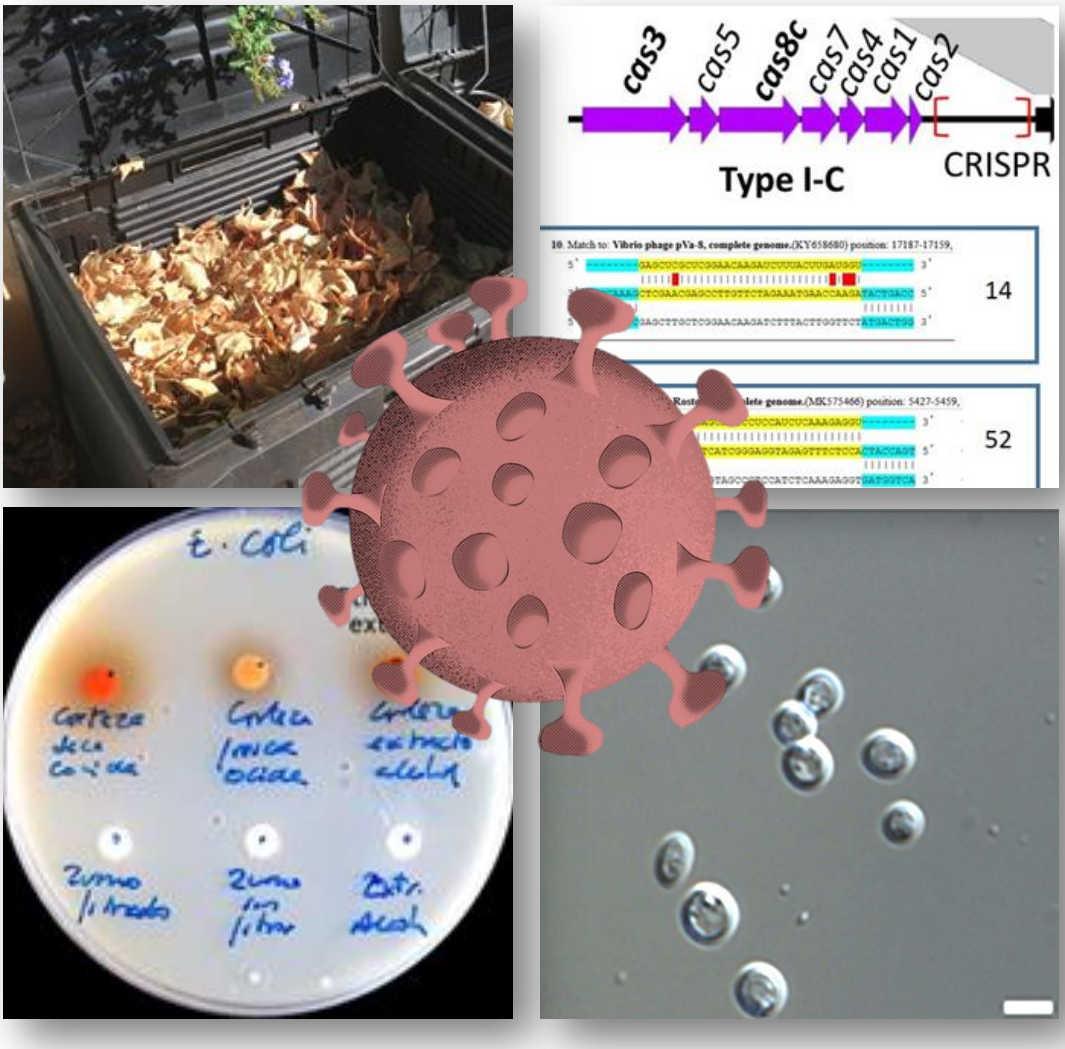
High School Students for Agricultural Science Research

Volume 9



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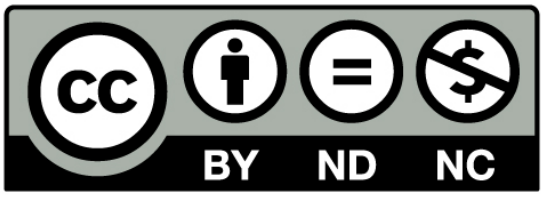

Index

EDITORIAL: Science education in pandemic times

Manuel Espinosa-Urgel

Science Outreach Unit. Estación Experimental del Zaidín. CSIC.

In December 1999, the film "*Girl, interrrupted*" was released. Based on an autobiographic book by Susanna Kaysen, it featured, among others, Winona Ryder and Angelina Jolie, who won the Best Supporting Actress Oscar for her role. The film tells the story of an 18-year old girl who spends several months confined (except for some unauthorized night excursions…) in a mental institution after a nervous breakdown. The title of the film could very well reflect the feelings of many of our teenagers during the COVID-19 crisis we are still slowly trying to overcome; classes and activities at school replaced by videoconferences, no hanging out with friends, confinement for several weeks, and economical difficulties in many homes. Life, in essence, interrupted, except for the access to internet resources and online social networks.

Among those activities that had to be stopped and re-shaped, were the Science education projects that we carry out each academic year with high school students and their teachers (PIIISA and Ciencia BaSe), and which constitute the backbone of this volume series. All of the projects were well underway and showing promising results when the virus stroke. This, and the enthusiasm of most of the participating students, who felt highly engaged with their research, made us find ways to allow them to complete the work as much as possible. Laboratory work had to be replaced by employing online resources, searching bibliography and discussing through videocalls. And above all, plenty of brain usage to interpret the data they already had and put them into writing, with the guidance of their teachers and the researchers involved.

This effort on all sides (students and their families, teachers and scientists) has crystalized in a series of articles, maybe with less experimental results than desired, but still remarkable under the circumstances. And perhaps most importantly, this volume is full of scientific thoughts written by the students, and reflected in the "My own ideas" section after each paper. As one could expect, the SARS-CoV-2 virus is present throughout these comments, along with the realization that science is not a bunch of arcane concepts carved into stone by old wise people, but a dynamic process in which they can get involved, as well as the key for our society to move forward. And that, after all, is the mission of our science education programmes: bringing science into the students' lives.

Against all odds, we proudly present Volume 9 of *High School Students for Agricultural Science Research*. Allow me to do so with a few lines by the American poet Walt Whitman, who was fascinated by scientific concepts such as Darwin's theory on evolution, yet was even more fascinated by the beauty of Nature, which luckily we can enjoy ourselves again after months of seclusion:

When I heard the learn'd astronomer,
When the proofs, the figures, were ranged in columns before me,
When I was shown the charts and diagrams, to add, divide, and measure them,
When I sitting heard the astronomer where he lectured with much applause in the lecture-room,
How soon unaccountable I became tired and sick,
Till rising and gliding out I wander'd off by myself,
In the mystical moist night-air, and from time to time,
Look'd up in perfect silence at the stars.

Walt Whitman – Leaves of Grass (1867 ed.)

How to know if a bacterium contains CRISPR elements?

Nicolás Amorós Monte[1*], Daniel Martín Jiménez[1*], Marta Nieves Vallejo[1*], Mª Dolores Puig Aguiar[1*] Sara López Laazibi[1], Marina Fijo Martín[1], Francisco Martínez-Abarca[2#]

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**All these students contributed equally to this work*

Highlights: *Students have learned how to identify the presence of strange structures in the DNA as indication of CRISPR-Cas Systems.*

Summary

Students have learned how to manage DNA sequence data using different programs and web pages to analyze them. Once having some practice we put our interest in finding the presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) elements in a bacterial genome non studied so far: the genome of the strain of *Vibrio vulnificus* VV5. This genome shows a complete CRISPR-Cas system in 12,5 kb of DNA locus. The characteristics of this locus are shown in this study. The fact that almost one half of the spacers of the CRISPR array present a match against mobile DNA (Plasmids and phages) suggests that is highly active being a good candidate for studying its biotechnological potential.

Keywords: *CRISPR, Clone manager, bacterial genomes*

INTRODUCTION

In 1993, F. Mojica and F. R. Valera researchers, studying *Haloferax mediterranei*, an archaeal microbe with extreme salt tolerance that had been isolated from Santa Pola's marshes, found an unexpected pattern on DNA segments in the genome [1]. Stretches of sequences of 30-bp long, repeated at regular distances (Fig. 1). Latterly other researchers found similar pattern in other bacterial genomes. These DNA segments were named afterwards CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). It took more than ten years to discover and assigned a biological role to these structures in the DNA. A role in immune defense against virus was proposed [2]. Mojica and collaborators found that these repetitive units were interspersed by short sequences that perfectly matches against sequences of bacteriophages (virus that affect to bacteria). Mojica suggested that CRISPR systems work as immune systems since the bacteria kept the record of previous infections to protect against identical viruses.

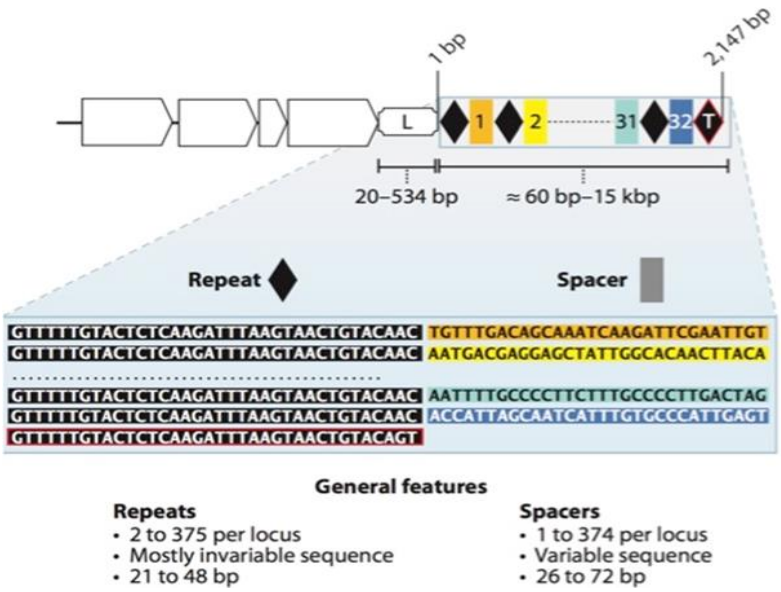

Figure 1. Diagram of a CRISPR-cas system. The leader sequence, between 20 and 534 nucleotides in length, contains the promoter sequence for transcription of the CRISPR array. The repeats (diamonds) are between 21 and 50 nucleotides in length (mean of 31 nt), while the spacers (colored squares) are between 17 and 84 nucleotides (mean of 36 nt). In the vicinity of the array, the presence of Cas genes (in white) is frequent.

Since then, a lot of studies have been achieved to decipher the complete mechanism of defense against virus of the CRISPR-System (Figure 2). The immunity mediated by CRISPR takes place in 3 stages: adaptation, expression and interference [3]. Cas1 and Cas2 proteins situated near of the Array are required for the acquisition of DNA spacer by the CRISPR locus (adaptation). The CRISPR array provides a precursor transcript (precursor crRNA) that is processed into short (mature crRNA) structured RNAs (expression), leading to the formation of crRNA-Cas effector complexes that recognize and bind complementary nucleic acids, resulting in degradation of the target molecule (interference). This last step, interference with

other nucleic acids (viruses) allow the opportunity for researchers to demonstrate that CRISPR might provide a powerful tool for cutting, and thereby editing, specific genomic loci [4]. Nowadays, biotechnological applications of CRISPR are the basis of what is called 'a la carte' genetic modification.

CRISPR-Cas systems are highly diverse, and currently exist different tools *in silico* to identify them and classify accordingly, based in the structure of the Direct repeats of the array, the characteristic of the adaptation complex formed and mainly by the set of proteins involved in the interference step.

