HIGH SCHOOL STUDENTS FOR AGRICULTURAL SCIENCE RESEARCH

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In this volume:

➪ **Proceedings of the 3rd Congress PIIISA - Estación Experimental del Zaidín**

➪ Winning article of the II EEZ Science Award (2013)

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High School Students for Agricultural Science Research

Volume III May, 2014

Scientific Editors

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FOREWORD / PREFACIO

Hardly could those around us admit that young people from our High Schools would be able to do top level scientific research. This book demonstrates that, in fact, it is possible. Inside, there are ten papers that summarize the work developed by ten scientific teams with professional researchers and secondary school students at the Estación Experimental del Zaidín (EEZ-CSIC).

Once again, our students have had the opportunity of learning about the latest techniques in molecular biology like DNA and protein extraction, DNA amplification using PCR, electrophoresis, gene sequencing, and knowing the most advanced instruments to watch the inner structures of life, as optical and electron microscopes. This is a challenge in which young people can go ahead of their own teachers at their high schools.

But PIIISA projects have other advantages as well. These pages include the Proceedings of the III PIIISA-EEZ Congress. Then, our students will have carried out one of the most important features of science: to expose their results to everybody. They are going to learn that science is something meaningless if it cannot be transmited to the society. And moreover, this volume will become their first published paper thanks to the EEZ.

And for all of these reasons we, as their teachers, have to thank the EEZ researchers for the important work performed with our students. Because they have been able to accept the challenge of becoming teachers of teenagers, of teaching complex concepts and techniques. And the most important fact: they have succeeded.

And in relation to our educative system we can discover new perspectives. At least, in its compulsory levels, it offers a basic formation that lets our students get on in our modern world. But we usually forget that in our hands we also have the scientists of the future, young people who, thanks to projects like PIIISA, are at the forefront and who are going to have a clear view of scientific research much greater that youngsters with the same age. We don't know if they will study in the future a scientific degree related to the research they have carried out now, but they know that after these experiences, their vision of science will be different, the won't see science with the same eyes as months ago when they came into the EEZ.

But we should not stop here; PIIISA spirit must spread to the rest of the society. PIIISA must be a way that leads to a change in the perception of science in society, a way that definitely launches our country to Science. And this way begins just now, with our students, with researchers, with us, their teachers, with their partners in the classroom, and even though, their families. Without science there is no progress; without progress there is no future. PIIISA shows that all of these objectives are possible. These pages demonstrate it.

Difícilmente aquellos que nos rodean llegarían a pensar que jóvenes de nuestros institutos pudieran ser capaces de llevar a cabo una investigación científica de alto nivel. Este libro demuestra, que de hecho, sí que es posible. Contiene diez artículos científicos que resumen el trabajo llevado a cabo por diez equipos constituidos por investigadores profesionales y por estudiantes de secundaria y bachillerato de institutos granadinos en la Estación Experimental del Zaidín (EEZ-CSIC).

Una vez más, nuestros estudiantes han tenido la oportunidad de aprender las últimas técnicas en biología molecular, entre ellas, la extracción de ADN y proteínas, la amplificación de ADN mediante la reacción en cadena de la polimerasa, la secuenciación de genes, o de conocer aquellos otros instrumentos que permiten ver las estructuras más íntimas de la materia viva, como los microscopios ópticos y electrónicos. Es este un reto en el que los jóvenes llegar a ir incluso por delante de nosotros, sus profesores de los centros educativos.

Pero PIIISA muestra además otras ventajas. Estas páginas son las Actas del III Congreso PIIISA-EEZ. En ese momento, nuestros estudiantes también habrán llevado a cabo otra de las

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funciones más importantes de la ciencia, la de exponer los resultados obtenidos. Ellos también aprenderán que la ciencia es algo sin sentido sino se transmite al resto de la sociedad. Y más aún, este volumen contendrá su primer artículo publicado, también gracias a la EEZ.

Es por todo ello por lo que, como profesores de estos jóvenes, hemos de reconocer a los investigadores de la Estación Experimental del Zaidín el importante trabajo llevado a cabo con nuestros estudiantes, ya que han sido capaces de aceptar el desafío de convertirse en profesores de adolescentes, de transmitirles técnicas y conceptos complejos. Y lo más importante: han tenido éxito.

Y también podemos ver nuevas perspectivas en relación a nuestro sistema educativo. Al menos, en su enseñanza obligatoria, ofrece una formación básica que permite a nuestros alumnado enfrentarse al mundo actual. Pero normalmente olvidamos que también tenemos en nuestras manos a los científicos del futuro, gente joven que, gracias a proyectos como PIIISA, están en la vanguardia y van a tener una visión mucho más clara que otros jóvenes de su edad. Desconocemos si en un futuro ellos estudiarán una carrera relacionada con la investigación que ahora han desarrollado, pero ellos son conscientes de que tras estas experiencias su visión de la ciencia será diferente; no verán la ciencia con los mismos ojos que hace unos meses cuando llegaron a la EEZ.

Pero no debemos detenernos aquí. El espíritu PIIISA debe extenderse al resto de la sociedad. PIIISA debe ser un camino que conduzca a un cambio en la percepción de la ciencia en la sociedad, una vía que definitivamente lance a nuestro país hacia la Ciencia. Y este recorrido comienza justo ahora, con nuestros estudiantes, con los investigadores, con nosotros, sus profesores, con sus compañeros de clase y, por supuesto, con sus familias. Sin ciencia no hay progreso; sin progreso no hay futuro. Y PIIISA demuestra que todos estos objetivos son posibles. Estas páginas lo demuestran.

Antonio Quesada Ramos

Coordinador PIIISA del IES Zaidín-Vergeles

As a secondary school teacher, every single day holds a new challenge. Now, it is time for this book to be introduced.

This Fourth Edition of the PIIISA 2014 Program on Agricultural Science Research summarizes the scientific stories of students' endeavor to do something special with a great enthusiastic encouragement.

At this point, I would like to mention a quote from Jean Paul Sartre who stated that "We are our choices". And our scientific choice begun last 5th of February, after some secondary students were assigned to several scientific investigation groups at the Estación Experimental del Zaidín. I have been fortunate over the past months to have worked with motivated secondary students and researchers, leading these last ones, important investigation groups on different agricultural fields.

Science teachers everywhere agree: teaching science, no matter the level, is hard work and it is also well known that in this process collecting, analyzing data and drawing conclusions are involved. But scientific knowledge is, at least partially, based on and/or derived from human imagination and creativity. All these essential ingredients have come about throughout the PIIISA Program, allowing the students could enhance their learning process on Science.

For all the above, I consider that engaging students in scientific investigations in a very fruitful context, like the Estación Experimental del Zaidín has promoted, is a perennial goal on Science education.

We hope to continue spreading the magic of Science in this unbroken chain.

So, let this page turner continue…

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Siendo una profesora de secundaria, cada día surge un nuevo reto. De ahí, que sea mi tarea hoy introducir este libro.

Esta Cuarta Edición del Programa PIIISA 2014 en investigación agrícola recoge las historias científicas de alumnado de secundaria empeñado en realizar algo especial con gran entusiasmo y aliento.

Llegado este punto, me gustaría mencionar una cita de Jean Paul Sartre que dice "Somos nuestras elecciones". De esta manera, "nuestra elección científica" comenzó el pasado 5 de febrero, después de la asignación de estudiantes a disitintos grupos de investigación científica en la EEZ. He sido afortunada en estos últimos meses por haber trabajado con estudiantes e investigadores motivados, encabezando estos últimos importantes grupos de investigación en diferentes campos.

Enseñar Ciencia es una tarea ardua a cualquier nivel y es bien conocido que en este proceso toman parte la recogida y análisis de datos, así como la discusión y redacción de unas conclusiones. Aunque el conocimiento científico, al menos en parte, está basado y/o deriva de la imaginación y creatividad. Todos estos ingredientes se han puesto de manifiesto a lo largo del Programa PIIISA, permitiendo que los estudiantes puedan potenciar su aprendizaje en Ciencia.

Por todo lo anteriormente expuesto, considero que animar al alumnado hacia la investigación científica en un fructífero contexto, como ha promovido la EEZ, es un objetivo perenne en la Educación sobre Ciencia.

Esperemos seguir esparciendo la magia de la Ciencia en esta ininterrumpida cadena.

Así que, dejemos que este libro apasionante continúe…

Carmen Plata
IES Montes Orientales

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Proceedings of the 3rd Congress PIIISA – EEZ

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Can we produce tomatoes with a pinch of salt?

(or how to reduce the negative impact of water and soil salinity on cultivated tomato)

Sofía Clemente[1], Ángela Maldonado[1], Patricia Vilchez[2], Encarnación Pérez[3], Ana López[4], Noelia Jaime-Pérez[5], Raquel Olías[5] & Andrés Belver[5*]

[1]*CDP Sagrado Corazón, Paseo de los Basilios nº 3 18008 Granada, Spain*
[2]*IES Montes Orientales, Carretera de la Sierra, 15, 18550 Iznalloz, Granada, Spain*
[3]*CDP Virgen de Gracia, Músico Vicente Zarzo, 18002 Granada, Spain*
[4]*IES ACCI, Buenos Aires 68, 18500 Guadix, Granada, Spain*
[5]*Departmento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*
[*]*Corresponding author:* ***e-mail:*** *andres.belver@eez.csic.es*

SUMMARY

Excessive soil salinity causes abiotic stress and consequently diminishes crop yields. In this study of cultivated tomato, we analyzed one of the mechanisms of salt tolerance operating in other plant models such as Arabidopsis and rice. This mechanism is based on the activity of Na^+ transporters belonging to subfamily HKT1, which determine genetic traits associated with salt tolerance in these plant models. These transporters drive Na^+ through the plant to tissues and subcellular compartments where it is not toxic and/or help its expulsion from the plant. In our lab, two tomato genes encode HKT1-like transporters have been identified. To test the importance of these transporters in the mechanism of salt tolerance in tomato, we have worked with two tomato near-isogenic lines (NIL) differing in terms of the HKT gene allele they contain (from *Solanum lycopersicum* and *S. cheesmaniae*), and different transgenic lines derived from these NILs in which each one of the HKT1;1/HKT1;2 allelic variants has been previously silenced by stable gene transformation. In this study, we provide the preliminary phenotype characterization for each genotype in order to determine which of these genes/alleles plays the most significant role in tomato salt tolerance measured as tissue growth in plants cultured in different media (Petri plates, pots and hydroponics). Results obtained may be the basis for future research in order to improve the tolerance of plant crops to salinity in water and soils.

INTRODUCTION (AND OBJECTIVES)

On a world scale, no toxic substance restricts plant growth more than salt. More than 800 million hectares of land throughout the world are salt affected [1]. In Spain soil salinity is a very serious problem for a great variety of crops, including tomato. High salt concentrations in the root growth media impose both an ionic and an osmotic stress to most plants [2]. The major ionic stress associated with high salinity is due to sodium (Na^+) toxicity. Under salinity conditions, Na^+ is taken up by roots, transported to shoots in the transpiration stream and accumulated in plant cells over time [2,3]. Because of its toxicity, Na^+ accumulation in the cytosol of plant cells results in progressive damage affecting negatively many physiological processes within the plant [2]. In addition, a high external Na^+ concentration also prevents the uptake K^+, leading to insufficient cellular K^+ amount for enzymatic reactions and osmotic adjustment [2,3]. Therefore the regulation of intracellular concentration of Na^+ and K^+ (homeostasis) in plant cells and tissues is a key mechanism in saline stress tolerance [3]. All these involves a network of processes regulating uptake, extrusion through the plasma

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membrane, compartmentation of salts into cell vacuoles and recirculation of ions through the plant organs, thus allowing the osmotic adjustment and maintenance of high K^+/Na^+ ratios in the cytosol of plants grown under salt stress. In order to control Na^+ (and K^+) homeostasis, plants have different Na^+ transporters to protect the plant against damage due to Na^+ accumulation: antiporters in the root that extrude Na^+ back to the soil in a mechanism coupled to H^+ transport (involving the SOS pathway) [4-7]; transporters that retrieve Na^+ from the transpiration stream avoiding the over-accumulation of Na^+ in the photosynthetic tissues (involving HKT transporters) [8-10]; and antiporters that sequester Na^+ in the vacuoles (involving NHX1 antiporters), along the electrochemical gradient generated by the H^+-ATPase and the H^+-PPase [2,11].

HKT1-like transporters are one of the most studied Na^+ permeable transporters which play an important role in Na^+ and K^+ homeostasis [9]. These Na^+ transporters, located at plasma membrane of parenchyma cells surrounding the xylem vessels, are responsible for unloading Na^+ from the xylem, thus preventing Na^+ accumulation in aerial parts and indirectly improving K^+ homeostasis in many plant species [8-10]. Recent studies have shown their crucial importance in salinity tolerance in both mono- and dicotyledonous species [10,12,13]. This makes HKT transporters a preferential target for the engineering of plant stress tolerance. However, salt tolerance in plants is a quantitative trait that could be determined by one or multiple genes. The identification of quantitative trait loci (QTLs) controlling this characteristic is of great importance in order to breed salt-tolerant crops [14, 15]. Using two tomato near-isogenic lines (NIL) differing in terms of the HKT gene allele they contain (from Solanum lycopersicum and S. cheesmaniae) showed that the connection between the allelic variants of tomato HKT1;1 and HKT1;2 and salt tolerance was unclear and mostly depended on salt tolerance criteria used [16]. Therefore, to test the above hypothesis and whether these transporters are important in the mechanism of salt tolerance in tomato we previously generated different transgenic lines derived from these NILs in which each one of the HKT1-like allelic variants has been previously silenced by stable gene transformation (Belver et al., unpublished results).

In this study, we provide the preliminary phenotype characterization for each genotype in order to determine which of these genes/alleles plays the most significant role in tomato salt tolerance measured as tissue growth in plants cultured in different media (Petri plates, pots and hydroponics).

MATERIALS AND METHODS

Plant material

We used two types of tomato seeds called NIL 14 and NIL 17 (Near-isogenic lines) only differing in terms of HKT allele. NIL 14 contains the HKT1-like alleles from the wild salt tolerant *S. cheesmaniae* and NIL 17 contains the *S. lycopersicum* alleles (supplied by Dr. MJose Asins, IVIA Valencia). The *S. cheesmaniae* allele makes it possible to store more sodium and less potassium in the aerial part of the plant when cultivated under saline conditions [15,16]. Given that RNAi silencing constructs are dominant traits, different silenced T1 lines of each HKT1-like gene were used (generated in collaboration with Dr Vicente Moreno's lab, IBMCP-CSIC-Univ. Politecnica de Valencia). As a control line, we used the non-silenced HKT Ti14 and Ti17 lines, which were also subjected to the whole gene transformation process.

Tomato plant growth conditions

Phenotypic evaluation of plants was performed using seedlings grown in solid medium in Petri plates, as well as plants grown in hydroponics and in pots. Seeds were sterilized by immersing

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them in an ethanol solution for two minutes in order to remove the gelatinous layer on the seed. Ethanol was eliminated and the seeds were washed using distilled water and were then immersed in a bleach solution for 20 minutes. The seeds were washed 4 times in sterile water and were left to soak all night at 25 °C. Finally, the seeds were stored at 4 °C for 24 hours. This last step was carried out in order to stimulate germination uniformly.
After sterilization, seeds were cultivated using 3 different techniques: Petri plates (non-transpiring conditions), pots and hydroponics (transpiring conditions).

Petri Plate culture: tomato seeds used were surface sterilized and germinated in Petri plates (10x10 cm) containing MS medium [17]. Cultivation was performed in an environmentally controlled chamber at 24°C/18 °C day/night and a 16-h light/8-h dark cycle with irradiation of 140 µmol m^{-2} s^{-1}. The seedlings were kept in these conditions for 5 days, after which it was transferred in sterile conditions in a laminar flow chamber to new plates containing MS medium supplemented with 150 mM NaCl for five additional days. We took pictures and we analyzed the fresh weight of aerial part and roots separately and determined dry weight.

Pot culture: With this technique we pretended growing plants in a similar way to normal conditions, using cocopeat, an inert substrate, as support for the plant. Apart from irrigation, in the greenhouse, we also controlled light conditions, temperature and humidity, so that experimental conditions could be reproducible for all experiments. These experimental conditions are normally very similar to the ones that are used normally in tomato culture before marketing. Sterilized seeds were grown individually and kept in a culture chamber at 25ºC in darkness and irrigated with water until the emergence of the cotyledons of the plant (5-7 days). Later, each plant where transferred to a pot with a cocopeat. They were cultivated in a greenhouse with a natural light irradiation supplemented with artificial light of 122 µmoles m^{-2} s^{-1}, with a relative photoperiod, temperature and humidity of 16/8 hours, 24 ^{o}C/18 ^{o}C and 40/55%, day/night, respectively. Watering was applied 2-3 times a week with a ¼ Hoagland solution [18]. When plants were in the vegetative stage of 6-leaves, we applied the saline treatment, consisting in irrigation containing 100mM of NaCl, during 15 days. We cultivated 6 pots per line with a plant per pot, three of them receiving the saline treatment and the others three only nutrient solution (control treatment). Growth analysis was monitored by photographic record, and the determination of the fresh and dry weight of the stem and leaves.

Hydroponics culture: This is a method where a liquid media with solution of mineral salts is used instead of soil. With this system is easier to control the different treatments we need to apply in our experiments and the root of the plant roots keeps clean and easy to sample. Sterilized tomato seed, were germinated in plastic boxes containing quartz sand sterile (inert support) for 5-7 days in darkness and at 24°C. Germinated seeds were cultivated in a growth chamber, with controlled temperature and humidity conditions. Seedlings were watered for one week with a 1/10 dilution of Hoaglands nutrients solution [18] and for another week with a ¼ dilution of the same nutrient solution. Four-leaves seedlings were transferred to 2,5-L pots (three plants for pots) and grown in a greenhouse under same conditions indicated for pots, in hydroponic system for 15 days in an aerated ¼ dilution of Hoagland solution, that was renovated every three days to avoid contamination. Ten days after hydroponic culture initiation, we applied the saline treatment, by adding 100 mM NaCl to the new ¼ dilution nutrient solution, the plants growing on it for 6 additional days. We used 2 pots with 3 plants per line), two of them receiving the saline treatment and the other ones only nutrient solution (control treatment). The growth analysis were carried out as for pot culture.

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Determination of tissue fresh and dry weight

We collected tissue samples from leaves, stems and roots of each plant after treatment. each sample was washed four consecutive times in deionized water to eliminate salt adhered to the surface of the tissues and we dried out with filter paper. Tissue samples were weighed in a balance to determinate the fresh weight. Each sample was oven dried at 70 °C for 48 hours between filter papers and weighed in a balance to obtain the dry weight.

Determination of Na^+ and K^+ content

We intended to determine the content of Na^+ and K^+ in the dry material but, unfortunately, we could not do it because of ICP-OE Service from EEZ was provisionally out of service.

RESULTS AND DISCUSSION

The results obtained in this preliminary assessment of the phenotype for HKT-silenced tomato lines were very interesting and will be confirmed in future experiments. These findings will be the basis for future research in order to improve the tolerance of plant crops to salinity in water and soils.

Data obtained in this project are not provided here as the research carried out is subject to the confidentiality rules imposed by the ongoing R&D National Project as well as a doctoral thesis. In addition, the materials used in this research are the result of collaborative work with two external laboratories, which are also subject to confidentiality rules and copyright. However, such research has proved useful to show students the overall objective of this PIIISA Project: "to show students interested in scientific research (or to make them interested in) what RESEARCH is and how it is carried out". Nevertheless, details of these results will be presented as an oral presentation and in poster form.

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MY OWN IDEAS

Sofía Clemente Olías, CDP Sagrado Corazón de Jesús, Granada
This Project has shown me how to work as a scientist such as thinking like one, organizing myself like one and working in a laboratory with a lot of scientific materials.
This may help my Project mates and me in our future careers in things like making decisions about what to study and even proposing new questions and problems to investigate. I have learnt plenty of techniques like how to prepare samples before the analysis of ion concentration.
If someone asked me if I would like to participate in this project again, I would say yes without hesitation.

Este proyecto nos ha enseñado a trabajar como un científico así como a pensar al igual que uno, organizarme como tal y a trabajar en un laboratorio con muchos materiales científicos.
Esta participación seguro que nos ayudará a todos en un futuro en cosas como qué queremos estudiar o incluso en plantearnos nuevos problemas y preguntas que desarrollar sobre este proyecto. He aprendido muchas técnicas como cómo preparar una muestra antes del análisis de la concentración de iones.
Si alguien me preguntase si volvería a participar en este proyecto, afirmaría sin pensármelo dos veces

Ángela Maldonado Ortega, CDP Sagrado Corazón de Jesús, Granada**.**
I have found the project PIIISA very positive because it has helped me to understand the research world and it has also given me theoretical and practical knowledge, which is the result of daily teamwork with the help of professional researchers.
I think that this experience will help me in the future, so thank you for the trust you have placed in me by allowing me to participate in this research project.

El proyecto PIIISA me ha parecido muy positivo porque me ha ayudado a conocer el mundo de la investigación además de aportarme conocimientos no sólo a nivel teórico, sino sobre todo

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práctico, ya que el resultado obtenido es fruto del trabajo en equipo realizado día a día gracias a la ayuda de nuestros investigadores.
Pienso que esta experiencia me va ayudar mucho en el futuro, por lo que agradezco la confianza que han puesto en mí para hacerme partícipe de este proyecto de investigación.

Patricia Vílchez Fernández, IES Montes Orientales, Iznalloz, Granada
In this project, we have had the opportunity to experiment for ourselves how scientific work is carried out and how to be a real researcher. During these months, with the help of professionals, we have learnt how to carry out an investigation.
We have had, for the first time, the chance to work in a laboratory, using the same resources and methods that researchers use normally. We also learnt to be patient and reliable to go on with the investigation successfully. This project has introduced us to the world of research, and enables us to clarify our future career expectations.
We now know how important research is for our lives and all the things it can give us to improve them.

Con este proyecto hemos tenido la oportunidad de experimentar por nosotros mismos como es el trabajo científico y ser como verdaderos investigadores. Durante estos meses, con la ayuda de profesionales, hemos aprendido cómo llevar a cabo una investigación.
Por primera vez hemos podido trabajar en un laboratorio, usando los mismos recursos y métodos que los investigadores utilizan normalmente. También aprendimos a ser pacientes y responsables para llevar a cabo una investigación con éxito. Este proyecto nos ha introducido en el mundo de la investigación, por lo que podemos ver más claramente nuestras expectativas para el futuro.
Ahora sabemos lo importante que es la investigación para nuestras vidas y todas las mejoras que nos aporta.

Encarnación Pérez Santiago, CDP Virgen de Gracia, Granada.
In this project, we have seen how researchers work in their laboratories and we have also had the opportunity to work with them. We have worked with some techniques we did not know such as growing plants in different mediums (pots, hydroponics and Petri dishes) as well as crushing plants in order to weigh them.
We also have learnt the importance of being patient and careful in a project and of not giving up if something goes wrong. And we have participated in writing the proceedings and in all the work involved.
So now, we have more information to think about our future careers.

En este proyecto hemos visto cómo trabajan los investigadores en sus laboratorios y también, hemos tenido la oportunidad de trabajar con ellos. Hemos trabajado con algunas técnicas que no conocíamos, moler las plantas para pesarlas y plantarlas en diferentes medios, como macetas o placa Petri.
También hemos aprendido la importancia de ser pacientes y cuidadosos en un proyecto y no rendirse si algo sale mal. Hemos llevado a cabo el proceeding y las tareas.
Así que ahora, tenemos más información para pensar en nuestro futuro.

Ana López López, IES ACCI, Guadix Granada.
In this project, we have seen how salt affects two types of tomatoes, with two different genes transporting sodium and we have cultivated this tomato plants in three different types of cultivation, for example, in pots, hydroponic systems and Petri plates. In my opinion, this project is a good idea because, apart from learning new concepts, it teaches us how to work in

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laboratories and how research is carried out by experts in this field. It was also interesting because I had never participated in any activity like this before and I have learned to use scientific tools.
I think that what I liked most was being able to participate in this project which I would not have had the opportunity to experience at my age anywhere else.

En este proyecto hemos podido ver como la sal afecta a las plantas, en este caso en dos tipos de tomate para dos genes que se encargan de transportar el sodio en las plantas y nosotros hemos cultivado estos tomates en diferentes tipos de cultivo, como por ejemplo, placas petri, macetas y en sistema hidropónico En mi opinión, este proyecto es una buena idea porque aparte de enseñarnos nuevos conceptos, nos enseña a trabajar en laboratorio y a saber cómo se realizan los proyectos de investigación. Este proyecto me ha parecido también interesante porque yo nunca había participado en alguna actividad como ésta y me ha proporcionado la oportunidad de trabajar con nuevas herramientas.
Lo que más me ha impresionado es poder realizar esta experiencia a mi edad ya que en ningún otro sitio cercano podre realizarla.

Thanks
We would like to thank all the research team made up of Noelia Jaime, Raquel Olías and Andrés Belver for all the help, time and support they have given us in order to make this project possible.

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Fructose signalling unbalancing and effect on Arabidopsis growth and the photosynthetic pigments content

Rafael Barnes Ontiveros[1*], Marina Fiñaga Cortés[2*], Francisco López Rodrigo[3*], Maria Elena Noguera Vilches[4*], Claudia Rodríguez Ibáñez[4*], Mariam Sahrawy Barragán[5] and Antonio Jesús Serrato Recio[5]

[1]*IES Francisco Ayala. Av Francisco Ayala, 18014 Granada, Spain.*
[2]*IES Federico García Lorca. San Ramón, 10 · 18194 Churriana de la Vega · Granada, Spain.*
[3]*IES Luís Bueno Crespo. Av de las Palmeras, s/n 18100 Armilla · Granada, Spain.*
[4]*CDP Sagrado Corazón. Paseo de los Basilios, 3 · 18008 Granada, Spain.*
[5] *Estación Esperimental del Zaidín. CSIC, Profesor Albareda 1, 18008 Granada, Spain*
[*]These authors contributed equally to this work
e-mail: *aserrato@eez.csic.es*

SUMMARY

Sugar biosynthesis, tightly regulated in plants, depends on photosynthesis as the only biochemical process able to fix atmospheric CO_2 in photoautotrophic organisms. In *Arabidopsis thaliana*, sucrose, glucose, and fructose are the most abundant soluble sugars. Sugar pools are fluctuating during the day/night cycle responding to plant requirements, as the growth stage or the response to diverse environmental stresses. For that reason, plants have developed signalling mechanisms to orchestrate the replenishment (synthesis) and mobilization (hydrolysis) of the different sugar pools. The disruption of these signalling pathways can provoke either resistance or sensitivity to sugars. In this work, we have characterized several *A. thaliana* lines earlier selected as putative fructose insensitive. Our results have showed that, though deficient for fructose sensing/signalling, these plants exhibit normal physiological parameters of root growth and photosynthetic pigment accumulation. Nevertheless, after performing growth assays in a high glucose medium, some of these Arabidopsis lines have also revealed to be glucose insensitive.

INTRODUCTION (AND OBJECTIVES)

As photoautotrophic organisms, plants generate their own sugars through the photosynthesis, one of the most important processes for the life. During the day, the energy (ATP) and reducing power (NADPH) supplied from photosynthetic electron transport is used by the Calvin-Benson cycle to convert atmospheric CO_2 and water to carbohydrates and oxygen, using sunlight as an energy source. The energy-rich sugar molecules are used by plants for their development and growth. To maintain the balance of metabolite and energy levels, organisms have developed sophisticated sensing and signalling mechanisms that underlie the physiological responses to cell metabolite fluctuations.

Since few years, sugar sensing and signalling has become important in its effects on plant growth and development [1]. Complex metabolic and hormonal signals are cross talking to integrate and transmit vital information for plant adaptation to the changing environment. For example, high glucose concentrations inhibit growth and the establishment of photosynthesis in seedlings. On the contrary, low sugar concentrations promote growth. Still, a great deal remains to be learned about the precise molecular mechanisms involved. In a previous screening carried out in our laboratory, several T-DNA insertion Arabidopsis lines have been

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selected as insensitive to high fructose concentrations (6%). In this work, we have characterized these Arabidopsis lines by analyzing some physiological parameters as the root-growth rate and the photosynthetic pigments content and tested whether, in addition to fructose, these lines are insensitive to other sugars as glucose.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana wild type (ecotype Columbia) and mutant plants were grown in soil in culture chambers under long-day conditions (16h light/ 8h darkness) at 22 ºC during the light and 20 ºC during darkness. *In vitro* plants were cultured on solid 0.5x Murashige and Skoog (MS) medium. The light intensity was 120 µmol m^{-2} s^{-1} .

Determination of photosynthetic pigments

After pigments extractions in methanol, the content of chlorophyll *a* (chla) and *b* (chlb), and carotenoids was spectrophotomerically quantified according to the method of Lichtenthaler and Wellburn (1983) [2].

RESULTS

Gene mutations leading to fructose insensitivity

Eight Arabidopsis mutant lines (homozygous T-DNA insertion lines from SALK laboratory) were selected as high-fructose concentration insensitive, according to a previous selection (unpublished data). An *in silico* search was developed to find out which genes were responsible for this resistance. The gene list is showed in Table 1. Genes coding for putative nuclear proteins (At3g10530, At5g48090, and At1g56240), putative oxidoreductases (At2g38080 and At1g15140), a putative heat shock protein (At3g62600), a putative stress protein (At2g47710), and a protein of unknown function seem to be involved in the resistance behavior. In line with our results, a nuclear protein (a transcription factor) has already been described as one important player in the fructose signalling pathway in *A. thaliana* [3]. As can be observed in figure 1, these Arabidopsis mutants show a wild-type phenotype at the seedling stage.

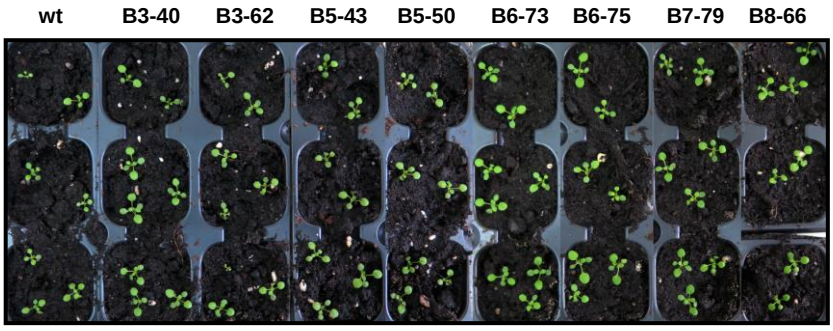

Figure 1. Phenotype of wild-type plants and *A. thaliana* mutant lines 14 days after germination (dag).

Determination of chlorophyll and carotenoid content

Photosynthesis homeostasis depends on a proper and balanced chlorophylls (chl) and carotenoids content. Moreover, deviations in the normal chl *a*/*b* ratio could suggest

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photosynthesis alterations [4]. In order to know whether these mutant lines have photosynthesis impairments, photosynthesis pigments were isolated from plants showed in figure 1 and spectrophotometrically quantified. In figure 2A, it can be observed a similar pigment content in all lines, including wild-type. However, chlorophylls ratio (figure 2B) of some mutant lines (B3 40, B5 50, and B6 73) are slightly different from wild-type, pointing out to possible alterations in photosynthesis that should be further investigated.

Analysis of the *in vitro* root growth

In order to know whether these Arabidopsis lines were developmentally affected, seedling root growth was tested in control (sugar-free) or in sucrose-containing (1% sucrose) solid media. Figure 3 shows that there were no significant differences among the lines analyzed neither in the free-sugar medium nor the sucrose-containing medium. As it could be expected, the rate growth in the presence of sucrose was higher than in the absence of this sugar (approximately 2 fold).

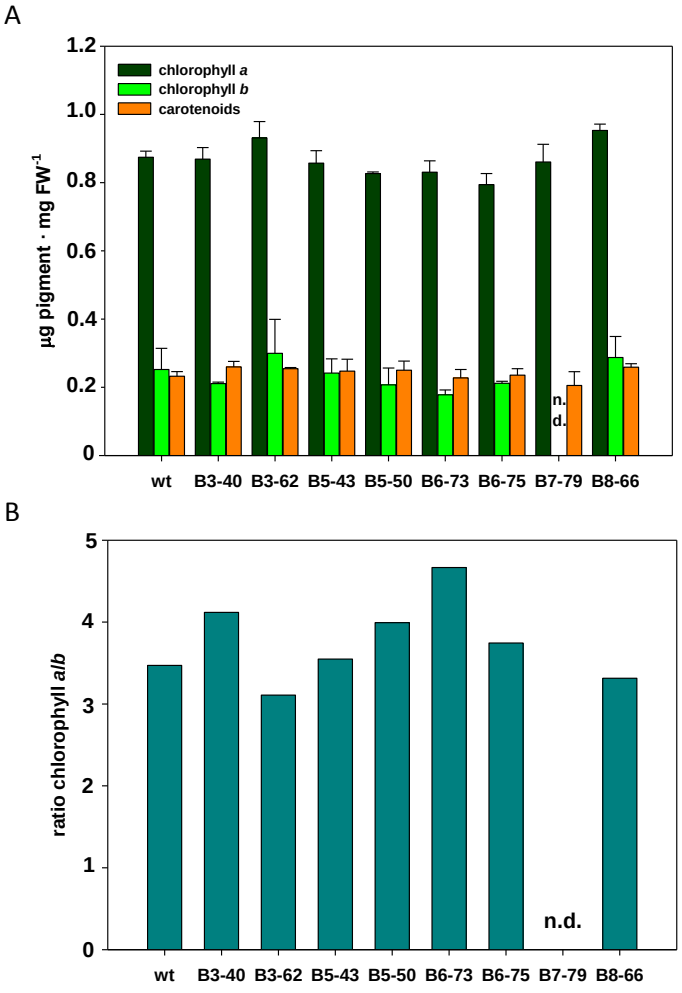

Figure 2. Photosynthetic pigments in *A. thaliana* lines grown in soil. A, Chlorophyll *a* and *b* and carotenoids content in 14-dag wild-type and mutant plants. B, Calculation of the chlorophyll *a*/*b* ratio for the different lines. At least five plants were analyzed for each measurement. n.d., not determined.

