of the Arabidopsis growth may be due to the diffusible exudates of *C. rigida* and the diffusible and volatile exudates produced by *A. auricula-judae*.

3. Our results in catalase activity suggest that hydrogen peroxide is perhaps involved in the signalling underwent during the association of these organisms.

ACKNOWLEDGEMENTS

This project was supported by FEDER funds and from the Spanish Ministry of Science and Innovation (projects AGL2012-32873 and AGL2011-26044).

REFERENCES

[1] Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review Plant Biology 55: 373-399

[2] Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254

[3] Eudes F, Comeau A, Rioux S, Collin J (1998) Phytotoxicity of eight mycotoxins associated with *Fusarium* diseases in wheat ears. Canadian Journal of Plant Pathology 22: 286-292

[4] Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Technical Communication. Commonwealth Agricultural Bureaux 22: 237-315

[5] Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7: 405-410

[6] Wang G, Tang W, Bidigare RR (2005) In: Natural Products Drug Discovery and Therapeutic Medicine Terpenoids As Therapeutic Drugs As Pharmaceutical Agents. (Zhang L, Demain AL, eds). Humana Press, New Jersey, United States of America

[7] Zonno MC, Vurro M (2002) Inhibition of germination of *Orobanche ramosa* seeds by *Fusarium* toxins. Phytoparasitica 30:519-524

MY OWN IDEAS

Leticia Castellano Sánchez, IES-Generalife, Granada

The PIIISA Project has been a great experience for me because I think I could learn a lot of things about biology and biochemistry, which are the subjects I like a lot and I would like to study something similar at the University. So, I think this experience is very good for this future.

Also, another advantage of this project is that young people like me can know other people with the same age and with the same interests. We can learn how the investigation's world goes, we can concern a bit about the effort made by all these people and the money invested in our country to the science.

I liked knowing the EEZ (Experimental Station from Zaidín) because I have seen the real research, I mean the proceeding, their facilities and the operation of a laboratory too. On the other hand, I want to thank to all the researchers and my tutors to make this project possible.

Finally, I only hope that PIIISA Project carry on existing for a lot of years and for a lot of young people.

El Proyecto PIIISA ha sido una gran experiencia para mí, porque creo que he aprendido un montón de cosas sobre la biología y la bioquímica, que son los temas que me gustan mucho y me quería estudiaren la Universidad. Por lo tanto, creo que esta experiencia es muy buena para este futuro.

Además, otra de las ventajas de este proyecto es que los jóvenes como yo pueden conocer a otras personas con la misma edad y con los mismos intereses. Podemos aprender cómo va el mundo de la investigación, y darnos cuenta el esfuerzo realizado por todas estas personas y el dinero invertido en nuestro país a la ciencia.

Me gustó conocer la EEZ (Estación Experimental del Zaidín) porque he visto la verdadera investigación, es decir el procedimiento, sus instalaciones y también el funcionamiento de un laboratorio. Por otra parte, quiero dar las gracias a todos los investigadores y mis tutores por hacer posible este proyecto.

Por último, sólo espero que el Proyecto PIIISA pueda continuar vigente durante un montón de años y estimule amucha gente joven.

Ana Rodríguez Ronchel, IES-Miguel de Cervantes, Granada

Taking part in this project has been, from my point of view, very interesting and motivating. This experience has allowed me to closely follow how science works and to know a little bit more about how scientists work in real-life. In addition, it was very exciting to investigate real situations, and I felt very good because we were

contributing to something interesting. I learnt a lot, especially in the meetings. I am sure this experience will help me to make a decision about my future. Fungi, plants and the relationships between them are much more complicated than I imagined. What I liked the most was having the experiments (the Petri dishes with the cultures) at home. I could see them every day and I felt like a real scientist, taking notes about all the changes and the evolution of the investigation. I also think that our project was one of the most attractive, it was very useful and offer many possibilities. I am very glad that we have obtained some interesting results.

La participación en este proyecto ha sido, desde mi punto de vista, muy interesante y motivante. Esta experiencia me ha permitido seguir de cerca cómo funciona la ciencia y saber un poco más acerca de cómo trabajan los científicos en la vida real. Además, fue muy emocionante investigar situaciones reales, y me sentí muy bien porque estábamos contribuyendo a algo interesante. He aprendido mucho, sobre todo en las reuniones. Estoy segura que esta experiencia me ayudará a tomar una decisión sobre mi futuro. Los hongos, las plantas y las relaciones entre ellos son mucho más complicados de lo que imaginaba. Lo que más me gustó fue llevarme los experimentos (los platos de Petri con los cultivos) a casa. Yo los veía todos los días y me sentí como una verdadera científica, tomando notas acerca de todos los cambios y la evolución de la investigación. También creo que nuestro proyecto fue uno de los más atractivos, que era muy útil y ofrece muchas posibilidades. Estoy muy contenta de que hayamos obtenido algunos resultados interesantes.

Isabel García Martin, IES-Alpujarra, Órgiva

The PIIISA of this year has seemed to me to be very interesting, with projects that were calling enough the attention to the young persons and has been very well coordinated.

Initially I was thinking that it was going to be a more boring project where we could not have worked with investigators and the reality was everything opposite, it has been a very interesting project where we have learned things that normally in the high schools are not taught and also it can help us to see the exits that a scientific career can have.

Also it is necessary to be grateful for the collaboration of the investigators since they have had patience great. I hope that there are more editions of the PIIISA because they are the only experiences where besides involving yourself in these types of projects you know new people and relate to persons of other high schools.

My project has been raised well and the necessary changes have been done in order that good results were arising, the work on the fungi and the seeds has met his fruits and I believe that it has been a good project with satisfactory results. I would not add anything to the project and neither I would suppress anything.

El PIIISA de este año me ha parecido muy interesante, con proyectos que llamaban bastante la atención a los jóvenes y ha estado muy bien coordinado.

Al principio pensaba que iba a ser un proyecto más aburrido donde no hubiésemos podido trabajar con investigadores y la realidad fue todo lo contrario ha sido un proyecto muy interesante donde hemos aprendido cosas que normalmente en los institutos no se enseñan y también nos puede ayudar para ver las salidas que puede tener una carrera científica.

También hay que agradecer la colaboración de los investigadores ya que han tenido mucha paciencia. Espero que haya más ediciones del PIIISA porque son experiencias únicas. Donde además de implicarte en estos tipos de proyectos conoces gente nueva y te relacionas con personas de otros institutos.