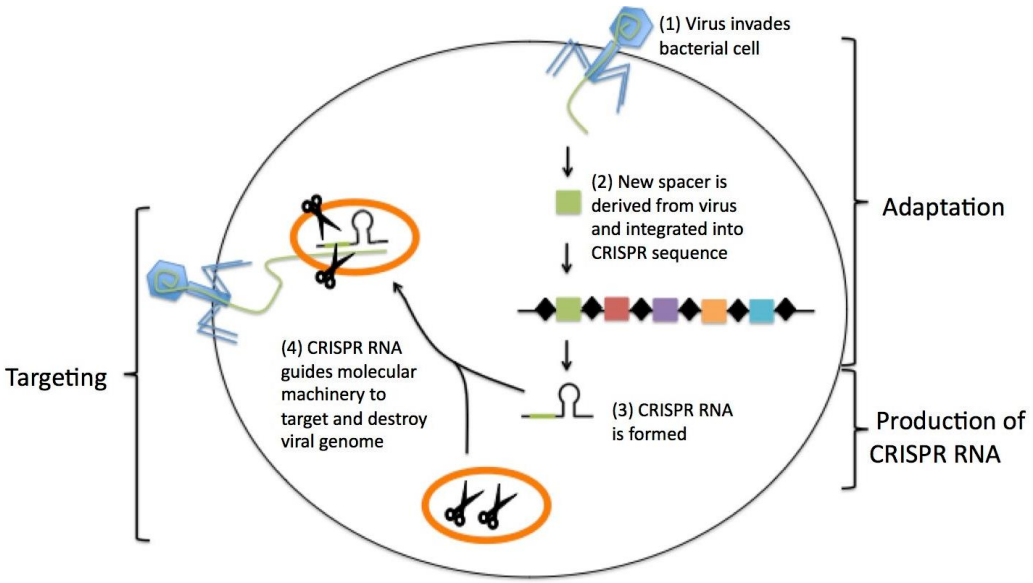

Figure 2. The immunity mediated by CRISPR takes place in 3 stages: adaptation, expression (production of the CRISPR RNA) and interference mediated by DNA targeting to destroy the Virus.

In this project we have learned how to work with DNA with the computer. From small DNA fragments (several Kb) to complete genomes of bacteria (Mb). In addition, we have acquired knowledge about tools for identification and characterization of CRISPR systems in genomes non studied so far. Finally, we could understand why CRISPR-Cas systems have become a tool for modifying genomes on demand with great potential in novel biotechnological applications.

MATERIAL AND METHODS

1. Genome availabity

Bacterial Genomes used in this study and training for learning how to search CRISPR system were obtained from NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/) containing currently more than 240.000 genomes of bacteria. The genome in our study: *Vibrio vulnificus* strain VV5 was kindly contributed by Carmen Amaro from the University of Valencia.

2. Clone manager program

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

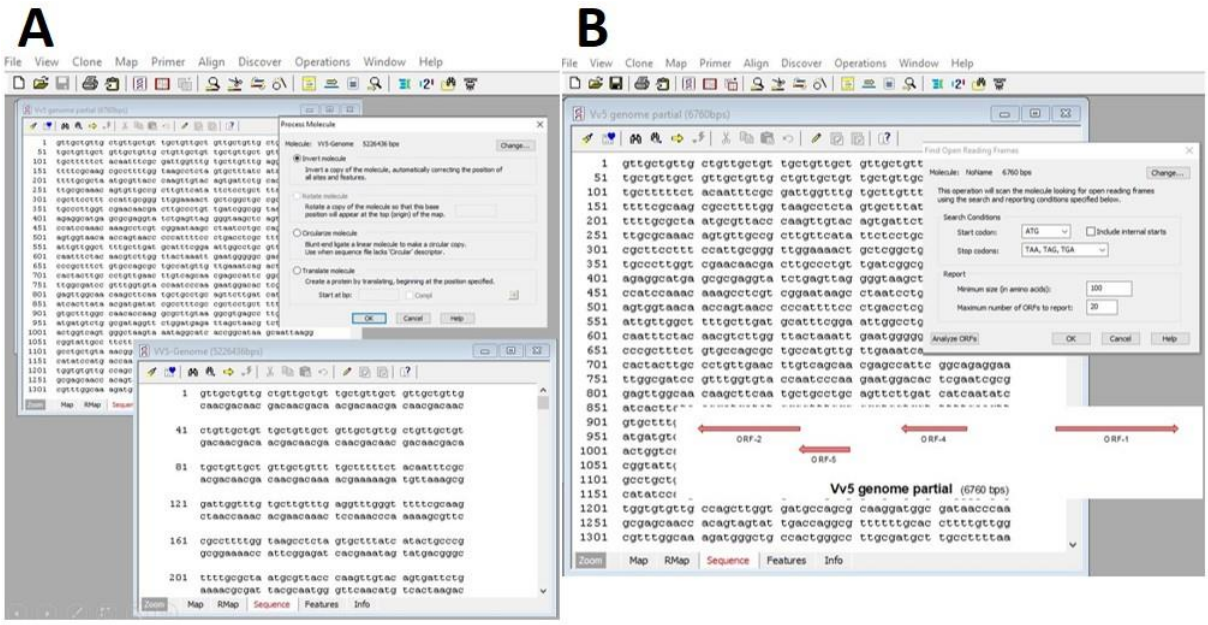

Figure 3. Examples of the tutorial of clone manager program: (A) 'Invert' Tool to work with the complementary strand of DNA. (B) 'ORF search' tool to find the Open reading frames of the sequence in order to identify and annotate every gene present.

3. CRISPR tools

1) The CRISPRCasFinder program enables the easy detection of CRISPRs and cas genes in user-submitted sequence data (allows sequences up to 50 Mo otherwise download standalone program). https://crisprcas.i2bc.paris-saclay.fr/ [5].
2) CRISPRSuite programs and tools currently consist of CRISPRTarget, CRISPRDetect, tracrRNAFinder and CRISPRBank developed in Otago university (New Zealand). A tool to predict and analyze CRISPR arrays. This tool has been used to understand the origin of the different spacers present in a particular array. http://crispr.otago.ac.nz/CRISPRTarget/crispr_analysis.html [6].

RESULTS AND DISCUSSION

1. *V vulnificus* VV5 genome.

Vibrio vulnificus is a pathogenic bacterium with curved morphology, Gram negative and oxidase positive. It is halophilic, so often present in coastal marine waters and within molluscs, crustaceans and fish. The pathology is produced by consumption of seafood, shellfish and raw or undercooked fish and exposure to contaminated water. VV5 isolate belong to a collection of bacterial isolates of sea water obtained of beaches close to Valencia. The genome has been sequenced by Illumina technology by C. Amaro group in the University of Valencia, and the subsequent assembly show a genome size of 5,226,436 bp.

2. Identification CRISPR Systems in *V. vulnificus* VV5 genome.

The complete genome to study was upload to the CRISPR finder platform in order to search for putative CRISPR Systems. The program identified 3 arrays but only one of them with high score identity level (Figure 4). In addition, this array was adjacent to a gene cluster of seven genes of a complete CRISPR Cas system of type IC. This system is closely related to other system previously described in a strain of other *Vibrio* species: *Vibrio navarrensis* [7].

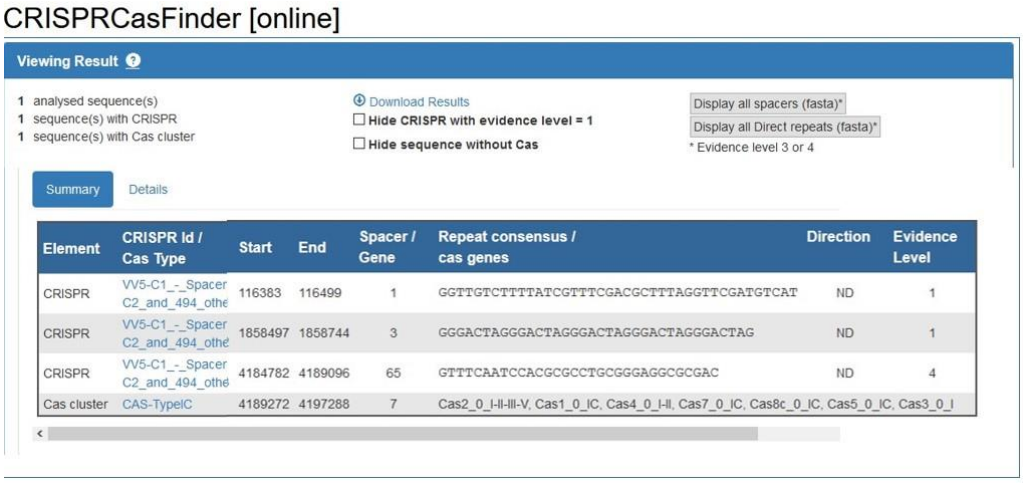

Figure 4. Analysis with CRISPRCasfinder tool of 5.2 Mb of *V. vulnificus* VV5 genome. The VV5 genome shows three different CRISPR Systems localized in different part of the genome. However, only one of them score with high evidence (4) since present 65 different spacers and a cluster of CAS protein just upstream of the array.

The array is formed by 65 direct repeats of 32 nt along 4.3 kb in length (Figure 5). The sequence: GTCGCGCCTCCCGCAGGCGCGTGGATTGAAAC form a characteristic palindromic structure. The cas operon is localized just upstream of the array (at 176 nt) and contains seven genes (in 8 kb of DNA sequence) involved in the different steps of the immune response. But, what is the history of the phage infection suffered by this strain?

3. Matching of spacers sequences.

A tool (CRISPRTarget) that predicts the most likely targets of CRISPR RNAs (http://bioanalysis.otago.ac.nz/CRISPRTarget) has been used for analyzing the 65 spacers found in this array. From the 65 spacers, 31 present matches against sequence contained in Genebank-Phage; RefSeq-Plasmid and IMGVR databases. Most of them present sequence against phages (some examples shown in Figure 5) and plasmids. The fact that almost 50% of the spacers present a positive hit map against exogenous DNA is a good indication that the system is active in this strain.