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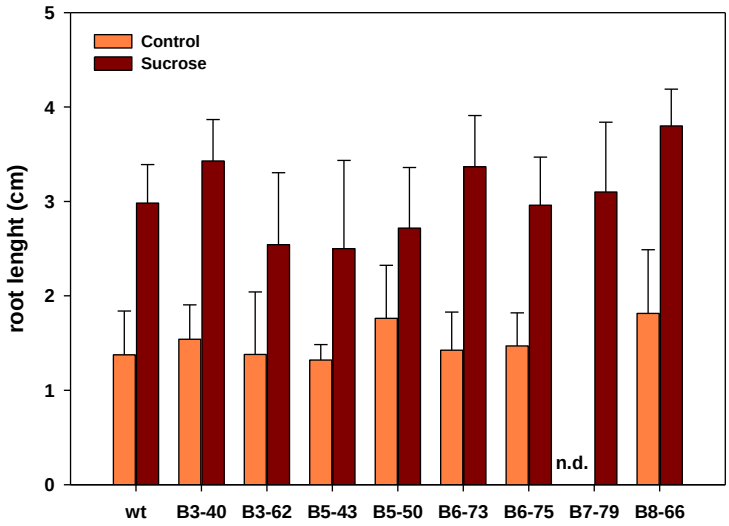

Figure 3. Root length of the Arabidopsis lines (wild-type and mutants) of 7-dag plants grown on 0.5x MS medium with or without sucrose (1%). Seedlings were grown vertically on the surface of hard agar plates. At least five plants were analyzed for each measurement.

Table 1. Identification of the loci containing the T-DNA insertions and their putative functions. Data were obtained from TAIR database (www.arabidopsis.org).

Assigned ID	AGI affected	Putative function
B3-40	At2g38080	Hydroquinone:oxygen oxidoreductase activity.
B3-62	At3g10530	Nuclear protein.
B5-43	At3g62600	Unfolded protein binding, heat shock protein binding.
B5-50	At5g48090	Nuclear protein.
B6-73	At1g56240	Nuclear protein.
B6-75	At1g15140	Oxidoreductase activity.
B7-79	At2g47710	Putative stress protein.
B8-66	At5g25750	Unknown function.

Determining sugar specificity of the putative signalling/sensing pathways altered in the different Arabidopsis lines

Mutations leading to gaining insensitivity to a high fructose concentration in the growth medium could be specific of this sugar or might be rather an unspecific resistance to several sugars. With the purpose of differentiating both possibilities, Arabidopsis mutant lines were grown in solid medium containing a high glucose concentration (6%) and compared with plants grown in the presence of the same fructose concentration. In figure 4 can be observed that lines B3-40, B5-43, and B6-73 are resistant to both sugars. Surprisingly, and on the contrary to our previous results, the lines B7-79 and B8-86 were fructose sensitive. Only Arabidopsis lines B3-66, B5-50, and B6-75 showed specific fructose insensitivity, corresponding to mutations in genes At3g10530 and At5g48090 (two putative nuclear proteins) and At1g15140 (a putative oxidoreductase), respectively. As expected, wild-type plants had highly impaired germination on the fructose medium while on the glucose medium the germinated seedlings showed white cotyledons (no photosynthetic activity).

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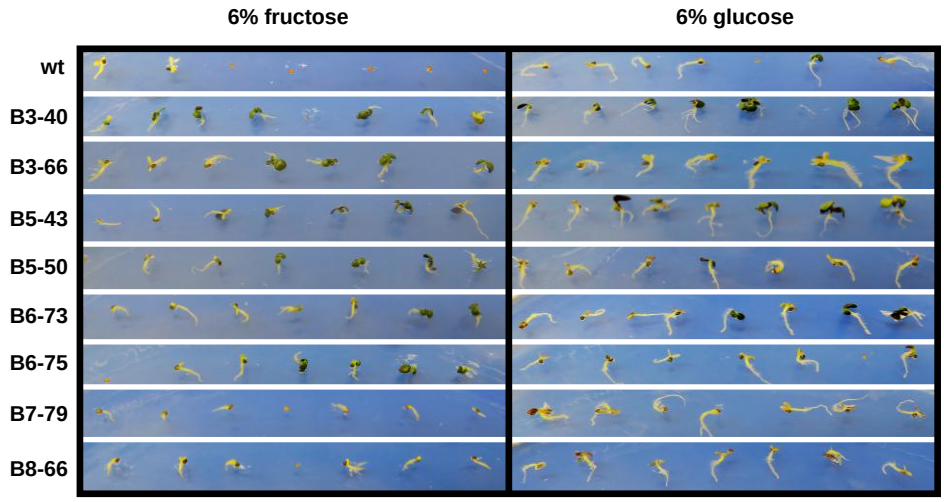

Figure 4. Fructose and glucose signalling. Arabidopsis lines were grown for 12 days on 0.5x MS medium containing 6% fructose or glucose.

CONCLUSIONS

[1] Despite the link between photosynthesis and sugar biosynthesis, our results show that disturbing sugar signaling does not necessarily affect Arabidopsis photosynthetic pigment content or the plant growth.

[2] In addition to being fructose insensitive, a 37.5% of the mutant lines also showed glucose insensitivity. The presence of 25% false positives in a second screening round suggests that environment factors could greatly affect the screening results.

ACKNOWLEDGEMENTS

This work was supported by "Programa Nacional de Proyectos de Investigación Fundamental" from "Ministerio de Economía y Competitividad" (BIO2012-33292). We also want to thank José Antonio Rojas González for its help during this work.

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MY OWN IDEAS

Rafael Barnes Ontiveros, IES Francisco Ayala, Granada
En este proyecto hemos estado estudiando el efecto de los azúcares sobre el crecimiento vegetal y contenido de las plantas en Arabidopsis thaliana. Durante nuestro proyecto hemos tenido que llevar a cabo diferentes tareas tales como: siembra de semillas en medios de cultivo, medición de raíces y recuento de hojas, determinación del contenido de las clorofilas.
Para poder conseguir los resultados que nos hacían falta para completar el proyecto, hemos tenido que aprender a usar el método científico, con todo lo que conlleva, y en especial, el uso de herramientas y técnicas de laboratorio.
Para mí poder participar en este proyecto de investigación ha supuesto una fantástica experiencia que me ha permitido conocer cómo es el mundo de la ciencia, de lo que estoy muy satisfecho porque estoy seguro de que en un futuro me ayudará a no partir de cero y elegir mejor.

Marina Fiñaga Cortés, IES Federico García Lorca, Churriana de la Vega
In this project we worked with the model plant *Arabidopsis thaliana*, both with wild type as with some derived lines.
During the first session we sow seeds on agar culture media containing different sugars: glucose, fructose and sucrose. We let them grow for about 10 days in a growth chamber with a photoperiod of 16 hours light and 8 dark and 22ºC of temperature. Then, we carried out a set of experiments in order to analyse the effect of different sugars on the plant physiology. These experiments consisted in counting the leaf number and quantifying the rosette biomass, determining the root-growth rate and the chlorophyll content. Finally, we statistically analysed the results and obtained conclusions.

En este proyecto hemos trabajado con la planta modelo Arabidopsis thaliana, tanto con la forma silvestre como con diferentes líneas derivadas de ésta.
En la primera sesión sembramos semillas en cajas de cultivo in vitro con agar que contenían diferentes azúcares: glucosa, fructosa y sacarosa. Cultivamos las plantas durante 10 días en una cámara de cultivo con un fotoperiodo de 16 horas de luz y 8 horas de oscuridad a 22ºC. A continuación analizamos el efecto de los azúcares sobre la fisiología de la planta. Estos experimentos consistieron en contar el número de hojas y cuantificar la biomasa de la roseta, determinar la tasa de crecimiento de las raíces y el contenido de clorofila. Finalmente se analizaron estadísticamente los resultados y obtuvimos una serie de conclusiones.

María Elena Noguera Vilches, CDP Sagrado Corazón, Granada
This experience has helped me to get more into the field of plant biology. This project has been amazing because I never imagined that we would be able to observe consequences over the plant physiology coming from small modifications in the DNA. This project showed how is the work in a laboratory and also how important is to follow the methodology, the order and, especially, the English language. I am very happy to have participated in this experience.

Esta experiencia me ha ayudado a profundizar en el campo de la biología de las plantas. Este proyecto ha sido sorprendente ya que nunca imaginé que seríamos capaces de observar consecuencias derivadas de pequeñas modificaciones en el ADN. Este proyecto me ha enseñado cómo se trabaja en un laboratorio de investigación y la importancia que tiene la metodología, el orden y, especialmente, el inglés. Estoy muy contenta de haber participado en esta experiencia.

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Claudia Rodríguez Ibáñez, CDP Sagrado Corazón, Granada

In this project we have studied the *Arabidopsis thaliana p*lant, both wild type as modified lines. Specifically, we have studied how different kind of sugars like glucose, sucrose or fructose affects the plant growth. To do that, we let grow the plants for 10 days in a culture house with 16 hours light and 8 dark with a temperature of 22 °C. After that, we compared the different plant lines and we did a detailed study generating graphs and obtaining conclusions. 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. Particularly, I have learned how is the work in a research laboratory.

En este proyecto hemos estudiado la planta Arabidopsis thaliana, tanto la variedad silvestre como otras variedades derivadas de la misma. Concretamente hemos estudiado cómo diferentes azúcares como la glucosa, la sacarosa o la fructosa puede afectar al crecimiento de las plantas. Para llevar a cabo este estudio se cultivaron plantas de Arabidopsis con un ciclo de luz de 16 h y de oscuridad de 8 h a 22ºC. Después se compararon las diferencias entre líneas y realizamos un detallado estudio generando distintas gráficas y obteniendo diversas conclusiones. Escogí este proyecto porque suscitó en mi mucho interés y curiosidad. La variedad de conocimientos adquiridos durante este trabajo me ha ayudado a entender e incrementar mi experiencia en temas académicos que desconocía total o parcialmente. Sobre todo he aprendido como se desarrolla el trabajo en un laboratorio de investigación.

Functional characterization of peroxisomal protein kinases in A*rabidopsis* plants

Ana I. Olmedo Moya[1], Andrea Sánchez Alonso[1], Gemma Beiro Valenzuela[2], Ana Carmen Villegas Buendia[3], Adrián Ollero del Peral[4], Alba Valdivia Ruiz[3,] Katiuska E. Cárdenas[5], Luisa M. Sandalio[5], María Rodríguez Serrano[5*]

[1] *IES Francisco Ayala, Avd. Francisco Ayala s/n 18014 Granada, Spain*
[2] *IES Juan XXIII, Camino de Santa Juliana s/n, 18006Granada, Spain*
[3] *IES Acci, Avda Buenos Aires 68, 18500 Guadix, Granada, Spain*
[4] *Colegio Virgen de Gracia, Calle Músico Vicente Zarzo, 18002 Granada, Spain*
[5] *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: maria.rodriguez@eez.csic.es

SUMMARY

Post-transcriptional modifications of proteins by phosphorylation is one of the most common mechanisms involved in regulating the activity, subcellular location and turnover of proteins. These processes are involved in sensing environmental cues and regulating the cell response to biotic and abiotic factors. Peroxisomes are an important source of signaling molecules such as reactive oxygen species (ROS), NO and hormones, and play a central role in regulating different metabolic pathways and cell response to stress conditions. The presence of kinases and phosphatases in peroxisomes has recently been reported although the protein target of this type of modifications and its role in the peroxisomal metabolism have not been identified so far. In this study, we analyze the role of the peroxisomal protein kinases, CPK1, PK1 and PK4, in the plant response to stress caused by Cd and their role in regulating catalase and glycolate oxidase activities by using *Arabidopsis* mutants deficient in these kinases (*cpk1, pk1* and *pk4*). The results obtained suggest that CPK1, PK1 and PK4 are not essentially involved in the cell response to Cd, although PK1 and CPK1 may regulate glycolate oxidase activity while CPK1 regulates ROS production under Cd treatment.

INTRODUCTION

Post-transcriptional modifications of proteins play an important role in the regulation of the catalytic activity of proteins, their stability, interactions between proteins, and subcellular location [1]. Among these post-transcriptional modifications, protein phosphorylation is one of the most important and is probably the best known modification in both prokaryotes and eukaryotes. The phosphorylation/de-phosphorylation of proteins is a reversible process regulated by kinases and phosphatases, respectively (Fig. 1). Annotation of protein families in *Arabidopsis* predicted the existence of approximately 1003 kinases, accounting for 4% of all proteins (Pfam; http://pfam.sanger.ac.uk/proteome?taxId=3702).

Phosphorylation/de-phosphorylation is one of the most common mechanisms used by the cell to regulate the activity and functionality of different proteins, their subcellular location, interaction with other proteins and protein turnover [1]. These processes are involved in perceiving changes in the cell environment and triggering signaling events which enable a rapid regulation of metabolic pathways in response to extracellular changes which include biotic and abiotic factors [1,2] (Fig. 1). Plants have developed these modifications as sophisticated and

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specific mechanisms for tolerating adverse conditions and for protecting themselves under stress conditions [2]. In recent years, the proteomics of cells, tissues and organisms have become essential to understand the mechanisms involved in the regulation of cellular responses to their environment.

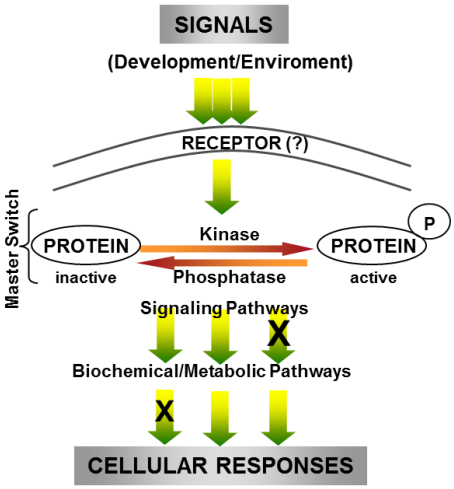

Figure 1. A schematic presentation of protein phosphorylation events which eventually control cellular responses [2].

Peroxisomes are subcellular organelles delimited by a single membrane that contain, as basic enzymatic constituents, catalase and hydrogen peroxide (H_2O_2)-producing flavin oxidases andare involved in very important metabolic pathways such as β-oxidation of fatty acids, the glyoxylate cycle, photorespiration, ureide metabolism, biosynthesis of plant β-oxidation of fatty acids and metabolism of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [3, 4]. The presence of kinases and phosphatases in peroxisomes has recently been reported which points to an important role for phosphorylation/de-phosphorylation in the regulation of these organelles [5, 6]. However, the protein target of these types of modification has not been identified so far. A recent phosphoproteomic study has identified some of the protein target of phosphorylation in peroxisomes, including glycolate oxidase (GOX), which produces H_2O_2, superoxide dismutase (SOD) and catalase (CAT), which areall involved in ROS metabolism [7].

In this study, we will analyze the role played by some of the protein kinases previously located in peroxisomes, CPK1, PK1 and PK4 [6], in the plant response to stress caused by a heavy metal. For that purpose, we will use *Arabidopsis* mutants deficient in these kinases and will measure plant growth over time in the presence of the metal. To attempt to determine the target of these kinases, the activity of some of the protein previously shown to be phosphorylated in peroxisomes will also be analyzed.

MATERIALS AND METHODS

Plant Material and growth conditions

Arabidopsis wild type (WT) col 0 and *Arabidopsis* mutants deficient in PK1 (*pk1,* Salk_112111C), CPK1 (*cpk1,* Salk_080155C) and PK4 (*pk4,* Salk_054351C) were obtained from The European *Arabidopsis* Stock Centre (NASC). Seeds were surface-sterilized and stratified at 4°C for 24 hours and grown in Murashige and Skoog medium (MS) 0,5x (control) and MS medium supplemented with $CdCl_2$50 µM in vertical plates. The plants were grown in a growth chamber (22 °C under a 16 h light/8 h dark regime) and kept for 12 days. Root length was measured

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every day using Image 3.0 software. The number of lateral roots and total fresh weight were analyzed after 12 days. Plants were also grown in soil and vermiculite (3:1) in a growth chamber at 21ºC/18ºC, 60%/50% relative humidity and 14 h of light and kept for 4 weeks. In adult plants, leaf area was measured using a Portable Area Meter Nod LI-3000.

Enzymatic assays

Catalase activity was measured spectrophotometrically as the consumption of H_2O_2 at 240 nm for 2 min at 25 °C. One ml of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10,6 mM H_2O_2, and 10 µL of extract [8]. Glycolate oxidase activity was measured spectrophotometrically according to Pazmiño et al. [8]. One ml reaction mixture contained 50 mM potassium phosphate buffer (pH 8.3), 3 mM EDTA, 10 mM phenylhydrazine and 5 mM glycolic acid. The reaction was initiated by adding the sample and measuring the formation of the glyoxylate-phenylhydrazone complex at 324 nm for 2 minutes.

ROS imaging

H_2O_2 was detected by incubation with 25 µm 2',7'-dichlorofluorescein diacetate (DCF-DA; excitation at 485 nm, emission at 530 nm) in 10 mM Tris-HCl (pH 7.4). Roots were incubated for 30 min at 37ºC as indicated by Sandalio et al. [9] and observed under a fluorescence microscope (Leica DMI600B). The fluorescence was quantified using the LAS AF lite software.

Protein and statistical analysis

Protein concentration was determined with the BIO-RAD Bradford Protein Assay kit (BIO-RAD) using bovine serum albumin (BSA) as standard. Data were subjected to one-way analysis of variance for each parameter. Differences were evaluated for significance by the t-student test.

RESULTS

Under physiological conditions, no differences were observed in terms of fresh weight between WT and p*k4 Arabidopsis* plants, while the growth of *cpk1* plants was slightly higher than that of WT plants, while, for *pk1* plants it was slightly lower (Fig. 2). The treatment with Cd induced a severe reduction in total fresh weight in all plants, WT and mutants, although for *cpk1* the reduction was smaller, as compared to the other plants (Fig. 2).

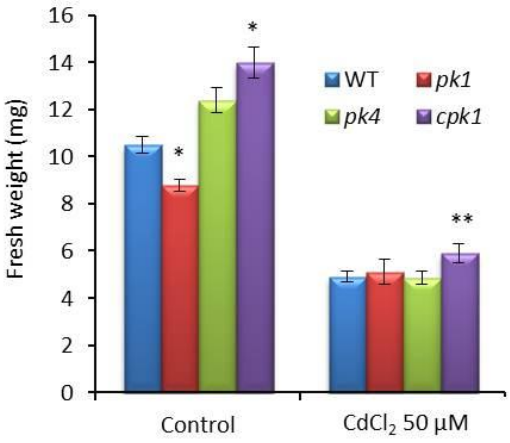

Figure 2. Cadmium effect (50 µM $CdCl_2$) on fresh weight of WT, *cpk1, pk1* and *pk4 Arabidopsis* plants. Values are means±SD. Asterisks indicate that values are significantly different (*, p<0.05; **, p<0.01).

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The time course analysis of root length in the presence and absence of the metal showed a similar pattern in WT and the mutants *pk1*, *cpk1* and *pk4* (Fig. 3). However, the length of *pk4* was significantly greater after 12 days of growth (Fig. 3-5). Treatment with Cd produced a severe reduction in root length, affecting both WT and the mutants in a similar way, although the metal affected *pk4* and *cpk1* to a slightly lesser degree (Fig. 3-5).

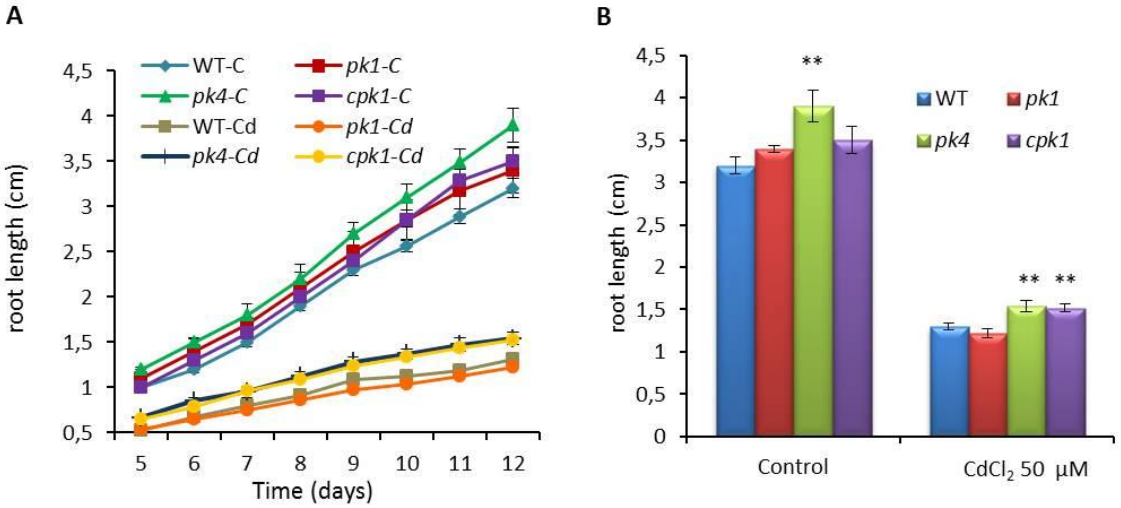

Figure 3. Time course analysis of cadmium (50 µM $CdCl_2$) effect on root length in WT, *cpk1, pk1* and *pk4 Arabidopsis* plants (A). (B) Effect of Cd treatment on root length after 12 d of treatment. Values are means±SD. Asterisks indicate that values are significantly different (*, p<0.05; **, p<0.01).

Under physiological conditions, the number of lateral roots was slightly higher in *pk4* and *cpk1*, while *pk1* did not show any difference as compared to WT. Treatment with Cd caused a reduction in lateral roots which was more pronounced in WT and *pk1* plants (Fig. 4).

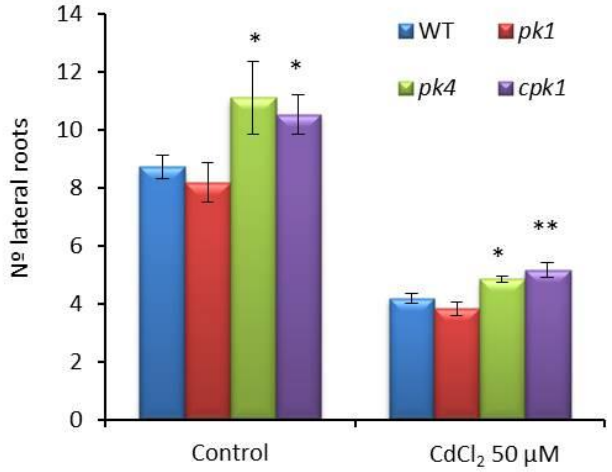

Figure 4. Cadmium effect (50 µM $CdCl_2$) on the number of lateral roots in WT, *cpk1, pk1* and *pk4 Arabidopsis* plants. Values are means±SD. Asterisks indicate that values are significantly different (*, p<0.05; **, P<0.01).

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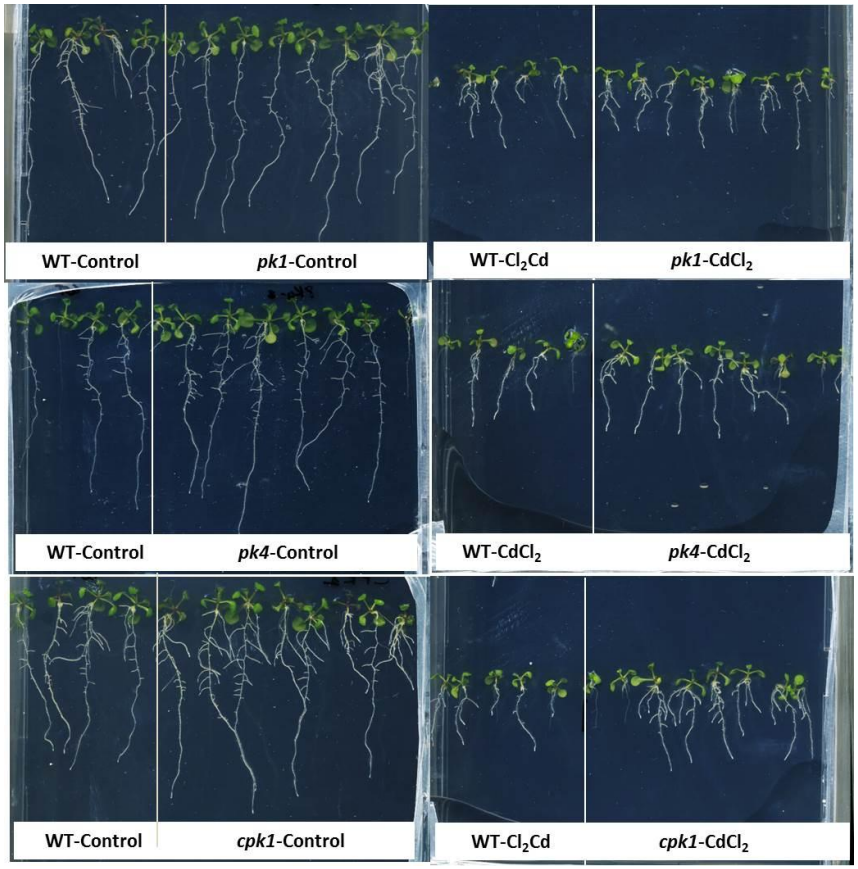

Figure 5. Phenotype of *Arabidopsis* WT and mutants *cpk1, pk1* and *pk4* grown in MS medium (control) and MS medium supplemented with 50 µM $CdCl_2$ after 12 days of treatment.

Analysis of leaf area in adult plants grown in soil for 21 days did not show any statistical differences between WT and *Arabidopsis* mutants deficient in PK1, CPK1 and PK4 (Fig 6).

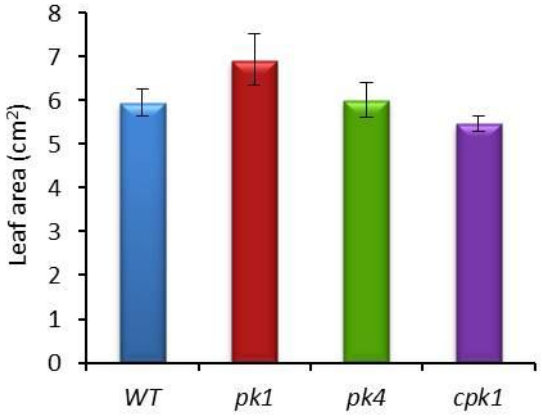

Figure 6. Leaf area of WT, *cpk1, pk1* and *pk4 for* 21 day-old *Arabidopsis* plants. Values are means±SD.

To determine the targets of the peroxisomal kinases analyzed in this study and taking into account that GOX and CAT have previously been demonstrated to be phosphorylated [7], the activity of these enzymes was analyzed in leaves from WT plants and the different *Arabidopsis* mutants. The analyses were carried out on adult plants (21 days old) and in the absence of Cd

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treatment. Catalase activity did not show any differences between WT and the mutants, which demonstrates that CAT is not regulated for either of these kinases (Fig. 7). GOX activity was similar in WT and mutants, although a statistically significant reduction of between 15-20 % was observed in *pk1* and *cpk1*, respectively. These results suggest that GOX may be partially regulated by these kinases (Fig. 8).

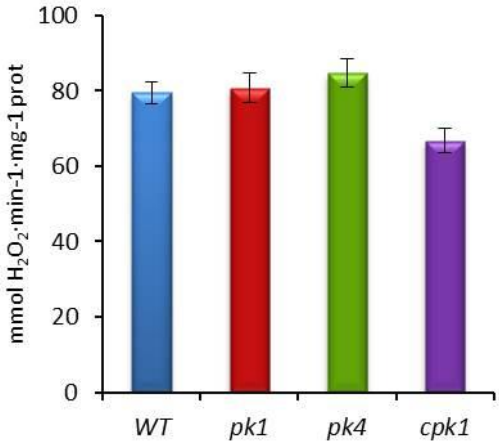

Figure 7. Catalase activity of WT, *pk1, pk1 and pk4* 21 day-old *Arabidopsis* plants. Values are means±SD.

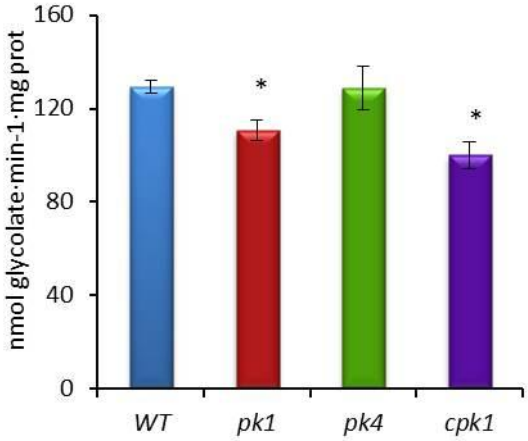

Figure 8. Glycolate oxidase activity of WT, *pk1, cpk1 and pk4* 21-day old *Arabidopsis* plants. Values are means±SD. Asterisks indicate that values are significantly different (*,p<0.05).

To check whether the changes observed in GOX activity affect the H_2O_2 production induced by Cd, H_2O_2 was analyzed by imaging its accumulation by fluorescence microscopy using DCF-DA. For that purpose, WT and *cpk1* plants were treated with 100 µM $CdCl_2$ for 24 h and the accumulation of H_2O_2 was imaged in roots. Figure 9 shows that, under physiological conditions in the absence of Cd, fluorescence caused by H_2O_2 was not visible in either of the plants, while, after treatment with Cd, a sharp increase in DCF fluorescence was observed in WT plants. However, in *cpk1* mutants, the fluorescence signal was considerably reduced (Fig. 9). These results demonstrate that CPK1 may regulate the production of H_2O_2 in peroxisomes. However,

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due to the sharp reduction observed in H_2O_2 accumulation after treatment in *cpk1* and the slight reduction observed in this mutant's GOX activity, it is possible to speculate that, in addition to GOX, others sources of ROS production regulated by this kinase could also be involved. Additional more in-depth research is required to identify the targets of peroxisomal kinases and to determine the role played by these kinases in the signaling and regulation of the peroxisomal metabolism.

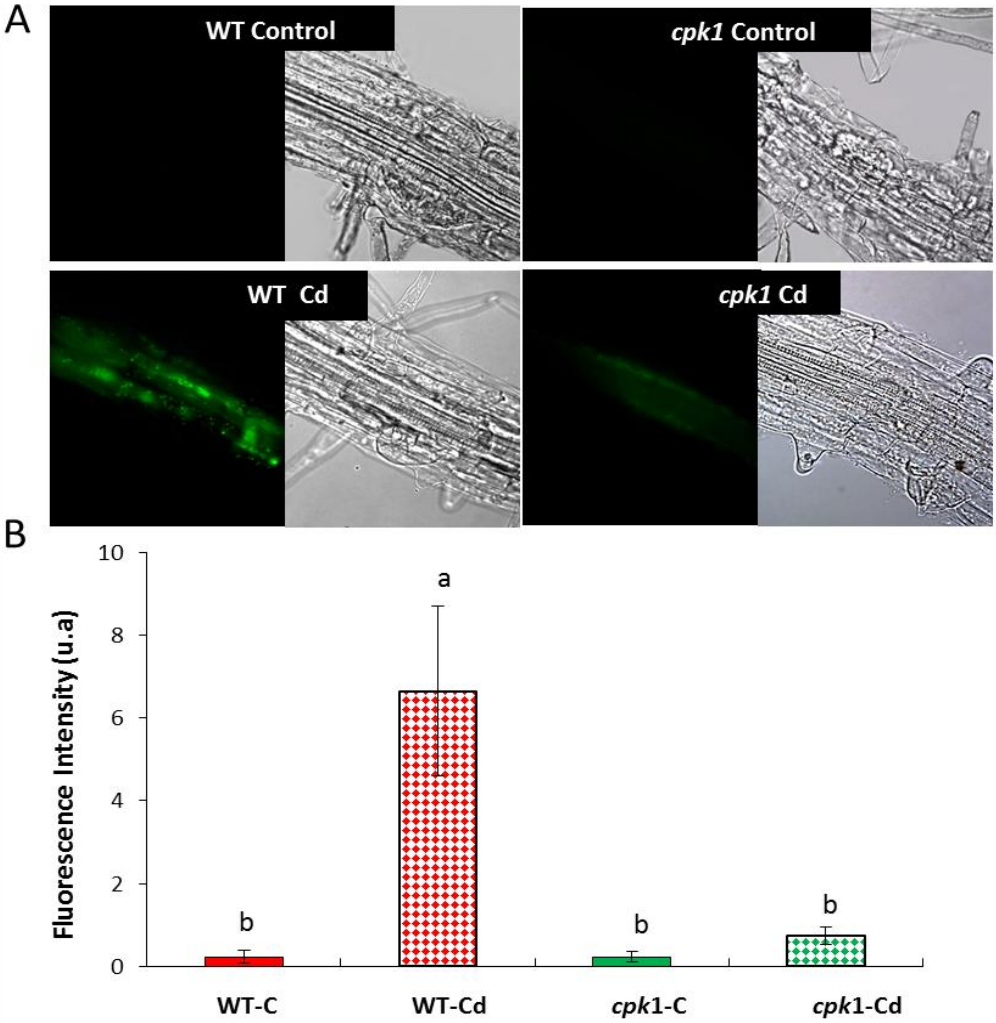

Figure 9. Imaging of H_2O_2 accumulation induced by Cd in roots of *Arabidopsis* WT and *cpk1.* Ten day-old plants were treated for 24 hours with 100 µM $CdCl_2$ and H_2O_2 was imaged by fluorescence microscopy using DCF-DA (Ex/Em: 485/530 nm; green, A). Root segments are shown by bright field(A). DCF-DA fluorescence was quantified in arbitrary units (a.u., B). Different letters indicate significant difference at $p< 0.05$.

CONCLUSIONS

1. The deficiency in PK1, CPK1 and PK4 does not affect the phenotype of *Arabidopsis* plants, although plants deficient in PK4 and CPK1 grew slightly more than WT.