Mi proyecto ha estado bien planteado y se han hecho los cambios necesarios para que surgieran buenos resultados, el trabajo con los hongos y las semillas ha dado sus frutos y creo que ha sido un buen proyecto con resultados satisfactorios. No le añadiría nada al proyecto y tampoco suprimiría nada.

Yolanda Torres Montero, IES-Alpujarra, Órgiva

Hello my name is Yolanda Torres, and I come from the Instituto IES Alpujarra. Sign to the project thinking that it was going to be entertained, and very interesting. And I have not been wrong. I have liked it very much. Not only I have known new people, but name of fungi and rare devices. I have been charmed with working with all, also I have been charmed being involved at the laboratories, since I want to study sciences.

Being in this project have achieved incredible results, and I've seen the result and the reaction of different seed and fungi

In my case, I used throughout the project, shared plates, and it is amazing how the fungus being on one side pass through the small barrier there, that separates one side from the other.

Hola mi nombre es Yolanda Torres, y vengo del Instituto IES Alpujarra. Me registré al proyecto pensando que iba a ser entretenido, y muy interesante. Y no me he equivocado. Me ha gustado mucho. No sólo he conocido a gente nueva, sino también el nombre de hongos e instrumentos de laboratorio. Me ha encantado trabajar con otros estudiantes, también me ha encantado estar en los laboratorios, ya que quiero estudiar ciencias.

En este proyecto se han logrado resultados increíbles, y he visto el resultado y la reacción de los diferentes hongos con las semillas.

En mi caso, he utilizado durante el proyecto placas compartidas, y es increíble cómo el hongo que está en un lado de la placa puede afectar a la planta que está en la otra parte de la placa.

Antonio María Montero Ruiz, IES-Fray Luis de Granada, Granada

The days we have spent together have been very good, I could not imagine how much fun could be science, also to meet people from other places. I have been very well and they treated me as another scientist but I lack a lot to be researcher. It has been a pleasure working with all the scientific group and I would like to repeat. Thank you very much for everything.

Bueno estos días que hemos pasado juntos han sido muy buenos, no podía imaginar lo divertido que podía ser la ciencia además de conocer a gente de otros sitios. He estado muy a gusto y me han tratado como a otro científico más aunque me falte mucho para serlo. Ha sido un placer haber estado con vosotros y me gustaría repetirlo. Muchas gracias por todo.

Lourdes Almagro Martín, IES-Miguel de Cervantes, Granada

I liked a lot the proyect, and it was also very interesting. I didn't imagine the world of science that way and seeing the people working in the different projects was amazing! I think that the machines and their prices were very interesting. It is an experience that I recommend to everyone.

Me ha gustado mucho el proyecto, además ha sido super interesante. No me imaginaba el mundo de la ciencia así y ver en primera persona a la gente trabajando en proyectos reales la verdad es que ha sido alucinante! Cuando nos hablaron de las maquinas tan complejas y de sus precios fue increíble! Ha sido una experiencia que recomiendo.

RIPENING OF PEPPER FRUITS

Cecilio V. Aranda[1], Irene García-Fernández[1], Irene Hernández-Quero[1], Santiago Sánchez-Requena[1], Carmelo Ruiz[2], Francisco J. Corpas[2], José M. Palma[2]

[1]IES Fray Luis de Granada, Huerta del Rasillo s/n, Granada, Spain
[2]Departamento de Bioquímica Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Granada, Spain

MY OWN IDEAS

Santiago Sánchez Requena

At first I was a little disappointed, since my project was something as simple as peppers, an issue that I sincerely did not attract first. Later when José Manuel told us, what interested me was also to learn new skills, use equipment and materials scientists, some as surprising as liquid nitrogen, etc ...

The working environment in the research centre really surprised me, especially the order, cleanliness (each utensil was thoroughly washed before use), the rigor, the structuring of tasks: sometimes very repetitive (write in the tubes test the sample name), etc. .... The relationships have also been very good creating a good working environment.

This experience has been enjoying a lot. Mainly due to near-patient treatment of José Manuel Palma, Carmelo Ruiz and other researchers. The project seemed very interesting to me to bring research students, offering alternatives so far unaware.

Al principio estaba un poco decepcionado, puesto que mi proyecto trataba de algo tan simple como los pimientos, un tema que, sinceramente de primeras no me atrajo. Posteriormente cuando José Manuel nos explicó de qué trataba me interesó mucho, además aprendí nuevos conocimientos, utilice aparatos y materiales científicos, algunos tan sorprendentes como el nitrógeno líquido, etc...

El ambiente de trabajo en el centro de investigación me sorprendió muy positivamente, especialmente el orden, la limpieza (cada utensilio era concienzudamente lavado antes de su uso), el rigor, la estructuración de las tareas: a veces muy repetitivas (escribir en los tubos de ensayo el nombre de las muestras), etc.... Las relaciones entre los compañeros han sido también muy buenas creando un buen ambiente de trabajo.

Esta experiencia me ha gustando bastante. Sobre todo gracias al trato cercano y paciente de José Manuel Palma, Carmelo Ruiz y demás investigadores. El proyecto me ha parecido muy interesante para acercar la investigación a los estudiantes, ofreciéndonos alternativas que, hasta ahora, desconocíamos.

Irene García Fernández

This is about the work I've done these months of research. I have been working with the peppers and antioxidants and I liked it; the experience was new to me and I had

never done anything like this or investigated and it is something I really like. We've been working a lot and I liked it because I liked to wear the lab coat and put peppers in jars, dissect them and investigate many things. I'm glad I got into this project because I learned a lot thanks to it and I had so much fun doing some afternoon to continue working. Although this year our work is not exposed I'm happy to do next year and that our efforts will be rewarded. When they told me the name of the project is not illusion but made me laugh when I saw what was and what we had to do each time I became interested more and more and I really like.

Voy ha hablar del trabajo que he hecho estos meses de investigación. Ha sido trabajar con los pimientos y sus antioxidantes y me ha gustado mucho la experiencia, ha sido algo nuevo para mi ya que nunca antes había hecho nada parecido ni investigado y es algo que me gusta mucho. Hemos estado trabajando mucho y eso me ha gustado porque me gustaba ponerme la bata y diseccionar pimientos meterlos en frascos e investigar muchas cosas. Me alegro de haberme metido en este proyecto porque he aprendido muchas cosas gracias a él y me he divertido mucho yendo alguna tarde a seguir trabajando. Aunque este año no se exponga estoy feliz de poder hacerlo el año que viene y que nuestro esfuerzo se vea recompensado. Cuando me dijeron el nombre del proyecto no me hizo mucha ilusión pero cuando vi de lo que iba y lo que teníamos que hacer cada vez me fue interesando más y más y me gusta mucho.