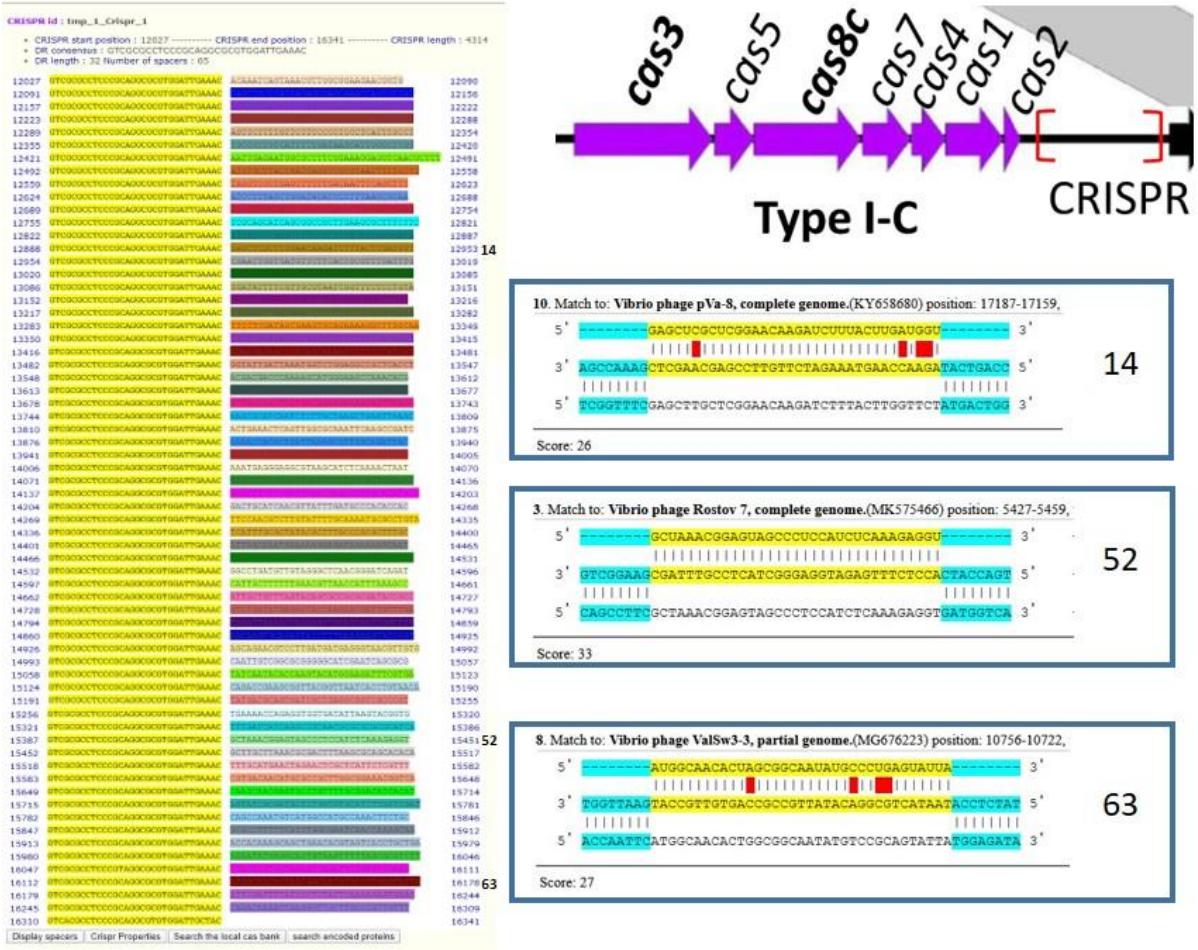

Figure 5: Characteristics of the complete CRISPR System found in VV5 genome. On the left, the CRISPR array complete with the Direct Repeats (yellow) and the diferent spacers (coloured). Three examples (spacers 14,52 and 63) of significative hits against protospacers found in vibrio phages (viruses) are shown. On top, the corresponding ORFs showing a complete CRISPRCas Sytem Type IC are indicated.

All these properties suggest that this CRISPR system is active and it can be a good candidate to be studied as biotechnological tool in genome editing.

CONCLUSIONS

1. One goal achieved was to learn and understand the information of a DNA sequence using clone manager program.
2. The *Vibrio vulnificus* VV5 genome presents a complete CRISPR System.
3. This CRISPR System is composed of 65 direct repeats together with a cas cluster system Type IC. The half of the spacers match against a virus which infect to *Vibrio*.
4. All these characteristics suggest it can be a good candidate for exploring as biotechnological tool.

Acknowledgements

This work was performed in the Microbiology and Symbiotic Systems department in the Estación Experimental del Zaidín – Consejo Superior de Investigaciones Científicas and **at student's home**. It was supported by research project BIO-2017-82244-P; Secondary School Institute Arjé in Chauchina Granada. Also we appreciate the coordination in the project of teacher D. Ramón Pérez Moreno. We are particularly indebted with the teacher Antonio Quesada Ramos for his help in developing our blog: https://eez-crispr.blogspot.com/.

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MY OWN IDEAS

Francisco Martínez-Abarca (Investigador)

The PIIISA Project of this 2019/2020 course will be difficult to forget, like so many things that have occurred. And it is that, in particular, the last months with COVID19 have been quite a challenge for students, teacher and Senior Researcher. The merit of this work is superior to those carried out in previous years. Congratulations to the students who have participated. Congratulations to their families and congratulations to the whole institute.

El Proyecto PIIISA de este curso 2019/2020, será dificil de olvidar, como tantas y tantas cosas que han sucedido. Y es que, en particular, los últimos meses con el COVID19, han sido todo un desafío para los estudiantes. El mérito de este trabajo es superior a los llevados a cabo otros años. Enhorabuena a los estudiantes que han participado. Enhorabuena a sus familias y enhorabuena al instituto en su conjunto.

Nicolás Amorós Montes (4º ESO)

It has been a quite interesting project in which we have been able to understand what is a genome and also biological data we did not know until now.
In this project we have learned to use tools derived from the clone-manager program that has helped us learn how define a gene in a bacterium; another informatics tool like 'crispr-finder' to find the crispr systems that exist in the genomes of bacteria and at last, the 'crispr-target' that serves to know through the 'spacers' which viruses have been faced to this particular bacterium.

Ha sido un proyecto bastante interesante en el que hemos podido conocer mejor lo que es un genoma y datos biológicos que desconocíamos hasta el momento.
En este proyecto hemos aprendido a utilizar herramientas como el clone-manager que nos ha ayudado a aprender cómo se conforma el gen de una bacteria otro como el crispr-finder para encontrar los sistemas crispr que hay en los genomas de las bacterias y el crispr-target que sirve para saber mediante los 'spacers' con los virus que se han encontrado la bacteria .

Daniel Martín Jiménez (1º Bachiller)

My expectations with this project have changed dramatically throughout its development. At first, it seemed to me to be too important an investigation for some simple high school students, but with the researcher's help, which is not trivial, we have managed to know what CRISPR systems are, which allow us to detect the viruses that have attacked the analyzed bacteria, and especially learning to detect them.
Throughout the project we learned about programs and websites that can be used for DNA analysis and search CRISPR elements, for example, Clone Manager or CRISPR-cas. But all of this is general and out of focus.
Our true goal was to investigate something that no one had ever investigated before. Detecting CRISPR systems in the *V. vulnificus* VV5 bacteria, which was not going to be an easy task. We had a lot of obstacles, such as technologies, the difficulty of this investigation even with an alarm state in the world (COVID19). A situation that led us to a confinement in our houses, which unfortunately paralyzed us, but only momently our investigation. Paco, the researcher, organized some online sessions for us, thanks to which we were able to finish our project.

This project, at personal level, has been a big challenge. I thought this research required people at university level, but, we, only high school students, have succeeded it. It has been an Incredible experience.

Mis expectativas con este proyecto han cambiado de una forma drástica. Al principio me pareció una investigación demasiado importante para unos simples estudiantes de bachillerato, pero con ayuda, la cual no es poca, del investigador hemos conseguido conocer qué son los sistemas CRISPR, que nos permiten detectar los virus que han atacado a la bacteria analizada, y sobre todo aprender a detectarlos.
A lo largo del proyecto conocimos los programas y páginas webs que se podían utilizar para la búsqueda de los elementos CRISPR, por ejemplo, Clone Mánager o CRISPR-cas.
Pero todo esto es en general. Nuestro verdadero objetivo era investigar algo que nadie antes hubiera investigado. Detectar sistemas CRISPR en la bacteria V. Vulnificus VV5, lo cual no iba a ser tarea fácil. Tuvimos un montón de obstáculos, como pueden ser las tecnologías, la dificultad de esta investigación o un Estado de alarma. Un Estado de alarma que nos llevó a un confinamiento, que por desgracia nos paralizó, pero momentáneamente la investigación. Paco, el investigador, nos organizó unas sesiones online, gracias a las cuales, hemos podido terminar nuestro proyecto.
Este proyecto, a nivel personal, ha sido una superación. Veía una investigación como para gente de una carrera universitaria, y, nosotros, alumnos de bachillerato lo hemos conseguido. Experiencia increíble

Mª Dolores Puig Aguiar (1º Bachiller)

For me, this project has been an experience that is really worth living. At first, when I was offered the opportunity to participate in it, I thought I was not going to be trained for something too important. With this project we have been able to learn what is a CRISPR system and all their biology, as well as the required informatics tools for searching, including Clone Manager program.
I think it is a privilege to have been able to participate in it since there are not many people with the possibility of carrying out such a project. The final objective was to detect all CRISPR systems in the *V. vulnificus* VV5 bacteria. Moreover, it has been difficult to finish the project due to the situation we find ourselves in. Being quarantined and confined to our homes made it more difficult for us to organize our meetings. Previously, they were at the Estación Experimental del Zaidín, but our researcher managed to contact us through videoconferences, thus being able to successfully conclude it. In my opinion, it has been a very interesting and entertaining experience.

Para mí, este proyecto ha sido una experiencia que realmente vale la pena vivir. Al principio, cuando me ofrecieron la oportunidad de participar en él creía que no iba a estar capacitada para algo demasiado importante.
Con este proyecto hemos podido aprender el concepto de sistema CRISPR y todo lo que éste conlleva, así como los programas necesarios para su búsqueda, entre ellos Clone Manager.
Creo que es un privilegio haber podido participar en él puesto que no son muchas las personas con la posibilidad de realizar un proyecto así.
El objetivo era detectar sistemas CRISPR en la bacteria V. vulnificus VV5.
Ha sido un poco complicado poder terminar el proyecto debido a esta situación en la que nos encontramos sumidos y el hecho de estar en cuarentena nos hizo más complicado retomar

nuestras reuniones, que se llevaban a cabo en la Estación Experimental del Zaidín, pero nuestro investigador consiguió contactar con nosotros mediante videoconferencias pudiendo así concluir lo que empezamos.
En mi opinión, una experiencia muy interesante y entretenida.

Marta Nieves Vallejo (1º Bachiller)

I remember the day our teacher Ramon said, who wants to join the PIIISA project?, and like crazy, my colleagues and I raising my hand with great emotion, and look where they chose us to participate. I really did not know what it was about, although Ramón explained it to us, I said yes but the truth is that I did not find out.
Well, that long-awaited day came we went to the project at the Zaidín Experimental Station, what most caught my attention was that building, it was old but very beautiful. I was very nervous because I did not know if I would like that project or not, within minutes our researcher showed us all the facilities: what bigger buildings! When he finished showing us those facilities he took us to our class and there he was explaining to us what this investigation was about. At first it seemed complicated to me, the truth was that I saw it for university students and I did not see myself qualified for that, but little by little I liked it more.
Thanks to our researcher, we have learned the concept of CRISPR, the programs and websites for his search such as Clone Manager, CRISPR-cas ... Our objective was to investigate something that no one was investigated, that is, to detect CRISPR systems in a bacterium with a name a little difficult, the bacterium *Vibrio vulnificus* VV5.
In my opinion it was somewhat difficult and, in addition, it came the covid-19 that prevented us from finishing this investigation at the Estación Experimental del Zaidin, but thanks to Paco (our researcher) we have been able to end it with videoconferences from home. I think it is a complicated project, but if you put enough time into it you can become a great researcher in CRISPR systems. It is a great experience because it is a world that I did not know and thanks to this project I have been able to know many things that I did not know previously.