2. PK1, CPK1 and PK4 do not appear to be significantly involved in the regulation of cell response to Cd treatment.

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3. Catalase activity is not the target of PK1, CPK1 and PK4, while GOX could be the target of PK1 and CPK1.

4. PK1 and CPK1 could be involved in regulating H_2O_2 accumulation and the photorespiration pathway where GOX is the key enzyme.

ACKNOWLEDGEMENTS

This work was supported by ERDF-co-financed grant BIO2012-36742 from MICINN, *Junta de Andalucía* (BIO-337) and FECYT.

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MY OWN IDEAS

Ana Isabel Olmedo Moya

This Piiisa Project has been a very worthwhile experience for me and I have learnt a lot without getting bored. I choose this subject as, in my view, it is really interesting. We have had very few compulsary sessions, although we had to do many different types of activities. If there had not beent any afternoon sessions, we could not have finished the project in time, which is why I think there should be a greater number of compulsary sessions in order to meet the deadline and to carry out the project more effectively. The researchers and scientists have been extremely helpful throughout the course of the project. During the project, we carried out many activities which I had not done before and everything was clearly explained. I am extremely grateful for the help provided by the scentists. To sum up, the experience has been amazing, interesting and fun and I have learnt many things.

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Garlic used as a preservative increases the reduced form of ascorbate in pepper fruits

Cecilio Aranda López[1], Irene García Fernández-Gaytán[1], Irene Hernández Quero[1], Santiago Sánchez Requena[1], Carmelo Ruiz[2], Francisco Javier Corpas[2], José Manuel Palma[2*]

[1]*IES Fray Luis de Granada, C/ Huerta del Rasillo, s/n, 18004 Granada*
[2] *Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology and Agri-Food, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*
[*]*Corresponding author, e-mail: josemanuel.palma@eez.csic.es*

SUMMARY

This work is a continuation of the one initiated by our group last season which dealt on the *Ripening of pepper fruits*. The main objective of this project is to design strategies which allow post-harvest and storage practices for pepper fruits conducting to extend their shelf life without lowering their nutritional value. In this work, an innovative procedure was set up with results implying improvement of quality of fruits. The potential repercussion of the proposed process is under a broader study for future transfer to the productive sector.

INTRODUCTION (AND OBJECTIVES)

Pepper is a plant which belongs to the family of the *Solanaceae*. Pepper varieties are classified as sweet and hot, based on the culinary purposes. The main pepper's producer countries are China, Mexico, Turkey, Indonesia, Spain and USA [1,2]. According to the shape, three main types of fruits are distinguished: California (square and short), Lamuyo (square and long), and Dulce italiano (long and narrow). From all of these types of peppers, the California one has been chosen for our experiments.

In this project, we have also used garlic. The properties attributed to garlic are almost innumerable: improves the body immune system, increases the body defences and the vitality, is an anticoagulant and improves blood circulation by dilating blood vessels; it is also a great antiseptic and expectorant, so it protects the respiratory system [3].

The ingestion of vitamin C helps to prevent illnesses. In fact, vitamin C deficiency in the human diet causes scurvy, but also cardiovascular diseases [4]. Pepper fruits have been proved to be a good source of vitamin C [5]. In a former work, the effect of incubation with garlic in the physiology of pepper fruits was studied [6]. In this work, the involvement of garlic in the post-harvest nutritional features of pepper fruits has been investigated.

MATERIALS AND METHODS

Plant materials and Experimental design

Pepper fruits (California type) used in this work were provided by *Syngenta Seeds, Ltd*, El Ejido, Almería, Spain, and garlic (purple type) was obtained from the farm *El Huerto del Tito*, Casas Nuevas, Granada, Spain.

The following experimental design was developed:

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1. Fruits were weighed individually and washed with distilled water and dried.
2. Then, they were numbered (1-30) for future identification.
3. Six groups of fruits were set:
   Group 0: fruits were chopped and frozen with liquid nitrogen until we used them for analyses.
   Group A: incubated with a master preparation of garlic (MPG).
   Group B: peppers without garlic incubation.
   Group C: peppers at room temperature until the end of the experiment.
4. The groups A and B were subdivided into two subgroups each one:
   Sub-group A1: Peppers with MPG in a cold room (6ºC; 14 hours of darkness).
   Sub-group A2: Peppers with MPG at room temperature (22ºC/14ºC, day/night; 14 hours of darkness).
   Sub-group B1: peppers without garlic in a cold room (6ºC; 14 hours of darkness).
   Sub-group B2: peppers without garlic at room temperature (22ºC/14ºC day/night; 14 hours of darkness).
5. Fruits from sub-groups A1, A2, B1 and B2 were weighed and analyzed at 11 and 25 days.

Assays

In all pepper fruits the protein concentration [7] and vitamin C content by HPLC-MS were measured. The volatile compounds in garlic were identified and determined by gas chromatography linked to mass spectrometry (GC-MS).

RESULTS AND DISCUSSION

Some of the results obtained in this work cannot be given here since they are within a broader investigation focused in the future transfer to the productive sector. Therefore, they are subjected to confidentiality as part of a potential patent.

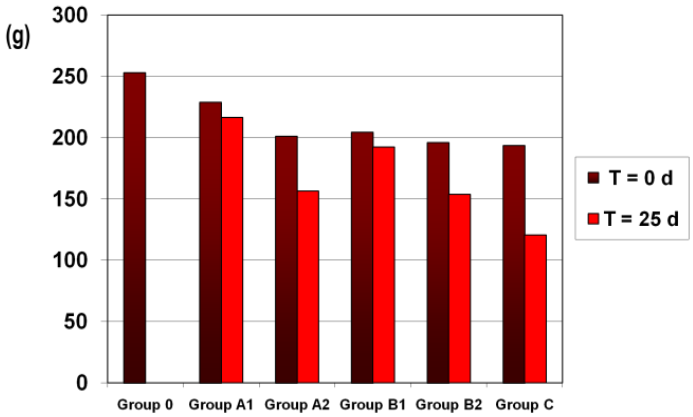

Figure 1. Effect of MPG and temperature on the fresh weight of pepper fruits.

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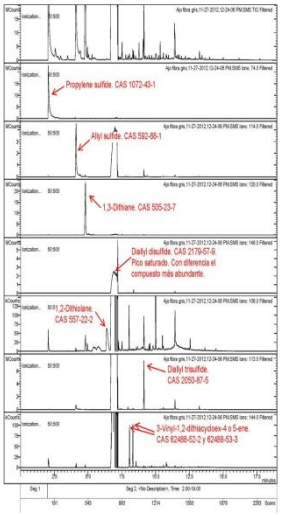

Número	Compuestos identificados en orden de elución	Número CAS	Area (cuentas)	% referido al más abundante
1	Propylene sulfide	1072-43-1	8,154E+07	2,98
2	Allyl mercaptan	870-23-5	3,304E+06	0,12
3	Methyl allyl sulfide	10152-76-8	3,377E+06	0,12
4	Dimethyl disulfide	624-92-0	8,643E+06	0,32
5	Allyl sulfide	592-88-1	7,744E+07	2,83
6	2,4-Dimethylthiophene	638-00-6	2,095E+07	0,76
7	1,3-Dithiane	505-23-7	1,870E+08	6,82
8	2-Propenyl, methyl sulfide	2179-58-0	1,916E+06	0,07
9	1-Propenyl, methyl sulfide	5905-47-5	2,869E+07	1,05
10	Diallyl disulfide	2179-57-9	2,740E+09	100,00
11(*)	2-Mercapto-3,4-dimethyl-2,3-dihydrothiophene	NIST 322301	2,054E+07	0,75
12	3-Vinyl-1,2-dithiacyclohex-4-ene	62488-52-2	8,514E+07	3,11
13	3-Vinyl-1,2-dithiacyclohex-5-ene	62488-53-3	1,029E+08	3,76
14	Diallyl trisulfide	2050-87-5	4,499E+07	1,64

(*) No se posee el CAS, en su lugar se ofrece el NIST

Figure 2. Separation and identification of volatile compounds from garlic. The analysis was done by combination of gas chromatography to mass spectrometry (GC-MS) at the Instrument Technical Service of the EEZ.

CONCLUSIONS

1. Garlic increases the vitamin C content (mainly the reduced form of ascorbate) and this improves the conservation of peppers fruits.
2. This methodology can be also applied to other agricultural produce.
3. This project could lead to a patent.

ACKNOWLEDGEMENTS

This work was supported by the ERDF-cofinanced grant AGL2011-26044 from the Ministry of Science and Innovation, Spain. HPLC-GC and LC-MS analyses were performed at the Instrument Technical Service of the Estación Experimental del Zaidín, CSIC, Granada. The coordination of the IESs' teachers who participated in the PIIISA program is also acknowledged.

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MY OWN IDEAS

Santiago Sánchez Requena

Empecé el proyecto PIIISA en 4º de la ESO, y nuestro grupo ha sido el único que ha continuado con el mismo proyecto durante 2 años dada su importancia, ya que podría desembocar en una patente.

El primer año nos dedicamos fundamentalmente al trabajo en el laboratorio. Me gustó bastante porque algunas veces parecía un científico, con la bata y los guantes, trabajando con bisturí y nitrógeno líquido. El trabajo de laboratorio es entretenido, e incluso divertido, es bastante metódico, en mi opinión; me sorprendió la excesiva limpieza para no contaminar las muestras. A lo largo de nuestra investigación hemos podido comprobar y realizar algunos de los pasos del método científico como: la elaboración de una pregunta, la creación de una hipótesis y la experimentación, siempre con la orientación de nuestro investigador José Manuel Palma.

Este último año nos hemos dedicado a la recopilación de los resultados para crear unas conclusiones que expondremos en la EEZ (Estación Experimental del Zaidín) y comunicaremos; Terminando así el ciclo del método científico. La divulgación la realizaremos mediante un Poster y la exposición, ambas en inglés. Esta parte ha sido más difícil y rigurosa, en ocasiones tediosa. Aunque creo que lo peor será cuando tenga que hablar en público y en inglés.

Esta experiencia me ha gustado bastante, ya que nos ofrece alternativas que hasta entonces desconocíamos y el hecho de que hayamos "colaborado" en la creación de una patente con solo 15 años me parece muy gratificante. Además me siento orgulloso de ser también los únicos que hemos continuado con el mismo proyecto 2 años seguidos. También ha servido para darme cuenta de lo difícil que es ser científico, ya que se necesitan muchos conocimientos, idiomas, curiosidad, dedicación, y una mezcla entre creatividad y razonamiento a la hora de la creación de las hipótesis. Quisiera darle las gracias a José Manuel Palma, Carmelo Ruiz, y otros muchos científicos por la paciencia que han tenido con nosotros.

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Seedless watermelons: from the microscope to the table through the greenhouse

Antonio J. Castro[1], Estefanía García[1], Trinidad Mª Caballero[2], Lidia Linares[3], Carlos Pérez[3], Andrea Piñar[4], Nieves Rivas[3], Nataly Vanessa Santillán[5], Krzysztof Zienkiewicz[1], Agnieszka Zienkiewicz[1], Manuel Jamilena[6], and Juan de Dios Alché[1*]

[1]*Department of Biochemistry, Cell and Molecular Biology, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*
[2]*IES ACCI, Avda. Buenos Aires 68, 18500 Guadix, Granada, Spain*
[3]*IES Montes Orientales, Carretera de la Sierra 31, 18550 Iznalloz, Granada, Spain*
[4]*IES El Temple, C/ Gloria Fuertes 2, 18130 La Malahá, Granada, Spain*
[5]*IES Zaidín-Vergeles, C/Primavera 26-28, 18008 Granada, Spain*
[6]*Department of Biology and Geology, University of Almería, Profesor Albareda 1, 18008 Almería, Spain*
[*]*Corresponding author:* ***e-mail:*** *juandedios.alche@eez.csic.es*

HIGHLIGHTS

- Pollen development in triploid watermelon plants is highly asynchronous.
- Microspores/pollen grains derived from triploid plants are larger than those from diploid plants and show degradation symptoms and abnormal cell wall development.
- As a consequence, pollen morphology from triploid plants is also variable.

SUMMARY

Seedless triploid varieties of watermelon (*Citrullus lanatus* var. *lanatus*) are very appreciated by consumers, but its production is limited because pollen donor diploid plants and insect-assisted pollination is required. To improve this process it is necessary to better study pollen biology aspects such as pollen viability and germinability during long-term storage, stigma receptivity period, etc. In this work we have compared the morphology and ultrastructure of triploid and diploid plant-derived pollen grains in commercial varieties using diverse microscopy techniques. We have not detected at this stage key macroscopic morphological differences between diploid and triploid flowers. Anther development within the triploid flower is highly asynchronous. Microspores from triploid plants are larger than those from diploid plants and showed symptoms of cytoplasmic degeneration. Pollen grains from triploid plants present different morphologies, contain three isodiametric pores but colpi are sometimes not well developed. Moreover, and also depending on the hydration stage, the pollen surface is sometimes smooth, without the characteristic reticulate pattern present in pollen grains from diploid plants. All these developmental features may lead to infertility of triploid plant-derived pollen.

INTRODUCTION (AND OBJECTIVES)

Some seedless varieties of watermelon (*Citrullus lanatus* var. *lanatus*) have additional commercial value because they are sweet and lack of seeds. Production of these fruits requires triploid (3n) plants to be pollinated effectively with pollen from diploid (2n) donor plants, a duty that is carried out by bees and bumblebees introduced in artificial hives in greenhouses

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[1, 2]. As result of double fertilization, triploid plants produce seedless fruits, a process called stenospermocarpy. However, continuous production of seedless watermelon fruits is challenging because: 1) the efficiency of the process is very low, 2) large amounts of viable pollen are required, and 3) donor-derived pollen production and triploid stigma receptivity must be synchronized [3]. Improving this process requires detailed knowledge of how pollen production takes place, how and when the female flowers reach their receptivity, and whether pollen is viable and can or cannot germinate. In this context, the main aim of this work was to characterize the morphological and ultrastructural characteristics of pollen grains from triploid watermelon plants and compare them with those of pollen grains produced by diploid donors.

MATERIALS AND METHODS

Plant material

Triploid and diploid male and female flowers of watermelon (*Citrullus lanatus* var. *lanatus*) were collected at different developmental stages in the greenhouses of the Fundación Finca Experimental UAL-ANECOOP (Almería, Spain). Macroscopic images of whole male and female flowers were taken *in situ* with a Nikon Coolpix 4500 digital camera (Nikon, Japan). Whole flowers were also observed after dissection under a Leica epifluorescence stereomicroscope M165FC (Leica Microsystems, Germany).

Sample preparation for microscopy

Whole flowers were fixed in 4% (w/v) paraformaldehyde, 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer at pH 7.5 overnight at 4°C. After fixation, samples were washed with several changes of cacodylate buffer, dehydrated in an ethanol series, embedded in Unicryl resin (BBInternational, Cardiff, UK) and polymerized at -20°C under ultraviolet light for two days. A second set of flowers were processed for paraffin embedding at the "Biobanco del Sistema Sanitario Público de Andalucía" (Granada, Spain).

Light microscopy (LM)

Semi-thin (1 µm) resin sections were obtained using a Reichert-Jung Ultracut E microtome (Leica Microsystems). Semi-thin (10 µm) paraffin sections were obtained using a hand operated-rotary microtome. The sections were placed on BioBond-coated slides, dewaxed (paraffin sections) and stained with a mixture of 0.05% (w/v) methylene blue and 0.05% (w/v) toluidine blue according to [4]. Finally, the slides were mounted with Merckoglass (Merck, Germany). Observations were carried out using a Zeiss Axioplan (Carl Zeiss, Germany) microscope. Micrographs were obtained using a ProGres C3 digital camera with the ProGres CapturePro 2.6 software (Jenoptic, LaserOptic Systems, Germany).

Confocal laser scanning microscopy (CLSM)

Whole mature pollen grains were dispersed in an anti-fading Citifluor (Sigma, USA)/water (1:1) solution and observed in a Nikon C-1 confocal laser scanning microscope (Nikon, Japan).

Transmission electron microscopy (TEM)

Ultrathin sections (70-90 nm) were cut on a Reichert-Jung Ultracut E microtome (Leica Microsystems), mounted on 200-meshed nickel grids and stained with 2% (w/v) uranyl acetate followed by 1% (w/v) lead citrate. Observations were carried out with a JEOL TEM-1011 (JEOL, Japan) transmission electron microscope at 80 kV.

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RESULTS

Flower morphology of triploid watermelon plants

Watermelon is a monoic species with unisexual flowers on the same individual. Macroscopic observations revealed significant differences in morphology between male and female flowers (Figure 1). Male triploid flowers contain five atypical stamens and possess a rudimentary unfertile ovary (Figure 1A).

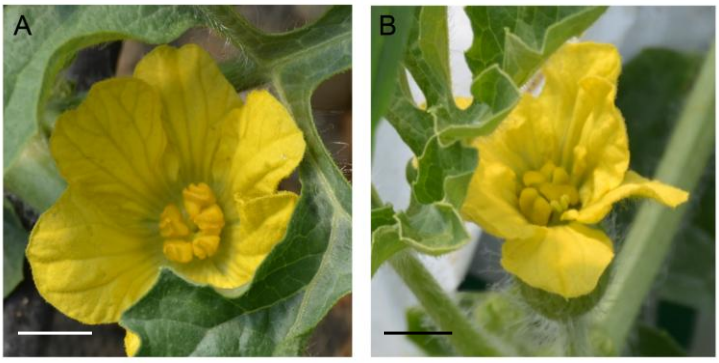

Figure 1. Unisexual flowers of triploid watermelon (*Citrullus lanatus*) plants at anthesis. A) Male flower. B) Female flower. Bars= 1 cm.

On the other hand, female triploid flowers have a tricarpelar and inferior ovary (Figure 1B). The ovary contains numerous orthotropous ovules and the placentation is of parietal type (Figure 2). We did not find noticeable morphological differences between diploid and triploid flowers.

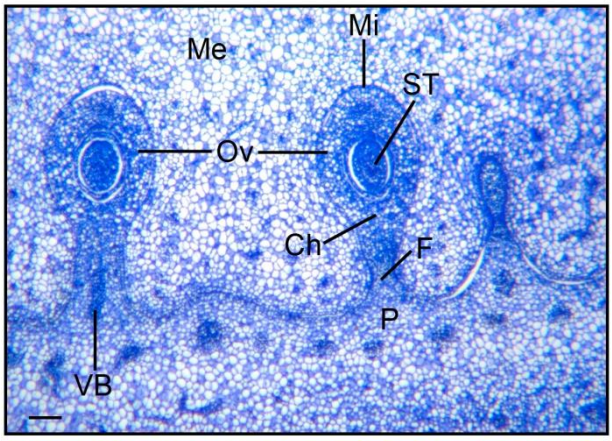

Figure 2. Morphological characteristics of a triploid watermelon ovary. Ch, chalaza; F, funiculus; Me, mesodermis; Mi, micropyle; Ov, ovule; P, placenta; ST, sporogenous tissue; VB, vascular bundle. Bar= 100 µm.

Watermelon pollen morphology

Anther development within the flower is asynchronous since different anthers contain microspore/pollen at different stages of development (Figure 3). Moreover, microspores from triploid watermelon plants are bigger than those from diploid plants (Figure 3E, H).

Pollen grains from diploid and triploid watermelon plants are tricolporate, and the pollen exine is of reticle-perforated type (Figure 4A). Pollen grains from triploid plants may vary in their morphology. They contain three isodiametric pores but colpi might be not well developed

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(Figure 4B). Moreover, the pollen surface is sometimes smooth, and may lack the characteristic reticulate pattern present in pollen grains from diploid plants (Figure 4B).

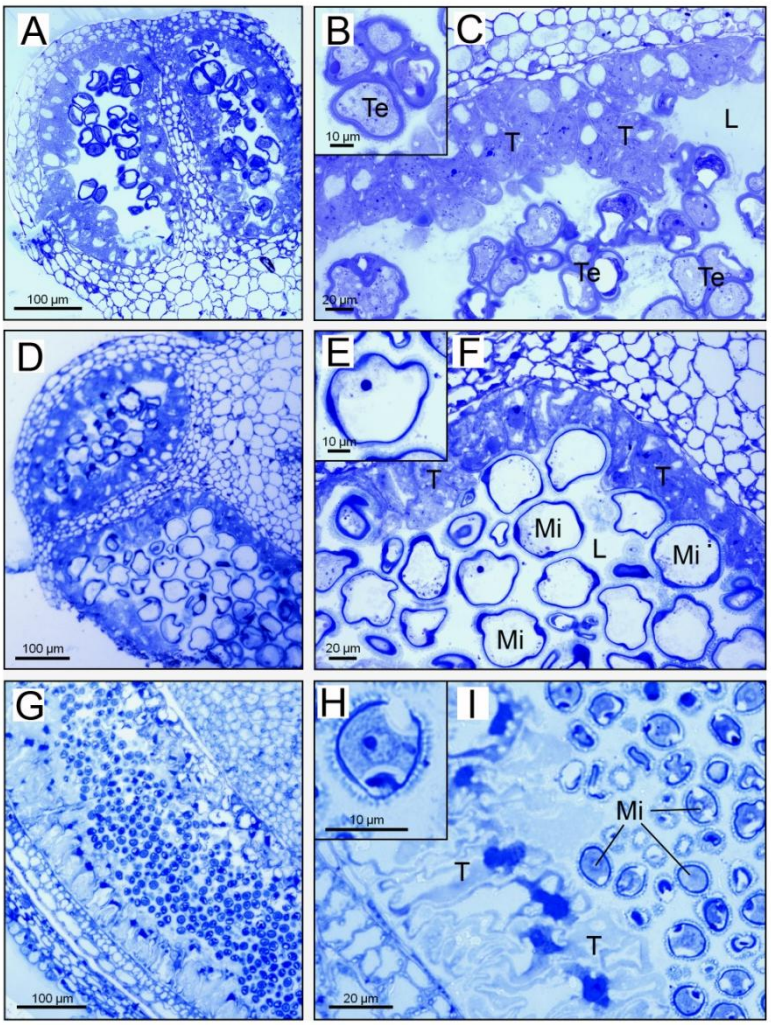

Figure 3. Comparison of anther development between diploid and triploid watermelon plants. A-F) Light microscopy toluidine blue-stained sections of two triploid anthers at the stages of tetrad (A-C) and microspore (D-F), respectively. G-I) Sections of a diploid anther at the stage of microspore. L, anther locule; Mi, microspore; T, tapetum; Te, tetrad.

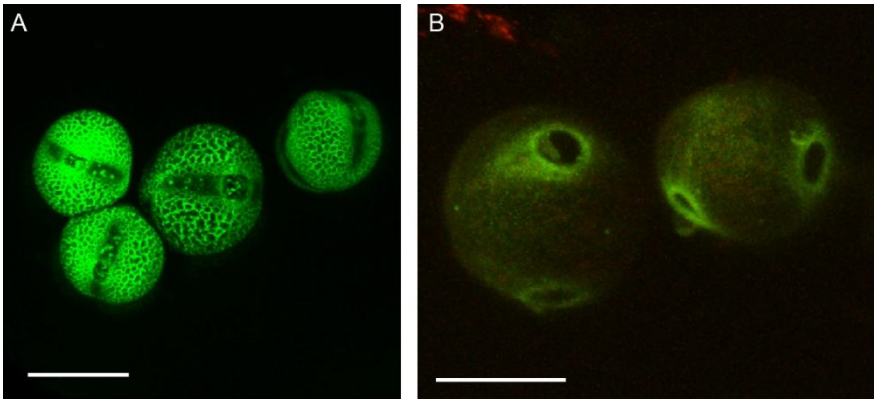

Figure 4. Different morphologies detected in pollen from triploid watermelon plants. Bars= 50 µm.

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Watermelon pollen ultrastructure

The exine comprises two layers, the outer sexine and the inner nexine (Figures 5A-B). The sexine is sculptured with numerous tecta and bacula. The cytoplasm shows numerous amyloplasts filled with starch granules (Figure 5B). Microspores from triploid plants showed symptoms of cytoplasmic degeneration (Figures 5C-D).

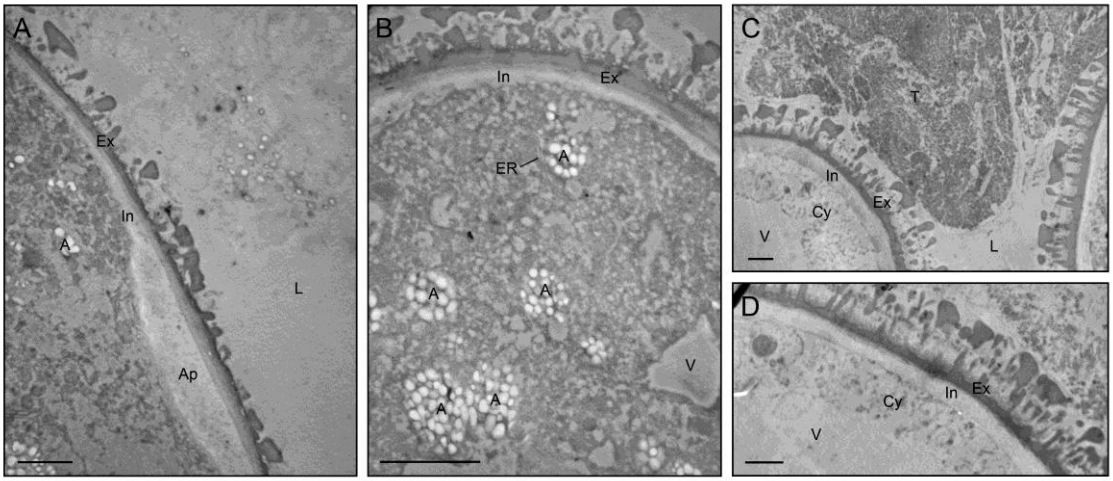

Figure 5. Ultrastructure of watermelon pollen grains. A-B) Ultrathin sections of maturing pollen grains from diploid watermelon plants observed with TEM. Sections were contrasted with uranyl acetate and lead citrate. C-D) Ultrathin sections of microspores from triploid watermelon plants. A, amyloplast; Ap, aperture; Cy, cytoplasm; ER, endoplasmic reticulum; Ex, exine; In, intine; L, anther locule; T, tapetum; MLC middle layer cell; V, vacuole. Bars= 2 µm.

CONCLUSIONS

1. **Pollen development within the flower of triploid watermelon plants is highly asynchronous.**
2. **Triploid plants-derived microspores/pollen grains are larger than those from diploid plants and show degradation symptoms and abnormal cell wall development.**

ACKNOWLEDGEMENTS

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MY OWN IDEAS

Trinidad María Caballero Sierra

First I want to thank all the researchers who have been with us, and to my Secondary School. This project has been a new experience because I had never used these kinds of microscopes. It was very nice to meet people who have devoted themselves to the investigation. They were explaining topics that we already learn in class; however, we were using this knowledge in a practical way. We have been using several microscopes that we had never seen, and for the first time we could use and handle them, thanks to the Experimental Center of the Zaidín. I didn't know very much on this topic, but now we known many things. What surprised me the most was to know that triploid watermelons do not have seeds, or just very little and small. I hope that with this project we will be able to improve the production in greenhouses. This project also served to reinforce our English skills. Finally and more importantly, I have meet new people and made very good friends.

Lidia Linares Parra

I can define this experience like unique, because working with plants in this way is not a game, nor easy. We have got the chance of sectioning, staining and observing the sections through different microscopes. The microscopes are impressive. With them, you can see the minimal details. Also we have seen the different stages of watermelon flowers. In the triploid watermelons (those without seeds), the pollen grains are alive during the early stages. However, pollen grains die when flower development progresses. Finally I would like to thank for this opportunity because it was wonderful to know the world of science, and to be able to work inside it.

Carlos Pérez Torres

Thanks to this fantastic project I have learn how to carry out a scientific research and the working methods of researchers. Our project dealt with triploid watermelons (seedless watermelons) and how to obtain them through the laboratory. We learn numerous working procedures, including sectioning, staining and dewaxing; moreover we have used different types of microscopes, which helped us to take pictures of flowers and different cell parts, using chemical products. Thanks for this experience, which helped me to know better the world of science.

Nieves Rivas Cañadas

To start with, I want to thank all people who have collaborated and worked in this project with us. This project helped us to know the world of science. Thanks to researches, we have carried our project "Seedless Watermelons". I think that all the instruments that are part of the CSIC's laboratories are incredible, particularly the microscopes. Also, we learnt to cut, stain, etc. flower samples. With the microscopes we could see the most complex details of different cells. One day we went to CSIC, we took pictures of the watermelon flowers and we managed to see the pollen grains very well. We have learnt a lot about cells, flowers, etc. Finally, I want to say that this is an amazing experience that all the people should have, and thank for this opportunity that we have had to know better the world of science, work with these incredible researches. Now, we like science a little bit more, because each day we can learn something new.

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Single mutations in a unique gene explain the non-mucoid phenotype in soil bacteria

[1]Fernández-Cruz, P.; [2]Guerrero-Dionisio, M.J., [3]Lara-García, M.; [4]Sánchez-Muñoz, M.; [5]Sánchez-Segovia, J. L.; [5]Ruiz-Rico, T. and [6*]Martínez—Abarca F.

[1]IES ACCI Avda Buenos Aires 68, 18500 Guadix, (Granada)
[2]IES El Temple. Gloria Fuertes, 2, 18130 La Malaha (Granada)
[3]IES Jose de Mora. Blas Infante 9, 18800 Baza (Granada)
[4]IES Francisco Ayala. Av Francisco Ayala s/n, 18014 Granada
[5]CDP Sagrado Corazón. Paseo de los Basilios 3, 18008 Granada
[6]Department of Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain
*[*]Corresponding author: **e-mail:** fmabarca@eez.csic.es*

HIGHLIGHTS

Colony Isolation and sequence analysis of a single mutation on a regulatory gene explain changes in the mucous phenotype of a soil bacterium. Students have been able to isolate non-mucous variants, amplify specific genes by PCR, obtain the sequence of the variants and finally, understand the consequences of the mutation observed.

SUMMARY

Soil bacteria of the species *Sinorhizobium meliloti* isolated from the field present a mucous phenotype derived of the presence of exopolysaccharide galactoglucan (EPSII). Frequently, the production of EPS II is lost growing the bacterial strain under laboratory conditions. It is known that a regulatory gene, *expR*, is a key gene to maintain this phenotype. We compare the frequency of EPS II lost among bacterial isolates from different geographical origin, finding significative differences between them. In addition, the sequence analyses of two non-mucoid variants show two novel mutations which have naturally been occurred on *expR* gene generating a truncated and probably non-functional protein.

INTRODUCTION

Life is conducted by a process that Charles Darwin defines as Natural Selection. But, the natural selection must be conducted over non-identical individuals (1). Differences between individuals are due to changes in their genetic content. At the beginning, all of these differences are the changes at the DNA level by DNA mutations. These changes are accumulated along evolution and it is believe that occurs slowly. But sometimes you can study in Laboratory conditions. This is especially true in procariotic systems (bacteria) where the growth and division occurs faster than in eukaryotic cells.

Our bacteria the study is a soil bacteria named *Sinorhizobium meliloti* that belongs to a group collectively referred to as rhizobia that together with leguminous plants contributes the largest input of combined nitrogen into terrestrial ecosystems (2). It is a genetically tractable model species for investigating rhizobial biology.

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S. meliloti contains all the genes required for the synthesis of the Exopolysaccharide (EPS). This compound can be clearly observed under growing on Agar plates at 30 ºC for two days giving a mucous phenotype (Figure 1). The EPS is formed by two types (I and II). The mucous phenotype is due to the EPS II component constituted by galactoglucan, a polymer of repeating galactose and glucose disaccarides with pyruvyl and acetil modifications (3). Some lab strains do not produce EPS II (Figure 1-B), and it has been described the presence of mutations that affect the regulatory gene *expR* (4,5). This regulatory gene control the expression of a large array of genes involved in free living and symbiotic functions such as transport of metal ions, motility, chemotaxis, … (6).

In this work, we aimed to obtain several *S. meliloti* isolates they are lost of the mucoid phenotype under laboratory culture and to analyze if the DNA sequence of their *expR* gene could explain such phenotype

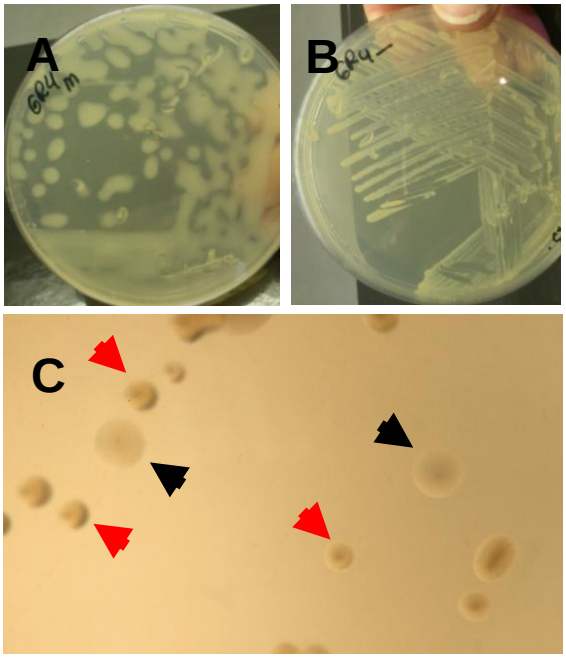

Figure 1. *Sinorhizobium meliloti* mucoid and non mucoid phenotypes. Morphology on plates of mucoid (A) and non mucoid (B) *S. meliloti* strains grown on TY media for 48 h at 28 ºC. In C, a detail under magnification where single mucoid (black arrows) and non-mucoid (red arrows) colonies can be observed.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions. The bacterial strains used in this work, their source and relevant characteristics are listed on table 1. Triptone yeast (TY) solid media were used for maintenance and grow at 28ºC *S. meliloti* strains.

Table 1. Bacterial strains used in this study.