Irene Hernández Quero

My name is Irene Hernández Quero and I have participated in the PIIISA Project; this project for me it has been a big experience in a personal and professional level , because to work in a professional laboratory , with scientists , people that really work in a laboratory, I think that I am a very lucky girl .

It has been six months that we have been working in this project and we have learnt a lot, but this I thank to Pepe Palma that he has been the person who from the beginning has been with us and he has learnt all.

Mi nombre es Irene Hernández Quero y he participado en el proyecto PIIISA, este proyecto para mí ha sido una gran experiencia a nivel personal y profesional, ya que el poder trabajar en un laboratorio profesional, con investigadores, personas que verdaderamente se dedican a esto es una suerte.
Han sido seis meses los que hemos estado trabajando en el proyecto y en los que he aprendido mucho, pero esto también se lo agradezco a Pepe Palma que ha estado con nosotros y es el que nos lo ha enseñado todo desde el principio.

Cecilio V. Aranda

PIIISA has become for me a interesting project and for that, I've learnt a lot about science. Also, I have had the opportunity to learn about the work of scientists. In my PIIISA project, I've studied peppers and their properties, and for me, this project is awesome. I'm very glad of PIIISA because it makes me to continue on my dreams for be a scientist or an engineer.

NOVEL 'GROUPS' OF *MASSILIA* IN THE DNA OF A BACTERIAL COMMUNITY OF SOILS

Elrajeh, L[1]., Fernández-Alfaro, I. [1], Gómez-Fernández, A. [2], Guzmán-Martín, J.L. [3], Megías, C. [4], Nogales-Gámiz, M. [2], Obeso-Benítez, P. [4], Rensch, J. [1], Urbano-Vaquera, A.[1], Zvonaru, A. [5], & Martínez-Abarca F. [6]

[1]*IES Miguel de Cervantes. Granada.*
[2]*IES Virgen de la Caridad. Loja.*
[3]*IES Padre Manjón. Granada.*
[4]*IES Zaidín-Vergeles. Granada.*
[5]*IES La Madraza. Granada*
[6]*Departmento de Microbiología y Sistemas Simbióticos Estación Experimental del Zaidín – CSIC – Granada, Spain*
[*]*Corresponding author: fmabarca @eez.csic.es*

HIGHLIGHTS

Description of novel Group of *Massilia* bacteria based on its 16SrRNA

SUMMARY

The DNA opens a new window for the identification of novel species in the Earth. Phylogenetic relationships among bacteria can be inferred from comparisons of their Small Ribosomal RNA subunit (16S rDNA) sequences. DNA from soil samples of Mexico was obtained and 16S rDNA was amplified. Our objective consisted in the study of a particular group of these sequences - 52 sequences corresponding to the genera *Massilia* based in the RDP-database. After a comparison of these sequences together with other *Massilia* 16SrRNA from databases eight different groups (clades) were formed. The analysis of the different changes at nucleotide level reveals a pattern of changes according to the predicted secondary structure of the 16S rRNA. Our data suggest the presence of novel group(s) of *Massilia* bacteria in our samples

INTRODUCTION

The earth is dominated by the life; in recent years a lot of studies have been focus to determine the biodiversity present in order to catalogue the number of species (Rosselló-Mora, 2005).

Table 1. Total number of described species at the end of XX century.

Group	*Described (x1000)*	*% Total*	*Estimated (x1000)*
Plants	270	15.4	300-500
Artropods	1065	60.8	2375-101200
Mussels	70	4	100-200
Nematods	25	1.5	100-1000
Protozoa	40	2.3	60-200
Algae	40	2.3	150-1000
Fungi	75	4.3	200-9900
Prokaryotes	4.9	0.3	50-3000
Viruses	4	0.2	50-1000
Others	115	6.6	200-800

Meanwhile the concept of specie is well defined for disciplines like Zoology (Animals), Botany (Plants), in Microbiology is a difficult task. Currently, it is accepted the names by a science denominated taxonomy (Rosselló-Mora, 2005).

Every discipline estimates the amount of species present (Table 1). There are kingdoms where a majority (60.8%) of the species are catalogued and identified; Artropods. However, in the world of microbiology this is not the case; only a tiny part, 0.3% are described. Note it is difficult to imagine that the number of species of bacteria in the earth should be 6-fold less than the Plant species or 200-fold less than Artropods: Aracnids, Mussels, Insects, (The most diverse phylum in the animal kingdom). As a consequence, a great majority (>99%) of novel bacterial species remains to be discovered (and described).

Our work will focus on the life present in the soil, particularly in the rhizosphere soil near of the plant roots where the number of bacteria present is estimated between 1 and 10,000 millions per gram. These numbers make the soil one of the most complex ecosystems in the Earth.

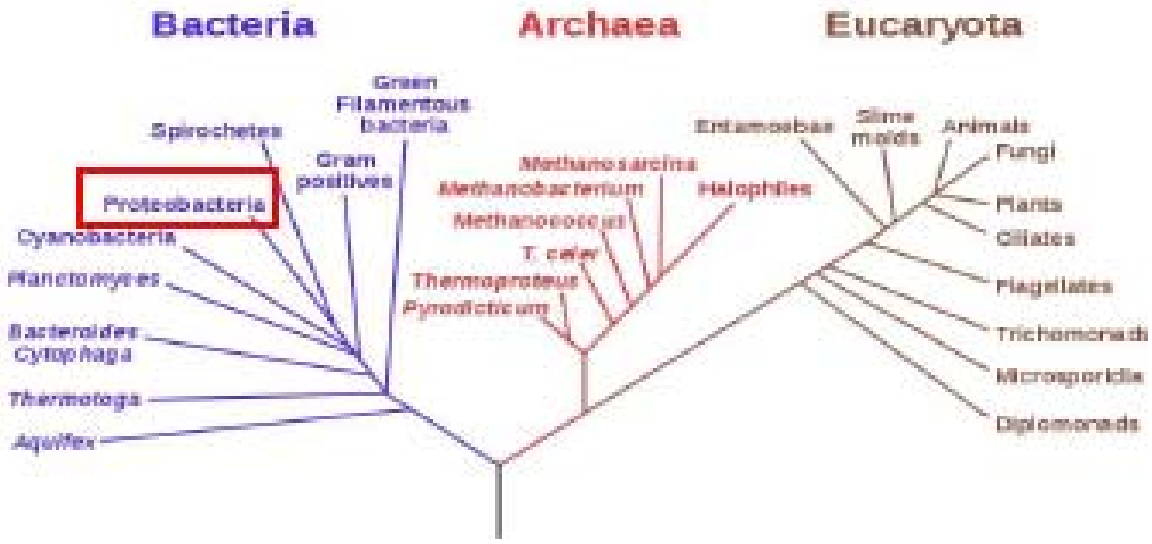


Figure 1: Phylogenetic tree based in the comparative analyses of the encoded gene for the small subunit ribosomal RNA.