Recuerdo aquel día que dijo la profesora, ¿quién quiere apuntarse al proyecto PIIISA?, y como locas mis compañeras y yo levantando la mano con una emoción grandísima, y mira por donde nos escogieron para participar. Yo realmente no sabía de qué iba, nuestro orientador Ramón nos lo explicó, pero no lo entendía, yo decía que sí pero la verdad que no me enteré.
Bueno pues llego ese día tan esperado que fuimos al proyecto en la Estación Experimental del Zaidín, lo que más me llamo la atención fue aquel edificio, era antiguo pero muy bonito. Estaba muy nerviosa porque no sabía si ese proyecto me gustaría o no, a los minutos vino nuestro investigador y gran persona Paco, nos enseñó todas las instalaciones, que madre mía que edificios más grandes, cuando termino de enseñarnos aquellas instalaciones nos llevó a nuestra clase o sala, allí nos estuvo explicando de que iba esta investigación. Al principio me pareció complicada la verdad lo veía para estudiantes de universidad y yo no me veía capacitada para eso, pero poco a poco me iba gustando más.
Gracias a nuestro investigador Paco hemos aprendido el concepto de CRISPR, los programas y webs para su búsqueda como Clone Manager, CRISPR-cas … Nuestro objetivo era investigar algo que nadie fuese investigado, es decir, detectar sistemas CRISPR en una bacteria con un nombre un poco difícil, la bacteria Vibrio vulnificus VV5.
En mi opinión fue algo difícil y encima llegó el gran covid-19 que nos impidió acabar esta investigación en la Estación Experimental del Zaidín, pero gracias a Paco lo hemos podido

finalizar con videoconferencias desde casa. Creo que es un proyecto complicado, pero si le pones bastante tiempo puedes llegar a ser un gran investigador de sistemas CRISPR, es una gran experiencia porque es un mundo que yo no conocía y gracias a este proyecto he podido conocer muchas cosas que antes no sabía.

Ramón Pérez Moreno (Coordinador PIIISA – IES Arje)

The PIIISA 2019 will go down in history as a year of infinite challenges. Its objective of showing secondary school students what research is and how it is carried out has been affected due to this global pandemic that has brought to the table the need to invest even more in Science.

Our students have had the opportunity to get involved in a project led by a scientist, Paco, incessant and very committed to involving them in research, to knowing first-hand what the scientific method consists of and how the research process is. And so much effort in this 'State of Alarm' has allowed us to obtain the results that are reflected in this report, but perhaps what I value most as a professor at IES Arjé and as a professor in another specialty non related with this project, is the imprint that all this process will undoubtedly stop fostering scientific vocations in our students. Finally, I want to thank D. Francisco Martínez-Abarca, our researcher of this PIIISA 2019 for his tenacity, patience with us and for that incessant spirit for transferring his love for research and science. Thank you.

El PIIISA 2019 va a pasar a la historia como un año de infinitos desafíos. Su objetivo de mostrar al alumnado de secundaria qué es la investigación y cómo se realiza se ha redimensionado a causa de esta pandemia mundial que ha puesto sobre la mesa la necesidad de invertir aún más en ciencia.

Nuestro alumnado ha tenido la oportunidad de involucrarse en un proyecto liderado por un científico, Paco, incesante y muy comprometido por hacerle partícipe por la investigación, por conocer de primera mano en qué consiste el método científico y cómo es el proceso de investigación. Y tanto esfuerzo en este estado de alarma, ha permitido obtener los resultados que en esta memoria se refleja, pero quizás lo que yo más valoro como profesor del IES Arjé y como profesor de otra especialidad que nada tiene que ver con este proyecto, es la impronta que todo este proceso sin duda va a dejar en fomentar vocaciones científicas en nuestro alumnado.

Por último, quiero dar las gracias a D. Francisco Martínez-Abarca, nuestro investigador de este PIIISA 2019 por su tesón, paciencia con nosotros y por ese espíritu incesante por trasladarnos su amor por la investigación y la ciencia. Gracias.

Biological insights of Estación Experimental del Zaidín (EEZ) bio-waste composting

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Highlights

- *Composting is a feasible methodology for bio-waste recycling.*
- *A significant loss of bio-waste dry weight was found during composting.*
- *Compost had more bacterial population than a soil.*
- *Composts did not have any phytotoxicity and could be used in agriculture.*

Summary

Bio-waste is defined as the biodegradable fraction of the waste produced at domestic, commercial or local levels, which include garden and park waste, food and kitchen wastes from households, restaurants or similar. It is characterised by an important content of water and an easily biodegradable organic matter, being mandatory a treatment before its disposal. Composting is a feasible methodology for bio-waste management, in which the organic matter is transformed by the own microorganisms presented in the raw materials. In this research, the feasibility of the bio-waste composting generated by Estación Experimental del Zaidín (EEZ) was studied. For that, two composting procedures (composting pot and bin) were assayed, and the degradation of the organic matter during the process, the relationship between temperature, moisture and bacterial population and the compost phytotoxicity were evaluated. Data confirmed 40% of dry weight loss of initial bio-waste during the process, probably related to CO_2 release during the organic matter degradation. Also, a relationship between temperature, moisture and bacterial population was found, being compost an organic material with more bacterial abundance compared to a soil. Finally, the maturity test of the obtained composts confirmed an absence of phytotoxicity, being ready for their use in agriculture.

Keywords: Bio-waste, composting pots, composting bin, organic matter, temperature, moisture, bacteria.

INTRODUCTION

Bio-waste is defined as the biodegradable fraction of the waste produced at domestic, commercial or local levels [1]. It includes green garden and park waste, food and kitchen waste from households and restaurants, and similar wastes produced at processing food plants [2]. The bio-waste production is variable in time and its main characteristics are high content of water and organic matter easily biodegradable, being mandatory a treatment before its disposal [3].

Composting is a feasible methodology for the bio-waste management. It is defined as a controlled bioxidative process, in which the organic waste is transformed by the own microorganisms of the raw waste [4]. Several phases can be observed during composting according to temperature evolution and microorganisms activity [5]: mesophilic phase (<40 ºC), thermophilic phase (40-60 ºC), cooling (60-40 ºC) and maturation phase (ambient), respectively. The bioxidative process (mesophilic and thermophilic phases) is the most active stage, where an important organic matter degradation, CO_2 emissions and microbial activity take place [5].

Microorganisms are one of the most important factor of soil biology [6, 7]. They constitute its living part and are responsible for the dynamics of soil transformation. Microorganisms modify soil properties and nutrient cycles, and can improve plants growth and development. Nowadays, bio-fertilizers or biostimulants based on soil microorganisms are commonly use in agriculture, due to they promote plant nutrition and growth. Also, composts can be a valuable source of plant growth-promoting microorganisms [8].

The aim of this project was to study the feasibility of bio-waste composting, with emphasis in its biological aspects. For that, the degradation of the organic matter during the process, the relationship between temperature, moisture and bacterial population, and the composts maturity were evaluated. Also, the bacterial diversity was assessed by microscopy.

MATERIALS AND METHODS

This study was carried out at the facilities of the Estación Experimental del Zaidín (EEZ), a research center of the Spanish Council for Scientific Research (CSIC) sited in Granada (Spain). The composting experiments were done at the EEZ Campus, an area of 2250 m^2 located in the city center, with several buildings and Departments including a restaurant, green zones and also, a botany garden.

1. Bio-waste.

The organic wastes used in this research were chipped tree prunings, fresh cut grass and food waste, all of them easily available at EEZ (Figure 1). The chipped tree prunings, mainly from *Platanus orientalis* and *Platanus occidentalis*, and the fresh cut grass were provided by the EEZ Gardening Service, and the food waste was supplied by the Restaurant staff.

Figure 1. Chipped tree prunings (a), fresh cut grass (b) and food waste (c) used for composting.

2. Composting.

Two different composting procedures were assayed: composting pots and a composting bin.

2.1. Composting pots.

In this experiment, eight 25 L-pots (Figure 2) were filled with a mixture containing equal volumes of chipped tree prunings (10 L) and fresh cut grass (10 L). Food waste was manually cut into small pieces with scissors and added to pots according to the following treatments (Figure 2):

- C (Control): No food waste was added.
- D1: 600 g of food waste was added.
- D2: 1200 g of food waste was added.
- D3: 2000 g of food waste was added.

Two replicates per treatment were done. The composting pots were placed in a greenhouse bench and once a week, were turned and checked for their humidity. The experiment started at 19 of December 2019 (T0) and finished at 26 of February 2020 (T1). At each time, a representative sample of 200-300 g was randomly taken for further analysis.

2.2. Composting bin.

In this experiment, a 450 L-composter (Figure 3) was filled with a mixture containing equal volumes of chipped tree prunings (200 L) and fresh cut grass (200 L), which was turned using a hand compost turner. The experiment started at 19 of February 2020 and one week later, the compost temperature rose up until thermophilic range (> 45 ºC). Then, a representative sample of 200-300 g was randomly taken from different bin locations for further analysis.

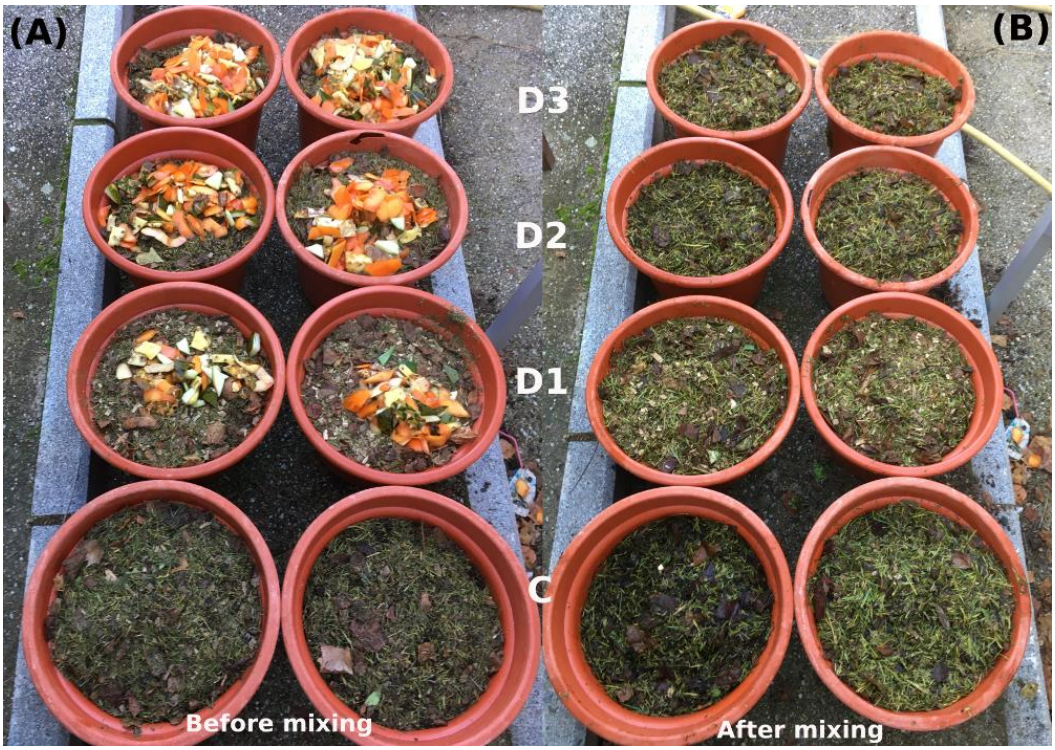

Figure 2. Composting pots before and after adding and mixing food waste according to each treatment: C (Control, no addition), D1 (600 g of food waste), D2 (1200 g of food waste) and D3 (2000 g of food waste).

Figure 3. The 450 L-composting bin and the hand compost turner used in this research.

3. Analysis.

3.1. Moisture.

Moisture was determined as the difference between wet and dry weights. Briefly, a representative sample of wet bio-waste (T0) or compost (T1) (100-200 g) was randomly taken and weighed in a precision balance. Then, samples were dried in an oven during 3 days at 70ºC until constant weight. Water content of each sample were calculated as followed:

$$\text{Moisture (\%)} = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100$$

Composting pot wet weights were estimated just before sampling, at T0 and T1, with a manual portable luggage scale.

3.2. Temperature.

Compost temperature was randomly measured at different composting bin locations by using a digital thermometer with a probe (Figure 4).

Figure 4. Digital thermometer with a probe used to measure compost temperature.