***S. meliloti* strains**	**Relevant characteristics**	**Reference or source**
AK21	Field isolate - mucous (Aral Sea region, Russia)	RIAM*
RMO17	Field isolate - mucous (León, Spain)	(7)
GR4 wt	Field isolate - mucous (Granada, Spain)	(8)
GR4.5.1.1	GR4 spontaneous non mucous variant	This work
GR4.5.1.2	GR4 spontaneous non mucous variant	This work
GR4.6.1.1	GR4 spontaneous non mucous variant	This work
GR4.6.3.1	GR4 spontaneous non mucous variant	This work
GR4.6.4.2	GR4 spontaneous non mucous variant	This work

*Culture collection of All-Russia Institute of Agricultural Microbiology (St. Petersburg, Russia).

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-Isolation of non-mucoid variants. A collection of single colonies were extended until dilution on Agar plates on TY media for 48º at 28ºC. Under binocular amplification non-mucous variants could be distinguished (Figure 1) and isolating to new plates. Two days later the non-mucous phenotype were confirmed.

-Total DNA extraction and PCR Reactions. A single colony of 1-2 mm of diameter was picked and suspended in 200 ul of Sarcosyl at 0.5%. After a fast spin (seconds) the supernatant was removed and the pellet suspended in 100 ul of H_2O, and boiled for 10 min. 2ul of this solution was the DNA template for PCR reactions. The PCR reactions were performed as following:

For *nifH* gene; the primers used were: NifH1 TTGAGCGATTTCTGACGCGT and NifH2: AAGCTATTTCGGTTGTTCGG.

PCR reaction (Vol -50 ul).		**PCR conditions:**
DNA (Bacterial lisate)-	2 ul	1-94ºC Enter
10x TpB	5 ul	2-94ºC 3 min
dNTP (2.5 mM)	5 ul	3-94ºC 30 s
NifH1 primer	1 ul	4-60ºC 30 s
NifH2 Primer	1 ul	5-72ºC 30 s
Taq enzyme	0.3 ul	6- goto 29
H20	35,7 ul	7-72ºC 5 min
		8- Hold (4ºC)

For *expR* gene; the primers used were: expR1 ATCCGATACCATGGGAGG and ExpR2: GGGCTGGCCGGATTC.

PCR reaction (Vol -50 ul).		**PCR conditions:**
DNA (Bacterial lisate)-	2 ul	1-94ºC Enter
10x TpB	5 ul	2-94ºC 3 min
dNTP (2.5 mM)	5 ul	3-94ºC 30 s
expR1 primer	1 ul	4-63ºC 30 s
expR2 Primer	1 ul	5-68ºC 3 min
Taq enzyme	0.3 ul	6- goto 29
H20	35,7 ul	7-68ºC 5 min
		8-Hold (4ºC)

-DNA Electrophoresis. 5 ul of every PCR reaction were loaded in an agarose gel at 0.8% and develop at 120 volt for 45 min. After a gel-red staining for 10 min, the agarose gel was visualized under uv light. Specific amplicons of the expected size were detected.

-DNA Purification and sequencing. The 45 ul of six selected amplicons were further purified to remove primers and salts using illustra MicroSpin S-300 HR Columns (GE Healthcare) following the specifications of the supplier. Two alicuots of 4ul with the corresponding primer expR1 and expR2, respectively were sent to the DNA sequencing service of the EEZ processed by the technician A. Salido Ruiz (http://www.eez.csic.es/?q=en/node/4110).

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-**DNA sequence analysis**. The comparison and analysis of the sequences were performed using Clone-Manager Program.

RESULTS AND DISCUSSION

All non-mucoid variants are derivatives of GR4 strain

It is known that under Laboratory conditions rhizobia and also other soil bacteria generates variants which the exo-muco-polysaccharide EPS II is lost (Figure 1). In order to determine if several strains show this property, we study if the frequency of no-mucoid variants was similar in three *S. meliloti* strains from different origin: GR4 and RMO17, from different Spain sites, and AK21 isolated from Russia in a site close to the Aral sea (Table 1).

Several individual single colonies of mucoid phenotype were plating on TY agar by single dilution in order to detect non-mucoids variants. After two days at 28ºC only non-mucoid variants were observed on isolates derivatives of the *S. meliloti* GR4 strain. AK21 and RMO17 plates under identical conditions did not show any single colony with non-mucoid phenotype. The irreversibility of the non-mucoid phenotype in these GR4 variants were confirmed by growing a second plate of the single colony isolated.

Crude extract containing total DNA of these non-mucoid variants were subjected to PCR reactions in order to confirm they are *S. meliloti* strains (*nifH* gene amplification) and the current state of the *expR* gene (*expR* gene amplification; Figure 2). It was included, as a positive control, a DNA amplification of the field isolate GR4 strain, which all of them were derived (Figure 2, lane 9). DNA Electrophoresis gel confirms that all non-mucoid variants are *S meliloti* strains (*nifH* gene amplification was positive) and in all of them a band of 1.2 kb corresponding to the expected size of the *expR* gene amplicon were obtained.

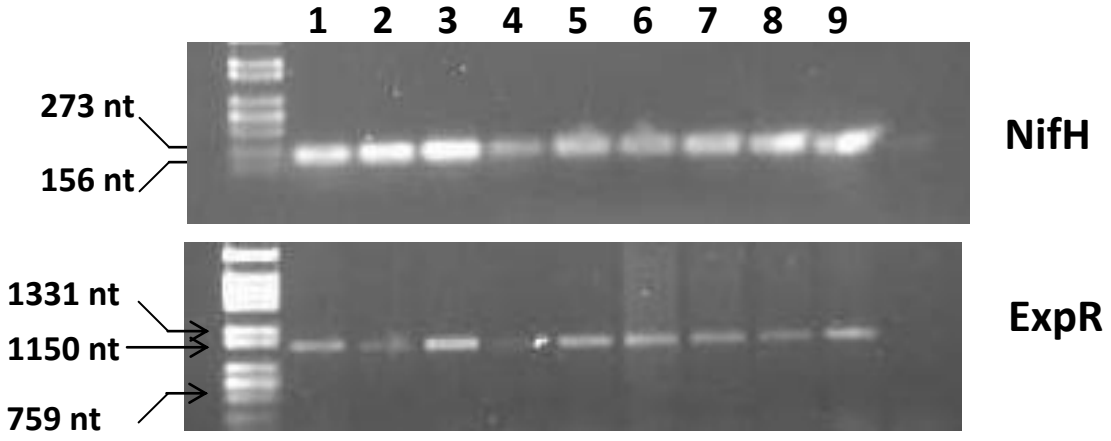

Figure 2. DNA Electrophoresis of the PCR amplicons of the *nifH* (above) and *expR* (below) genes obtained from non mucoid variants of *S meliloti* GR4 (1-8) described on table 1. 1-2 lanes derived from colony GR4-5 and 3-8 derived from colony GR4-6. Lane 9 derived from the mucous control *S. meliloti* GR4 field isolate.

Two single mutations are found in the expR *gene of non mucoid isolates.*

The *expR* gene amplicon of two (5.1.1 and 5.1.2) and three (6.1.1, 6.3.1 and 6.4.2) colonies from each group of GR4 variations from independent single colonies were chosen for further DNA sequencing. After DNA cleaning the sequence of the 738 nt corresponding to the *expR*

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genes were obtained (Figure 3).

Two mutations, 'G' to 'T' at 337 and 565 nt positions of the *expR* gene for the GR4-5 and GR4-6 group were found. No other mutations were observed in the rest of the sequence encoding the expR protein. In order to interpret the significance of such mutations, the analysis of the open reading frame of the DNA containing the mutations indicates those two different stop codons were generated. In the mutant T337 a GAG codon of a Glutamic amino acid (E in the code of single letter) is transformed to the stop codon UAG ('TAG' in DNA letters); meanwhile the mutant T565 changes a GAA codon (again for Glutamic amino acid) to the stop codon UAA. In both cases a truncated non-functional protein of 112 and 188 aa for T337 and T565, respectively, was generated (against 246 aa of the expR wildtype).

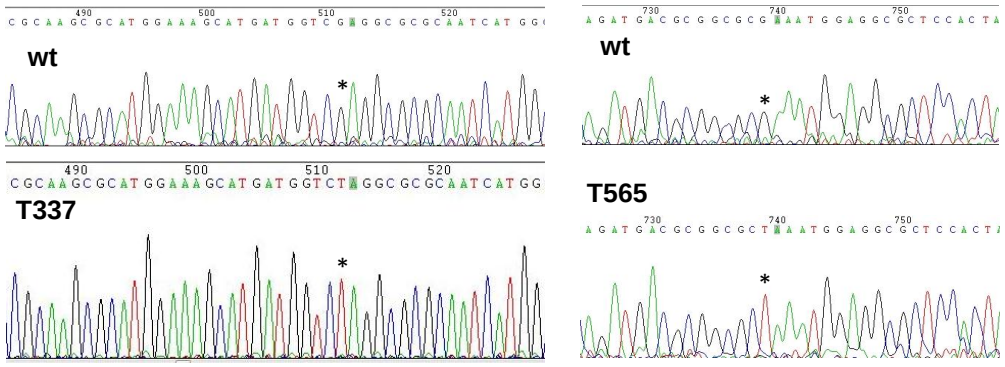

Figure 3. Electropherograms showing the single mutations detected. DNA electropherograms corresponding to *expR* amplicons of the wildtype (wt) and the two mutants: GR45.1.1 (T337) and GR46.1.1 (T565). The nucleotide mutated is indicated in arterisk.

The fact that two different mutations in the same gene are associated to the non-mucoid phenotype probably indicates, in agreement with the literature, that *expR* is a key gene in this process (3, 5). In addition, these two mutations were different among them and non-reported previously which it is an indication they were generated during the PIIISA project experiments (in two weeks or less).

The frequency and detection of such mutations indicates that this process is highly dynamic and it is a good example of how a single nucleotide change can generate drastic phenotypic changes.

CONCLUSIONS

1.- *Sinorhizobium meliloti* is a soil bacteria which easily change from mucoid to non-mucoid phenotype in laboratory culture.

2.- *S. meliloti* GR4 strain shows more frequently non-mucoid variants than AK21 and RMO17 strains.

3.- Single mutations T337 and T565 present in the *expR* gene of the mutants Gr4-5.1.1 and GR4-6.3.1 respectively affect deeply the *expR* gene and probably explain the non-mucoid phenotype.

4.-*expR* gene is a good candidate to analyze the mucoid and non-mucoid phenotypes.

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MY OWN IDEAS

Jose Luis Sánchez Segovia

When I first was in front of such a select audience and working so closely with trained and capable people, not only to perform a job, but also to convey their knowledge and set goals with youths our age, I thought we could do something grandiose , at least shocking to other teenagers who are in this situation like me. That initial fear of the first day, inexperience and ignorance facing the laboratory , microbiology or material used when talking about genetic material, was surpassed in each session, and so I could harden with the issue, always being careful not to lose any detail. We must be careful when we are in contact with microorganisms, and even smaller, macromolecules. It is an enthralling world that most people don't understand, and although we do, not everyone can boast about the privilege of having it in front of their eyes. Seeing the changes of our project step by step , being intrigued to know what will be your next mission, listening disappointments because some parts have failed or the euphoria of knowing that we have reached the limit more than we had expected from a project in our category, are sensations that only can be lived with PIIISA. That is why I am proud to have selected this project in the forefront of my options, because it's worthy, and more challenging and promising than it may seem at first. Practices with the bacterium S*inorhizobium meliloti* , gloves, magnifier, pipette , the PCR , or the Scottish groove are just some of the steps of this production that I can mention and that have amazed me doing much

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more enjoyable the time dedicated to the project. I have to include my thanks to Francisco Martínez Abarca, our researcher that has known how to deal with our shyness, faults and remembered us the right way when we forgot something. All my expectations have been reached, and it has been more than an honor to have been part of the group, that between sweat and laughs, has managed to study how do mutations occur…

Miguel Sánchez Muñoz

At the beginning of this experience, I was hoping to do something big and I think we have far exceeded the level of large, even huge. The nerves that plagued me at first have become patience, effort and willingness to work. From the first day I took the bacteria plaques to home and watched every morning knew this project was going to be spectacular.

At first, we presented this project as "learning how to mutate the genes of a bacterium", but what they have achieved is to see how mutate genes of our bacteria; bacteria that we have grown and observed by ourselves. What started pick up colonies, continued to search for non-mucous bacteria on a agar plate; extraction of DNA from these bacteria, DNA amplification and subsequent electrophoresis to finally obtain the sequence of the amplified fragment, which it was analyzed.

Of all the bacteria we got isolate only analyzed the sequence of six of them. In any laboratory study, you should analyze and choose what and what not to study. Every isolates belonged to the species GR4, five of them were non-mucous and a mucous control, which would verify the gene mutated as we study: the ExpR gene.

After studying the DNA sequences of these bacteria we conclude that ExpR gene mutations reduce the number of amino acids with a stop codon. Now we prepare for the presentation of the project, wanting to do well and show what we have achieved, and even gain the final exposition. Come on, We can!

Al empezar esta experiencia, esperaba hacer algo muy grande y creo que hemos superado con creces el nivel de grande, incluso el de enorme. Los nervios que me atormentaban al principio se han convertido en paciencia, esfuerzo y ganas de trabajar. Desde el primer da en el que me llevé la placa de bacterias a casa y que observaba cada mañana sabía que esto iba a ser espectacular.

Al principio, nos presentaron este proyecto como "aprender cómo mutan los genes de una bacteria", pero lo que hemos conseguido es ver cómo mutan los genes de nuestras bacterias; bacterias que hemos crecido y observado nosotros. Lo que empezó con pinchar colonias, se continuó con la búsqueda de bacterias no mucosas en una placa de Agar; con la extracción de ADN de esas bacterias así como la amplificación de ese ADN y su posterior electroforesis para finalmente obtener la secuencia de ese fragmento amplificado, la cual analizamos.

De todas las bacterias que conseguimos aislar sólo analizamos la secuencia de 6 de ellas. En todo laboratorio hay que estudiar y elegir lo que analizar y lo que no analizar. Todas ellas pertenecían a la especie GR4, cinco de ellas eran no mucosas y un control mucoso, con el cual comprobaríamos como ha mutado el gen que estudiamos: el gen ExpR.

Tras estudiar las secuencias de ADN de estas bacterias llegamos a la conclusión de que las mutaciones del gen ExpR reducen el número de aminoácidos con un codón de parada. Ahora nos preparamos para la exposición del proyecto, con ganas de hacerlo bien y de demostrar todo lo que hemos conseguido, e incluso ganar la exposición. ¡Podemos!.

Marina Lara García

For me PIIISA project has been, essentially, decisive, since this was the final push to clear my head and make me see this is what I want to devote myself in the future. I started this project with the ideas a little lost, especially in the final results, but has been surpassed. I've been a very good experience. One of the things I liked most it was the practice that we have carried out in the laboratory. I had performed laboratory studies previously, but not at this level, and

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much less in Researcher Institute. I have learned many things about a topic related to microbiology, which I'm sure will continue to use if I head in the world of science, so it has been quite productive.
Regarding the work of the project itself, I must say I have been impressed to be able to carry out, or at least try something like this. I've learned to use the methods that scientists daily apply in microbiology, and that it was exciting. At the beginning, the project sounded too 'typical research coming out on television when they discover something important', but, I had never worked in this way. I've realized that expanding a bit my knowledge acquired in school, I could understand and follow the thread and participate in this project, which has also made me realize that there are times that although we do not have much knowledge, really are quite spacious and with them we can do great things.
I'm glad to have participated in this project and not in another. It has made me see that experimentation is truly exciting, and more on the environment as good as we have had in recent months. Next year, I hope to participate again, but in my research project carried out next year, one of the most similar possible to this one.
In conclusion, it has been a truly exciting and productive project.

Para mí el proyecto PIIISA ha sido decisivo, ya que este ha sido el último empujón para aclararme las ideas y hacerme ver que es esto a lo que quiero dedicarme en un futuro. Empecé este proyecto con las ideas un poco perdidas, sobre todo por el resultado final, pero ha sido superado con creces. Me he llevado una muy buena experiencia. Una de las cosas que más me ha gustado ha sido el desarrollo del proyecto que hemos llevado a cabo en el laboratorio. Ya había trabajado en el varias veces, pero no a este nivel, y ni mucho menos, en una estación experimental. He aprendido muchas cosas sobre el tema relacionado con la microbiología, las cuales estoy segura que voy a seguir utilizando si me encamino en el mundo de las ciencias, por lo que ha sido bastante productivo. Respecto al trabajo del proyecto en sí, he de decir que he quedado impresionada de poder llevar a cabo, o al menos intentar, algo como esto. He aprendido a convivir con los métodos que los científicos de microbiología utilizan a diario, y eso ha sido apasionante. Al principio me sonaba demasiado a 'las típicas investigaciones que salen en televisión cuando descubren algo importante', ya que yo nunca había trabajado de esta manera, pero me he dado cuenta de que ampliando un poco mis conocimientos adquiridos en el instituto, he sabido entender, seguir el hilo y participar en el desarrollo del proyecto, lo cual también me ha hecho darme cuenta de que hay veces en las que aunque creemos que no tenemos demasiados conocimientos, en verdad son bastante amplios y con ellos podemos hacer grandes cosas. Me alegro de haber participado en este proyecto y no en otro de los solicitados, incluso de los que pedí antes de este. Me ha hecho ver que la experimentación es algo verdaderamente apasionante, y más en el ambiente tan bueno como el que hemos tenido en estos meses. El año que viene, espero poder volver a participar, si no en el proyecto que lleve mi investigador a cabo el año que viene, en uno de los más similares posible. En conclusión, ha sido un proyecto verdaderamente entusiasmante y productivo.

Maria José Guerrero Dionisio
This project is very interesting. At first sight seemed that was going to be very difficult, I was scare a bit because I thought that I can't do this and I am not going to understand this, but when I started the project I saw I can do this without problem. It doesn't cost anything do it. On top of that it help me with biology, because in the high school I was giving just this theme.
What more I have liked is when we went to the laboratory and make it I same, and to see that there was happening rightly what they had said to you in advance.
I surprised it when we made the "electroforesis" I believed that I was not going to go out for us but it went out for us well.
In conclusion I have liked it very much, and I have learnt very much also.

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Este proyecto me ha parecido muy interesante. Al principio parecía que iba a ser muy difícil, te asustas un poco porque piensas que puede que no lo sepas hacer y los demás sí o no que no vas a entender lo que haces y yo creía que no me iba a enterar de nada, pero no, ha sido todo lo contrario no me ha costado mucho, y además me ha ayudado bastante con biología, porque en el instituto estaba dando justo ese tema.
Lo que más me ha gustado ha sido el ir al laboratorio y hacerlo tú mismo, y verlo como sucedía exactamente lo que nos habían explicado con antelación.
Cuando fuimos a la habitación para ver si nos había salido la electroforesis yo me sorprendí porque creía que no nos iba a salir pero si nos salió bien.
En conclusión me ha gustado mucho este proyecto, y también he aprendido mucho.

Teresa Ruiz-Rico

Microbiology is the science responsible for the study of microorganisms, small living creatures also known as microbes. It is dedicated to study the organisms that are only visible through a microscope: prokaryotes and simple eukaryotes. Microbes are considered all microscopic forms of life; these may be formed by a single cell (single cell) as well as small formed by equivalent cell cellular aggregates (without gradual differentiation), these can be eukaryotic (nucleated cells) such as fungi (cells not defined) core as bacteria.
However traditional microbiology has been especially occupied with pathogens from bacteria, viruses and fungi , leaving other microorganisms in disciplines like Parasitology and other categories of biology.
This is one of the projects as well as many others, it seems difficult, as 16 year-olds know not to see through a microscope, perform PCRs ... and many other things that perhaps are difficult to write or think. All my teammates like me, start one of the projects we thought would be ideal for us, one of our choices, ie, a project that we believe that we would serve, because maybe in a few years, we would not want to spend to take an interest in this or just learn and learn new subjects.
I remember that none understand anything, but slowly we were going trying, getting, practicing ... I'm satisfied with the effort because this project requires more hours and we all tried to do our part and go longer. But it is a job that in my point of view will result from generation to generation and if it works this way, someday discover something very important.

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 solo visibles a través de un microscopio: organismos procariotas y eucariotas simples. Son considerados microbios todos los seres vivos microscópicos , estos pueden estar formados por una sola célula (unicelulares), así como pequeños agregados celulares formados por células equivalentes (sin diferenciación gradual);estos pueden ser eucariotas (células con núcleo) tales como hongos (células sin núcleo definido) como las bacterias. Sin embargo la microbiología tradicional se ha ocupado especialmente de los organismos patógenos entre bacterias , virus y hongos , dejando a otros microorganismos en manos de la parasitología y otras categorías de la biología.
Este es uno de los proyectos al igual que otros muchos , que parece difícil , ya que chicos de 16 años no sabemos ver por un microscopio , realizar PCRs ... y otras muchas cosas que quizás son difíciles de escribir o pensar. Todos mis compañeros al igual que yo , comenzamos uno de los proyectos que pensamos que sería el ideal para nosotros , uno de nuestras elecciones , es decir, un proyecto que consideramos que nos iba a servir , porque quizás dentro de unos años , no querríamos dedicar a esto o simplemente interesarnos por aprender y conocer nuevas materias.
Me acuerdo que ninguno entendíamos nada, pero poco a poco lo íbamos intentando , consiguiendo , practicando ... Estoy satisfecha por el esfuerzo realizado ya que este proyecto

requiere más horas y todos hemos intentado poner de nuestra parte e ir más tiempo.
Pero es un trabajo que bajo mi punto de vista tendrá un resultado y si generación tras generación se trabaja de esta manera, algún día descubriremos algo muy importante

Paula Fernández Cruz
The project PIIISA 2013-2014, has been a unique experience, because I had never experienced before. It was very interesting that we ourselves used laboratory materials, which have led to bring at home, petri plates and we have seen in the first person as growing, and differentiating colonies, isolate them and get a clone.
Sinceresly, I found it hard to the project on time. Living in another town with my parents leaving their jobs late, it has prevented me going to the extra meetings, and therefore, I lost things in sessions I would have liked to be present.
Nevertheless, it has been a nice experience, because although it has cost us, not everyone can say, that at 16 years old, has participated in a RESEARCH; and although at the end I missed it, the whole project has gone smoothly and I believe that although we have not done all that our coordinator wanted to do, what we have done has finally gone successfully.

El proyecto PIIISA 2013- 2014, ha sido una experiencia única, pues nunca antes la había vivido.
Ha sido muy interesante el que hayamos usado nosotros mismos materiales de laboratorio, que nos hayamos llevado a casa placas petri y que hayamos visto en primera persona como crecían, y diferenciar las colonias y separar y obtener un clon.
Sinceramente, me ha resultado difícil ir al día, ya que el vivir en otro pueblo y el que mis padres salieran tarde de trabajar me ha imposibilitado el ir a las reuniones extras, y por lo tanto, me perdía cosas, en concreto, el momento en el que clonaron, momento en el que me hubiese gustado estar presente.
También, dábamos temas complejos, y a veces a mi y a mis compañeros nos costaba un poco seguir el hilo sin perdernos en algunos momentos.
A pesar de todo, ha sido una experiencia bonita, porque aunque nos ha costado, no todo el mundo puede decir que con 16 aňos ha participado en una investigación, y aunque el final me lo he perdido, todo el proyecto ha ido sobre ruedas, y creo que aunque no hayamos hecho todo lo que nuestro coordinador quería hacer, lo que hemos hecho, ha salido de forma correcta.

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Use of plant extracts to block bacterial biofilm formation

Jose A. Lizana[1], Sergio López[2], Ana Marchal[2], Úrsula Serrano[3], Diego Velasco[1] and Manuel Espinosa-Urgel[*4]

[1]*IES Alhambra, Beethoven 2, 18006 Granada, Spain*
[2]*IES Francisco Ayala, Avda. Francisco Ayala s/n, 18014 Granada, Spain*
[3]*CDP Compañía de María, Avda. Fuerzas Armadas s/n, 18014 Granada, Spain*
[4]*Estación Experimental del Zaidín. CSIC. Profesor Albareda 1, 18008 Granada, Spain*
[*]*Corresponding author:* ***e-mail:*** *manuel.espinosa@eez.csic.es*

SUMMARY

We live surrounded by bacteria; in fact, in only one gram of soil we can find millions of bacterial cells. Our body houses more than 10^{14} bacteria. Even though some of these microorganisms can cause us problems, such as caries, actually most of them help in the proper functioning of our organism. Generally, bacteria coexist setting up communities associated to solid superficies, this is to which we refer as biofilms, that serve as a survival strategy. This type of formation cause serious sanitary problems for both humans and animals. Nowadays, chemical or natural compounds able to block this formation are looked for. In this project, we have set out how to use extracts of different plants with the purpose of testing their effects against biofilms of two bacterial species: *Escherichia coli* and *Pseudomonas putida*.

INTRODUCTION

Bacteria are single-cell prokaryotic organisms, that is to say, they can only be observed with a microscope and they are constituted by only one autonomous cell that has no nuclear membrane. They are widely spread all around Nature; we find them in all environments with life possibility: air, water, land, inside the human body, animals and plants. Even some bacteria have been found in meteorites. Their seniority on earth is very noticeable, since the most archaic fossils known are bacteria. Depending of environmental circumstances, bacteria can suffer modifications that are generally not definitive, because when the causes that motivated them disappear, bacteria return to their normal unicellular forms and properties.

In nature, generally we find bacteria living in communities associated to solid surfaces, called biofilms. This fact was already reported by Arthur Henrici in 1933, when he observed that most aquatic microorganisms were not in the form of individual cells swimming freely but aggregated over solid submerged surfaces [1]. However, in the last decade the importance of biofilm formation as a microbial survival strategy has been recognized in a generalized manner, as well as its huge impact on many human activities [2,3]. The ability of microorganisms to colonize solid surfaces in the biofilm form is a serious problem in human and animal health as these populations are more resistant to antibiotic action [4]. This fact allows pathogenic bacteria to survive and wait for the occasion to invade a new host, or to multiply when the immune system decreases its activity. Thereby, the importance of biofilms in infections arising from prostheses and medical implants, keratitis associated to contact lens wearing, or colonization of lung tissue in patients with cystic fibrosis has been highlighted [5]. Biofilms can also generate deterioration of materials and cause health risks in the food industry. Nevertheless, not all is negative; the ability of microorganisms to colonize solid surfaces has been taken advantage of in industrial process, such as the case of wastewater treatment.

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All these factors, especially its clinical importance, explain the great interest in studying biofilms and the search for new naturally or chemically synthesized compounds capable of blocking its formation. One promising approach is to identify compounds of plant origin that can have antimicrobial or antibiofilm effects. Throughout human history, many plants have been used because of their healing effects. One example is *Aloe vera*. In historical documents from the ancient Romans, Greeks, Indians and Arabs it is possible to read about its medicinal use to treat several diseases, such as skin or hair diseases, or even about its use in the embalming process. Aloe vera has proven to be quite useful in cases of acne and bronchitis. Chamomile (*Chamaemelum nobile*) flowers, are another paradigm of traditional remedy, in the form of infusion, to treat eye irritation and infection. Another well-known example is garlic (*Allium sativum* L.). It has been used in almost every culture. In ancient history, this plant was known for its beneficial effects on controlling heart diseases, bites, intestinal parasites and tumors, and also for being a great meat and fish preservative. Some of its presumed or known properties are: it improves blood circulation, it is a bactericide because of its high sulphur content, it protects against cancer and is a great skin sanitizer in case of bites, fungi or burns. Recently, it has been reported that a compound present in garlic, ajoene, can interfere with signaling beween bacterial cells [6], and by this mechanism it could possibly block infection and alter biofilm formation.

In this project we have focused on the effects of several plant extracts on biofilm formation, taking advantage of the botanical diversity at the Estación Experimental del Zaidín or using commercial dry plant material. We have identified plants that reduce biofilm formation by two different gram-negative bacteria.

MATERIALS AND METHODS

Bacteria and culture medium used

To check the extracts' inhibition capacity upon biofilms we tested them with two different bacteria:

- *Escherichia coli* MG1655. It is an inocuous laboratory strain, but some strains of this species are of medical interest because they cause several intestinal illnesses [8].
- *Pseudomonas putida* KT2440. It is a plant-beneficial bacterium that can colonise different environments [9]. Certain *Pseudomonas* species, such as *P. aeruginosa*, are opportunistic human pathogens.

Cultures were grown were grown at 30°C (*P. putida*) or at 37°C (*E. coli*) in LB medium [10].

Plant extracts

Plants used in this work are summarized in Table 1. Certain plant materials are not identified in the table for results protection reasons. We collected most plant material (leaves, fruits, petals...) from the gardens at the Estación Experimental del Zaidín. Besides, we included other plants according to their medical attributes, based on information found on the internet. These were obtained from commercial preparations as dry material, or from potted plants. Each extract was assigned a number.

To obtain the extracts, we put the collected material (between 0.5 and 5 g) in sterile tubes with 10 ml of an etanol:water solution (1:1). We added 10 glass beads (3 mm diameter), to help in the grinding. The tubes were agitated in a vortex at maximum velocity for two minutes and we took the extract solution out with a syringe. We used Whatman filters (0.45 µm) to remove particles and ensure the extracts were sterile.

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Table 1. Extracts used in this work

Extract number	Common name	Scientific name	Material	Known attributes or traditional medicine uses [7]
1	Cypress	*Cupressus sempervirens*	Leaf	antiseptic
2	Undisclosed			
3	Lavender	*Lavandula stoechas*	Leaf	antiseptic
4	Rose	*Rosa sp.*	Petals	treatment of bronchial infections
5	Cherry laurel	*Prunus laurocerasus*	Leaf	antispasmodic
6	Sago palm	*Cycas revoluta*	Leaf	-
7	Rose	*Rosa sp.*	Leaf	-
8	Wall cotoneaster	*Cotoneaster horizontalis*	Leaf (Fruit)?	-
9	Olive	*Olea europaea*	Fruit	-
10	Undisclosed			
11	Olive	*Olea europaea*	Leaf	reduction of fever
12	Aloe vera	*Aloe vera*	Leaf	treatment of skin diseases
13	Chamomile	*Chamaemelum nobile*	Dry flowers	treatment of eye inflammation
14	Ginkgo	*Ginkgo biloba*	Dry leaves	circulatory system
15	Lime flower	*Tilia sp.*	Dry leaves	sedative

Biofilm assays

Biofilm formation was studied in plastic 24-well plates or in glass test tubes. In 24-well plates, 1 mL of liquid LB medium was inoculated with 5 µl of an overnight culture of the chosen bacterium and we added increasing volumes of extract. As a control, the same volumes of ethanol:water (1:1) were used. Plates were incubated at 30°C or 37°C for 4, 7 or 24 h and then the liquid was removed and the biofilms stained. In test tubes, we mixed 2 mL of liquid medium (LB), 20 µL of extract and 40 µl of an overnight culture of the chosen bacterium. As a control, 20 µL of ethanol:water (1:1) was used instead of the extract. The tubes were incubated with orbital agitation at 30°C. After 2h, the cultures were taken and photographed on a black background. We then removed the liquid of the tube and stained the biofilms as described in the next section before photographing on a white background. This process was repeated at 3 and 5 hours.

Staining techniques

To visualize and quantify biofilms we used a 0.4% crystal violet solution, a dye that colours bacteria's polysaccharides. 1.5 mL or 5 mL of crystal violet were added to each well or tube, respectively. We let the solution rest for 10 minutes and then the dye was removed. The tubes and plates were washed three times with water and allowed to dry. The intensity of the violet colour that remains on the tube/plate is indicative of the amount of bacterial biomass attached to the surface. To obtain quantitative data, the dye was solubilized with 70% ethanol and colour intensity was measured in a spectrophotometer as absorbance at a wavelength of 540 nm (A_{540}).

Microscopy

Biofilm structure was studied using phase contrast microscopy. For *E. coli*, the crystal violet stained plates were directly observed with an inverted microscope (Euromex). In the case of *P. putida*, cultures were grown in LB in 6-well plates with extract or control (ethanol:water) with a 40 mm glass coverslip placed in the well. We let biofilms form on the coverslip for 6 hours and then we observed them with a Zeiss microscope.

Swimming motility

Bacterial motility was tested in Petri plates with semi-solid medium (LB + agar 0.3%), containing plant extract or ethanol:water as control. 2 µl of a grown culture were inoculated in the center of the plate and then plates were incubated at 30°C for 16 h. Swimming halos were visualized, and their diameter was measured.

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RESULTS AND DISCUSSION

Biofilms on glass

First we tested the effect of adding extracts on *P. putida* biofilm formation in glass tubes (40 µl of extract in 2 ml). Bacterial cultures were observed at different times and then stained with crystal violet as described in Materials and Methods. *P. putida* normally forms a ring on the tube surface, in the area where there is more oxygen. Results obtained with some extracts are shown in Figure 1. At two hours, the biofilm with extract 2 is a bit more diffuse than in the control. With extracts 6 and 10 results are similar to the control. Extracts from *Olea europaea* and *Aloe vera* at this time produce a diffuse biofilm that covers all the surface in contact with the culture. At three hours, without staining there is less biomass visible on the surface with extracts 2, 9 and 10 than in the control. After staining, with extract 2 some of the biofilm has shed. With extract 9 we observed spread of biofilm and less biomass. There is less staining with extract 10 than in the control. No clear differences were seen with extracts 6 and 12.
Finally, at five hours, clear reduction in the attached biomass is only observed with extract 10. Surprisingly, at this time *O. europaea* extract seems to cause increased adhesion.

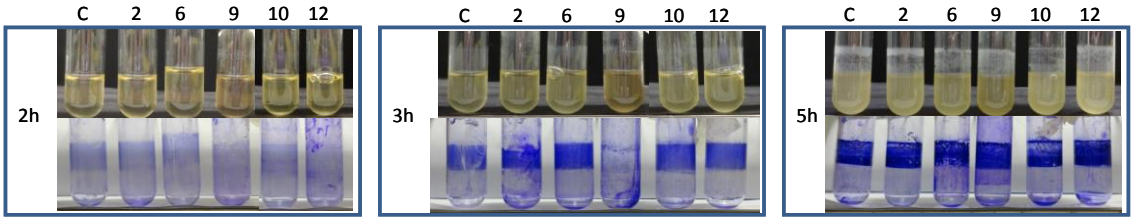

Figure 1. Biofilm formation by *Pseudomonas putida* in glass tubes in the presence of plant extracts or ethanol:water as control (C).