Traditional methods based on culture isolation of microorganisms are not sufficient to describe what soils or other ecosystems contain. In the last years the access to the DNA has opened a new window of characterization of the diversity. In particular one gene has been used for taxonomic and phylogenetic classifications: the sequence of the Ribosomal RNA. A tree of life can be constructed based only on the DNA sequence variations of this molecule between the different forms of life (Figure 1). Bacteria, Archaea and Eukaryotes form the three big kingdoms of life forms present in the Earth. This RNA forms part of the Ribosome, a key organelle for all life organisms. They are the machinery of translation of the proteins. All independent form of life must transform messenger RNA to proteins. They are a complex molecular machine constituted of two subunits: The large subunit is composed by two molecules of ribosomal RNA (rRNA), one of 5S and another of 23S, and 34 proteins. The small subunit is formed only by one molecule of 16S rRNA, and 21 proteins.

A lot of studies of diversity estimation in bacteria are based on the 16S rRNA (Rodicio 2004). This has had an enormous repercussion on bacterial taxonomy, leading to the currently applied system of classification, and allowing a rapid and precise identification of bacteria. Amplification of the gene to be sequenced is a method currently used for rapid bacterial

identification. And several databases are based on this molecule for rapid bacterial identification (Cole et al 2009).

Our objective consisted in the study of a particular group of 16S rDNA sequences obtained by DNA amplification of samples from Mexico soils and corresponding to bacteria of the genera *Massilia* (Torres-Cortes et al. 2012)

MATERIALS AND METHODS

DNA sampling

DNA's were obtained from soil sampling in the central south of Mexico in the Tehuacán-Cuicatlán Reserve (Torres-Cortés et al 2012). PCR amplification of the V4, V5 variable regions of the 16S rRNA gene using Universal primers U519F and U926R were performed as showed in Figure 2.

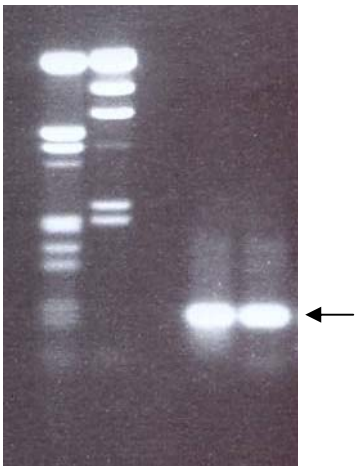


Figure 2. DNA electrophoresis of 16SrDNA Gene Amplification using U519F and U926R primers (arrow) used for 454 FLX Titanium sequencing.

These amplifications were subjected to massive sequencing using 454 FLX sequencer obtaining 28000 sequences which were assigned to phyla by the Ribosomal Data base (RDP-II classifier), using an 80% confidence threshold.

We chose 53 of these reads previously assigned to bacteria of the Family β-oxalabacteraceae for our Analysis.

Computer Program

Clone Manager Professional Suite version 6.0 for sequences treatments was the bioinformatic program used to familiarize with DNA sequences. This program allows to get based in any DNA sequence, its reverse, search immediately any DNA sequence within; with the Compare tool you can search any similarity between two sequences. The alignment tool establishes a distance of similarity between any groups of sequences.

RESULTS AND DISCUSSION

Once familiarized with the clone-manager program we chose 53 of these reads previously assigned to bacteria of the Family β-oxalabacteraceae for further analyses. The most related sequences described in databases related to this group were bacteria of the genera *Massilia*.

A phylogenetic tree was constructed showing the formation of eight monophyletic traits (clusters) within these 53 sequences (Fig. 3). The detail analyses of the alignment of the different clusters allow distinguishing single nucleotide changes some of them present in more than one read of the cluster.