3.3. Bacteria isolation and growth.

For bacteria isolation, 1-3 g of wet compost were aseptically placed in a 50 mL sterile tube. 30 mL of saline solution (NaCl 0.9%, w/v) was added to the tube and mixed by vortexing during 5-10 min. After that, the extraction was sedimented by gravity during 10 min.

For bacterial growth, 30 µL of the above extraction was added to Petri dishes, containing a general growing solid media for bacteria (TSA medium). The inoculation was done by adding 2 mm-glass balls to Petri dishes and by manually shaking during 1 min (Figure 5). Then, glass

balls were removed and Petri plates were incubated at 28 ºC during 24 h. After growth, bacterial morphology was checked by using a light microscopy.

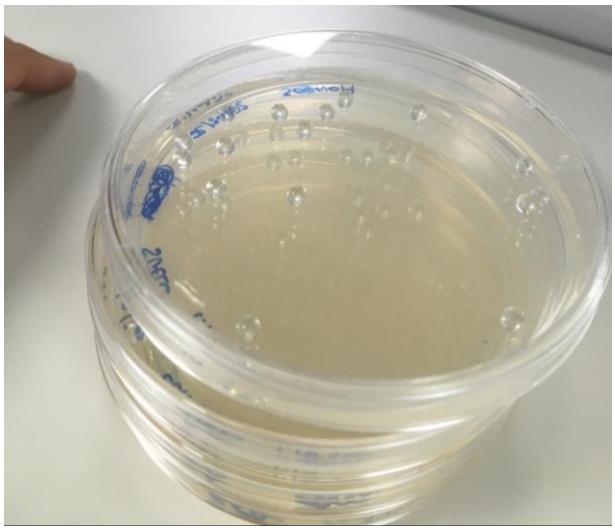

Figure 5. Bacterial inoculation of Petri dishes with 2 mm-glass balls.

3.4. Compost maturity.

Compost maturity was assessed with a seed germination test after adding a compost water extract. Briefly, 1 g of wet compost were placed in a tube containing 20 mL of tap water. Tubes were mechanical shaken during 2 h and were centrifuged at 5,000 *g* during 10 min . After that, 1 mL of the supernatant was added to a Petri dish, containing a slide of paper and 10 seeds of cress (*Lepidium sativum*) (Figure 6). Then, cress seeds were grown at room temperature in darkness during one week.

In order to compare compost phytotoxicity, a set of cress seeds were germinated with only tap water (control assay) and another set with a water extraction of a soil located close to the composting bin.

Figure 6. Compost maturity assessed with cress (*Lepidium sativum*) seeds germination test after adding a compost water extract.

RESULTS AND DISCUSSION

Organic matter degradation during composting.

Figure 7 shows the weight of composting pots at the beginning (T0) and at the end (T1) of the experiment. According to these data, pots dry weights at T0 were ranged between 1.2-to-1.7 kg and two months later (T1), were close to 0.9 kg. These reductions represented losses of 42, 29, 43 and 41 % of dry weight for C, D1, D2 and D3 composting pots, respectively (Figure 8). Part of these losses might be explained due to the release of some gases like CO_2 during the degradation of the organic matter produced by microorganisms activity during composting [9].

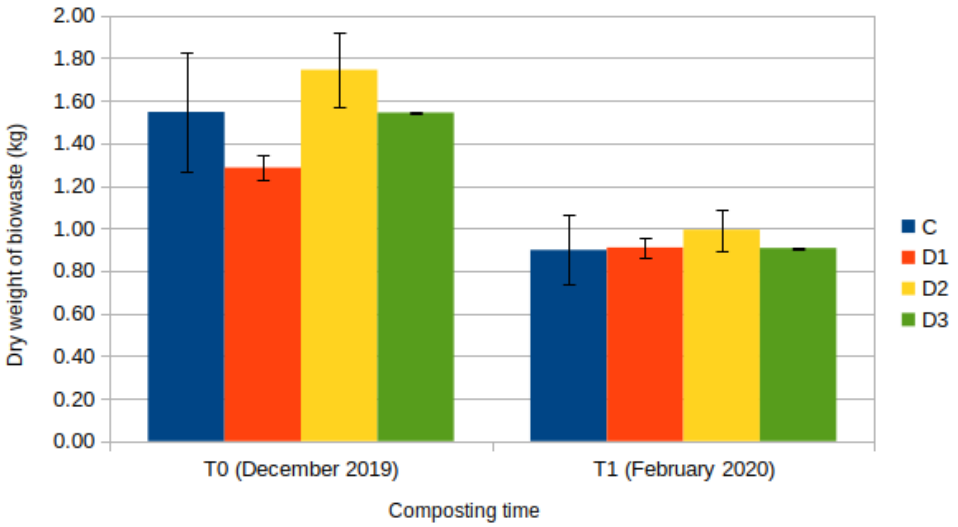

Figure 7. Dry weight of composting pots at the beginning (T0) and at the end (T1) of the experiment. Treatments are: C (Control, no addition), D1 (600 g of food waste), D2 (1200 g of food waste) and D3 (2000 g of food waste).

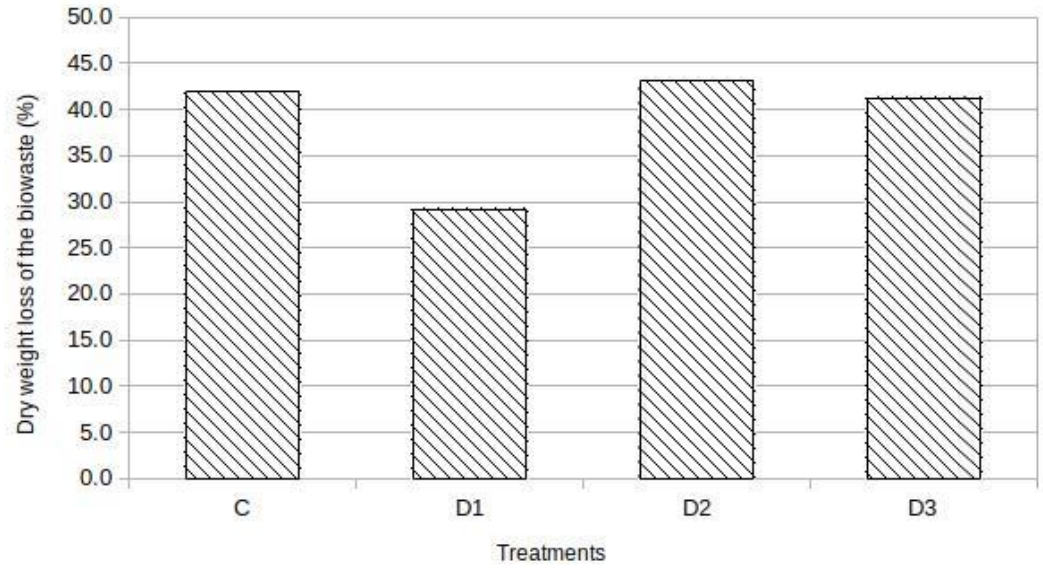

Figure 8. Dry weight loss (%) of composting pots during the experiment. Treatments are: C (Control, no addition), D1 (600 g of food waste), D2 (1200 g of food waste) and D3 (2000 g of food waste).

Relationship between temperature, moisture and bacterial population of compost.

As mentioned before, composting is a biological methodology for the organic waste treatment [4]. During this process, an important organic matter degradation occurs, especially due to the metabolic activity of a complex microbial population presented in the raw materials [5]. In order to know the role of microorganisms during composting, temperature, moisture and bacterial population were checked in a composting pile at thermophilic phase (the most active), and compared with a soil located close to the composting bin. According to Figure 9a, compost temperature was close to 50 ºC, 5-fold higher than the soil. This result confirms that compost was biologically active [5]. Also, the compost moisture was consistently higher than water content found in the soil (Figure 9b), being water content an important factor for the microbial development. As expected, bacterial population in compost was much notable than in the soil (Figure 9c). These findings suggested that a direct relationship between temperature, water content and bacterial population was found in the compost, being biologically more active than the soil.

Finally, some of the isolated bacteria were observed under a light microscope. As it can be shown in Figure 10, bacterial colonies had different morphology (forms and shape), which confirm that the compost biodiversity was relevant [5].

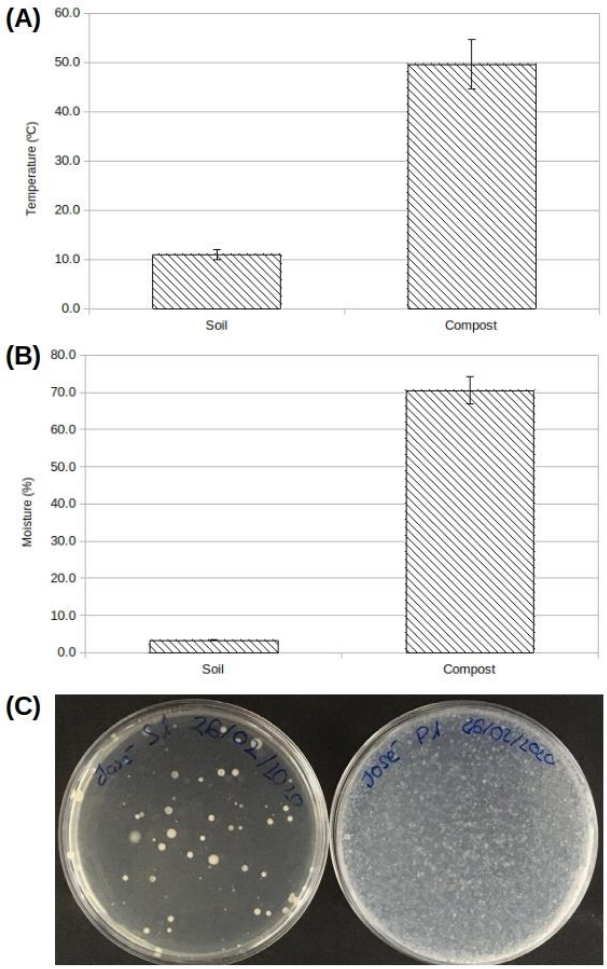

Figure 9. Temperature (A), moisture (B) and growth of bacterial population (C) in soil (left) and compost (right) at thermophilic phase.

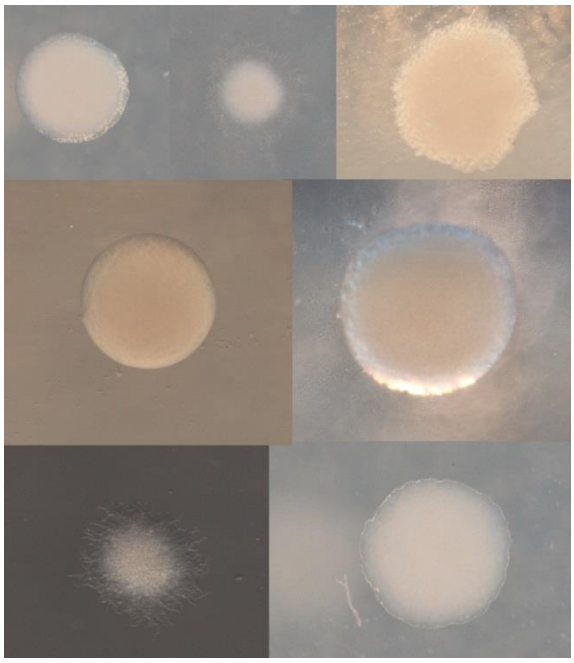

Figure 10. Different morphologies of bacteria isolated from thermophilic compost.

Maturity of composts.