Next we tested the effect of increasing concentrations of plant extracts on biofilm formation by *E. coli* in glass tubes. 40, 60 or 80 µl of extract were added to 2 ml of medium and cultures were incubated for 16 h. Biofilms formed were stained with crystal violet. Results with two extracts are presented in Figure 2. Both of them blocked completely biofilm formation when 80 µl were added.

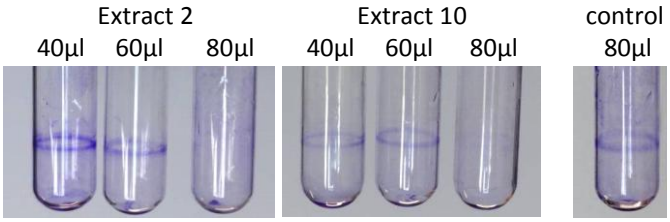

Figure 2. Increasing concentrations of extracts 2 and 10 block biofilm formation by *E. coli*.

Biofilms on plastic: quantification

We tested biofilm formation by *P. putida* in 24-wells plates to measure at the same time the effect of several extracts and concentrations. To do this we stained biofilms formed after 7 hours with crystal violet, and then solubilized the dye with ethanol 70%. We then measured absorbance at 540 nm in a spectrophotometer to quantify the color intensity. The amount of dye is proportional to the biomass attached to surface. Results are shown in Figure 3.

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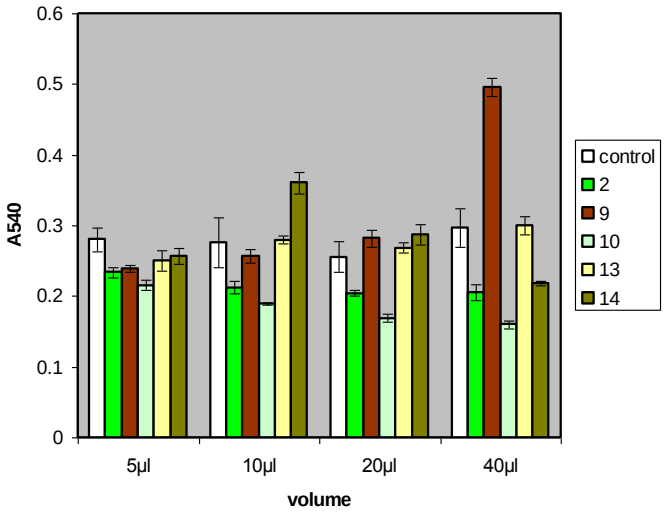

Figure 3A. Biomass of *P. putida* attached to plastic surface after 7 hours of growth in the presence of increasing amounts of extracts, or of ethanol:water as control.

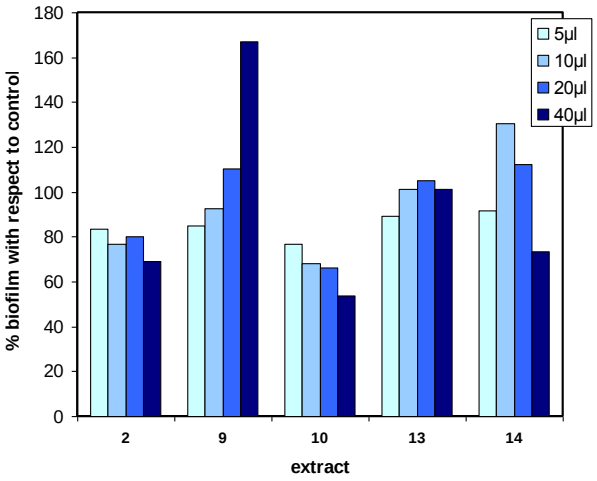

Figure 3B. Results from figure 3A are represented as relative biofilm formed with each extract and concentration, compared to the same volume of ethanol:water (value of 100%).

Extract 2: We observe how the amount of biofilm decreases with increasing volume of extract in relative absorbance values. It is without a doubt the 2nd more effective in these tests.
Olive (fruit): Although low doses tend to slow the progression of biofilm (10 µl, 20 µl), with increasing volume of extract its formation is stimulated.
Extract 10: It is the most efficient extraction of those analyzed. As increasing extract is supplied, the biofilm diminishes, reaching 55 % reduction (with 40 µl of extract).
Camomile: It is the extract that has less effect on the biofilm. It does not reduce or stimulate it.
Ginkgo biloba: At low doses (5 µl) it has no effect to the biofilm. When the quantity increases (a number between 10 µL and 15 µl), it tends to stimulate the formation to a certain point, from which it has a inhibitory power.

With this information we put attention to the two extracts that appear to give better results: 2 and 10. It was with them with which we did the following test with *E. coli*. We measured the percentage of biomass relative to the control in two time periods, at 4 h and 7 h. The results were very similar to the previous test (Figure 4): Extract 10 gives the best results in both periods (55% vs. 71% at 4h and 56% vs. 74%. at 7h). Through the use of this extract we could reduce the biofilm by almost half at four hours and seven hours.

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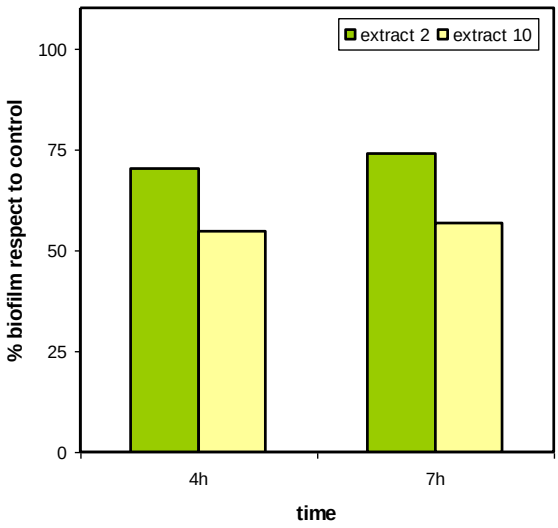

Figure 4. Biomass of E. coli attached to plastic at different times of growth in the presence of extracts. Results are represented as relative biofilm formed with each extract, compared to the ethanol:water control at that time (value of 100%).

Observation of biofilms under the microscope

We used the microscope to observe the structure of the biofilms formed by *E. coli* and *P. putida* (Figure 5). For *E. coli* we observed biofilms on plastic that were stained with crystal violet, and for *P. putida* we used contrast phase microscopy to observe biofilms on glass coverslips.

In *E. coli*, in the case of the control most areas are covered with biofilm, with areas where bacteria are very compacted. With extract 2 the results are good (the colored areas are the minority) but with extract 10 they are excellent (much less biofilm), although some areas show also compacted aggregates of cells. With other extracts such as that of olive fruit there is hardly difference with the control (result not shown).

In *P. putida* the results are similar, but extract 10 gave irregular results with most areas with very few bacteria and some others showing compact aggregates (not shown).

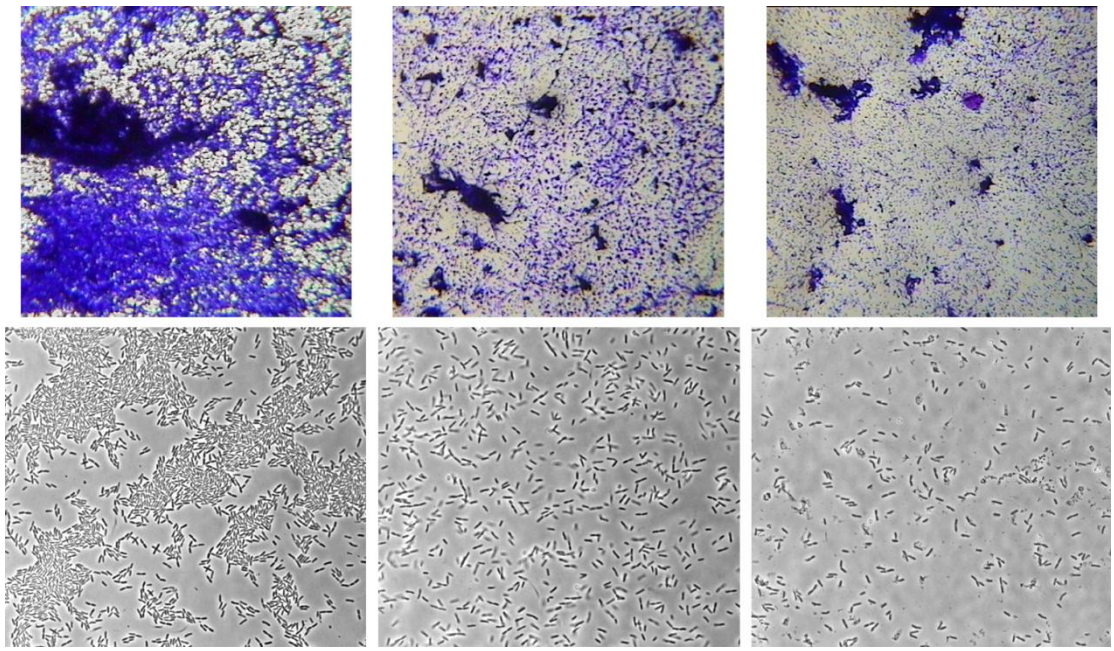

Figure 5. Microscopy analysis of biofilms of *E. coli* (top) and *P. putida* (bottom). Left: control without extract. Center: extract 2. Right: extract 10.

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Swimming motility

Biofilm formation starts with bacteria swimming towards a surface. We tested if plant extracts altered motility of *E. coli* in plates with a semi-solid medium (LB + agar 0.3%), where bacteria can swim, forming a halo from the point of inoculation. Results are presented in Figure 6. With extract 10 the size of the halo is smaller, indicating less motility. Olive and ginkgo stimulate the expansion of the bacteria, whereas extract 2 has no apparent effect.

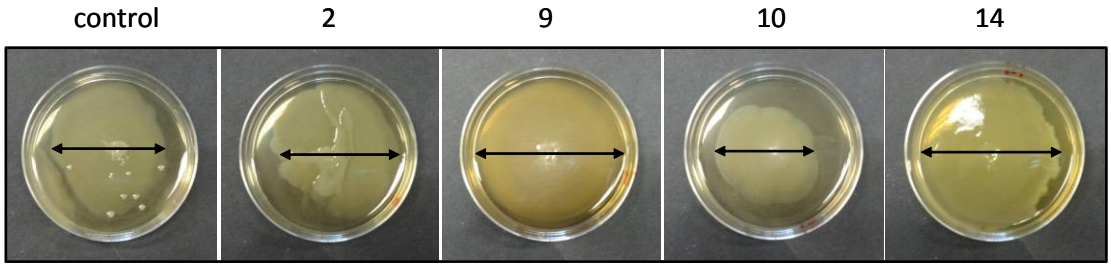

Figure 6. Swimming motility of *E. coli* in the presence of different extracts.

CONCLUSIONS

We have identified two plant extracts that have inhibitory effect on bacterial biofilms on different surfaces. Future experiments are required to study these extracts in detail and their potential as antimicrobials.

ACKNOWLEDGEMENTS

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MY OWN IDEAS

Diego Velasco IES Alhambra

This project has been a great experience because I could learn a lot about microbiology and how scientists work with different types of bacteria in the laboratory.

In our project we worked with two types of bacteria and thirteen types of plants. I've learned about the scientific method and I'm very satisfied with our work and results, it's true that only two extracts have a real and important effect in the inhibition of biofilm but my research team have done a good work. Also we learned to use some of the laboratory equipment such as pipettes and microscopes.

In conclusion, I think PIIISA is a great idea for made young children get closer to the scientific comunity. This project can help us to improve our knowledge of the scientific methods, materials and experiments.

José Alberto Lizana IES Alhambra

My experience in PIIISA 2014 has been very positive. This project opened the doors to enter a well equipped laboratory where there are many researchers working, and we have also worked in this laboratory. We have had the opportunity to work as if we were the researchers of truth, being in first person with laboratory and working with him. PIIISA opened me the mind to my future studies and also to mark me a goal in my career. I hope this project to proceed, and I recommend everyone try to access PIIISA, because I assure you that you will love.

Ana Marchal IES Francisco Ayala

The PIIISA project has seemed to me a very interesting, useful thing which shouldn't stop being organized. In my opinion, it means an opportunity to put into practice the theory taught at the high school, to strengthen that knowledge, furthermore it also shows us how one part of the day-to-day laboratory work is, which helps us to know more about this job.

I've learnt that patience, for example, is something required and indispensable in this job, as the team working. Also the constancy is very important because there will be days when you will get excellent results nevertheless, it might also be days when results won't be as good as the others. Despite of that, you have to keep on trying and trying.

On my project in particular, I believe that the idea of using plants against the micro organism action is something very useful and beneficial. I also found curious the fact of plants have been used against diseases for a thousand years.

Úrsula Serrano CDP Compañía de María

I am really grateful for being given the opportunity of participating in the PIIISA 2014 project. It has been an unique opportunity to be able to participate in activities related to the microbiology field, with professional researchers, suitable equipment and real research projects. Researchers have helped us with loads of patience and dedication, showing us some lab working techniques and how interesting science can be.

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Polyacrylamide gel electrophoresis: a powerful tool in the food-processing sector

Nancy Álvarez Mittelmann[1], Eva Diago Saavedra[1], Eva Mª García Calvo[1], Azahara Montero Méndez[1], Juan Carlos Pardo Carmona[1], Sandra Pérez López[1], Estefanía Soriano Calvo[1], Cristina Tortosa Jiménez[1], Carmelo Ruiz[2], Francisco Javier Corpas[2], José Manuel Palma[2]

[1]IES Zaidín Vergeles, Avda. Primavera s/n. Granada
[2]Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology and Agricultural Food. Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, CSIC, Granada.
e-mail: carmelo.ruiz@eez.csic.es

SUMMARY

Polyacrylamide gel electrophoresis (PAGE) is an analytical and powerful technique widely used in research to analyze proteins and nucleic acids. In this project, this technique has been used to study the protein pattern in different organs (leaves and fruits) and plant species. Specifically, we used pepper and pea plants, which have agronomical interest, and Arabidopsis as a model plant. Two different applications of PAGE, designated as SDS-PAGE and non-denaturing PAGE, useful to investigate the pattern of polypeptides and to assay in situ the enzymatic activities, particularly superoxide dismutase (SOD), were developed, respectively. The results show that each plant sample has a specify protein pattern and the number and type of SOD isoenzymes varies depending on the organ and the plant species.

INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is an analytical and powerful technique widely used in research for proteins and nucleic acids. Nowadays, there are two main types of gel electrophoresis: one dimension and two dimensions. One dimension PAGE includes **SDS-PAGE** which is the most widely used electrophoresis technique to separate proteins primarily by mass. In this type of electrophoresis the samples are heated, so proteins are denatured by breaking of the disulfide bonds and simultaneous treatment with SDS (sodium dodecyl sulfate), an anionic detergent, gives negative charge to each protein in proportion to its mass. Thus, when a current is applied, all SDS-bound proteins in a sample will migrate through the gel towards the positively charged electrode. Proteins with less mass migrate more quickly through the gel than those with greater mass because of the sieving effect of the gel matrix. On the other hand, non denaturing PAGE, also called **native PAGE**, separates proteins according to their mass/ charge ratio. This technique allows detecting *in situ* some enzymatic activities. In the case of two-dimensional PAGE (2D-PAGE), proteins are separate by sequentially by their isoelectric point in the first dimension, also called isoelectric focusing (IEF), and by the mass in the second dimension, as in the SDS-PAGE.

In this project, several of these techniques have been used to study the protein pattern in plant samples from different origins. In particular, we have used pepper and pea plants which have agronomical interest and *Arabidopsis thaliana* as a model plant. Thus, we have two different applications of PAGE designated as SDS-PAGE and non-denaturing PAGE which are useful to distinguish the pattern of polypeptide and to assay *in situ* an enzymatic activity, specifically superoxide dismutase (SOD).

Superoxide radical ($O_2^{\cdot -}$) is generated as a by-product in aerobic organisms from a number of physiological reactions such as the electron flow in the chloroplasts and mitochondria and from some redox reactions in cells. It can react with hydrogen peroxide (H_2O_2) to produce hydroxyl radical ($\cdot OH^-$), one of the most reactive molecules in the living cells. Hydroxyl radical can cause the peroxidation of membrane lipids, breakage of DNA strands, and inactivation of enzymes in cells (for reviews, see Bowler

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et al., 1992; Mehdy, 1994). To ameliorate the damage caused by hydroxyl radical formed from superoxide radical and hydrogen peroxide, organisms have evolved mechanisms to control the concentration of the two reactants. Superoxide dismutase (SOD) is a group of isozymes functioning as a superoxide radical scavenger in the living organisms. The reaction catalyzed by SOD is as follows:

$$O_2^{-} + O_2^{-} + 2H^{+} \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

There are three types of SOD isozymes which are classified according to the metal at the catalytic center, copper and zinc (CuZn-SODs), manganese (Mn-SODs) or iron (Fe-SODs). The most abundant SODs in plants are the CuZn-SODs, which are found mainly in the cytosol and chloroplasts. SOD is an important enzyme family in living cells for maintaining normal physiological conditions and under several stress conditions.

SPECIFIC AIMS

1. Protein extraction and separation from different organs and plant species using denaturing and non-denaturing polyacrylamide gel electrophoresis.
2. After SDS-PAGE and protein staining, analysis of protein pattern in plant samples
3. After non-denaturing PAGE, analysis of superoxide dismutase (SOD) and identification of number and type of the different SOD isozymes present in the plant samples.

MATERIALS AND METHODS

Plant materials and growth conditions

Pepper (*Capsicum annuum* L., type Dulce italiano) and pea (*Pisum sativum* cv Lincoln) plants of approximately 30-days were grown in aerated optimum nutrient solution (Airaki et al. 2011). *Arabidopsis thaliana* ecotype Columbia seeds were germinated and grown on soil plus vermiculite (1:3). Plants were grown in a growth chamber at 24 °C, under a 16 h photoperiod and a light intensity of 190 µE m^{-2} s^{-1} for a period of 4 weeks. Pepper fruits were purchased at the local market (Fig. 1).

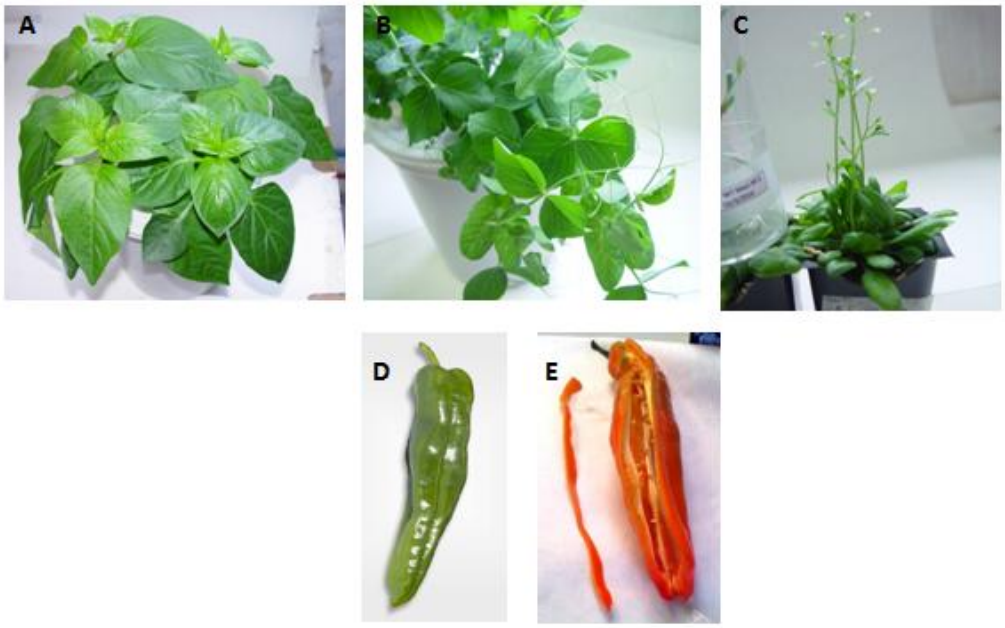

Figure 1. Phenotype and appearance of the plant materials used in our experiments. (A) Pepper plants. (B) Pea plants. (C) *Arabidopsis* thaliana plants. (D and E) Pepper fruits.

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Crude extracts of plant tissues

The plant samples were homogenized (relation 1/3; w/v) in assay tubes in buffer 50 mM Tri-HCl, pH 7.8 containing 10% (v/v) glycerol, 0.1 mM EDTA and 0.2 % (v/v) Triton X-100 (Table 1). Then, samples were filtered through two nylon layers and centrifuged at 27,000 g for 26 min at 4 °C. The obtained supernatants were used for the assays.

Table 1. Plant material used

Sample	Fresh weight (g)	Buffer (mL)
Green pepper	0.68	2.04
Red pepper	1.78	5.34
Pepper leaves	1.06	3.18
Pea leaves	0.98	2.94
Arabidopsis leaves	0.49	1.47

Protein concentration

Protein content was determined according to the method of Bradford (1976) using the commercial Bio-Rad Protein Assay (Hercules, CA) kit, with bovine serum albumin (BSA) as standard. In this method the Bradford dye (Coomassie Brilliant Blue G-250) forms a complex with the proteins and the increase of absorbance at 595 nm is proportional to the amount of bound dye (Bradford, 1976).

The calibration curve was prepared with using 0, 3, 6, 12 and 18 µg BSA in a final volumes of 800 µL. Then, 200 µL of the BioRad reagent were added given a final volume of 1 mL (Fig. 2). The plant samples (pea, pepper and Arabidopsis) were prepared in similar volume with adequate dilutions. After 10 to 15 min, the absorbance at 595 nm was measured in a spectrophotometer Beckman. The concentration was determined usinf the calibration curve (Fig. 2).

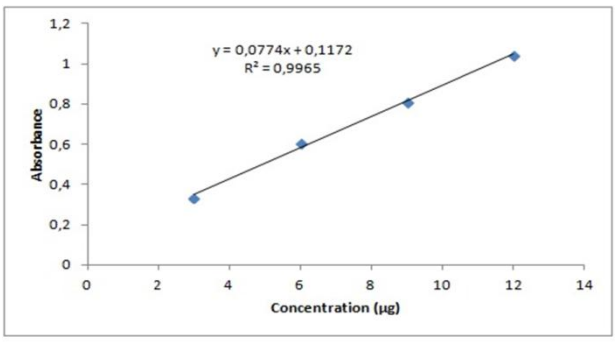

Figure 2. Calibration curve obtained by method of Bradford using BSA as standard

SDS-PAGE and protein staining with Coomassie Blue

Four polyacrylamide gels were prepared in a Mini – Protean III equipment of Bio Rad (Fig. 3), according to the indications given in Table 2. The electrophoresis samples were prepared in a loading buffer made with 125 mM Tris – HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.006 % (w/v) bromophenol blue and 10 mM DTT, and the protocol indicated in Table 3 was followed.

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Before loading, samples were heated in boiling water for 5 min.

After that, the gels were run at 100 V for 15 – 20 min; then, we applied 200 V for 45 min. The electrode buffer used for the cathode was 2.5 mM Tris – HCl, pH 8.3, 0.192 M glycine and 0.1% (w/v) SDS. The anode buffer was similar but without SDS. The amount of sample loaded onto gels depended of the sensibility of the staining method that we used.

Gels were stained with 0.1% (w/v) "Coomassie Brillant Blue" R-250,. This was prepared in 50% (v/v) methanol and 10% acetic acid . The staining of gels was run for for 30 min. Then, gels were clarified with 40% (v/v) methanol and 10% (v/v) acetic acid, until blue band appeared over a colourless background.

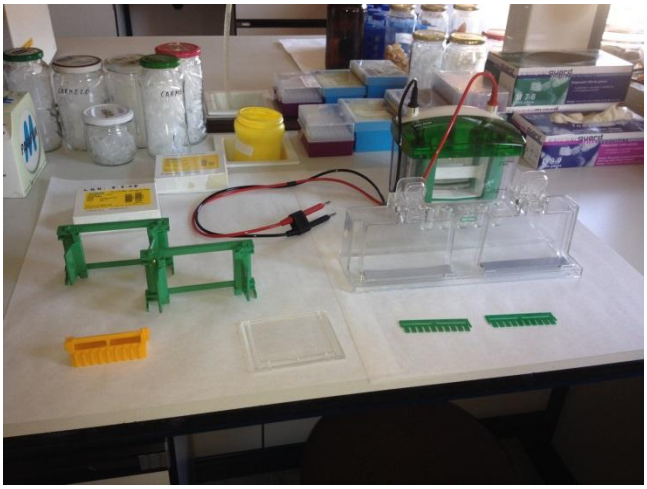

Figure 3. Components of a vertical electrophoresis cell (BioRad) used in our experiments

Table 2. Gel composition for running and stacking gels in SDS_PAGE.

	Running gel (12 %)	Stacking gel (4 %)
30 % Acrylamide	3.7 mL	0.50 mL
4 × Running gel	2.3 mL	-
4 × Stacking gel	-	1.00 mL
10 % (w/v) SDS	90 µL	40 µL
50 % (v/v) Glycerol	0.9 mL	-
Milli-Q H_2O	2.1 mL	2.46 mL
VACUUM 10 min		
10 % (w/v) Ammonium persulfate	50 µL	50 µL
TEMED	5 µL	10 µL

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Table 3. Samples distribution in for loading SDS-PAGE gels. SDS, sodium dodecyl sulfate. M, Molecular Weight Markers.

1st well	2nd well	3rd well	4th well	5th well	6th well	7th well	8th well	9th well	10th well
-	M	SDS	Pea leaves	Pepper leaves	Green pepper fruits	Red pepper fruits	Arabidopsis leaves	SDS	-
-			30 µL	30 µL	30 µL	30 µL	30 µL	30 µL	-

Non-denaturing PAGE and superoxide dismutase activity

In this electrophoresis, proteins were exposed to migration under native conditions. Proteins migrate depending on their charge, size and form.

We used a BIO Rad Mini-Protean III equipment where gels of 6.5 x 8 cm and 1.5 mm of thickness were made. Gels were prepared with a 10 % (w/v) polyacrylamide concentration of in Tris-HCl 377 mM, pH 8.9 buffer, as indicated in Table 4. Sample homogenates were prepared in 10 % (v/v) glycerol and 0.006 % (w/v) bromofenol blue and loaded directly onto gels as shown in Table 5, . We used an intensity of 15 mA for gel during 30 min, and then 25 mA until the blue of bromofenol reached practically the end of the gel. The electrode buffer used was 38 mM glycine, adjusted to pH 8.2 with the gel buffer.

SOD isoenzymes were identified in gels by a photochemical method. Gels were stained with 2.45 mM NBT for 20 minutes with constant agitation, followed by an 15 minutes incubation in a solution of µM 28 riboflavine, 28 mM TEMED in 50 mM potassium phosphate buffer (pH 7.8). The incubation were performed in darkness. Developing of gels was carried out with visible light during 15 min at room temperature.

Table 4. Gel composition for non-denaturing PAGE.

Gel 10 %	
30 % Acrylamide	9.00 mL
Gel buffer (1.5 M)	6.75 mL
Milli-Q H_2O	9.85 mL
VACUUM 10 min	
7 % (w/v) Ammonium persulfate	1.45 mL
TEMED	100 µL

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Table 5 Samples distribituion in non-denaturing PAGE

A.

1st well	2nd well	3rd well	4th well	5th well	6th well	7th well	8th well	9th well	10th well
-	-	Pea leaves	Arabidopsis leaves	-	Pepper leaves	Green pepper fruits	Red pepper fruits	-	-
-	-	40 µL	50 µL	-	50 µL	50 µL	50 µL	-	-

RESULTS

SDS-PAGE and protein staining

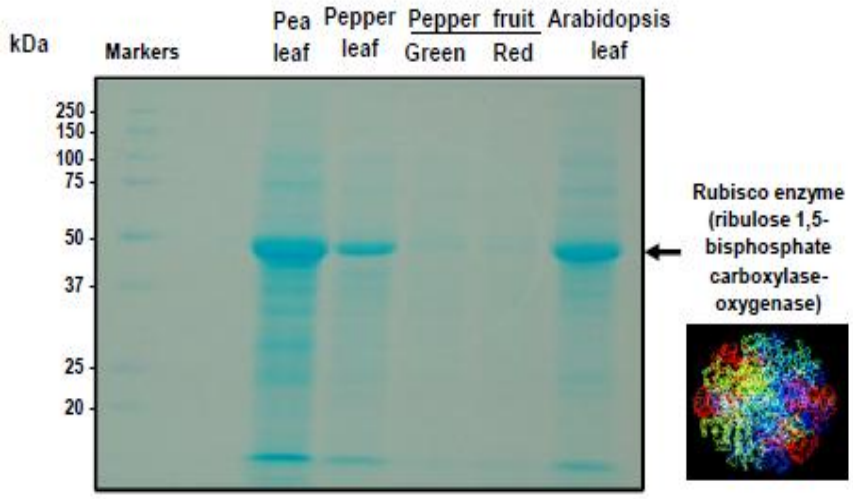

Figure 4. Electrophoretic polypeptide patterns under denaturing conditions using different types of plant samples. The migration of the most abundant protein in leaves (Rubisco) is indicated.

Non-denaturing PAGE and superoxide dismutase (SOD) activity

Figure 5. Electrophoretic pattern of SOD isozymes from distinct plant samples.

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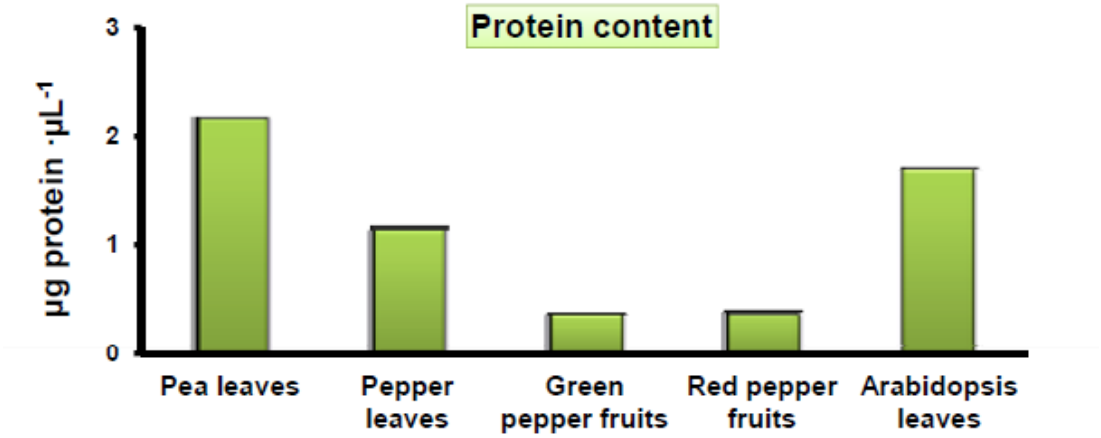

Figure 6. Protein concentration (expressed as µg/ µL) of the five plant samples studied in this work.

CONCLUSIONS

1. The protein content in fruits is lower than in leaves and this could be due to the higher water content in pepper fruits.
2. The protein pattern is different depending on the plant species and the analyzed organs. The most prominent band in leaves is the Rubisco which is present in chloroplasts and participates in the photosynthesis.
3. We have identified until four superoxide dismutase (SOD) isoforms in pepper fruits but this pattern changes depending of the analyzed organs and the plant species.

ACKNOWLEDGEMENTS

This work was supported by the ERDF-cofinanced grant AGL2011-26044 from the Ministry of Science and Innovation, Spain. Antonio Quesada Ramos, Professor at the IES Zaidín-Vergeles, Granada is specially acknowledged for his continuous assistance to develop our work.

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MY OWN IDEAS

Nancy Álvarez Mittelmann, IES Zaídin-Vergeles

A mí personalmente, me parece fantástica toda esta tecnología desarrollada y aplicada a moléculas orgánicas para poder analizarlas. Con el desarrollo de la tecnología cada vez nos vamos adentrando en el mundo de lo más pequeño para poder entender y saber cómo funcionamos.

Ésta fue una experiencia positiva para mí ya que fue mi primer contacto del mundo de la investigación. Tuve el privilegio de trabajar junto a investigadores y corroborar que la investigación es primordial para el desarrollo de un país. El futuro es la biotecnología y dentro de las técnicas que engloba, la electroforesis es una de ellas.

En este proyecto, como el título indica, la electroforesis es de gran utilidad en el sector agrícola-alimentario, dado que podemos aplicar este proceso para identificar proteínas. El DNA contiene la información necesaria para sintetizar proteínas, por tanto, con la identificación de las proteínas, podemos diferenciar a qué gen pertenece. Por supuesto, este proceso se complica dado que un gen puede dar lugar a varias proteínas.

Centrándonos de nuevo en la idea que estamos tratando en este caso con hortalizas y vegetales, si por ejemplo, podemos identificar una proteína que desarrolle una característica de resistencia a la sequía (un mal muy frecuente dada nuestra climatología española), podríamos ser capaces de incorporar esta resistencia a otras plantas, que a su vez daría lugar a un ahorro enorme en el agua en el sector de la agricultura. Esta técnica, de poder realizarse, también se podría extrapolar a casos en donde las hortalizas a tratar sean vulnerables a plagas y a su vez, un menos uso de fertilizantes que provocan impactos medioambientales en el suelo.

Eva María García Calvo, IES Zaidín-Vergeles

Si uno de los objetivos del proyecto PIIISA es acercar el estudio de las ciencias al alumnado e incentivarles en la investigación, lo consiguen con creces.

Este proyecto me ha abierto la mente a un nuevo campo de estudio que en un principio no me llamaba mucho la atención. Las plantas, su proceso de maduración, la elección de muestras, el tratamiento cuidadoso y escrupuloso con ellas, poder "ver" las proteínas que tienen y cuantificarlas, y además aprender a usar las herramientas adecuadas para descubrir hechos desconocidos. Es apasionante el mundo de la investigación, ahora lo sé.