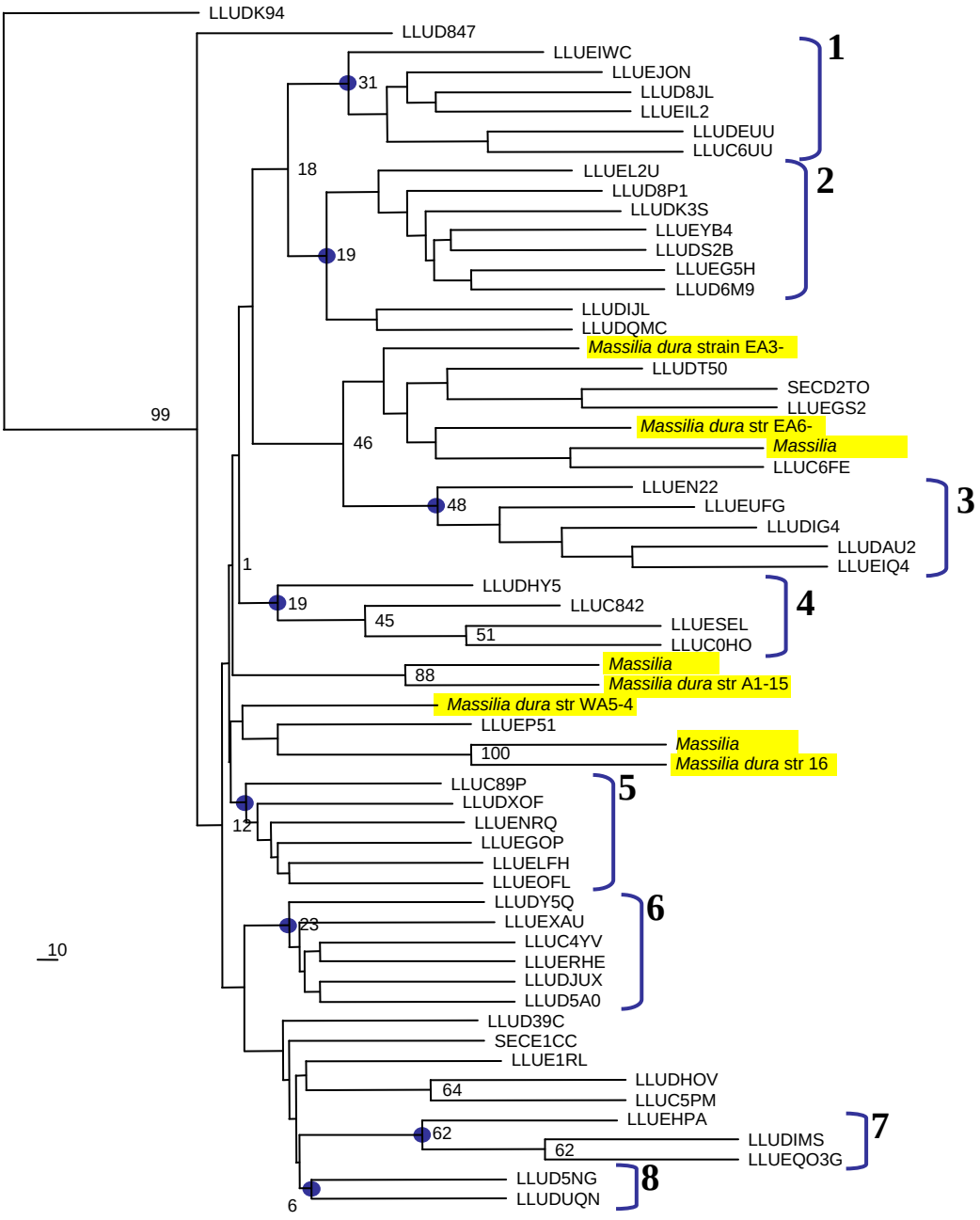


Figure 3: Topology of a consensus phylogenetic tree of 16SrRNA gene reads of *Massilia. Massilia* genera, species, or alternative sequences identified in databases are indicated as yellow boxes (Pictures). The 53 *Massilia* reads form clusters (1-8). Scale bar=0.1 changes/site.

Two of these single substitutions have an interesting characteristic. They are localized in one of the domains of the rRNA structure, the domain P21+1 described in the rRNA ribosomal of *Escherichia coli* (Figure 4).

The folding of the RNA molecule containing this domain reveal that this particular substitutions were compensated in one of the clades formed. The clade 3 contains a change of A-U but all of the rest of the clades contain an interaction G-C. These results reinforce the idea that the changes at nucleotide level and groups observed are not due to sequencing artifacts but reflect the biodiversity of our samples.

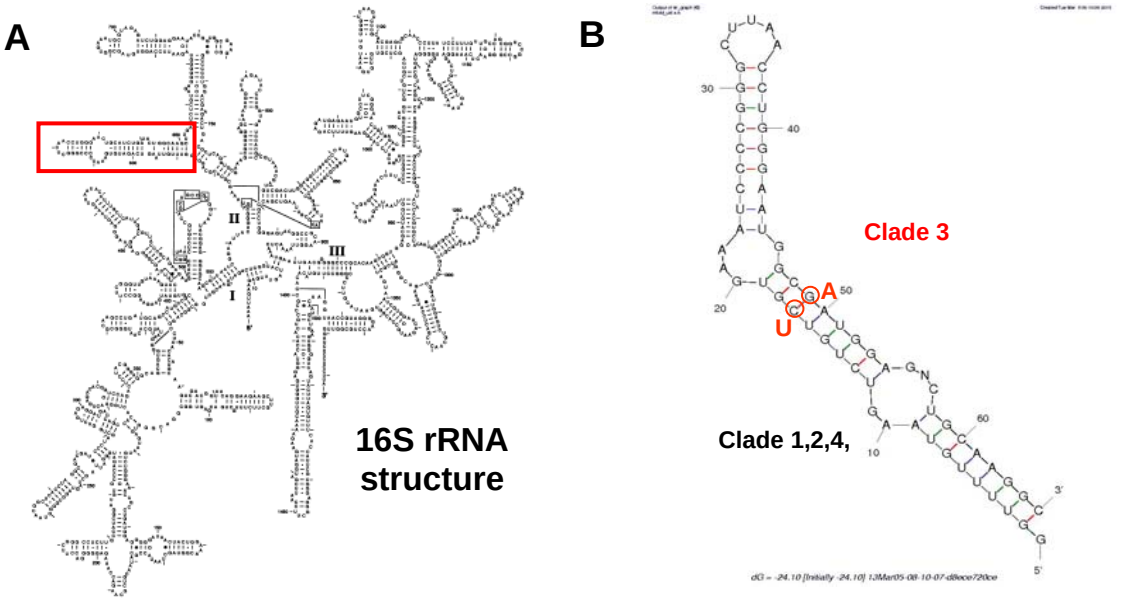


Figure 4. (A) 16S rRNA secondary structure. P21+1 domain is highlighted as a red box. (B) Single mutations as compensatory changes in the stem-loop 21+1 of the RNA structure present in the clade 3 (U-A change) and distinct form clades 1,2 and 4.

CONCLUSIONS

The comparison of 52 sequences of 16SrRNA belonging to *Massilia* bacteria group reveal 8 clades of similarity. Some of them are distantly related to Massilia described ribosomal RNA sequences. The analyses of these 8 groups reveal changes of nucleotides in specific positions. Two of these substitutions are localized as compensatory changes in the stem-loop 21+1 of the RNA structure. From these data we can infer the presence of novel group(s) of Massilia bacteria in our samples.

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 mainly by research project MICINN Consolider-Ingenio 2010. CSD2009-00006; Secondary Schools Institutes: Miguel de Cervantes, Zaidín-Vergeles, Padre Manjón y Madraza de Granada Capital and Virgen de la Caridad de Loja. Also we appreciate the coordination in the project of teachers Esperanza Vaquera-Heredia, Mª del Mar Martín Molina, Ana León, Mª

Concepción Martínez, Javier Cáceres and particularly to Antonio Quesada Ramos for his help in developing our blog: http://eezbioinformatica.blogspot.com.es/.