The disposal of organic waste can produce an important impact in the environment, especially to soil and plants. In order to know compost maturity, phytotoxicty was assessed with a germination test based on cress seeds. According to Figure 11, no differences in seeds germination between tap water (used as control) and the compost water extract were found. Similar results were obtained when a soil water extraction was used. These findings indicated that composts at T1 had no phytotoxicity and could be used in agriculture as organic amendments or fertilisers (Figure 12).

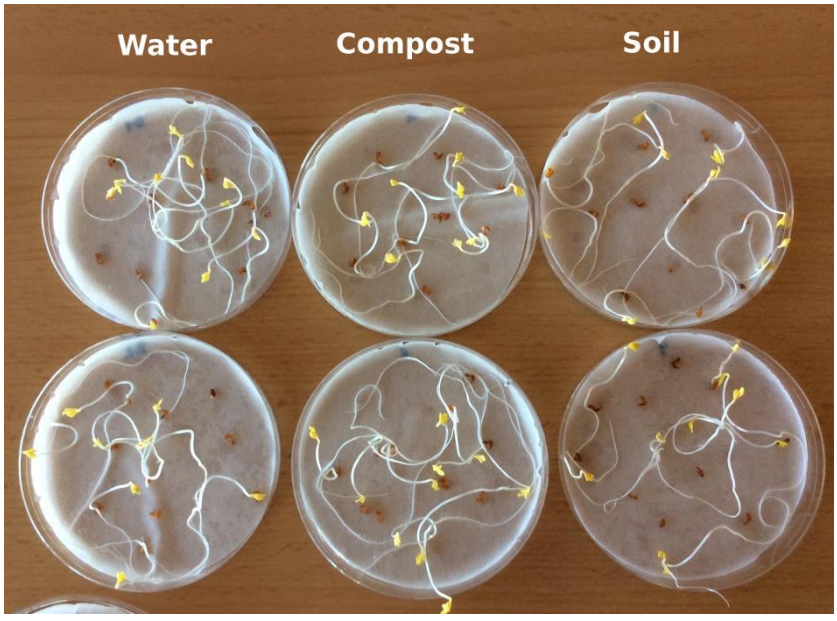

Figure 11. Cress (*Lepidium sativum*) germination with tap water (used as control), compost water extract and soil water extract, respectively.

Figure 12. Aspect of T1 compost used in the germination test.

CONCLUSIONS

1. Composting is a feasible methodology for the EEZ bio-waste treatment.
2. An important reduction in the dry weight of bio-waste occurred during the process, probably related to CO_2 release.
3. A direct relationship between temperature, moisture and bacterial population was found in a thermophilic compost.
4. Compost is more biologically active than a soil, showing an relevant bacterial diversity.
5. The composts had no phytotoxicity and could be used in agriculture.

Acknowledgements

The authors strongly thank to Manolo (EEZ Gardening Service) and José (EEZ Restaurant) for their contribution in this project.

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MY OWN IDEAS

José María Díaz

Teniendo en cuenta todas las actividades que hemos ido realizando a lo largo de todo el proyecto, en general, me ha encantado. Incluso teniendo en cuenta también toda la crisis del coronavirus todos los miembros del equipo hemos sido capaces de seguir adelante con el proyecto, y eso ha sido nuestra decisión, no era obligatorio en ningún momento, por tanto, es de lógica imaginar que todos seguíamos entusiasmados. La pregunta es la siguiente: ¿Qué nos motiva a seguir adelante? ¿Qué se podría cambiar para mejorar aún más?

Desde mi punto de vista, las veces en las que mejor me lo he pasado han sido los momentos en los que me veía a mi mismo sumergido en lo que podría ser un futuro próximo, es decir, una especie de flashforward a lo que significa en un futuro trabajar como un científico, ya sea de biologo, quimico etc… Esa sensación de la que estoy hablando solo me ha surgido 2 o 3 veces, las demás simplemente me sentía de otra forma en la que había alguien tomando las decisiones enfrente de mi, sin ser yo mismo autónomo. Nada me ha empujado a ir yo por mi cuenta hasta que vino el Covid, que hubo una especie de parón en el grupo y ya tuve yo que dar mis propias opiniones…

Con todo esto quiero decir que se nos debería dar aún más autonomía a la hora de investigar algo, fijar un objetivo claro que sea de principio a fin, y dejarnos a nuestro aire, a no ser que nos veamos encerrados, de esa forma seremos nosotros los que tomaremos las decisiones, como hacen los verdaderos científicos, que normalmente no tienen a nadie que sepa todo enfrente, y cuando acabe el proyecto y nos demos cuenta de que de verdad hemos sido nosotros los que manejabamos el ajedrez y no las fichas de éste se nos verá con otra cara.

Esa autonomía se puede conseguir si de verdad se reflexionan opciones, que las hay de sobra, pero eso no está en mi mano, prueben una sesión próxima de Piiisa a darles un objetivo claro al alumnado y que ellos mismos intenten sacar un resultado usando los conocimientos que han aprendido en la escuela, quizás de esta forma algunos aprendan de que el colegio sirve de verdad, y puedan ponerse las pilas por la motivación que les pueda surgir antes del fin de su carrera académica.

Dando por acabada ya la crítica constructiva, agradezco de corazón a todos los organizadores de este proyecto, que son capaces de crear tal actividad que puedas sentirte de verdad un científico, y eso es muy muy complicado de conseguir.

Yusuf Coletti

Según mi opinión este proyecto me ha parecido bastante interesante en aspecto de probar muchas herramientas y mucha práctica que aparte de la información, los datos recibidos, hemos aprendido como funciona todos los instrumentos y todas las nuevas maquinarias de los laboratorios aprendiendo básicamente el uso de las herramientas más simples y algunas complejas. La información que adquirimos seguramente se nos olvide al cabo de unos años a no ser que trabajamos en esto, pero la experiencia y el tiempo en este ámbito científico ser crucial para identificar si te gusta trabajar en este ámbito o prefieres trabajar en otro.

Aparte adquirimos conocimientos de como hacer un buen compost ya que, aunque parezca fácil tiene su grado de complicación. Yo, por ejemplo, he hecho un compost con mi padre en este confinamiento y gracias a lo básico aprendido en PIIISA ha salido muy bien.

Solo he visto un punto negativo y es la falta de conocimientos básicos sobre el proyecto ya que dedicamos un par de sesiones en aprender los conceptos básicos y a algunos como a mi no me han quedado del todo claro.

Pero por lo demás nos lo hemos pasado muy bien y Germán nos ha enseñado a la perfección los aspectos de la vida científica el día a día de un científico investigador. Además de presentarnos a grandes científicos de su departamento viendo de reojo sus respectivos experimentos.

Essential oils as antimicrobial agents against plant pathogenic bacteria

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Summary

The main objective of this research was to evaluate the effectiveness of some commercial essential oils from different plants against *Pseudomonas syringae*, *Xanthomonas campestris* and *Dickeya dadanti*, three of the most important plant pathogenic bacterias. We also studied the effect of this substances against *Bacillus megaterium* and *Escherichia coli*. Antimicrobial was investigated by the agar disk diffusion method. Essential oils from *Cimbopogon martinii*, *Cinnamomun camphora*, *Lavandula hybrida*, *L. latifolia*, *Satureja montana*, *Thymus mastichina* and *T. zygis* inhibited the growth of all the tested microorganisms. The stronger activity was shown by *S. montana* and *T. zygis*. Carvacrol and thymol, the compounds that define the chemotype of these two plants have been confirmed as bactericide in several studies. *P. syringae* was the most sensitive microorganism sensitive to essential oils. On the contrary, X. campestris was resistant to eight essential oils. Our results confirm that essential oils, mainly those obtained from *S. montana* and *T. zygis* must be investigated as a promising source of antimicrobial agentes against plant pathogenic bacteria.

Keywords: Essential oils, antimicrobial activity, *Pseudomonas syringae*, *Xanthomonas campestris*, *Dickeya dadantii*, *Satureja montana*, *Thymus zygis*.

INTRODUCTION

Plant diseases are responsible of important economic loss in agriculture and even in biodiversity. The main common cause of most of the plant diseases are infections by bacteria, fungi and viruses. Up to now, antibiotics have been the most used treatment against plant pathogenic bacteria; however, it has been recommended to reduce their use due to several reasons. One is the possibility of emerging pathogenic strains resistant to those antimicrobial products; other is the risk of the transmission of antibiotic resistant genes to other plant pathogens [1] or even human and animal pathogenic microorganisms. On other side, the accumulation of these chemical compounds may result in undesirable effects to the environment as they can turn into toxic residues difficult to biodegrade [2]. Therefore, new strategies are required to control plant pathogenic bacteria.

In this sense, there has been an increased interest in studying the antimicrobial properties of essential oils. These are complex mixtures or substances as terpenoids, phenolic compounds, alcohols, aldehydes, alkaloids, etc. extracted from different parts of the plants with an important biological activity. Some of them have antimicrobial, antiviral or antifungal activity [3]. Essential oils are considered safe products and easily biodegradable; consequently, they have been researched as potential natural antimicrobials. Lavender, thyme, peppermint, cinnamon, clove, eucaliptus, sage and tea tree are examples of plants whose antimicrobial activity has been demonstrated [4].

Antimicrobial activity of some essential oils against plant pathogenic bacteria has been researched. Ghalem [2] reviews the susceptibility of the top ten pathogenic species selected as result of their scientific and economic importance in plant diseases [5]. *Pseudomonas syringae*, *Ralstonia solanacearum*, *Agrobacterium tumefaciens*, *Xanthomonas oryzae*, *X. campestris*, *X. axonopodis*, *Erwinia amilovora*, *Xylella fastidiosa*, *Dickeya dadanti* and *Pectobacterium carotovorum* were sensitive to essential oils of plants like *Mentha piperita*, *Lavandula angustifolia*, *Cymbopogon martini*, *Eucaliptus globulus*, *Thymus vulgaris*, *Syzygium aromaticum* or *Satureja hortensis*, to name but a few.

In our laboratory we have carried out a project devoted to evaluate the antimicrobial activity of some commercial essential oils against three plant pathogenic bacteria: *Pseudomonas syringae*, *Xanthomonas campestris* and *Dickeya dadanti*. We have also studied the effect of these compounds on *Bacillus megaterium* and *Escherichia coli*, two strains widely used in our laboratory in previous projects.

MATERIAL AND METHODS

1. Essential oils samples

Two batches of essential oils have been used in our research. Preliminary experiments were carried out with nine samples present in our laboratory as part of its equipment from a long time ago. They were manufactured by Destilerías García de la Fuente and the only

information we had about them was the common name of the plants they were extracted. Scientific names were estimated from common denomination. The rest of the essential oils were purchased to Labiatae (https://labiatae.com). They are listed in Table 1. Information about chemotypes and main uses was taken from the catalogue of the supplying company.

Family	Species	Chemotypes	Main uses, folk uses
Lamiaceae	*Hysoppus officinalis*	1-8 cineole (eucalyptol)	Antiseptic
	Lavandula hybrida	Linalyl acetate	Antiseptic
	Lavandula latifolia	Linalool	Antiseptic
	Mentha piperita	Menthol	Antiseptic
	Pogostemon cablin	Bulnesol, pachoulol	Antiseptic
	Rosmarinus officinalis	α-pinene, camphor	Pain reliever
	Salvia offinalis	α-thujone	Estrogenic
	Satureja montana	Carvacrol	Bactericide
	Thymus mastichina	1-8 cineole (eucalyptol)	Antiseptic
	Thymus zygis	Thymol	Antiseptic
Geraniaceae	*Pelargonium graveolens*	Citronellol	Antiseptic
Rutaceae	*Citrus bergamia*	Limonene	Antiseptic
Poaceae	*Cymbopogon martinii*	Geraniol	Antiseptic, fungicide
Lauraceae	*Cinnamomum camphora*	1-8 cineole (eucalyptol)	Bactericide, antiviral
Cupressaceae	*Juniperus communis*	α-pinene	Diuretic

Table 1. Essential oils used in our assays supplied by Labiatae, chemotypes and main ethnobotanical applications.