Siendo alumna de un ciclo de FP, agradezco el esfuerzo por parte de la Estación Experimental del Zaidín del CSIC, del departamento de bioquímica, biología celular y molecular de plantas y de los investigadores Carmelo Ruiz, Francisco Javier Corpas y José Manuel Palma por hacer posible que esta experiencia haya llegado a nosotros. Si hubiera sido posible, habría participado en él mucho antes y espero que este proyecto dure muchos años y siga acercando la ciencia a nuestros futuros y pequeños Einsteins.

"Escucha, mira, huele, toca, gusta… el mundo está ahí para descubrirlo."

Azahara Montero Méndez, IES Zaidín Vergeles

Este proyecto nos ha dado la oportunidad de realizar una técnica de electroforesis en geles de poliacrilamina y aplicarla al análisis bioquímico y molecular de productos hortofruticulas permitiendo la obtención de un panel de marcadores proteicos de calidad.

Me siento orgullosa de haber participado en este proyecto, ya que nos han dado la oportunidad de adentranos de lleno en el mundo de la investigación, así como de usar sus instalaciones, material y medios especializados.

Creo que el proyecto PIIISA es una gran oportunidad de mostrar a los jóvenes el mundo de la ciencia y la investigación.

Por último, destacar que nos hemos sentido muy cómodos con los investigadores del CSIC, y agradecerles su esfuerzo y el tiempo que nos han dedicado para hacer posible este proyecto.

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Internalization of the anti-carcinogenic IBB1, a major Bowman-Birk isoinhibitor from soybean (G*lycine max*), in HT29 colon cancer cells

Marta Soria[1], Patricia Porras[2], Francisco J. Moya[3], Nerea Sánchez[4], Belén Rodríguez[5], María L. Ruiz[5], Juan de Dios Alché[6], María del Carmen Arques[6], Antonio J. Castro[7] and Alfonso Clemente[6*]

[1]*CPD Lux Mundi. Calle de Durán, 18199 Cájar, Granada, Spain*
[2]*CPD Sagrado Corazón. Paseo de los Basilios 3, 18008 Granada, Spain*
[3]*IES Fray Luis de Granada. Calle Huerta del Rasillo s/n, 18004 Granada, Spain*
[4]*IES La Zafra. Calle Vicente Aleixandre 6, 18600 Motril, Granada, Spain*
[5]*IES ACCI. Avenida Buenos Aires 68, 18500 Guadix, Granada, Spain*
[6]*Department of Biochemistry and Physiology of Animal Nutrition* and [7]*Department of Biochemistry and Molecular and Cellular Biology of Plants; Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*
[*]*Corresponding author:* ***e-mail:*** *alfonso.clemente@eez.csic.es*

HIGHLIGHTS

- IBB1, a major serine protease isoinhibitor of the Bowman-Birk family from soybean, exerts an anti-proliferative effect on HT29 colon cancer cells in a dose-dependent manner.
- IBB1 crosses the membrane of HT29 colon cancer cells over a time-course of 1 h, being localized in cytoplasm.

SUMMARY

Protease inhibitors of the Bowman-Birk type, a major protease inhibitor family in legume seeds, which inhibit potently trypsin- and chymotrypsin-like proteases, are currently being investigated as colorectal chemopreventive agents. Although the therapeutic target/s and the action mechanism/s of Bowman-Birk inhibitors (BBI) have not yet been elucidated, the emerging evidence suggests that BBI exert their chemopreventive properties *via* protease inhibition; in this sense, serine proteases should be considered as primary targets in early stages of carcinogenesis. In this work, we have demonstrated that IBB1, a major protease inhibitor of the Bowman-Birk family in soybean (*Glycine max*), exerts anti-proliferative effect in human colorectal HT29 cancer cells at concentrations higher than 15 µM, in a dose dependent manner. By using confocal microscopy, we have demonstrated that IBB1 is taken up by HT29 colon cancer cells in a time-dependent manner, being the bulk of the internalized protease inhibitor localized in the cytoplasm where might interact with their potential therapeutic target/s.

INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of cancer-related mortality worldwide, with over 1.2 million new cases diagnosed globally per year [1]. In recent years, substantial evidence has pointed to the link between dietary patterns and lifestyle in primary prevention and control of CRC. Within this framework, there is a growing interest in naturally-occurring serine proteases of the Bowman-Birk family due to their potential chemopreventive and/or therapeutic properties which can impact positively in pathological disorders, including cancer, muscle atrophy and neurodegenerative diseases [2]. In particular, several studies suggest that

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dietary BBI from different legume sources are effective at preventing or suppressing radiation- and chemical carcinogen-induced transformation *in vitro*, as well as carcinogenic and associated inflammatory disorders within the mammalian gastrointestinal tract [3, 4]. Soybean BBI exerted a protective role in dimethylhydrazine (DMH)-treated rodents when ingested at low concentrations (10 mg/100g diet), decreasing the frequency and incidence rates of colorectal tumours [5]. In patients with active ulcerative colitis, intake of BBIC -a protein extract of soybean enriched in BBI- was associated with a clinical response and induction of disease remission, as assessed by the Sutherland Disease Activity Index [6]. By using colon cancer cells as models, we are currently investigating the action mechanism/s by which BBI might exert a chemoprotective effect in early stages of colorectal carcinogenesis. A significant concentration- and time-dependent decrease in the proliferation of colon cancer cells, following treatment with BBI from several legume sources, including pea (*Pisum sativum*) [7], lentil (*Lens culinaris*) [8] and soybean [9] has been reported; neither BBI affected the growth of non-malignant colonic fibroblastic CCD18-Co cells. We demonstrated that such suppressive effect on growth of colon cancer cells was related to their intrinsic ability to inhibit serine proteases [10]. In order to gain insight about the action mechanism of BBI as colorectal chemopreventive agents, the aim of this study was to determine if IBB1, a major protease isoinhibitor from soybean, is internalized by HT29 colorectal adenocarcinoma cells facilitating the inhibition of intracellular target proteases.

MATERIALS AND METHODS

Materials

BBI from soybean, trypsin (type III) from bovine pancreas, N-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE), high-glucose Dulbecco's modified Eagle's medium (DMEM), neutral red (NR) and additional high-grade chemicals for cell culture were obtained from Sigma. The Cy5 fluorochrome was purchased from GE Healthcare. The human colorectal adenocarcinoma HT29 cell line was supplied by the Cell Bank of the Scientific Instrumentation Centre at the University of Granada (CIC-UGR). Culture flaks and flat bottom ninety-six-well microtitre plates were purchased from Corning Costar and Nunc, respectively. All other chemicals were of analytical grade.

Isolation of soybean BBI isoinhibitors

IBB1, a major BBI isoinhibitor from soybean, was purified from commercial soybean BBI consisting in a mixture of IBB1 and IBBD2 [9]. The mixture was fractionated on a MonoS 5/50 GL cation exchange column (GE Healthcare), connected to an AKTA FPLC system (GE Healthcare), using a linear gradient of 0-0.22 M NaCl in 25 mM sodium acetate buffer, pH 4.4, at a flow rate of 1 ml/min. The elution was monitored at 280 nm and 0.5 ml fractions were collected. Measurements of trypsin inhibitory activity (TIA) of eluted samples were carried out in flat-bottom microtitre plates by using BAPNA as specific substrate, and assay products measured at OD_{405nm} as previously described [11]. The unbound sample, containing both trypsin and chymotrypsin inhibitory activity –measured by using BTEE as specific substrate [12]-, was dialysed extensively against distilled water and freeze-dried until use.

Identification of IBB1 by peptide mass fingerprinting

The unbound freeze-dried sample (10 µg) was dissolved in NuPAGE® lithium dodecyl sulphate sample buffer (Invitrogen) and separated by electrophoresis on Novex 12% Bis-Tris pre-cast gels using NuPAGE® 2-N-morpholine-ethane sulphonic acid (NuPAGE, MES, Invitrogen) as running buffer. Immediately before use, the sample was reduced with dithiothreitol (DTT) and NuPAGE antioxidant added to the upper buffer chamber to prevent reduced proteins from re-oxidation during electrophoresis. The electrophoretic band was excised from Colloidal Blue

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(Invitrogen)-stained gels and subjected to in-gel trypsin digestion. Peptide fragments from digested proteins were desalted and concentrated using C-18 ZipTip columns (Millipore) and then directly loaded onto the matrix-assisted laser desorption/ionization (MALDI) plate, using α-cyanohydroxycinnamic acid as the matrix for MALDI-MS analysis. MS spectra were obtained automatically in a 4700 Proteomics Analyzer (Applied Biosystems) operating in reflecton mode with delayed extraction. Peptide mass data were used for protein identification against the MS protein sequence database (www.matrixscience.com).

Fluorescent covalent labeling of IBB1 protein

IBB1 was covalently labeled with the Cy5 fluorochrome using the minimal labeling protocol according to manufacturer´s instructions. This method ensures that only a single lysine residue per protein molecule is labeled, avoiding a major effect on its protease inhibitory activity. Briefly, 200 µg of freeze-dried IBB1 was dissolved in 200 µl of lysis buffer (30 mM Tris-HCl, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, pH 8.5), and mixed with 1,600 pmol of dye and leave on ice for 30 min in the dark. The reaction was stopped by adding 4 µl of 10 mM lysine followed by incubation for 10 min at 4 °C on the dark. Samples were then centrifuged through an Amicon® Ultra-0.5 3K device (Millipore) at 14,000*g* for 30 min. The concentrated Cy5-labeled IBB1 protein (~50 µl) was diluted in ultrapure H_2O to a final volume of 500 µl and then centrifuged again as above. This step was repeated three times and the final retentate was transferred to a microcentrifuge tube and stored at −80 °C in the dark until use. To check the quality of labeling, 1 µg of Cy5-labeled IBB1 protein was electrophoresed by SDS-PAGE according to standard procedures and visualized in a Pharos Molecular Imager (Bio-Rad) by exciting at 635 nm. The specificity of the fluorescence signal was confirmed by scanning the same gel at 532 nm.

Western blotting

A polyclonal Ab was generated by immunizing a healthy rabbit using purified IBB1 as antigen. The immunization and antiserum collection was carried out by Biomedal SL. The polyclonal anti-IBB1 Ab was affinity-purified by using a protein-A column (BioRad) following standard protocols. In order to evaluate its specificity, equals amounts of IBB1, IBBD2 and Kunitz inhibitor from soybean were separated by SDS-PAGE and electrotransferred to a PVDF membrane using a Trans-Blot® Turbo™ Transfer System (BioRad). When the transfer was completed, the nonspecific binding sites were blocked by immersing the membrane in 1% (w/v) BSA in Tris-buffered saline (TBS), 0.3% (v/v) Tween 20 (TBST) at 4 °C for 2 h. The membrane was washed with TBST and then incubated using the rabbit polyclonal anti-IBB1 Ab (diluted 1:1000) at 4 °C overnight. After three washes with TBST, the membrane was incubated with a secondary goat anti-rabbit IgG Alexa Fluor 488-conjugated Ab (diluted 1:2500) for 2 h at 4 °C in the dark. The image was visualized in a Pharos Molecular Imager (Bio-Rad).

Cell viability assays

Human colorectal adenocarcinoma HT29 cells were maintained by serial passage in 75 cm^2 plastic culture flasks. HT29 cells were cultured in DMEM, supplemented with 5% (v/v) fetal bovine serum, 2 mM glutamine and 1% (v/v) antibiotic-antimicotic solution (Sigma), all at final concentration. Optimal assay conditions for colonic cells were reported previously [7]. Briefly, ninety-six-well microtitre plates were inoculated at a density of 2,000 HT29 cells *per* well in 200 µl of growth media. Plates were incubated under 5% CO_2 in humidified air for 24 h to allow the cells to adhere to the wells. Purified IBB1 was dissolved in growth media at a range of concentrations (15-93 µM) and added to the cells under sterile conditions. Control cells received no IBB1. At the end of the growth period (96 h), the viability of HT29 cells was assessed by the NR (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) cytotoxicity assay, based on the ability of viable uninjured cells to incorporated and actively bind NR, a supravital dye, into lysosomes. Cells were stained with NR solution (2 h at 37 °C), followed by cell fixation [0.5% (v/v) formaldehyde, 0.1% (w/v) $CaCl_2$ for 30 s] at room temperature. Plates

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were washed by two brief immersions in PBS (0.01 M-sodium phosphate buffer, 0.15 M NaCl) and the dye extracted from the viable cells using an acidified ethanol solution [50% (v/v) ethanol, 1% (v/v) acetic acid] at 4 °C overnight. The absorbance of the solubilized dye was quantified at 550 nm using a BioRad Model 550 microplate reader (BioRad). Cell viability data, expressed as a percentage of the values determined for control cells grown in the absence of IBB1, were obtained from at least three independent experiments, having each at least three technical replicates. The concentration of IBB1 that reduced cell viability by 50% (IC_{50}), as compared with untreated controls, was calculated by non-linear regression fit using the GraFit software (Erithacus Software Ltd.). The data were analysed statistically by the Bonferroni's test to compare means and statistical significance was set at $P<0.05$.

Time-lapse confocal laser scanning microscopy

To study the internalization of IBB1 into human colorectal adenocarcinoma HT29 cells, we carried out time-lapse confocal laser scanning microscopy (CLSM) experiments in triplicate. For this purpose, HT29 cells were grown *in vitro* as described above. A 1µ-slide microscopy chamber (Ibidi) was inoculated at a density of 25,000 HT29 cells per well in 700 µl of growth media. Microscopy chambers were incubated overnight under 5% CO_2 in humidified air to allow the cells to adhere to the surface of the chamber. Fifty µg of Cy5-labeled IBB1 was added to the microscopy chamber and immediately placed on a C1 confocal laser microscope (Nikon). Z-series images of HT29 cells were recorded at different time (min) intervals after the onset of the experiment and processed with the software EZ-C1 Gold v2.10 build 240 (Nikon). Fluorescence images were obtained by exciting the sample with a red diode (633 nm). The signal corresponding to the Cy5-labeled IBB1 protein was visualized as discrete red fluorescent spots.

RESULTS AND DISCUSSION

Purification and molecular characterization of a major soybean BBI isoinhibitor, IBB1

Commercial soybean BBI, consisting in a mixture of IBB1 and IBBD2, was fractionated by MonoS cation exchange chromatography. The elution pattern of IBB1 and IBBD2, monitored by TIA measurements, are shown in **Fig 1**. At pH 4.4, IBB1 was not retained by the MonoS column, whereas IBBD2 was bound and eluted as a single broad chromatography peak in the range 0.04-0.13 M NaCl. The chromatographic fractions containing IBB1 were pooled and analysed by SDS-PAGE, showing a single band of appropriate molecular mass (12 kDa) (**Fig 2**, lane 1). The polyclonal Ab recognized the electrophoretic band corresponding to IBB1 (**Fig 2**, lane 2). Further studies by peptide mass fingerprinting were carried out in order to confirm the identity of IBB1. The purified protein, corresponding to the unbound chromatographic peak, was identified as Bowman-Birk proteinase inhibitor (Swiss-Prot entry: IBB1_SOYBN). The amino acid sequence of IBB1 is shown in **Fig. 3**, where the peptide sequences that contributed to protein identification by MS are indicated. Like others BBI proteins, IBB1 contain 14 Cys residues in conserved positions [3], with Lys and Leu in position P_1 in the N- and C-terminal inhibitory domains, respectively. In agreement with the identity of the P_1 residues, IBB1 inhibited both trypsin and chymotrypsin (data not shown). IBB1 was labelled with Cy5 fluorochrome in order to determine if the protease inhibitor is internalised by HT29 colorectal adenocarcinoma cells in a time-dependent manner. Cy5-labelled IBB1 was electrophoresed by SDS-PAGE and visualized in a Pharos Molecular Imager by exciting at 635 nm (**Fig 2**, lane 3); only an electrophoretic band was visualized, revealing the effectiveness of the minimal labelling protocol. To confirm the specificity of the signal, the same gel was also scanning at 532 nm (**Fig 3**, lane 4) in which the signal was completely abolished.

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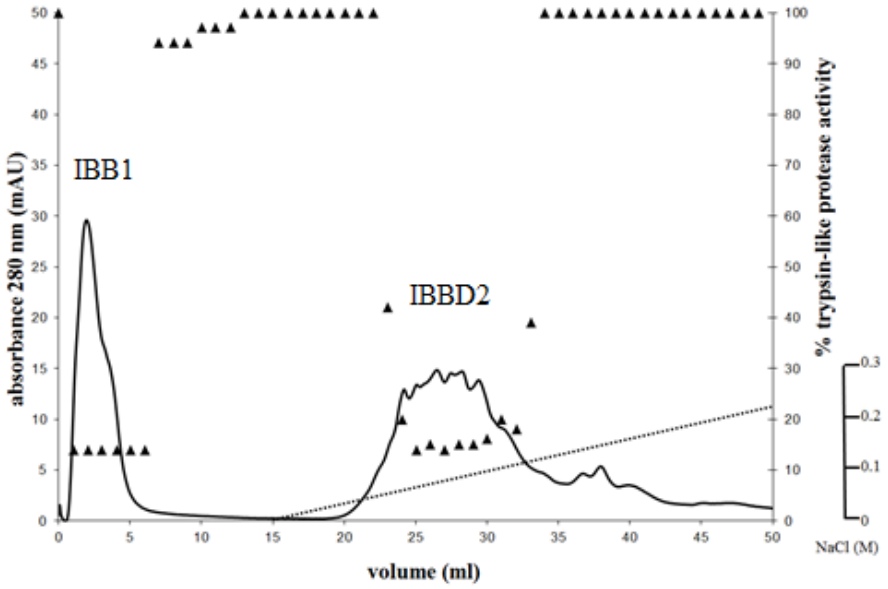

Figure. 1. Elution profile of a mixture of Bowman-Birk iso-inhibitors, IBB1 and IBBD2, from soybean on a MonoS 5/50 GL cation exchange column. Absorbance (mAU) at 280 nm of the chromatographic elution and the linear gradient of NaCl (0-0.22 M) are shown (solid and dotted lines, respectively). Using BAPNA as specific substrate, the trypsin inhibitory activity, measured on every fraction, is shown.

Effects of IBB1 on the proliferation of human colorectal adenocarcinoma cells HT29

The effect of IBB1 on the growth of human colon adenocarcinoma HT29 cells was determinated by comparing the cell viability of cells cultured in the absence or presence of IBB1 (15-93 µM), monitored by the cytotoxic NR cell assay. IBB1 exerted a significant effect on growth of HT29 cells at concentrations as low as 15 µM. A stadistically significant ($p<0.05$) and dose-dependent decrease of the growth of HT29 colon cells was observed (**Fig 4**); the IC_{50} value for IBB1 was 47 ± 5 µM, in agreement with those obtained for BBI from others plant sources, including lentil [8] and pea [10].

Internalization of IBB1 into human colorectal adenocarcinoma cells HT29

HT29 human colorectal adenocarcinoma cells were incubated with about 50 µg of Cy5-labelled IBB1 for 50 minutes (**Fig 5**). Fluorescent images were obtained by exciting the sample with red diode (633 nm), being the signal corresponding to IBB1 visualized as red fluorescent spots. Although additional biochemical assays are necessary, these results clearly demonstrated that IBB1 is internalized by HT29 cells. To further analyse the IBB1 internalization dynamics, images were taken at different time intervals (19, 32, 43 and 54 minutes). Labelled IBB1 crossed the cellular membrane of HT29 cells very rapidly and was gradually accummulated, forming fluorescent patches randomly distributed across the cytoplasm (**Fig 6**).

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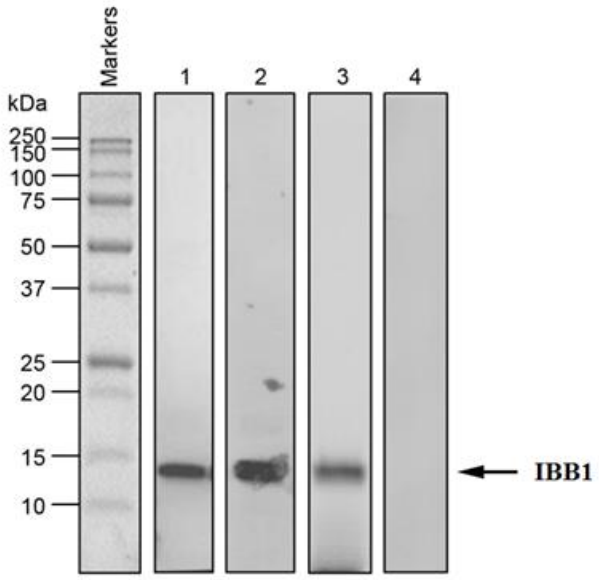

Figure 2. Electrophoretic characterization and fluorescent covalent labelling of IBB1. ***Lane 1***, analysis of the electrophoretic mobility of IBB1 by SDS-PAGE. One microgram of purified protein was loaded on a 4-20% gradient gel. A single band of about 12 kDa was visible on the gel after Coomassie staining. Molecular weight markers are shown on the left. ***Lane 2***, IBB1 protein was blotted to a PVDF membrane and probed with a rabbit polyclonal anti-IBB1 Ab followed by a secondary goat anti-rabbit IgG Alexa Fluor 488-conjugated Ab. The primary Ab was able to bind to the IBB1 protein. Negative control with preimmune serum did not show any signal (data not shown). ***Lanes 3-4***, fifty micrograms of IBB1 were covalently labelled with the Cy5 fluorochrome using the minimal labelling protocol. One microgram of Cy5-labelled IBB1 was electrophoresed by SDS-PAGE and visualized in a Pharos Molecular Imager by exciting at 635 nm (lane 3). The specificity of the signal was confirmed by scanning the same gel at 532 nm (lane 4).

IBB1 *DDESSKPCCDQCACTKSNPPQCRCSDMRLNSCHSACK*SCICA**LS**YPAQCFCVDITDFCYEPCKPSEDDKEN

Figure 3. Identification of IBB1 protein by peptide mass fingerprinting. The protein band from the Commassie-stained gel (Figure 3, lane 1) was excised and processed for MALDI-TOF/MS analysis. Amino acid sequences of inhibitory domains are underlined. The reactive peptide bond sites are in bold. **K** (Lysine) determines specificity for trypsin whereas **L** (Leucine) determines specificity against chymotrypsin. The peptides that contributed to protein identification are indicated in italics.

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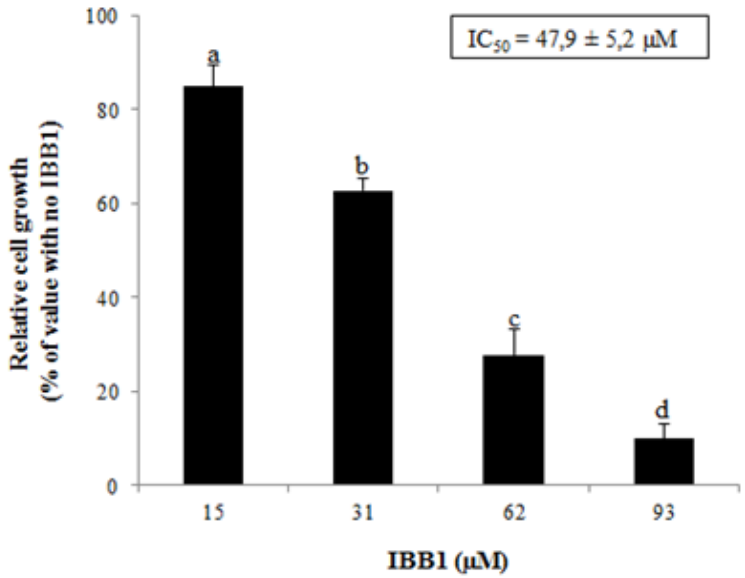

Figure 4. Effect of IBB1 on the *in vitro* growth of HT29 human colorectal adenocarcinoma cells. Growth media were supplemented with concentrations of IBB1 in the range 0-93 µM and cells harvested after a period of 96h. Data are means of three experiments, each having three technical replicates; bars represent SD. Mean values with different letters were significantly different ($p<0.05$; Bonferroni's test).

Soybean BBI has been shown to inhibit, specifically and potently, the chymotrypsin-like proteasomal activity in MCF7 breast cancer cells *in vitro* and *in vivo* [13]. The ability of soybean BBI to inhibit the proteasomal activity in intact MCF7 cells reveals that the cellular membranes are permeable to soybean BBI facilitating the inhibition of intracellular target proteases. Soybean BBI has been demonstrated to be taken up by colonic epithelial cells in a time-dependent manner, being the bulk of the internalised protease inhibitor present in the cytosol in active form [14]. These authors showed that soybean BBI was present in the cells for 12 h following 2h incubation. It has been also reported that soybean BBI is internalised into NIH/3T3 mouse embryo fibroblastic cells and is localized in the nucleus, even after simulated gastrointestinal digestion [15]. More recently, confocal microscopy studies have demonstrated that black-eyed pea (*Vigna unguiculata*) BBI crosses the membrane of breast MCF7 cancer cells, likely *via* endocytosis, and co-localizes with the proteasome in cytoplasm and in nucleus, inhibiting the chymotrypsin-, trypsin- and caspase-like activities of the 20S proteasome [16]. In order to determine the mechanism of internalization of BBI in human hepatoma Hep G2cells, fluorescein isothiocyanate-labelled buckwheat (FITC-BTI) (*Fagopyrum esculentum*) protease inhibitor was used [17]. FITC-BTI colocalised with labelled transferrin implying that BTI enters Hep G2 cells by clathrin-dependent endocytosis. Further studies to determine the internalization mechanism and correct localization of BBI in colon cancer cells will be relevant in order to identify serine proteases as potential therapeutic targets.

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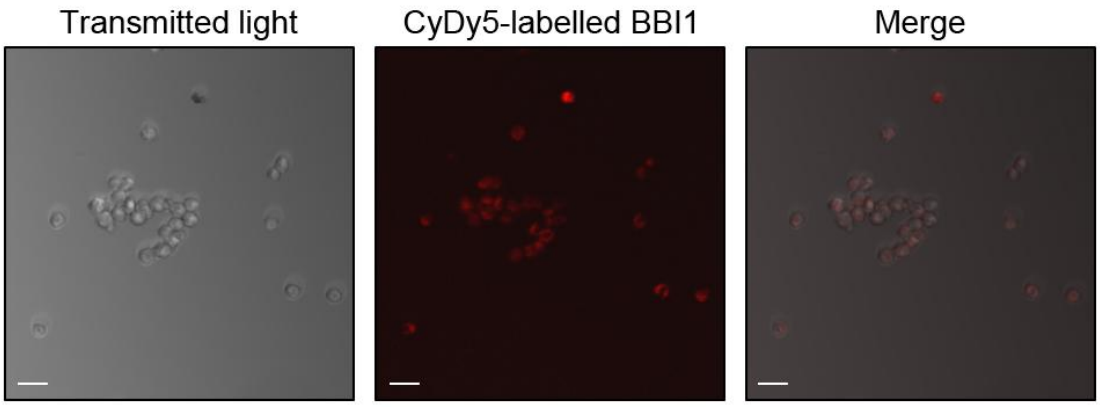

Figure 5. Internalization of IBB1 into human colorectal adenocarcinoma HT29 cells. About fifty micrograms of Cy5-labelled IBB1 were added to the culture medium. Transmitted light (left), fluorescence (middle) and merge (right) images represent a Z-stack projection of 20 optical sections of a group of HT29 cells cultured *in vitro* for 50 min. Images were captured using a confocal laser scanning microscope C1 (Nikon). Fluorescent image was obtained by exciting the sample with a red diode (633 nm). Red fluorescence corresponds to IBB1. Bars= 50 µm.

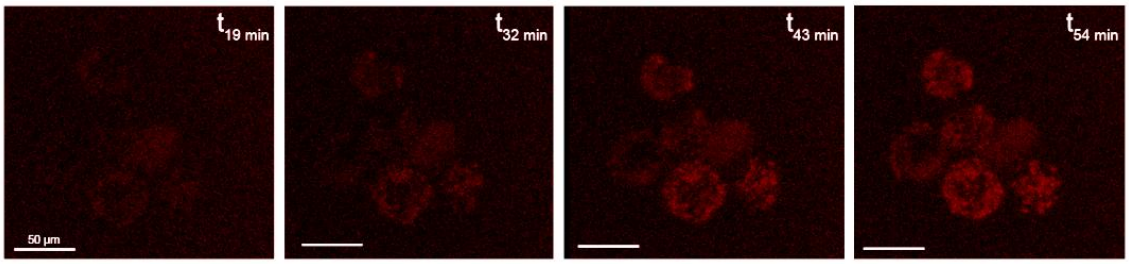

Figure 6. Analysis of IBB1 internalization dynamics in human colorectal adenocarcinoma HT29 cells using time-course CLSM microscopy. About fifty micrograms of Cy5-labelled IBB1 were added to the culture medium. Transmitted light (A) and fluorescence images (B-D) represent Z-stack projections of 20 optical sections of a group of HT29 cells cultured *in vitro*. Images were taken at different time (min) intervals using a C1 confocal laser scanning microscope (Nikon). Fluorescence images were obtained by exciting the sample with a red diode (633 nm). The signal corresponding to IBB1 was visualized as red fluorescent spots. We observed that the labelled protein entered the HT29 cells very rapidly (data not shown) and gradually accumulated, forming fluorescent patches randomly distributed across the cytoplasm. Bars= 50 µm.

CONCLUSIONS

1. **IBB1, a major Bowman-Birk isoinhibitor from soybean, exerts anti-proliferative effect on HT29 human colorectal adenocarcinoma cells.**
2. **IBB1 crossed the membrane of HT29 colon cancer cells over a time-course of 1 h and is localized in cytoplasm, where might interact with their potential therapeutic targets.**

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ACKNOWLEDGEMENTS

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MY OWN IDEAS

Marta Soria

Hello, my name is Marta and I am studying in the Lux Mundi School, in Granada and I have been involved in the PIIISA project. This project has given me the opportunity to know in depth a little part of the scientific world. I have enjoyed this scientific project so much, being an awesome experience. I have worked in a research group having incredible people and everybody gets along very well with each other. I have made really good friends, and I wouldn't have met them if I had not been part of the project. Being in the laboratory was an awesome experience and I felt super comfortable when I was working there. It was my favorite part of

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the project without a doubt! The project gives you the idea of how do people work in the laboratories. When I heard the word 'science' I used to think of a laboratory, but I have learnt that there are so many things besides the fact of being in a laboratory using goggles. I don't know yet what I want to study. However, being part of the PIIISA project has helped me to have it a little bit clearer. Hopefully one day I will be part of this huge scientific world. I'm so thankful I had this opportunity as it has made me grow. It has helped me to put my shyness aside. It has taught me a very important idea, and it is that if you want to fulfill your dreams you must run after them. You have to work really hard. If you really want to achieve your goals the effort will worth it. And the most important thing in life: you should never give up just because you failed the first time.

Patricia Porras

I'm Patricia Porras and I participated in PIIISA Project 2014. Before enrolling, I heard a lot of good things about PIIISA, so I didn't hesitated in becoming part of this. In my opinion, PIIISA is a fantastic opportunity for young people who want to study a scientist career. I learnt a lot here, such as laboratory techniques, working in a team a how a scientist's life is. In addition, PIIISA helped me to practice my English and it taught me how to explain what I did and the results of my work. At last, I want to thank PIIISA for give me this opportunity, the researches for thinking all time about us and my teammates for doing this experiment more joyful.

Francisco J. Moya

Initially I chose this project because it seemed very interesting and is closely related to what I want to study in a few years so since I thought this was going to serve me to know more closely the procedures, tools and discipline of scientific research, although at first I was a little frightened by the thought of having to make a presentation to many people in the same field of study. My first day on the project PIIISA research center Zaidin was fabulous, I felt like a little boy in a sweet shop , as the days passed I was giving me realize that my interest in matters related to my project was greater than I thought. Why not? Undoubtedly imagine me working on this, discovering new drug against deadly diseases and going to conferences around the world. Are already drawing conclusions from our research and about ten Congress expected the PIIISA in which we will reveal these fantastic data obtained after much effort. Now I can say I do not feel those nerves that I felt at the beginning of the project and no doubt I would repeat next year, because it has been a very constructive and didactic experience in which I have met wonderful people and researchers fantastic which have enabled us better understand these materials. I want to thank everyone who has made this project could take over.

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Use of vermitea for removing pesticides from soil

Laura León Rejón[1], Belén García Trigueros[2], María Rosales Reyes[3], Carlos Soto Paniza[4], Celia Cámara Pérez-Vela[4], Celia Cifuentes[5], Esperanza Romero[5], Laura Delgado-Moreno[5*]

[1]*IES Juan XXIII. Camino Santa Juliana s/n, 18007 Granada, Spain*
[2]*IES Padre Manjón. Gonzalo Gallas s/n, 18003 Granada, Spain.*
[3]*IES Alhambra. Beethoven 2, 18006 Granada, Spain.*
[4]*IES Alpujarra. Avd. Lora Tamayo s/n, 18400 Orgiva, Granada, Spain.*
[5]*Department of Environmental Protection , Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*
[*]*Corresponding author:* ***e-mail:*** *laura.delgado@eez.csic.es*

HIGHLIGHTS

- Vermitea, a liquid extract from vermicompost, contents high biological activity and dissolved organic carbon.
- Vermitea may be used as a bioremediation method to enhance pesticides degradation in soil.
- High concentration of vermitea added to soil may have a negative effect on soil biological activity.

SUMMARY

Tea obtained from vermicompost (vermitea) is currently used as liquid fertilizer but its potential to degrade pesticides in soil has never been studied. In the current study we evaluate the effect of tea from vermicompost of olive cake to degradate pesticides of different hydrophobicity: imidacloprid, diuron, tebuconazole and oxyfluorfen. For the degradation study, a non-amended soil and soils amended with two dosage of vermitea were incubated for two months. Results indicate that despite the dehydrogenase activity was higher in soil amended than in non-amended soil, addition of vermitea to soil does not significantly increase the soil ability to degrade the pesticides studied. In soil amended with vermitea at high dosage, the remaining concentration of diuron and oxyfluorfen at the end of the incubation time was significantly ($P< 0.01$) higher than in non-amended soil suggesting a negative effect of the vermitea on the microbial activity. These preliminary results indicate the necessity of improving the vermitea elaboration process adding nutrients during the aeration to increase its biochemical quality and the content of microorganisms to favor their potential to degrade organic contaminant such as pesticides.