REFERENCES

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M. (2009) The Ribosomal Database Project: improved alignments and newtools for rRNA analysis. Nucleic Acids Res. 37, D141–D145.

Rodicio, M.R., Mendoza, M.C. (2004). Identificación bacteriana mediante secuenciación del ARNr 16S: fundamento, metodología y aplicaciones en microbiología clínica. Enferm Infecc Microbiol Clin 22: 238-245.

Roselló-Mora, R. (2005). El concepto de especie en procariotas. Ecosistemas 12 : 11-16.

Torres-Cortes, Millán, V., Fernández-González, A.J., Aguirre-Garrido, J.F., Ramírez-Saad, H.C., Fernández-López, M., Toro, N., Martínez-Abarca, F.(2012). Bacterial community in the rhizosphere of the cactus species *Mammillaria carnea* during dry and rainy seasons assessed by deep sequencing. Plant and Soil. 357: 275- 288.

MY OWN IDEAS

Admina Zvonaru, IES La Madraza, Granada

Microbiology is the science for studying microorganisms. It's dedicated to study organisms that are visible thanks to the microscope (prokaryotic and eukaryotic organisms). This discipline is very important in many areas, highlighting in medicine, because it help us to understand the pathology they cause in the body, since more diseases are related to fungi, parasites, …; in the soil because there are microorganisms that contribute to the wealth and fertility of the soil, in feeding because we study the microorganisms that are in the food, to know if they are pathogenic or not. I can say that Microbiology is very important for all of us.
When I chose this project, I wasn't much guided. I didn't know what I would do in it. This project allowed me the opportunity to see where does a microbiologist works, how he/she works, the tools that uses and discover more about Science's world. Every time I hear the word DNA, I think in all the living beings that exist, either visible or not. I've always been fascinated with researches dealing with DNA. I always wanted to know more about it. In my opinion, the study with DNA analysis it's very useful and it help us to identify if there's any genetic change that has been made in the DNA of that living being, to discover if any living being it's a new species.
To build a new hypothesis it's not an easy task, because when it's being constructed you might have new questions and then you have to find an answer for these novel questions and your hypothesis may change. Also it is possible your hypothesis was wrong. Communicate the results of a scientific work it's important because it can change the science, upgrade it, to have more knowledge about it and be able to resolve problems. In my opinion, any scientist must be organized, hard-working and ambitious person. He/She should want to discover and know more and more about Science. Also I think that it should be a calm person, who loves and enjoys its work and above all it should be a person who does not give up very easily.
Sincerely, I'm very happy to have chosen this project, because it made me know and go in depth in Science, it made me want to be involved in Science, but I have not make a decision for what part of Science I want to go in depth yet.

La Microbiología es la ciencia encargada de estudiar los microorganismos, más bien conocidos como microbios. Se dedica a estudiar los organismos que son visibles gracias al microscopio(organismos procariotas y eucariotas).Esta ciencia es muy importante en muchos lugares, destacando en la medicina, porque nos ayuda a entender la patología que causan en el organismo, ya que la mayoría de las enfermedades están relacionadas con bacterias, hongos, parásitos….,en el suelo porque existen microorganismos que contribuyen a la riqueza y fertilidad del suelo, en la alimentación porque se estudian los microorganismos que hay en los alimentos para ver si puede ser patógeno o no y para muchísimas mas cosas.
Con esto puedo decir que la microbiología es muy importante para todos nosotros. Cuando elegí este proyecto, no estaba muy orientada, no sabia exactamente que es lo que haría en el. Al principio, me pareció difícil, porque habían cosas que yo desconocía,

pero aun así no me rendí. Este proyecto me ha dado la oportunidad de poder ver en donde trabaja un microbiólogo, como trabaja, las herramientas que usa y descubrir más sobre el mundo de la Ciencia.
Cada vez que oigo la palabra ADN, pienso en todos los seres vivos que existen, tanto visibles como no. Siempre me han fascinado las investigaciones sobre y con el ADN, siempre quise saber más sobre el tema. En mi opinión, el estudio mediante análisis del ADN es muy útil y nos ayuda mucho para identificar si hay algún cambio genético que se haya hecho en el ADN de ese ser vivo, para descubrir si el ser vivo es una nueva especie y para mas cosas.
El construir una hipótesis no es fácil, porque al estar construyéndola te pueden surgir preguntas y entonces tienes que buscar una solución a esas preguntas y puede que tu hipótesis cambie. También esta el hecho de que no sea correcta.
Comunicar los resultados de un trabajo científico es importante porque puede cambiar la Ciencia, mejorarla, tener más conocimientos sobre ello y poder resolver problemas.
En mi opinión, un científico debería ser una persona ordenada ,trabajadora, que sea ambiciosa y que quiera descubrir y saber mas y mas de la Ciencia, también pienso que deber ser una persona tranquila ,que le guste su trabajo y lo disfrute ,y sobretodo que sea un persona que no se rinda fácilmente.
Sinceramente, estoy muy satisfecha de haber elegido este proyecto ,porque me ha hecho saber y profundizar mas en la Ciencia, me ha hecho querer estar involucrada en la Ciencia, aunque no quita el hecho de estar indecisa sobre que parte de la Ciencia quiero profundizar.

Linn Elrajeh AL Royolah, IES Miguel de Cervantes, Granada

This year I entered the project PIIISA thank my physics teacher, it was she who informed us all. And we had to choose three projects; one of them was the CSIC microbiology, in which we enter. At first, everything was strange and we did not understand anything, but throughout the year we were going understanding and experiencing things. We met new people, and our coordinator, Francisco Martinez-Abarca. Our coordinator is very friendly, and whenever he was always for all kinds of doubts and he explained things. In this project we learned things in life, DNA sequences, the size of the ribosome, phylogenetic trees, sequence comparison using the program we are using “clone manager'', and finally we might have discovered a new species of bacteria with the bioinformatic results we did in computers, and in Congress that will we show that there may be a new species, only lack experience and deeper. Everything we have experienced was based on the thesis of Gloria and soil samples of Mexico. They were very curious things that anyone does not know. And I am very proud to complete this project with clear ideas and thanks to our coordinator Paco, to me it makes me sad end. This project promises, and the Congress will do great.

I thank my coordinator Paco, my teacher, and the people who agreed to enter into this project PIIISA

Cristina Megías Melero, IES Zaidín-Vergeles, Granada

Microbiology is a part of science very important and extensive, because the smallest microorganisms take importance in the course of the life. This science involves know and discovery how are the organulles and molecules that are present in our environment.