2. Bacterial strains

Three pathogenic plant bacteria and two control strains have been tested against essential oils. *Pseudomonas syringae*, *Xanthomonas campestris* and *Dickeya dadanti* are considered among the top ten most important pathogen microorganisms for plants [5]. *Bacillus megaterium* (gram positive) and *Escherichia coli* (gram negative) are two strains usually cultivated in our laboratory in previous projects; they were chosen regarding the differences in their cell wall. All the microorganisms were provided by the Estación Experimental del Zaidín (CSIC).

3. Antibacterial activity evaluation: Disk diffusion method

Twenty mililiters of TSA (triptone soya agar) medium were poured into sterile Petri dishes as a basal medium. 5 ml of semisolid medium (triptone soya broth + agar 0,6%) at 42ºC were inoculated with 100 microliters of liquid culture (TSB or LB) of the bacteria to be tested and spreaded on the basal solid medium.

The antimicrobial activity has been evaluated by the disk diffusion method. Whatman sterile filter paper disks (6 mm diameter) were soaked into essential oils. We let them to dry on filter paper to remove the excess of the product and deposited them on the plates prepared as we have indicated before. The diffusion of the agent result results in a gradient of the antimicrobial. When its concentration becomes so diluted it can no longer inhibit the growth of the test bacteria, resulting in a demarcated inhibition halo. Estimation of inhibitory activity has been made measuring the total area of that halo and subtracting the surface of the filter paper disks.

RESULTS

In order to assess the potential of essential oils as antimicrobial we carried out some preliminary experiments with samples present in our laboratory. We had no information about them but the common name and the company that extract them. Antibacterial activity was tested against all the cited bacteria. A representative experiment is shown in Figure 1.

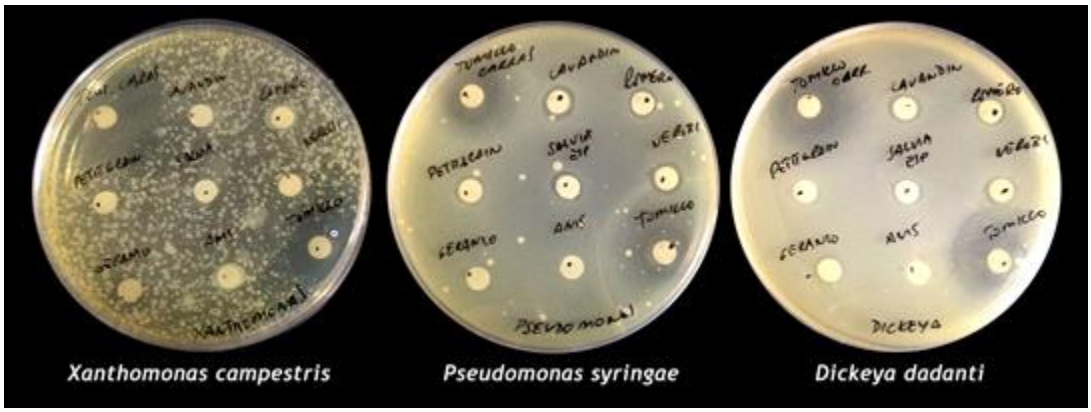

Figure 1. Antimicrobial activity of essential oils (DGF) against plant pathogenic bacteria.

The essential oils and results are shown in Table 2. The most sensitive bacteria among the plant pathogenic ones was *P. syringae*; on the contrary *X. campestris* was only inhibited only by essential oils from *Thymus vulgaris* and *T. zygis*.

Only essential oils from *T. zygis* and *T. vulgaris* inhibited the growth of all the tested microorganisms. Except for *X. campestris* the best antimicrobial potential was observed in *T. zygis*. On the contrary, the essential of "anis" only inhibit the growth of *B. megaterium*.Regarding non pathogenic plant bacteria, *B. megaterium* was the most sensitive to essential oils, inhibited by all of them but that one obtained from flowers of *Citrus aurantium* (neroli). *E. coli* was resistant to essential oils extracted from flowers of *C. aurantium* and anís (probably *Pimpinela anisum* or *Illicium verum*, bottle labelled with common name*)*. The extension of the inhibition halos was bigger with *B. megaterium* than *E. coli*. Essential oils were more effective against that gram positive bacteria.

Essential oils (DGF)	*Bacillus megaterium*	*Escherichia coli*	*Xanthomonas campestris*	*Pseudomonas syringae*	*Dickeya dadantii*
Lavandula hybrida **(Lavandín)**	172,8	35,3	-	50,3	35,3
Rosmarinus officinalis **(Romero)**	148,4	4,9	-	22	35,3
Salvia sp. **(Salvia española)**	50,3	10,2	-	22	-
Thymus vulgaris **(Tomillo carrasqueño)**	678,6	285,9	104,5	285,9	351,9
Thymus zygis **(Tomillo)**	678,6	285,9	22,0	502,7	678,6
Citrus aurantium, **flowers (Neroli)**	-	-	-	22,0	35,3
Citrus aurantium**, leaves (Petitgrain)**	172,8	22,0	-	22,0	-
Pelargonium sp. **(Geranio)**	462,6	84,8	-	+/-	-
Anís (*)	148,4	-	-	-	-

Table 2. Antimicrobial activity of essential oils (DGF: Destilerías García de la Fuente). Bottles labeled with common name in Spanish. (*) Anís essential oil may proceed from *Pimpinela anisum* or *Illicium verum*. Numbers show the area of inhibition expressed in square millimeters.

We have tested the antimicrobial activity of the fifteen essential oils obtained from the company Labiatae. The results are shown in Figure 2 and Table 3. Seven were active against all the tested bacteria. The biggest inhibition halos were observed with *T. zygis* and *Satureja montana*. On the contrary, essential oil from *Pogostemon cablin* did not present activity against any microorganism. Low activity was observed in essential oils of *Citrus bergamia*, *Mentha piperita*, *Juniperus communis*, *Hyssopus officinalis* and *Pelargonium graveolens*.

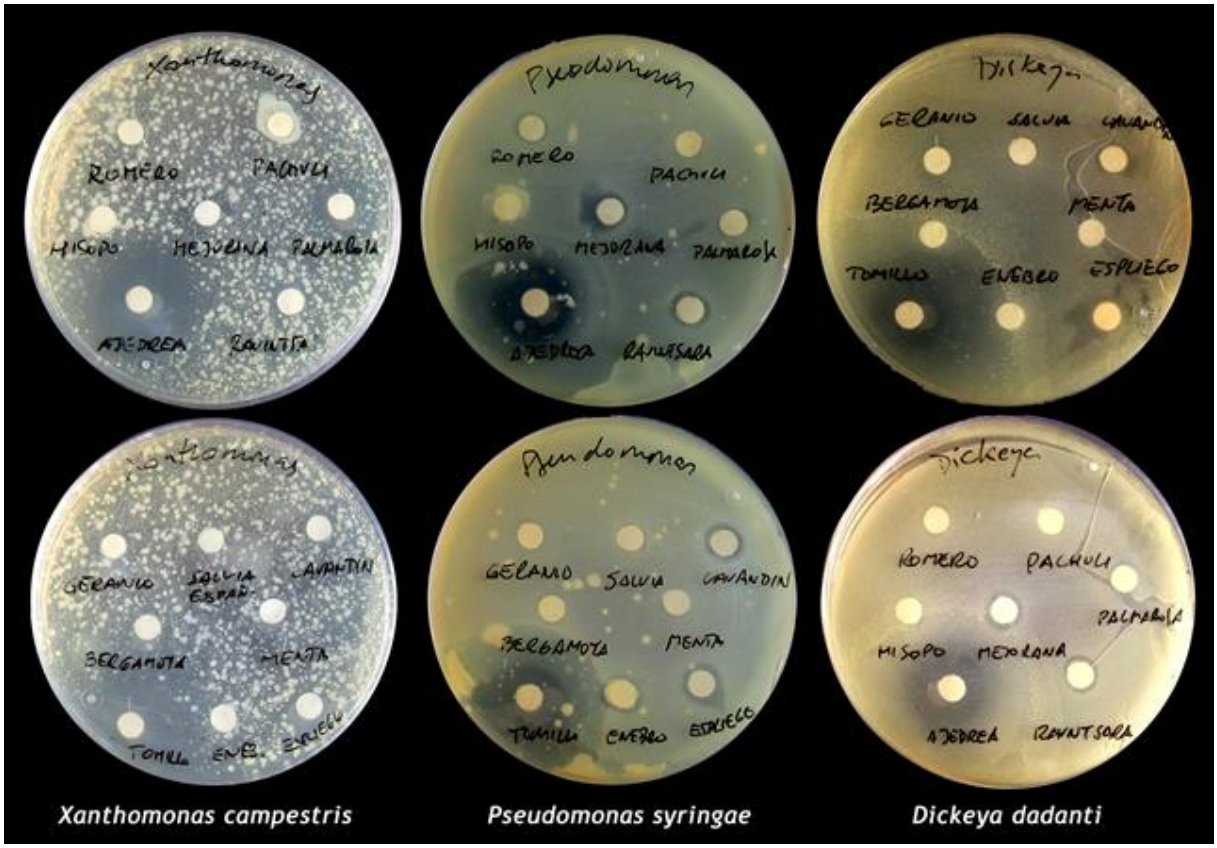

Figure 2. Antimicrobial activity of Labiatae essential oils against plant pathogenic bacteria.

Essential oils (Labiatae)	*Bacillus megaterium*	*Escherichia coli*	*Xanthomonas campestris*	*Pseudomonas syringae*	*Dickeya dadantii*
Hyssopus officinalis (Hisopo)	10,2	-	-	22	-
Lavandula hybrida (Lavandín)	50,3	10,2	50,3	50,3	50,3
Lavandula latifolia (Espliego)	66,8	10,2	84,8	50,3	84,8
Mentha piperita (Menta)	22	-	-	-	-
Pogostemon cablin (Pachuli)	-	-	-	-	-
Rosmarinus officinalis (Romero)	10,2	-	-	22	22
Salvia officinalis (Salvia)	22	-	-	10,2	22
Satureja montana (Ajedrea)	933,8	424,1	678,6	989,6	989,6
Thymus mastichina (Mejorana)	50,3	22,0	50,3	50,3	50,3
Thymus zygis (Tomillo)	989,6	462,6	502,7	1228,4	678,6
Pelargonium graveolens (Geranio)	22,0	-	-	-	84,8
Citrus bergamia (Bergamota)	-	-	-	10,2	-
Cymbopogon martinii (Palmarosa)	22,0	50,3	172,8	22,0	50,3
Cinnamommun camphora (Ravintsara)	22,0	35,3	50,3	22,0	50,3
Juniperus communis (Enebro)	-	-	-	35,3	-

Table 3. Antimicrobial activity of Labiatae essential oils. Numbers show the area of inhibition expressed in square millimeters.

Among pathogenic bacteria, *P. syringae* was the most sensitive microorganism, affected by 12 essential oils. On the contrary, *X. campestris* was the most resistant; its growth was only inhibited by seven. With respect to non pathogenic bacteria, *B. megaterium* was more sensitive to essential oils than *E. coli*. It was affected by twelve compounds while *E. coli* was sensitive to seven. The size of the inhibition halos was bigger in cultures with *B. megaterium*.

As a whole, the most important antimicrobial activity was observed in essential oils coming from *Satureja montana* and *Thymus zigys*.