INTRODUCTION (AND OBJECTIVES)

The presence of pesticides in the environment and their impact on human health and ecosystems are big concerns all over the world. In the majority of the world´s countries, standards to preserve the quality of the hydric resources have been implanted. The European Union legislation has initiated more stringent data requirements concerning ground and surface water contamination by pesticides (Directivas 2000/60/EC, 2006/118/EC). In order to reach the standards of quality of waters, and to fulfill those normative, it is necessary to develop techniques to reduce and prevent the origin of the contamination. Current research is focused on developing environmental friendly and low cost bioremediation strategies. Tea

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obtained from vermicompost, also known as vermitea, which is used to enhance agricultural productivity, may be applied to soil as a detox agent to minimize pesticide contamination. Vermitea is a liquid extract obtained from the mature vermicompost by aeration. It contains soluble nutrients and a diversity of beneficial microorganisms that increase the soil quality and the plant fertility [1, 2]. Nowadays, there is an increasing interest for using vermitea to increase the agriculture productivity and reduce the use of chemical fertilizer. However, the application of vermitea to minimize contamination causes by organic contaminants has been scarcely studied. Chiang (2013) introduced the use of vermitea as a remediation tool in the context of oil contaminated soil. The soil application of vermitea causes an increase of the microbial activity and thus it may enhance the microbial degradation of organic compounds such as pesticides. Also, the high content of vermitea´s dissolved organic carbon (DOC) may cause desorption of the sorbed fraction of organic contaminants to the soil solution [4] increasing their bioavailability and enhancing their degradation. Thus, vermitea may become an important tool for bioremediation strategies and management risk, especially for most hydrophobic organic contaminants which should render more adsorbed into the solid phase and thus less bioavailable but with high affinity for DOC [5, 6].

The aim of the current study is to test tea obtained from vermicompost of olive cake by aeration as a bioremediation tool to enhance the biological degradation of pesticides.

MATERIALS AND METHODS

Soil and vermicompost

The soil was collected from an agriculture field in Granada (Spain). Before analysis, the soil was air dried and sieved to 4mm. The soil had a silty clay loam texture (34.0% clay, 56.0% silt and 10.0% sand) and it contained 44.1% $CaCO_3$. The soil field capacity was 33.2%.

Vermicompost of olive cake was obtained by vermicomposting a mixture of wet olive cake and manure in the ratio 4:1 by worms of Eisenia Andrei over 6 months and 2 months more for maturation and drying.

Pesticides

One insecticide: imidacloprid (99.9% purity); two herbicides: diuron (99.5% purity) and oxifluorfen (99.9% purity); and one fungicide: tebuconazole (99.5% purity) were selected as representative pesticides used in olive culture. Table 1 shows the environmental fate parameters of these pesticides. All the chemicals were supplied by Sigma-Aldrich (St. Louis, MO). These chemicals were dissolved in acetone separately as stock solutions. All other solvents and chemicals used were of HPLC grade.

Tea extraction

Vermicompost of olive cake and manure (4:1) (25 g) were weighted into a nylon bag with a pore size of 50 µm. A glass vessel was filled with 250 mL of distillated water and aerated two hours before adding the bag containing the vermicompost. Once the bag was introduced into the water, the sample was aerated at 20ºC in the dark for 24 hours. Four replicates were carried out. After this time, the bags were removed and the extracts or vermiteas were mixed and analyzed. Table 2 contains some vermitea properties. Biological oxygen demand (BOD), pH and conductivity were measured using the Cyberscan PCD 6500 meter (Thermo Scientific, Landsneer, Netherland). Dehydrogenase activity was determined using iodonitrotetrazolium formazan (INTF) as substrate, as described by García et al. [8]. Briefly, 1 mL of vermitea was added with 0.2 mL of distillated water and 0.2 mL of 0.4% INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h at 22 ºC in the dark. The INTF formed was extracted with 5 mL of a mixture of 1: 1.5 ethylene chloride: acetone by shaking vigorously for 2 min. Samples were centrifuged at 3500 rpm for 15 min. The INTF was measured in a

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spectrophotometer at 490 nm. For the water soluble carbon determination, 0.5 mL of vermitea were added with 0.5 mL of distilled water, 1 mL of 1N $K_2Cr_2O_7$ and 2 mL of H_2SO_4. Sample was digested at 150ºC for 30 minutes and then measured in a spectrophotometer at 590 nm.

Two dosages of vermitea were used for the degradation study: i) a high dosage obtained as explained above from a 1:5 vemicompost: water ratio (w:v); and ii) a low dosage obtained by 1:2 (v:v) dilution of the vermitea at high dosage with distillated water.

Table 1. Chemical structure and selected physicochemical properties of the pesticides studied [7].

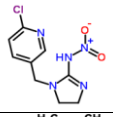
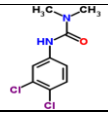
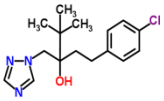
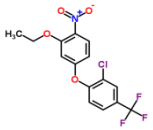
	Chemical Structure	Water Solubility (mg L^{-1})	Log K_{ow}
Imidacloprid		610.0	0.57
Diuron		36.40	2.85
Tebuconazole		36.00	3.70
Oxyfluorfen		0.12	4.47

Table 2. Physicochemical properties of vermitea obtained from 1:5 vermicompost: water ratio (w:v).

	BOD[a]	pH	Conductivity (mS cm^{-1})	Dehydrogenase activity (µg INTF $g^{-1}h^{-1}$)	WSC[b] (mg L^{-1})
Vermitea	7.98	7.56	3.94	76.48	1206.86

[a]BOD: biological oxygen demand.
[b]WSC: water soluble carbon

Degradation study

Soil samples (50 g) were spiked with 4 mL of an acetone solution containing a mixture of the pesticides studied. Final pesticide concentration in the soil was 5 µg g^{-1}. The samples were air dried in a fume hood to eliminate the solvent and homogenized by shaking in an end over end shaker for 5 minutes. The soil moisture content was adjusted to 80% of the soil field capacity adding ultra-pure water (S) or tea at low (STL) and high (STH) dosages. Samples were incubated in the dark at 20ºC for 60 days. Moisture content was maintained by regular additions of ultra-pure water. Immediately after pesticides application and at fixed intervals thereafter four samples from each treatment were removed and analyzed for pesticide concentration, dehydrogenase activity and dissolved organic carbon.

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Extraction and analysis of pesticides

The extraction of pesticides from the soil samples was accomplished using the QuEChERS method. An aliquot of 7.58 g (equivalent to 6 g dry weight) of the homogenized sample was weighed into a 50 mL centrifuge tube and 3 mL of acetonitrile was added. The mixture was vortexed for 1 minute. Then, sample was added with 1.2 g of a mixture of salts which contained sodium citrate (15.4%), sodium hydrogencitrate sesquihydrate (7.7%), magnesium sulfate (61.5%) and sodium chloride (15.4%) (Agilent Technologies, Santa Clara, CA). The sample was immediately vortexed for 1 min to avoid agglomeration of salts and followed by centrifugation at 3000 rpm at 10ºC temperature for 5 min. An aliquot of 1 ml of the supernatant was diluted with 1mL of water. Recoveries of the extraction method ranged between 86% and 104%, depending on the pesticide. The pesticides were analyzed by HPLC-DAD (series 1100, Agilent Technologies, Santa Clara, CA) on a Zorbax RX-C8 column (5 µm, 2.1 x 150 mm) (Agilent Technologies, Santa Clara, CA) connected to an Eclipse XDB-C8 (5 µm, 2.1 x 12.5 mm) precolumn (Agilent Technologies, Santa Clara, CA). The mobile phase was acetonitrile and water adjusted to pH 3 with sulfuric acid. In order to get good separation of each analyte a solvent gradient was used from 20% to 70% of acetonitrile. The flow was set at 0.2 mL min^{-1}, the injection volume to 20 µL, the oven temperature to 40ºC, and the detector wavelengths to 270 nm for imidacloprid, to 210 nm for diuron and oxyfluorfen and to 215 nm for tebuconazole. Retention times were 8.1, 16.4, 20.5 and 25.7 min for imidacloprid, diuron, tebuconazole and oxyfluorfen, respectively.

Dehydrogenase activity

An aliquot of 0.5 g of soil sample was used for dehydrogenase activity determination. Dehydrogenase activity was carried out as described above using the method described by García et al. [8].

Water soluble carbon

Soil samples (2 g) were added with 10 mL of distillated water and extracted by agitation in a bath at 50 ºC for 1 h. After this time, samples were centrifuged at 3500 rpm for 10 min and filtered through filter paper. 1 mL of the extract containing the water soluble carbon was digested at 150ºC with $K_2Cr_2O_7$ and H_2SO_4 as explained above. Samples were measured in a spectrophotometer at 590 nm.

RESULTS

Degradation of the pesticides studied in soil and soil amended with vermitea followed a first order kinetics (Table 3). The coefficients of regression were greater than 0.83 for all soil samples and significant ($P< 0.05$), thus indicating that the assumption of first-order kinetic was acceptable.

In soil, the remaining concentration values of diuron and imidacloprid decreased faster than those of tebuconazole and oxyfluorfen as indicated by their higher degradation rate constant (K_d) and half-lives values (Table 3). At the end of the incubation time, the soil concentration was 49, 25, 74 and 66% of the initial amount of imidacloprid, diuron, tebuconazole and oxyfluorfen, respectively (Figure 1). The high hydrophobicity of tebuconazole and oxyfluorfen (Log $K_{ow}\geq$ 3.7, Table 1) should render these chemicals more adsorbed to solid phase which protects them of being microbially degraded. In contrast, a significant fraction of diuron and imidacloprid, more water soluble (Table 1), may be present in the pore water where they can be degraded by soil microorganisms.

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Table 3. Kinetic parameters obtained from the simple first order equation fit for pesticide residual concentration in soil (S) and soil amended with tea at low (STL) and high (STH) dosages.

		C_0 (%) ± sd	K_d (d^{-1})x10^2 ± sd[a]	R^2	$t_{1/2}$ (d)
S	Imidacloprid	82.2 ± 5.6	0.93 ± 0.26	0.88	77.01
	Diuron	82.9 ± 4.6	2.07 ± 0.30	0.97	34.66
	Tebuconazole	101.7 ± 5.1	0.51 ± 0.17	0.83	138.62
	Oxyfluorfen	106.6 ± 2.9	0.56 ± 0.09	0.95	86.64
STL	Imidacloprid	85.5 ± 4.3	1.11 ± 0.21	0.94	69.31
	Diuron	86.0 ± 2.9	2.04 ± 0.18	0.99	36.48
	Tebuconazole	104.9 ± 2.8	0.62 ± 0.09	0.96	115.52
	Oxyfluorfen	107.0 ± 3.0	0.76 ± 0.10	0.97	99.01
STH	Imidacloprid	82.9 ± 5.6	0.91 ± 0.29	0.85	86.64
	Diuron	84.5 ± 8.1	1.61 ± 0.46	0.88	53.32
	Tebuconazole	103.3 ± 4.8	0.41 ± 0.16	0.78	173.28
	Oxyfluorfen	105.2 ± 4.6	0.75 ± 0.16	0.93	138.62

[a]Standard deviation (n=4)

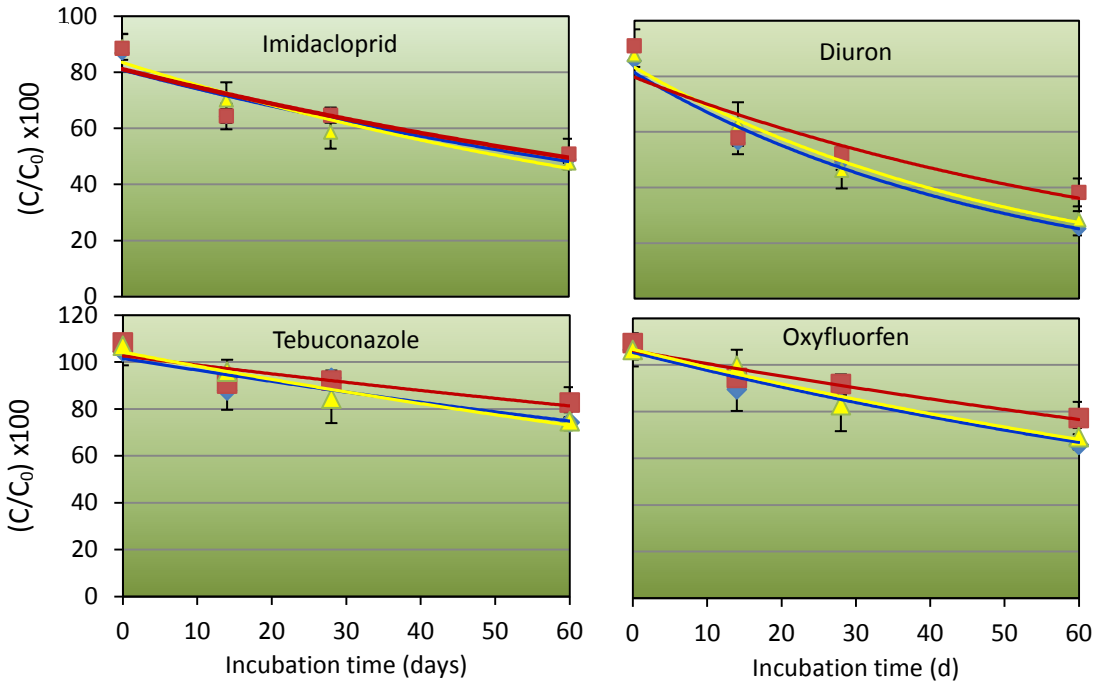

Figure 1. Pesticide degradation in soil (◆) and soil amended with vermitea at low (△) and high (■) dosages. Experimental data were fitted to a first order equation. The vertical lines represent the standard deviation in each sample (n=4).

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The addition of vermitea to soil did not increase its overall ability to degrade the four pesticides considered in this study. Thus, at the end of the incubation time, the concentration of the different pesticides in STL was not significantly different ($P > 0.05$) from that in S (Figure 1). Addition to soil of vermitea at high dosage did not have any effect on imidacloprid and tebuconazole degradation (Figure 1). However, at the end of the incubation time, the remaining concentrations in soil of diuron and oxyfluorfen were significantly higher ($P \leq 0.04$) in STH than in S (Figure 1). These results suggest an inhibitory effect of vermitea on the microbial activity of the soil.

Dehydrogenase activity has been used as indicator to estimate the total microbial activity of soil [9, 10]. The highest dehydrogenase activity was observed at 15 days of incubation in non-amended and soil amended with vermitea (Figure 2). STH presented the highest dehydrogenase activity at this time as it was expected because its highest microbial mass and higher water soluble carbon content (Figure 3). However, after 15 days of incubation, dehydrogenase activity in STH decreased markedly regard to STL (Figure 2) indicating that high concentration of vermitea may have a negative effect on soil microorganisms as indicated above. Despite the higher microbial activity of soil amended with vermitea regard to non-amended soil during the first month of incubation, in general, pesticide degradation was not significantly different between treatments suggesting that the vermitea´s microorganisms may have preferred the more easily degradable organic compounds of the vermitea as a carbon and or nitrogen source instead the pesticides. Other authors have considered this hypothesis previously for olive cake and different organic amendments [11-13].

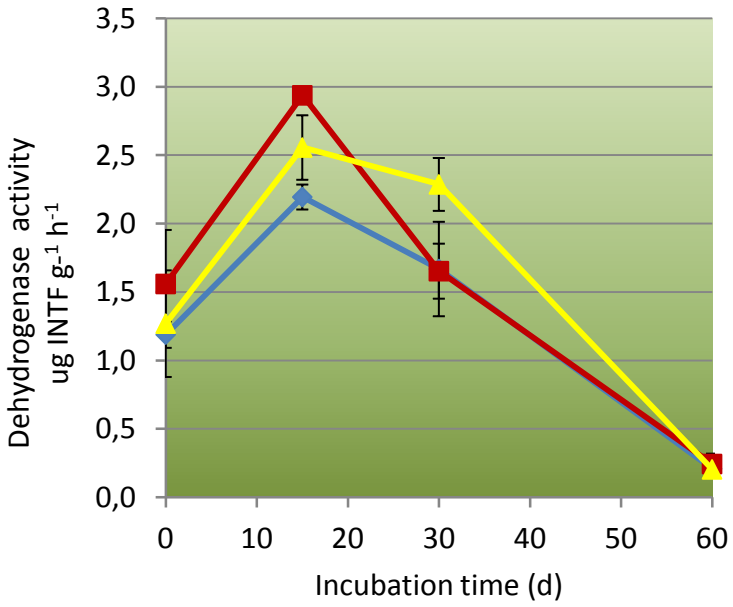

Figure 2. Dehydrogenase activity in soil (◆) and soil amended with vermitea at low (△) and high (■) dosages. The vertical lines represent the standard deviation in each sample (n=4).

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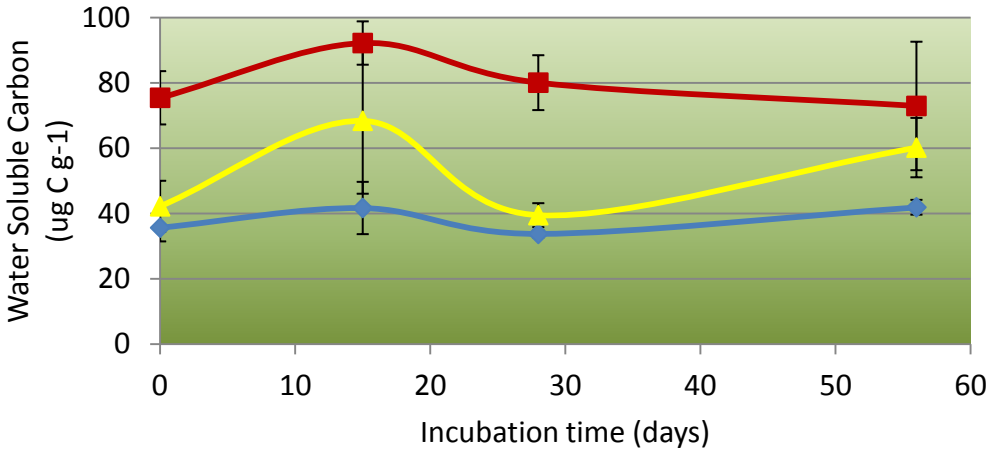

Figure 3. Water soluble carbon in soil (◆) and soil amended with vermitea at low (△) and high (■) dosages. The vertical lines represent the standard deviation in each sample (n=4).

CONCLUSIONS

1. **Imidacloprid and diuron were the pesticides more degraded. Degradation of tebuconazole and oxyfluorfen, more hydrophobic compounds, is limited by sorption to soil particles.**
2. **Addition of vermitea to soil did not modify the soil degradation capacity for pesticides.**
3. **The addition of vermitea to soil stimulated general microbial population activity without concurrent increases in pesticide degradation, suggesting that the specific microbial populations responsible for degrading the pesticide were not stimulated or that microorganisms prefer more labile compounds present in vermitea as nitrogen and carbon sources rather than pesticides.**

ACKNOWLEDGEMENTS

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MY OWN IDEAS

Laura León Rejón

This experience has been very pleasant, because I like everything related to nature and this has been very interesting. At first, the entire hypothesis seemed to me reasonable, if the tea removes contaminants from people, why could not it do the same from an agricultural soil? After doing the experiment with different doses of vermitea we have found that addition of high dosage of vermitea to soil may have a negative effect on microbial activity. If I were asked about defining Piiisa project with a single word I will have to say: RESULTS. Although the project has not gone as expected, we have found great things about how the soil affects the pesticide and its impact. This has been an unforgettable experience and, of course, everyone should experience it because it really worth it.

Esta experiencia ha sido muy fructífera, ya que a mí me gusta mucho todo lo relacionado con la naturaleza y éste ha sido un proyecto muy interesante. En un principio, todas las hipótesis me parecían razonables y poco a poco el conocimiento llevó a la hipótesis de que si el té elimina sustancias contaminantes de las personas, ¿por qué no se podía hacer con el suelo?. Después de hacer el experimento con distintas dosis de té hemos descubierto que éste, en dosis altas, puede tener un efecto negativo para la actividad microbiana.

Si alguna vez me preguntasen que cómo definiría PIIISA en una sola palabra, ésta sería: resultados. Aunque nuestro proyecto no haya salido como esperábamos hemos descubierto gran cantidad de cosas sobre los plaguicidas y la tierra. Ésta es una experiencia inolvidable que debería experimentar todo el mundo.

Belén García Trigueros

The existence of pollution in the environment and its consequences for people´s health and ecosystems is not something not everybody is aware of. The aim of this project is to test the efficiency of vermicompost “teas” made of organic residues in order to obtain a faster degradation of pesticides used in fields for cultivation.
I decided to choose this project because the idea of being able to provide new environmentally friendly techniques seemed very interesting to me, especially when it comes to two vital elements such as soil and the water.

Despite not achieving our initial expectative and success in this work at first, it has given me the opportunity to participate fully into a real scientific investigation process. Surely, scientists’ efforts will eventually lead to the achievement of new techniques which will make our intended objective a reality.

I knew about PIIISA through my high school center and our teachers encouraged us to participate.

My experience has been truly satisfactory, though I have worked in areas which are not exactly the ones I would like to keep on working in the future. I think there are very different projects that offer students the possibility of learning about the scientific method.

La presencia de contaminantes en el medio ambiente y su repercusión en la salud humana y los ecosistemas es una realidad que no suele tenerse en cuenta. El objetivo del proyecto que

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hemos desarrollado es determinar la eficacia de "tés" de vermicompost procedente de residuos agrícolas orgánicos para una degradación más rápida de los plaguicidas utilizados en los suelos de cultivo.

Elegí este proyecto porque me pareció muy interesante la idea de poder contribuir a desarrollar técnicas que cuiden el medio y recursos tan importantes como el suelo y el agua.

Aunque no hemos obtenido los resultados esperados en un principio, el proyecto me ha dado la oportunidad de sumergirme de lleno en la investigación científica. Estoy segura de que con el tiempo y el esfuerzo de investigadores se obtendrán técnicas que cumplan con el objetivo que intentábamos alcanzar.

Conocí PIIISA a través de mi centro educativo donde los profesores nos animaron a participar.

Mi experiencia al desarrollar este proyecto ha sido muy satisfactoria, a pesar de que trataba materias que no me gustaría seguir trabajando en un futuro. Creo que hay una gran diversidad de proyectos que ofrecen a los estudiantes la posibilidad de acercarse a la investigación.

María Rosales Reyes

The PIIISA project has meant to me a great opportunity to know how scientists make research and to understand why research is useful for our society. This project is a great advantage for those who want to pursue any field of research.

To be honest I considered this project at the last place because I prefer other projects more related to biomedicine or research related to disease and human body but also it has helped me to learn and this project has been as interesting as it could have been any other related to what I want to study.

Although we have not obtained the results expected at the beginning, it was a project of great interest to which I have shared time and desire with great colleagues which have been interested in our work a lot. I would truly like to repeat this experience and I think Piiisa project is an excellent and with great future project since it gives a great opportunity to somehow young people feel like scientists.

El proyecto PIIISA ha significado para mí una gran oportunidad para poder ver como se investiga y para lo que sirve la investigación. Este proyecto es una gran ventaja para todos aquellos que quieren dedicarse a cualquier campo de investigación.

Siendo sincera cogí este proyecto de los últimos porque mi dirección va más hacia la biomedicina o investigaciones relaciones con enfermedades y el cuerpo humano pero también me ha servido este proyecto para aprender y ha sido igual de interesante que podría haber sido cualquier otro relacionado con lo que quiero estudiar.

Aunque en nuestro proyecto no hemos obtenido los resultados que pretendíamos ha sido un proyecto de gran interés en el cual he compartido tiempo y ganas con unos grandes compañeros los cuales se han volcado mucho en nuestro trabajo. Me encantaría repetir esta experiencia y veo muy bien y con gran futuro a este proyecto ya que da la gran oportunidad de alguna manera de sentirse como un científico.

Carlos Soto Paniza

In this project, we have studied how pesticides are biologically degraded in the soil by adding tea obtained from vermicompost (vermitea) which we have used to provide greater resistance of plants to diseases.

To check the effectiveness of the vermitea we have tested the degradation of pesticides in soil of three different ways:

- Soil added with vermitea at high dosage.
- Soil added with vermitea at low dosage.
- Soil added with water.

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In this project we have learned to use scientific tools which I have not known so far, we have to learn how to weight accurately and precisely, to measure the pH and various other techniques ...

I decided to participate in the Piiisa project to try to understand how scientists work in a laboratory and also to know the instrument and other tools used in research and the science procedure. I found the Piiisa project very interesting because I have had the opportunity to know new people and colleagues. Although, I have to complain about the number of mandatory visits. I think we need more visits in order to perform the work with more time and dedication.

En este proyecto hemos estudiado como degradar biológicamente los plaguicidas en el suelo por adición de tés de vermicompost los cuales hemos empleado para dotar de mayor resistencia a las plantas frente a enfermedades.

Para comprobar la eficacia del vermité hemos probado la degradación de plaguicidas en suelo de tres maneras diferentes:

- Suelo adicionado con vermite a dosis alta.

- Suelo adicionado con vermite a dosis baja.

- Suelo adicionado con agua.

En este proyecto hemos aprendido a utilizar herramientas científicas no conocidas para mi hasta el momento, hemos a prendido a pesar de manera exacta y precisa a medir el pH y diversas cosas más...

Decidí entrar en el proyecto Piiisa para intentar entender más como es el trabajo en un laboratorio y conocer aparatos herramientas e infinidad de cosas que se pueden hacer en él, y de paso observar cómo sería una investigación importante con sus resultados y procedimientos. Me ha parecido muy interesante haber conocido este proyecto por haber conocido nuevas personas y compañeros con los que he trabajado el proyecto. Aunque hay una pega, que es la falta de más visitas obligatorias para poder realizar los trabajos con más tiempo y dedicación para la los alumnos que están en este proyecto.

Celia Cámara Pérez-Vela

I will start saying that the PIIISA project is an interesting project that I personally had not heard until 2014. I am very proud to have the possibility of participating in something like this, and have met all the people who have helped me with it.

I think the organization of this project is pretty good, but I also think it could be better with the ideas as follows:

- Increase the number of visits required, because with only three of these visits students with greater difficulty (whether economic or by the distance of their homes) will be absent in meetings to advance the project they've been assigned.
- If the period of the investigations was longer it would be really great for doing more things on each research.

On the other hand, the willpower and the desire of students to engage in this project are essential. The research voluntarily chosen by the students would significantly increase their involvement in that project.

Talking about my investigation group, we have reached interesting results, though it was not what we wanted to get, we have taken several positive conclusions from it, and this enables guided by another way to have what we want to achieve, which is the elimination of pesticides in soil. I have to say that I have learned a lot of it, and it would be excited to repeat something like this. The only problem is that I live far away from the site where we have the visits, so I could not contribute all that I wanted. Because of that I think the number of visits required should increase.

Overall, I think the project has been a good idea for development work and commitment to young students, and I hope it to continue being like this for a long time.

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Empezaré diciendo que el proyecto PIIISA es un interesante proyecto del que personalmente no había oído hablar hasta 2014. Estoy muy orgullosa de tener la posibilidad de participar en algo así, y de haber conocido a personas que me ayudaron con ello.

Creo que la organización del proyecto está bastante bien, pero también creo que podría mejorarse con las siguientes ideas:

Incrementar el número de "visitas obligatorias", ya que únicamente con tres de estas visitas, los estudiantes con mayor dificultad (ya sea económica o por la distancia de sus casas) se ausentarán en las demás visitas voluntarias para avanzar en el proyecto al que se les haya asignado.

Si el periodo de investigación fuera más largo, sería genial para investigar más en cada proyecto.

Por otro lado, la fuerza de voluntad y el deseo de los estudiantes de involucrarse en este proyecto son esenciales. La elección voluntaria de la investigación a realizar por cada estudiante, supondría una involucración significativa de éste en el proyecto.

Hablando sobre mi grupo de investigación, hemos logrado interesantes resultados, a pesar de que no era lo que queríamos conseguir en un principio, hemos obtenido de ello conclusiones positivas, lo que nos permite guiarnos por otro lado para llegar a lo que queremos lograr, que es la eliminación de plaguicidas en suelos. Tengo que decir que he aprendido mucho de ello, y sería emocionante repetir algo así. El único problema es que vivo lejos del sitio en el que se realizan las visitas, así que no he podido aportar todo lo que habría querido. Es por eso que creo que el número de visitas obligatorias debería aumentar.

A pesar de todo, creo que el proyecto ha sido una buena idea para el desarrollo en trabajo y compromiso de los jóvenes estudiantes, y espero que siga siendo así durante mucho tiempo.

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-Winning article of the II EEZ Science Award-

The importance of our environment: a qualitative study of water and soil in Iznalloz (Granada, Spain)

Bueno, Mª Belén; Cuesta, Lucía; Fresneda, Antonio; Garrido, Juan Miguel; González, Cristian; Gutiérrez, Álvaro; Morales, Pedro; Rivas, Mª Carmen; Ruiz, Marta; Vílchez, Patricia; Ortiz, Marisa[1*]; Plata, Carmen[2*]

I.E.S. Montes Orientales, Carretera de la Sierra s/n. 18550-Iznalloz-Granada
Corresponding authors: [1*]maluorma@gmail.com; [2*]biologiamencia@gmail.com

HIGHLIGHTS
environmental study, water and soil quality, soil fertility, uncontrolled dumping areas.

SUMMARY

Understanding and preserving our environment and spreading the information found about the richness of our cultural and natural background in Iznalloz (Granada, Spain) is essential for the development of our students. Therefore, by applying the scientific method and focusing our research on the surrounding fields through a water and soil quality analysis, we have obtained knowledge of how farming activities or even uncontrolled dumping of waste prove to be substantial in the modification of the quality of our environment. Thus, the presence of polluting elements in water like nitrates, phosphates or ammonia, can be attributed to a number of factors such as some farming routines.
Olive trees, for instance, are located in fields with high cationic exchange capacity. High levels of microorganisms have been found to cause the high rate of fertility of these soils. On the other hand, the tests done on the wastelands which are too often used as uncontrolled dumping areas rather than farming fields show significant reductions in the amount of beneficiary parameters mentioned above.
The whole educational community can benefit from the results and learning processes of this research in terms of a higher environmental awareness.

INTRODUCTION (AND OBJECTIVES)

The fundamental principles of the European environmental policies are conservation, protecting and improving the quality of the environment, protecting personal health, and finally, the prudent and rational use of natural resources. (Art. 130R of the Treaty of the European Union). At the national level, a large number of organisations have implemented the European Union norms, the most recent of these organisations being "*La Dirección General de Calidad y Evaluación Ambiental y Medio Natural"* (The Directorate General of Quality and Environmental Assessment and Natural Environment).This organisation was established in 2012 with the purpose of proposing, elaborating and programming national plans regarding pollution prevention and control, as well as pollution's environmental impact. This includes pollution indicator systems and the evaluations to which natural spaces should be subjected (Art.4 point 1).

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Among the different elements that form our environment, water quality and soil fertility levels have been protected since the initiation of these kinds of policies (1,2), which is due in part to the obvious relation not only with environmental quality, but also with human health itself (3,4). However, official reports, such as the one of 2006, have continuously shown that the water quality objective remains incomplete. Specifically, the water quality of various streams showS the presence of metals such as Se, Zn and Cr, as well as other toxic organic substances (5).

This same concern is evident when we analyze the situation of Andalusia, and while the 2012 reports of the "DMA (Directiva Marco del Agua)" [Water Framework Directive (WFD) network], under the Hydrographic Confederation of the Guadalquivir (6), identify degrees of improvement in the sections analyzed compared to that of 2007 (former ICA network). The number of runoff and oils that continue to appear have increased compared to the 2011 report. The most frequent impact was that the selected sections of river showed 66.3% residue present in the water. This impact becomes more serious in 10.9% of the sections, as in the areas near the river there are legal and illegal dumping areas.

The use of agricultural pesticides also contributes to pollution, and the presence of these pollutants in waters that return to natural sources compromise their later use, making the treatment of this wastewater a priority.

Soil quality is closely linked to water, especially when dealing with agricultural soils. Knowing their composition and treatment is crucial for improving its productivity and can correct possible contamination. According to *Informe sobre Calidad y Evaluación Ambiental* (2010) (7) of the Ministry of Agriculture, Nutrition and the Environment, the number of preliminary status reports of contaminated soils (IPS) received in the CCAA until 2009, amounted to 67,307, of which 11,130 correspond to Andalusia. Analysing soil pH level, the cation exchange capacity, and the degree of microbiological activity allow us to know not only the pollution index but the soil fertility.

The conservation of the environment is everyone's responsibility, but above that is the responsibility of solving problems that human activity generates. No policy related to the environment will succeed as long as society is not aware of the need to develop an environmental education that brings us back to a respect for nature. But for this we need to know what problems we are causing, which may have consequences for the environment and our own health and, of course, how we can prevent and solve such actions.

This conviction leads us to propose a research project that brings us to the environment of students themselves, as we are aware of the proximity of the students' school, therefore, the place in which they live must be the primary laboratory and object of analysis. This connection awakens more interest and enthusiasm among the students, helping them to learn about their immediate environment, but the learning process will make students become aware of the necessity to respect, care and dissemination of natural and cultural richness of the environment.

The quality and water use, its impact on the soil type and the use of it, influence and connection with the use of natural resources and the need to educate the implementation of the "three Rs" (recycle, reuse and reduce) allows students to not only learn from their environment, but translate that learning into an integrated and scientific project.
Thus, the main objectives of this research project are to ascertain the quality of water and soil of Iznalloz (Granada, Spain) for possible contaminants and investigate their origin and understand the need to disseminate the results, showing the research as an essential form of

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progress and improvement of our environment.