In our project we worked with the DNA and bacterias, but aren't necessary being in a laboratory and use the microscope for work about bacterias and know that bacteria are what we are seeing. For this we only need a sequencing of DNA and introduce them on a database.

When I started at this project I really didn't think that we can obtain these results and now I think something completely different. While you are working, you may find some things that you didn't expect, but these small changes sometimes can make you have something better than you expected. And the experimental work consists in them, search, study, explore and discover something that you can do confirm. But you haven't done anything if you don't publish your results. Publish your result are very important, because then anybody can read this scientific work and help you with your work, this is something elemental in a good scientific work.

I think that a scientist must be a person very organized, patient and hard-working. Also he have a good relation with his teams members and take initiative for start new projects and know that a investigation need time and effort to discover something, communication between the members and be clear about you purpose.

In this project I have learned that the science is something complicated but hard work you can get really interesting things. Not everything is as we suppose, and the microbiology can be fun. Finally, I have to say that in this project I learned many things and that is an experience I will never forget and I take many things.

La Microbiología es una parte muy importante de la ciencia y extensa, debido a que los microorganismos más pequeños tienen importancia en el curso de la vida. Esta ciencia implica conocer y descubrir cómo son los orgánulos y moléculas que están presentes en nuestro entorno. En nuestro proyecto hemos trabajado con el ADN y bacterias, pero no es necesario estar en un laboratorio y utilizar el microscopio para trabajar sobre bacterias y saber que bacterias son las que estamos viendo. Para ello sólo necesitamos una secuenciación de ADN e introducirlas en una base de datos.

Cuando comencé a trabajar en este proyecto, realmente no pensaba que podíamos obtener estos resultados y ahora creo algo completamente diferente. Mientras que estas trabajando, puedes encontrarte algunas cosas que no te esperabas, pero estos pequeños cambios a veces pueden hacer que tengas algo mejor de lo que esperabas. Y el trabajo experimental consiste en ello, buscar, estudiar, explorar y descubrir algo que puedas confirmar.

Pero no has hecho nada si no publicas tus resultados. Publicar el resultado es muy importante, porque entonces todo el mundo puede leer este trabajo científico y puedes ayudarle con su trabajo, esto es algo elemental en un buen trabajo científico.

Creo que un científico tiene que ser una persona muy organizada, paciente y trabajadora. También él tiene una buena relación con los miembros de sus equipo y tomar la iniciativa para iniciar nuevos proyectos y saber que una investigación necesita

tiempo y esfuerzo para descubrir algo, la comunicación entre los miembros y tener claro que el propósito.

En este proyecto he aprendido que la ciencia es algo complicado, perol trabajando que puedes conseguir cosas realmente interesantes. No todo es lo que suponemos, y la microbiología puede ser divertida. Por último, tengo que decir que en este proyecto he aprendido muchas cosas y es una experiencia que nunca olvidaré y me llevo muchas cosas.

Jaro Rensch, IES Miguel de Cervantes, Granada

Bioinformatics is a scientific field where biology, informatic and technology fuse revealing a new and independent discipline. There are three main branches in bioinformatics:
a) The development of new algorithms and statistics for establish connections between members of big data groups.
b) The analyses and interpretation from some types of data including nucleotide and aminoacid sequences, protein dominations and structures.
c) The development and the implementation of tools for allow and use the different types of information.

In this project we went into the second field. Our mission was basically comparing RNA samples, in particular from the 16S rRNA, from possible new species of *Massilia*, with the data from the Database. Probably you may say it's easy to stay opposite a computer, and I can't blame you for it. I thought alike until I began to explore this world nearly. Perhaps at the start we had thousands of DNA sequences, we finally reduce them to eight groups, and we analyzed nearly 52 sequences, which we compared with the existing data about bacterial species, which could gave us a clue about that what we were searching.
It wasn't an easy work, maybe it seems boring, but to do our job we must learn about computer programs, and a lot about microbiology, you must be interested in your work and about that what around us.
However the most difficult in investigation isn't confirm or study your theories. The most difficult is to convince those who listen to you, what you say is real and true. You must show your ideas and your hypotheses without pictures or photos; with a theory which everybody can understand.
After we were sure about our own ideas and discoveries about new specie of *Massilia,* based our hypotheses mainly in two changes which where in a DNA sequence, and which couldn't be chance, we could say we were right about our hypothesis.
Now our mission is to transmit next investigation and scientists our conclusions. This part is very important in bioinformatics and, main in investigation. Show your conclusions in order to give them future projects, which can use them.

La Bioinformática es el campo de la ciencia en la cual la biología, la informática y la tecnología se fusionan en una sola disciplina. Dentro de la bioinformática hay tres ramas principales:

a) El desarrollo de nuevos algoritmos y estadísticas para establecer relaciones entre miembros de grandes grupos de datos.

b) El análisis y al interpretación de varios tipos de datos incluyendo secuencias de nucleótidos y aminoácidos, dominios proteicos y estructuras de proteínas.

c) El desarrollo y la implementación de herramientas que permitan acceso y manejo eficientes de diferentes tipos de información.

En este proyecto nos hemos adentrado en el segundo campo. Nuestra misión ha consistido básicamente en comparar muestras de ARN, más concretamente de 16SrRNA tomadas de una posible nueva especie de Massilia, con los datos existentes en las bases de datos. Cualquiera diría que es un trabajo fácil, delante de un ordenador, y no puedo culparte por ello. Yo pensé igual hasta que me sumergí en el mundo de la bioinformática.

Aunque inicialmente había varios miles de secuencias de ADN, las conseguimos reducir a ocho grupos, y finalmente analizamos unas 52 secuencias, que comparamos con datos existentes de especies bacterianas, que podrían darnos una pista sobre lo que buscábamos. Aun así no fue tarea fácil, pues por aburrido que pueda sonar hay que saber manejar determinados programas, y sobre todo hay que tener ganas de saber más sobre lo que nos rodea, aunque no podamos verlo directamente.

Sin embargo lo más difícil de la investigación, no es comprobar, estudiar o afirmar tus hipótesis, sino poder convencer a quien te oye de que lo que dices es cierto y de que además puedes demostrarlo, no con un dibujo, ni una foto de microscopio, sino con una hipótesis que pueda explicar lo que afirmas, de forma que todos puedan entenderte.

Después de convencernos de que habíamos encontrado una nueva especie de Massilia, basándonos principalmente en dos cambios que se daban en una secuencia del ADN, y que no podían ser casuales, hemos establecido que lo que decíamos, era cierto. Ahora nuestra misión es transmitir esto a futuras investigaciones. Esto es una importante parte de la bioinformática y en general de la investigación, publicar las conclusiones para que otros proyectos puedan aprovecharlas.

José Luis Guzmán Martín, IES Padre Manjón, Granada

I love participate in this project, I always have fascinate the world of the microbiology, the virus, bacteria, the DNA… and I had never thought that we can discover new species due to the RNA. This task has been one of the things that fascinate me more of this project.

I have to admit that at first I was disillusioned because I won't use a microscopy, but sooner I forgot it when I learnt a new way to discover novel bacteria species. I do not think that in other projects that I request I could learn so much as in this project. In addition we have luck of have an investigator that is at the same time patient, likeable and enthusiastic.

I have learnt too all the process that we have to do to get the samples with we work and in a short visit, I have the opportunity to see an electronic and a confocal microscopy.

In conclusion this has been a big experience and it has reaffirmed me my opinion of want to dedicate me to the investigation.

Me ha encantado estar en este proyecto, siempre me ha fascinado el mundo de la microbiología, los virus, las bacterias, el ADN… y jamás hubiese pensado que se pudiesen descubrir nuevas bacterias gracias al ARN, esto ha sido una de las cosas que más me han fascinado de este proyecto.

He de admitir que al principio estaba desilusionado por no utilizar un microscopio, pero pronto me olvidé de ello cuando aprendí una nueva forma de poder descubrir nuevas especies de bacterias. No creo que en los otros proyectos que solicité hubiese podido aprender tanto como con este. Además hemos tenido la suerte de tener un investigador que es a la vez simpático, paciente, y entusiasta.

También he aprendido todo el proceso que hemos de hacer para conseguir las muestras con las que trabajábamos y he tenido la oportunidad de ver el funcionamiento de un microscopio confocal y otro electrónico

En conclusión esta ha sido una gran experiencia y me ha reafirmado mi opinión de querer dedicarme a la investigación.

Maria Nogales Gámiz, IES Virgen de la Caridad, Loja

Microbiology is the science to study microorganisms, small live forms visible under microscope. Include fungi, protists and prokaryotes. However, traditionally has been involved mainly in pathogens, bacteria, fungi and viruses.

It has been a smart project. I have feel comfortable, I have learned and it has been a nice experience and a motivation for studying sciences. I could not participate in all programmed sessions due to our institute was in Loja, but I have followed all the discussions with the help of my teacher.

DNA is a nucleic acid which encodes the genetic instructions for the development of all forms of life and responsible of the transmission of the hereditary characters. It maintains the information for building all the components of the cell, proteins, RNA. The segments encoding such information are named as genes. The analysis of DNA allows comparing microorganisms and reinforcing ideas in which we can have got doubts.

Working in Research and in experiments generate a lot of discoveries for improve our life conditions and to save lives. To construct any hypothesis, first you have to put your attention in any non common process, then ask your self a lot of questions you can answer in order to solve and reinforce such hypothesis. It is important communicate the results of a scientific work because other researcher must confirm our results or to detect any non-reproducible experiment. It gives more consistency to the result of the experiments.

A good researcher must have interest in his work. He must do a hard work, good concentration, avoid any fails and put attention in all he does. He must be a good student, demonstrate competence and scientific skills. Additionally he must do the research in a good centre; good machines, High quality to perform their research accordingly with the best results.

La microbiología es la ciencia encargada del estudio de los microorganismos, seres vivos pequeños también conocidos como microbios. Se dedica a estudiar los organismos que son sólo visibles a través del microscopio: organismos procariotas y

eucariotas simples. Son considerados microbios todos los seres vivos microscópicos, estos pueden estar constituidos por una sola célula (unicelulares), así como pequeños agregados celulares formados por células equivalentes (sin diferenciación celular); estos pueden ser eucariotas (células con núcleo) tales como hongos y protistas, procariotas (células sin núcleo definido) como las bacterias. Sin embargo la microbiología tradicional se ha ocupado especialmente de los microorganismos patógenos entre bacterias, virus y hongos, dejando a otros microorganismos en manos de la parasitología y otras categorías de la biología.

Para mí ha sido un buen proyecto. Me lo he pasado bien, he aprendido y me he llevado una buena experiencia y una motivación para estudiar ciencias. Yo no he podido asistir a todos las sesiones que se han programado porque soy de Loja, pero igualmente lo he seguido y con la ayuda de mi profesora he conseguido que me vaya muy bien en este proyecto.

El ADN, es un ácido nucleico que contiene instrucciones genéticas usadas en el desarrollo y funcionamiento de todos los organismos vivos conocidos y algunos virus, y es responsable de su transmisión hereditaria. El papel principal de la molécula de ADN es el almacenamiento a largo plazo de información. Muchas veces, el ADN es comparado con un plano o una receta, o un código, ya que contiene las instrucciones necesarias para construir otros componentes de las células, como las proteínas y las moléculas de ARN. Los segmentos de ADN que llevan esta información genética son llamados genes, pero las otras secuencias de ADN tienen propósitos estructurales o toman parte en la regulación del uso de esta información genética. Por esto es muy importante analizar el ADN, para comparar y poder afianzar ideas en las que podamos tener dudas.

Trabajar investigando y experimentando es muy importante para la vida de todos los seres vivos. Gracias a muchos descubrimientos se han podido salvar vidas y se puede vivir en mejores condiciones de vida.

Para construir una hipótesis primero tienes que encontrar algo que sea extraño y en ese momento hacer miles de preguntas, las cuales estés interesadas en responder. Cuando sepas las preguntas que tienes diseñas como podrías resolverlas.

Es importante comunicar los resultados de una trabajo científico ya que así otras científicos pueden comprobar si todo es cierto y no ha habido ningún fallo en el experimento. Así da más consistencia al resultado de los experimentos.

Todo buen científico debe tener muchas ganas e interés en lo que quiere trabajar, debe trabajar duro con mucha concentración no cometer ni un fallo, poner atención en lo que hace. Debe tener buenos estudios, para demostrar que es competente y vale para se científico. Trabajar en un centro donde haya muchas maquinarias, de buena calidad, para trabajar mejor y obtener muy buenos resultados.