MATERIALS AND METHODS

The first step taken was to analyze and apply the scientific method, specifically, to understand the steps one must follow in any investigation for that work to be considered scientific. For that, we visited and analyzed the webpage http://www.sciencebuddies.org/science-fair-projects/project_question.shtml. Then, we focused on the web https://trello.com/¿ which permitted us to open a previously closed online communication channel by uploading and sharing information, as well as organising the different steps necessary for the project.

All samples studied in this work were taken in different sites from Iznalloz (Granada, Spain). We proceeded to locate the *illegal* dump sites around the town; areas whose toxic substances could affect the water and soil quality of our town. Pictures were taken of the selected sites and data about possible toxic substances and its origin were gathered using an especial data collection chart (8). These areas would be selected for sampling water and soil for its further chemical and microbiological analysis.

Preparation of water sampling program

There were three aims:

1) quality control
2) characterization of specified parameters (pH, chromium, chlorine, copper, cyanide, iron, nitrate, phosphate, silica, sulphur)
3) identification of sources of contamination

To achieve these aims, the following materials were used:
For sampling:
Latex Gloves
Plastic containers with a minimum capacity of 300 ml with screw on lids, ensuring complete closure
Adhesive labels to identify containers
Pen
Thermometers for taking water temperature

For the documentation phase:
Pens
Field Notebook
Data collection quadrant
Camera

Sampling was carried out in different types of sources: natural springs, rivers, public fountains and different potable water faucets:

In the surface sources (springs and rivers), samples were taken using latex gloves while holding the plastic containers in hand but away from mouths to avoid contamination. The bottles were immersed completely, filling and rinsing three times with water from the same source that we were going to sample. Once assured that the containers were not contaminated, we immersed them about 30 cm below the surface, sampling upstream to prevent an excess of floating material. In regard to choosing a sampling point sufficiently indicative of the quality and characteristics of the water, samples were taken neither too close to the shore nor far from the

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spring. Plastic containers were completely filled, closed and labelled while keeping away from excessive light and heat. Water temperature was taken and then data was collected and put into the quadrant.

Sampling in wells without pumps was done by hand, using gloves and without touching the opening of the container. Once again, the plastic containers were rinsed with water from the same well. Touching the walls of the well was avoided, in order not to contaminate the sample. Samples were taken from the surface water of the well. In wells with pumps, the pump was turned on for one minute before taking the sample, which was collected using a hose whose opening was set one meter below the surface. The containers were closed and labelled, then the water temperature was taken and the data quadrant was filled.

Finally, when sampling of potable water taps we wash our hands to avoid contamination. Furthermore we cleaned the tap itself, for which the filter was unscrewed and the mouth of the faucet was cleaned disinfected with an alcohol wipe. The water was allowed to run for 2 minutes to ensure that there would not be any deposits from the pipes in the final samples. We took the plastic container, with care not to touch the opening, and we placed it under the tap and took the sample. The vessel was sealed and labelled, then the data quadrant was filled with the collected data.

All samples were kept away from heat and light until the laboratory analysis, which was undertaken no more than 96 hours after the moment in which the samples were taken. All the samples were labelled immediately, stating the sample number, the date, time, place, and the name of person who took the sample.

The labels went along with a quadrant in which the information about the sampling site was recorded, as well as on-site observations. Specifically, the recorded information was the name and place of the sample site, the time and date the sample was taken, the weather conditions in the moment of taking the sample, and the recent weather conditions, such as if it had rained strongly in prior hours. The colour, smell and clarity of the water were also recorded, or lack of clarity in cases in which the water was turbid or even muddy. Any other significant observations were also noted, such as the presence of rubbish or fish.
Lastly, we photographed the site and the sampling process.

This water sampling method was performed at 11 different sites:

-Three were potable water taps in the high, medium and low level areas of the town, in order to analyse any possible changes in the quality or composition caused by the flow of water itself.
-The public fountain of the traffic circle of Iznalloz; a round fountain that serves to distribute traffic, and therefore could present pollution.
-Water spout located near the sports centre, near one of the areas with the presence of an illegal dump.
-The Well of Prado San Juan del Río; a private property well located in an area of olive groves. It was therefore interesting to investigate possible contamination by agricultural activity.
-The Periate Well; the same as the above well; it is located in an area of olive cultivation. We decided to test its level of pollution with the previous one.
-Ventorrillo River; located in an illegal dumping area.
- The Cañada Hermosa River; we wanted to test the water quality in relation to the Ventorrillo River.
-The Well in the Llano de la Valentina (a plain); a private well located in an area of olive cultivation.

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-Fuentezuelas Natural Spring: This spring is located next to the main road of Iznalloz, and as such it is an area with continuous traffic. The population of Iznalloz has a custom of collecting and drinking water from the spring.

In the school laboratory, the chemical analysis of the different water samples proceeded, using the Labs-Aids Inc. Kit and protocol titled "Introducción cualitativa a la polución del agua" (Qualitative Introduction to Water Polution). Our use of it was made possible by the University Rovira i Virgili de Tarragona (Ref. 19) through the scientific educational project APQUA.

Preparation of Soil Sampling Program

This preparation was carried out with three objectives:

1) To analyze the soil fertility and potential productivity, based on chemical and microbiological soil analyses.
2) To determine characterizing parameters (pH, cation exchange capacity, the need for adding lime (calcium carbonate) to the soil, optical 400x microscopic study of soil micro-organisms)
3) To identify the possible sources of pollution.

To do this, the following materials were used:

For taking samples:
- Latex gloves,
- clean plastic bucket to collect the sample,
- small spade or shovel to remove the soil,
- Plastic bags that have not contained fertilizer in order to save the labled samples.
- Adhesive labels to identify the containers.
- pens.

For the documentation phase:
- pens,
- field notebook,
- quadrant for data collection,
- camera

As when performing the water analysis, different sites were chosen to sweep the widest possible space and make more accurate conclusions about the soil quality in the area. The selected points are located in areas of olive groves as well as meadows and landfills, and they were collected and located on a topographic map of the location. Care was taken so that the materials and tools used in sampling were clean and free of pollutants that would affect the sample taken.

The sampling procedure was as follows:
- Approximately 3 cm of the soil surface was scraped at each point in order to clean and remove residue of fresh organic matter, road dust and other artificial contaminants.

- A hole was dug in the shape of a " V " the width of a shovel and this earth was left on one side , while a second stroke of approximately 15 cm thickness was used for sampling. The edges were discarded by making a cut with a knife, and the rest was put in a bucket or large bag.
Upon carrying out not only a chemical analysis but also a microbiological one, it was necessary to take several samples from each sampling point, and reserve those designated for the

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microbiological analysis for a week at room temperature with water until reaching its optimum moisture level without saturating, before proceeding to the analysis.

- Bags were identified with the identification label and closed securely. On the label of each of the samples, the Sample No. was recorded, along with the date, time, and the geographic coordinates at which the samples were taken, in order to later put the name of the farm or place of sampling and the name of the person who collected the sample on the topographic map.

- The labelling of the sample was supplemented with the quadrant in which the information about the site of sampling and in situ observations were recorded. Specifically the information recorded was: Name and location of the sampling site, date and time of sampling, geographic coordinates, depth of sampling, weather conditions at the time of taking the sample conditions and recent colour of the soil, the name of the person responsible for sampling and any other significant observations. Finally, we photographed the site and the sampling process and we located the site on the topographic map.

This sampling procedure was performed in six points:

-The olive grove next to the cemetery, to analyze the fertility of its soil.

-Valentina Olive Grove, compare their features and potential productivity with those of the grove above.

-The illegal dumping site named El Pilar, to determine the extent of pollution.

- The meadow by the cemetery, to characterize their parameters and potential soil productivity
-The Faragüit ravine and the ground in front of the school in order to compare the obtained data obtained after the microbiological analysis.

Once the samples we collected proceeded to chemically and microbiologically analyse soil samples in the laboratory of our secondary school using a kit and protocol from Lab-Aids inc. "Biology and chemistry of the soil," made possible by the Rovira i Virgili University of Tarragona (Ref. 32) through the APQUA educational science project.

RESULTS

Chemical Analysis of Water Samples

The results obtained after completing the chemical analysis of various parameters in water samples in our laboratory are shown in the ANNEX OF TABLES, Table 1 (Identification of Water Samples) and Table 3 (Water Samples Chemical Analysis Results). Discussion of the data obtained is as follows:

Ammonium nitrogen

Well ventilated surface waters usually contain little ammonia; Water from natural streams that are little polluted usually have no more than 0.10 mg NH_3/L. Higher levels of ammonia are indicative of recent contamination. The main source of contamination of ammonia is sewage. In the waste water, the ammonia comes from the breakdown of the urea, $CO(NH_2)_2$.
Furthermore, it is known that rainwater, due to dissolution of nitrogen from the atmosphere, may have traces of this compound (9).

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Our analysis of samples conducted show the following results for this parameter **(Table 3)**:
There is a slight positive (a small change of coloration in the colorimetric test) in samples from the **source of the roundabout of Iznalloz** (sample 2) and the **well of the Valentina Plain Olive Grove** (sample 8). A possible explanation for these results would be the possible presence of agricultural wastes (animal excrement, garbage, fertilizers) in the case of water from the well located in the grove (10).

In the literature reviewed (11), we found that natural concentrations in groundwater and surface water of ammonia are usually less than 0.2 mg/L, but anaerobic groundwater may contain up to 3 mg/L and intensive farming can generate much higher concentrations in surface waters. The cement mortar used to coat pipes can also produce ammonia contamination. The latter reason could add small amounts of this compound to the water of the source analysed, which was taken at the roundabout near the secondary school.

pH

The origin of the pH in the water can be natural or artificial. As a natural cause, carbon dioxide is dissolved from the atmosphere, and, more fundamentally, can be found in the infiltration of the earth as a result of respiration of living organisms, as well as respiration and photosynthesis of aquatic organisms.

Organic acids, including humic acids, are also common in the waters, the latter owe their origin mainly to forest mulch, which is washed by water runoff.

Among others, calcium carbonate is one of the primary basic constituents found. This compound affects the pH of the water because it is able to react with dissolved carbon dioxide to form calcium bicarbonate, which is soluble, producing a buffer system.

Although the pH does not tend to directly affect the consumers, it is one of the most important operative parameters of water quality, and the optimal value is generally from 6.5 to 9.5 [According to data from the OMS (12)].

The colorimetric analysis of the samples placed the pH values in our samples between **7-9**, as shown in **Table 3.**

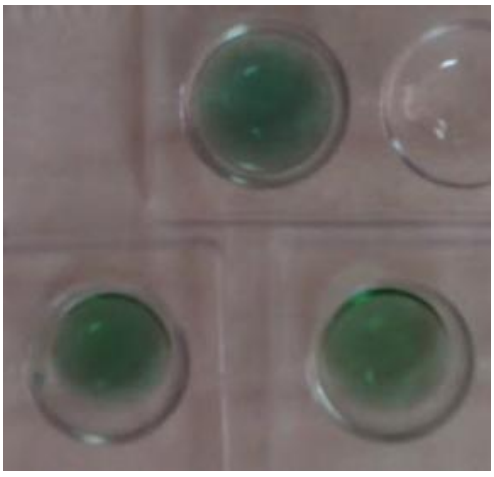

Figure 1
An example of the results of the colorimetric test in three water samples

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Water has a natural pH of about 7, i.e., neutral. Living organisms require a level which is between 6 and 9.
As seen from our results **(Table 3)**, in several samples the pH is alkaline. In water, the alkalinity is produced by the presence of a high concentration of carbon molecules in mineral suspension. Water with high alkalinity is said to be "hard." The mineral compound that is caused is calcium carbonate, from rock, such as limestone or dolomite leaching or ground calcite. This would explain the alkalinity of the water in the area, given the limestone geology of the study area according to the GEOLOGICAL MAP OF SPAIN-IZNALLOZ (Geomineral Technological Institute of Spain)(13).

Chlorine

The disinfecting action of chlorine in water derives from its high oxidizing power in the chemical structure of bacteria cells, destroying the normal biochemical processes of development. Environmental conditions that optimize the outcome of this disinfection are chlorine concentration, pH, temperature and contact time.
The main characteristic of chlorine for use as a disinfectant is its continued presence in water as residual chlorine. The Spanish technical-health regulations determine that water intended for human consumption must have a minimum concentration of free or combined residual chlorine or other disinfectant as follow:

Residual chlorine available in water		
pH	Minimum concentration of free residual chlorine (ppm, 10 minutes)	Minimum concentration of combined residual chlorine (ppm, 1 hour)
6	0.2	1
7	0.2	1.5
8	0.4	1.5
9	0.8	--
10	0.8	--

Furthermore, chlorine not only acts as a disinfectant, but also reacts with other components present in the water, such as ammonia, iron, manganese and other substances that produce odours and flavours, improving water quality.

On the other hand, an excessive concentration of chlorine in water causes immediate rejection by the consumer. It is not harmful to health, but it gives a very strong and unpleasant taste to the water if its concentration exceeds 0.5 ppm (14).

The results in our sample **(Table 3)** suggest a positive for Chlorine in the sample from the water supply of the Iznalloz sports center. This being the only positive data in our analysis could be explained by the fact that the water of this sample is close to a chlorination point in the town, and the process could have taken place shortly before we took the sample.

Chromium

Hexavalent chromium (which rarely occurs in drinking water in its trivalent form) is carcinogenic, and it is necessary to determine and ensure that drinking water it is not contaminated with this metal (15). This metal is naturally in water, soil and rocks. It is also found in crops and as an element remaining in agricultural soils. In addition, there are trace levels of chromium in the environment, which come from industrial activity (16).

The presence of chromium in drinking supplies can be found as a result of industrial waste, especially from chromium salts used to prevent equipment corrosion, as chromates are added

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to refrigeration water. Perhaps this could be explain the only positive sample in our analysis, tap water from the higher part of our town **(Table 3)** or it could be a false positive and we have to repeat the analysis after collecting a new sample at this point.

Copper

Copper is a metal of high interest when considering the quality of drinking water because it has a dual nature; it is an essential metal for humans and can, in both deficiency and excess, cause harmful health effects. The essential character of copper comes from its incorporation of a large number of proteins with catalytic and structural purposes. Its biochemical toxicity, when it exceeds the homeostatic control, comes from its effects on the structure and function of various biomolecules.

Copper is a widely used metal worldwide due to the following desirable qualities: durability, ductility, malleability, and electrical and thermal conductivity. Many of the materials used in distribution systems for drinking water faucets or pipes contain copper as the main component or as part of alloys.

The use of copper pipes in drinking water distribution varies widely around the world. The presence of copper in drinking water may be of natural or anthropic origin. The latter is as an affect of leaching/corrosion due to the physical and chemical characteristics of the water matrix that comes into contact with materials containing copper (17).

None of our samples proved to be positive in this parameter (table 3).

Cyanide

There may be presence of cyanide in some foods, particularly in some developing countries, and sometimes in drinking water, primarily as a result of industrial pollution.

In this case no cyanide was detected in any of the samples analysed **(Table 3).**

Iron

Iron is one of the most abundant metals in the earth's crust. It is present in natural fresh water at concentrations of 0.5 to 50 mg/l. There may also be iron in water consumption due to the use of iron coagulants or the corrosion of cast iron or steel pipes during water distribution.
No reference value for iron in drinking water is proposed [according to the WHO (12)]. In our analysis, all samples were negative for detection of iron in water **(Table 3).**

Nitrates

Figure 2 and **Table 3** show the results obtained for this parameter.

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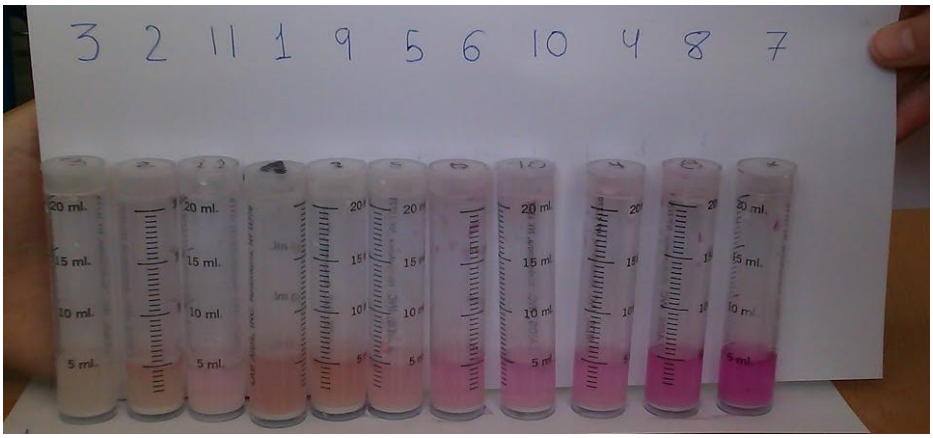

Figure 2
Results of colorimetric qualitative study of nitrate in water samples

The Results in approximate % of nitrate concentration are shown in Figure 3.

Nitrate is mainly used in inorganic fertilizers. The concentration of nitrate in groundwater and surface water is generally low, but can become high from the filtration of agricultural runoff or due to contamination by human or animal waste as a result of the oxidation of ammonia and similar sources. The area where the results are positive in this parameter are agricultural areas, which may explain the presence of nitrates.

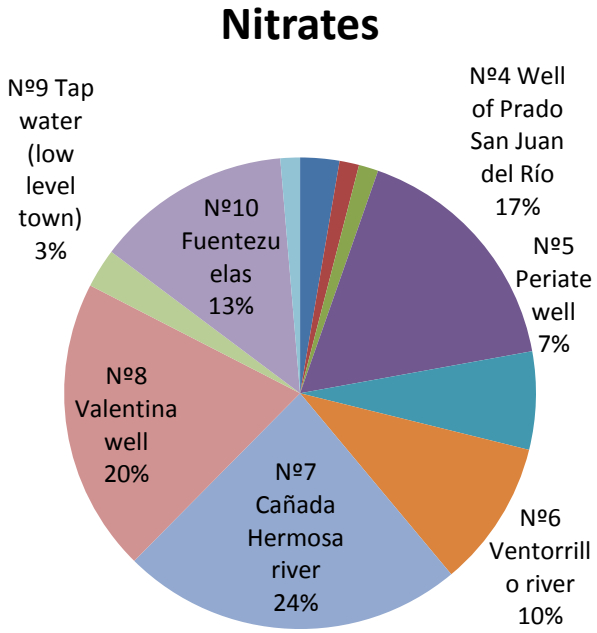

Figure 3
Results of the colorimetric study in approximate % of nitrate in water samples

The presence in most countries of nitrate concentrations in drinking water from surface water do not exceed 10 mg/L, although the levels of nitrate in well water often exceed 50 mg/L, (18), (19).

Phosphate

The only water sample that was found positive was the Valentina Well (Table 3). The origin of such presence can be very varied, but the most common reason is contamination by detergents for washing clothes or cleaning in general. The use of fertilizers, compost or pesticides that include phosphates may affect positively the detection of these compounds, this fact is due to the percolation effect of these products to natural aquifers (20). The Figure 4 shows the results of our analysis.

Figure 4
Results of the qualitative colorimetric study of phosphate in water samples

Figures 5 and 6 show the results obtained.

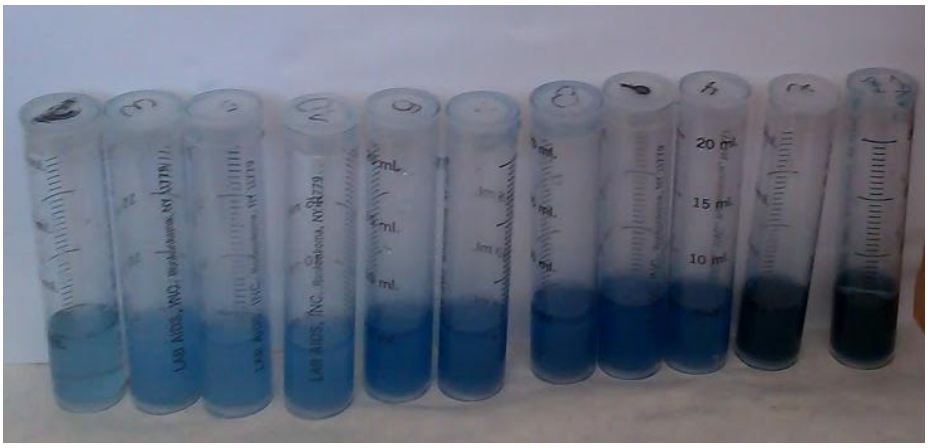

Figure 5
Results of the qualitative colorimetric study of Silica in water samples

We can explain the presence of silica as a consequence of erosion from rocks containing silica (clays). The eroded silica then becomes suspended particles in natural water sources, in colloidal or polymeric state, and as silica acids or silicate ions. These data are explained due to the Geology of the zone (13), where Sierra Harana is composed of clay mainly, dated from the Triassic period.
The silica content in natural water is usually between 1.0 and 30 mg/L, but it is not uncommon to find concentrations of 100 mg/L or even 1000 mg/L in some brackish water and high seas (21).

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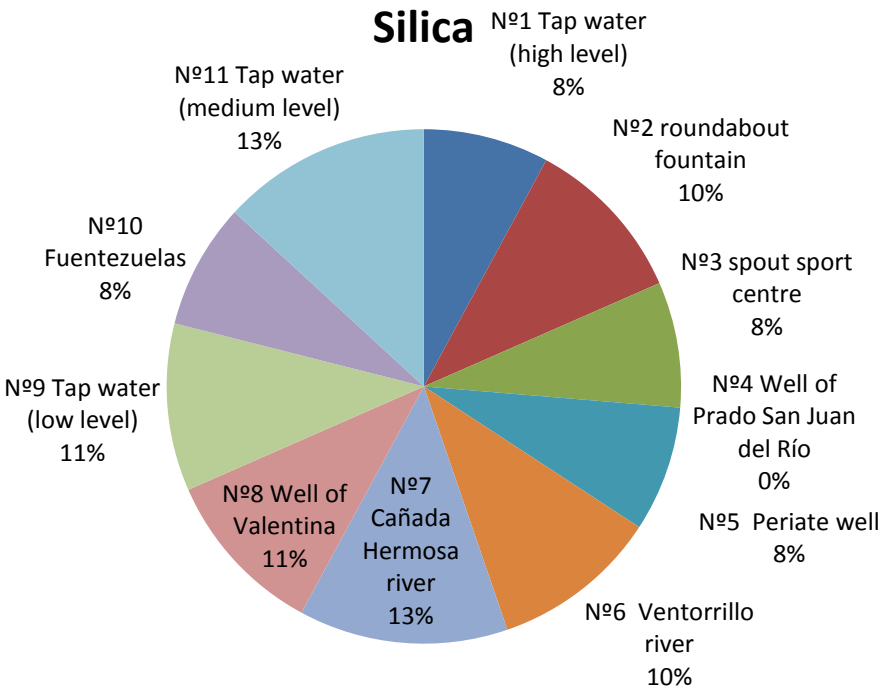

Figure 6. Results of the qualitative colorimetric study of Silica in water samples

Sulfur

The only positive result is found in the source at the Iznalloz Roundabout **(Table 3).**
Hydrogen sulfide is a gas with an unpleasant odour characterised as a "rotten egg" and is detectable at very low concentrations (below 0.8 g/m^3) in the air. It is formed by hydrolysis of the sulphides in water. However, the concentration of hydrogen sulfide in water consumption is usually low because the sulfides are oxidized rapidly in well oxygenated waters. Therefore, although no reference value is proposed, hydrogen sulfide should not be detectable in drinking water for taste or odour (12).

The Sulfur in the water samples may come from the following sources (22):
- Acid mine drainage
- Sewage: In some areas where the water is stagnant, all the oxygen has been used and, in its place is hydrogen sulfide. e.g. water from the bottom of stratified lakes and reservoirs.
-Waste water: sulfide produced by bacterial sulfate reduction.
- Other sources: the paper industry, petrochemical, tanneries and slaughterhouses.

Since the presence of sulfides in surface waters, and well-oxygenated water in general, is scarce, none of the above sources is responsible for the positive result of the roundabout sample. However, there are artificial sources of sulfur, as in the case of incomplete combustion (23). When combustion is performed with oxygen shortage, sulfur fossil fuel is converted into H_2S, and carbon into CO. The location of the roundabout is a constant passage of vehicles, which could be the reason why there is sulfur in the water from this source.

Chemical Analysis of Soil Samples

After conducting a chemical analysis of various parameters on soil sampling in our laboratory, the results obtained are shown in ANNEX TABLES, Table 2 (Identification of soil samples) and Table 4 (Chemical analysis results of soil samples). Discussion of the data obtained are as

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follows:

pH and need for addition of lime (calcium carbonate)

In soil with pH lower than 7, the yield of most crops can increase if lime is added to the field. In our case, the pH of all soil samples is between 7-8, and therefore there is no need to add lime **(Table 4) (Figure 7).**

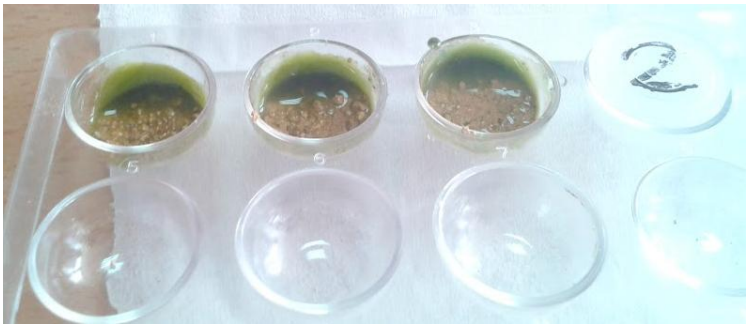

Figure 7: Example colorimetric method to measure pH in soil (sample 2)

Cation exchange capacity (C.E.C.)

The cation exchange capacity is an important measure of fertility and productivity potential of soils. Organic matter has a high C.E.C., so therefore, soils with a high content of organic matter generally have a C.E.C. than that of soils with a low content of organic matter. Moreover, soil pH affects the C.E.C., as highly acidic soils retain a high percentage of hydrogen ions, whereas soils with a favourable pH of 6 to 8 (neutral) retain a high percentage of calcium ions. Soil texture (clay concentration) also affects the C.E.C. Thus, clay soils show high and desirable C.E.C. values. But again, the organic matter plays a vital role in soil texture (24), (25).
The diagram below **(Figure 8)** explains this concept in detail.

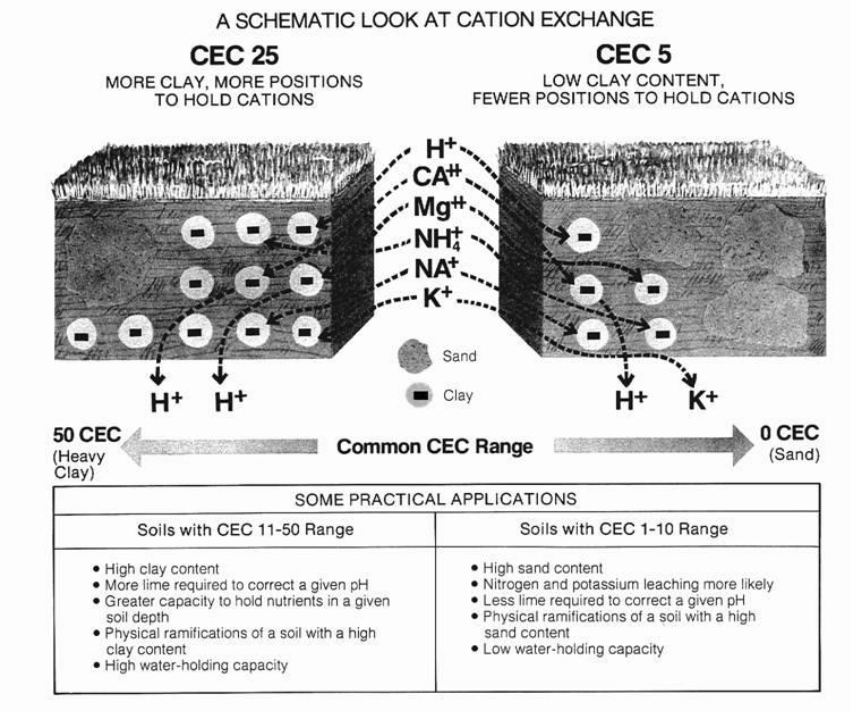

Figure 8. Schematic diagram of cation exchange in soils

The values obtained in our laboratory tests, are shown in **Table 4** and **Figure 9.**

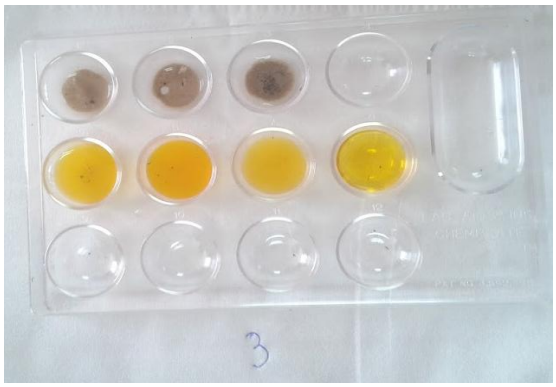

Figure 9.
Example C.E.C. testing method (Sample 3)

Soils with greater estimated C.E.C. correspond to the olive crop samples near the cemetery and on the Valentina Plain. Thus there is a correspondence in the study area between crops (olive trees in this case) and the highest estimated values of C.E.C. since soils with high C.E.C. typically have high clay and/or organic matter. These soils are considered more fertile as they can retain more nutrients. The soils analyzed with the scarcest estimate of C.E.C. correspond to areas of meadow and illegal dumps. ("El Pilar" dump site).

Microbiological Analysis of Soil Samples

In the soil samples analyzed **(Figures 10a, 10b)** possible diplococci, streptobacilli, fungi, plant roots and a large population of actinomycetes were found in some samples.

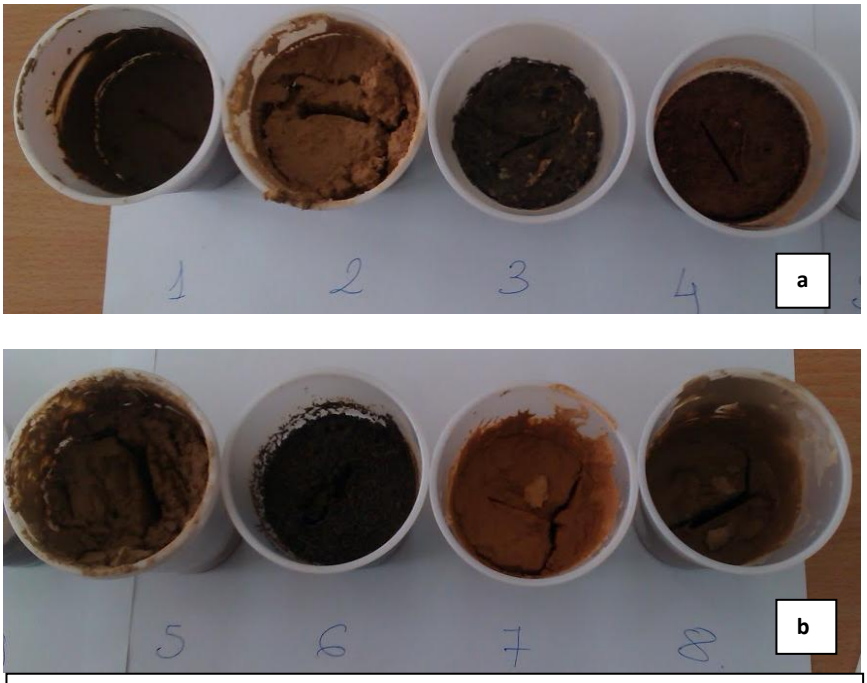

Figure 10
Soil samples after incubation for one week at room temperature for the microbial analysis. (Samples 1,2,3,4, 5, 6,7,8)

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According to the literature review (26,27,28,29), the actinomycetes do not tolerate acidic pH, as its density is the inverse of the hydrogen ion concentration. Species of the genus *Streptomyces* do not proliferate at a pH lower that 5.9, and in acidic soil the ratio of actinomycetes is less than 1% of the total microbial population. Nevertheless, there are varieties resistant to acidity (27,28,29), which grow best in micro-environments with pH values ranging between 7 and 8, which corresponds to our observations.

Our observations have led us to conclude that the most active soil, from the microbiological point of view, corresponds to **sample 4** (Valentina Olive Grove), where the frequency of possible streptobacilli, actinomycetes and fungi (optical microscope 400x) surpasses that of other samples. The soil with a lower presence of micro-organisms corresponds to **sample 3** ("El Pilar" Dump Site), although it is in this sample in which microscopic nematodes were found (**Figure 11).**

Figure 11
Samples with the highest (4) and lowest (3) microbial content.

CONCLUSIONS

This research, completed by the students of year 4 of E.S.O. (Secondary/High School Programme, bilingual section), has highlighted the importance of the scientific method as applied to environmental study of Iznalloz (Granada, Spain) and its surroundings. After the chemical analysis of water and soil samples and microbiological analysis of the latter, we have seen how agricultural or farming activities and uncontrolled discharges may affect the conditions of our environment.

Thus, the presence of ammonium nitrogen and phosphates in the Well of the olive grove in Valentina Plain and nitrate in water samples from streams and wells in areas adjacent to the town of Iznalloz confirm water contamination at these points given farming that occurs in them.

The richness of the silica in our waters (supply network in the city and surrounding wells and streams) is due to the presence of clays dating from the Triassic period as one of the geological components of the Sierra Harana Mountain. In this way we confirm the connection, from the chemical point of view, between geosphere and hydrosphere.

The most fertile soils (with a high cation exchange capacity) are dedicated to cultivation, mainly in olive groves, while soils with fewer values for this parameter are areas left uncultivated (meadows and the "El Pilar" Dump Site).

The microbiological study in our soil samples has identified a wealth of actinomycetes, including micro-organisms, due to the pH measured in these samples (pH 7-8). The exception is found in the "El Pilar" Dump Site, where the incidence of micro-organisms was drastically reduced.

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ACKNOWLEDGEMENTS

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