DISCUSSION

Our results demonstrate that essential oils can have an important antimicrobial activity. Of all we have tested, we must highlight the activity of *T. zygis*, *T. vulgaris* and *S. montana*. Bactericidal activity of these compounds has been described by several authors [6,7,8]. On the contrary, essential oils like *Rosmarinus officinalis* or *Pogostemon gablin* did not show the important activity reported in other researches [9,10]. The bactericidal activity of essential oils depends on their chemical composition, determined by the plant genotype and the influence of the environment [6]. This variable proportion of chemical components in different plants of the same species defines the chemotype. Normally the most abundant component is responsible of the activity of the extract but it is possible that other in less proportion, or the synergy relationships between different ones reinforce their effect exhibiting more activity than the sum of each one independently. This can explain the difference in antimicrobial activity observed in essential oils from plants of the same species.

The chemotypes of *T. zygis* and *S. montana* are defined, respectively, by thymol and carvacrol (Table 1). Essential oils with high antimicrobial activity contain a high proportion of these phenolic compounds [4,7,11,12,13].

Our experiments show that the antibacterial activity of essential oils is much more evident against *B. megaterium* than against the rest of the tested bacteria. In other words, we have always found more defined and bigger inhibition halos against this bacterium than against the other microorganisms. *B. megaterium* is a Gram-positive bacterium. In contrast, the rest of the tested microorganisms (*E. coli*, *X. campestris*, *P. syringae* and *D. dadanti*) are Gram-negative. The key differences between these microorganisms are that Gram-positive bacteria have a thicker peptidoglycan layer and Gram-negative ones have an outer membrana with a complex lipopolysaccharyde.

It has been suggested that the bactericial activity of essential oils involves multiple targets within the cell. All of them present hydrophobicity, consequently they can react with lipids on the bacterial cell membrane. When its integrity is altered there is an increase in permeability that alters cells structure and breaks homeostasis. It has been demonstrated that a mixture of thymol and carvacrol, both of them present in the chemotypes of *S. montana* and *T. zigys* we have tested, causes and increase permeability of cell membrane [14].

The outer membrane of Gram negative microorganism is rich in lipopolisaccharide that is almost impermeable to essential oils. They also have hydrolitic enzymes in the periplasmic space, the region between the outer membrane and the cell wall, able to metabolize antimicrobial substances. In Gram positive bacteria, lipophilic components of essential oils can damage the cell membrane directly.

Antibiotic resistance in plant pathogenic bacteria becomes an important problem when antibiotics have been used for a long time. Alternative treatments requiere new bactericides with different modes of action [1] and plants are good candidate to provide them. Wińka et al. [4] propose that the antimicrobial activity of essential oils is weaker compared to synthetic compounds and antibiotics. However, their low toxicity level as well as their natural origin makes them an interesting alternative to compounds that are able to promote the development of resistance to antimicrobials in microorganisms and also be harmful to the environment. The findings described in this research, with compounds with great activity like those of *Thymus* and *Satureja montana*, confirms that essential oils must be investigated as a promising source of antimicrobial agents against plant pathogenic bacteria.

Acknowledgements

We wanted to thank our teacher Antonio Quesada for proposing projects like this that will allow us to have a much better development and that we see science from other points of view, our high school for accepting proposals of this type and Manuel Espinosa for giving us the opportunity to be even closer to an experiment, for being so kind and helping us. With

Antonio and Manuel we have felt quite welcome and the atmosphere has been very good. We are truly grateful for these opportunities and for trusting us. Also thanks to the Zaidín Experimental Station for inviting us to be among them and to see laboratories, ways of working, etc.

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MY OWN IDEAS

Paula Duro Muñoz

After finishing this project I can say that my classmates and I have learned and matured a lot. When we started the project we weren't aware of all the things we could discover. Now that the end has arrived and we think about it, we should feel really proud of ourselves.

Throughout these months, before we had to stop working, I have learned a lot of essential oils, their uses and how to use them, how important they are…

As I said before, we couldn't know how many things these oils could do. I didn't even know what they were and now, thanks to this project, I've had the opportunity to see how they act against pathogen bacteria, which is amazing.

Personally, I think I'm very lucky to participate in these activities because they are not the typical way of learning that we all know (books, exams…). When we started it, I was sure that it would be an interesting experience as it was the last year, and I wasn't wrong.

With all these experiments you can test yourself because you have to interpret the results, prepare the experiments by yourself with your classmates… just like a real scientist. At the beginning, you can think that working on a project like this is too difficult because you have to face a lot of difficulties. For example, for me I think that interpreting the results was the most difficult part because sometimes it wasn't so clear.

That's why, when you participate on something like this, you grow a lot as a person, because it lets you know yourself more, and as a scientist, because you become familiar with the environment of a laboratory.

There are two things that I like a lot of this kind of projects. One is that all the experiments that we have made, are made in a common high school by some students of bachillerato, and we are not aware of the repercussion that this can have. The other one is that, for example, impregnating the discs with the oils and wait for them to attack our bacteria, takes some time. Maybe we do it on a Wednesday and then we have to wait a week to see the results. That makes us get excited to go to class, so it's not that boring as it usually is.

From a scientific point of view, we have contributed to the antimicrobial studies more than we think. With this project, we intended to learn the importance of the antimicrobial products nowadays. Science is always changing and evolving as the planet and their living beings are. For a few years people have been using too much antibiotic products. Because of that, the bacteria around the world that we try to fight against gets used to these products and gets even stronger over time. Due to that, some of the antibiotics we usually use are becoming unuseful and that's why it's important to promote these experiments, because they can be the solution to the huge problem we are making misusing the antibiotics we've got.

From now on, I would obviously like to continue investigating on this, because I've realized how big is the problem we are facing.

Because of quarantine we couldn't finish the project as we wanted so, if the next year we could continue with it, I think it would be truly interesting to try the oils that have had the most antimicrobial activity on plants that have been infected by the three plant pathogen bacteria we have tested to see if they are as useful as we have discovered with the project.

Also, I would like to try the essential oils that had less activity on new bacteria because, maybe, they can surprise us.

Mónica Carrascosa Sánchez

This project has been a great experience, with which i have learned a lot together with my classmates about how to work in a laboratory, the use of natural alternatives such as antimicrobials against plant pathogens, the importance of antibiotics and their good use, knowing how to look information, assessing the results and more things that they are useful for a posible future in science and in life.

It is the first time that I carry out a project of this type and, to be honest, at the beginning I felt that everything was going to be too big for me, however, I have really enjoyed learning and becoming aware of a very important subject now. This project has helped me develop skills as working with bacteria and drawing conclusions that are necessary to do a research; believe a little more in myself and get closer to what I want to do in a future, science. Therefore, I find it very interesting and important to carry out this type of process in teaching because they motivate you to continue investigating and with them to acquire certain competences, which you have to apply in a future work ,which cannot be learned through the theory that it is given in books, which of course is crucial also to know what you are doing in practice. In addition, it is a great opportunity and very interesting to work with a researcher and professionals on your side because you learn more and it makes you more interested and take the project more seriously.

Speaking less in my personal experience, this project is very important because it talks about a topic a little-researched topic, treating plant pathogens with natural alternatives that cause less damage to the environment than those currently used. A difficulty for this research has been the Pandemic of Covid-19, which forced us to stay at home and have to continue working only with the bibliography. But with this pandemic we have realized how important the existence of research like ours is and, above all, that more research should be done on it because, as I have said previously, it is not a much-researched subject. It would also be very interesting to investigate the antiviral activity of essential oils because of the situation we are living with Covid-19.

Furthermore, having talks as Sonia Anaya gave us about antibiotics is very necessary so that in addition to creating indispensable projects, citizens become aware of and use antibiotics correctly to help curb this growing resistance. This talk helped us to motivate us more to continue with the research.

Antonio David Zamora Toro

I think these projects are very necessary in educational centers. We can be in a normal class and learn, but doing things like this makes us develop so much our skills, that we have a more professional and scientific vision, it makes us think, it challenges us.

The project brings me maturity, brings me knowledge and ultimately personal development. When you finally, put together all the pieces that you have been making over the past months and see a report, you feel proud.

You learn science from the most basic as it is to ask yourself questions and try to give them answers. Also, I think that from projects like this one can get great ideas from us, it is the advantage of not having the limit that a normal class has. We are thrown into a problem and we face it with what we can think of and propose.

I personally am very grateful for these opportunities, because thanks to them I learn in a very different way, I see different points of view and I analyze much more. It’s true that it’s not

easy, writing something more professional, thinking about possible causes of a result, proposing new experiments... And that is the good thing, which is not easy. At the beginning it costs more because we are not used to this type of classes and I think it is the fault, we should have more projects like this, that make us grow.

Regarding proposals on this project and future, I think that we could decide the theme, what we want to investigate, whenever possible. Fortunately we have the help of scientists like Manuel Espinosa and that gives us opportunities to see laboratories, do certain experiments, etc. I think that apart from being able to write an article, we could present our ideas and results at the institute or conferences to give more visibility to projects like this. And finally, experimenting with other types of bacteria and essential oils can also help to increase the interest in this research since they would be new with unknown and perhaps promising results.

Nuria Barroso Vacas

In my opinion the project has been very interesting. I've never been involved in anything like this before, but I can say it's been a great first experience. On top of everything I've learned intellectually, it's a great way to learn how to work as a team and how important that is. And focusing on the subject of the project, maybe at the beginning of the year someone couldn't have taken it as seriously as we are now with the pandemic that we are experiencing globally, but I think that this situation has given us more enthusiasm to continue with our project and helped us to be aware of the importance of this one.

And if it had been possible to continue experimenting, I would very much have liked to have continued advancing with the samples that had more activity and to have tried many others for the same.

Elena Rodríguez Comerón

I personally believe that this project has been and is yet another opportunity to learn more about the infinite world of science. It has given me the ability to know how to investigate and deepen in order to find an answer to the many questions that this project has made us elaborate. In conclusion, I would like to contribute that this project has made me reconsider and think that many of the solutions to many questions in life are in science, since, for example, we have been able to appreciate and work as simple essential oils or extracts of plants can combat various bacteria, thus providing us with the truth of the antimicrobial effects of these plants. In the end, we have been able to verify that something so simple, at the same time, can be so elaborate, and so beneficial for everyone. As a new contribution to the project, we could continue to instigate these antimicrobial effects by treating them with other types of bacteria and thus testing the antimicrobial reach of these plants, or conversely, obtaining new extracts and new oils from other plants in order to observe their evolution with the same bacteria that we've been working with.

Gonzalo Rodríguez López

My personal assessment has been droll and I have found it very interesting. Nowadays we have a problem and the it is that scientists are not discovering new antibiotics against multiresistant microorganisms. With this kind of experiments, we can help to find in the future new antimicrobials.

I have learned how to differentiate between two essentials oils with different results. I found very interesting and useful making this type of experiments because you can learn a lot of things like for example how to extract essentials oils from common plants and test them against microorganisms.

Víctor Manzano Guerrero

After all the work done, I have come up with various ideas. Firstly I would try ravintsara with other bacteria since it has not had any antimicrobial effect. Secondly thyme and savory have a great effect against both bacteria so I propose to use them against other bacteria to see various effects and hence propose some antomicrobials or even antibiotics.

I think it has been a very interesting and useful work and I would like to continue it someway.

Julia Martínez Barranco

This project is an opportunity we are given to learn in a different way, in a more pleasant way for us, being more practical and motivating us to continue with our research. It is also an advance that we contribute to science because we do not know the results that we can come out and we can be very helpful. We learn to work as a team in a responsible way, to organize ourselves, to think more looking for possible answers etc.

As we all know halfway through the project, a pandemic has reached us that has forced us to continue with our results from our homes, analyzing results and proposing options that always help. What we wanted to achieve was to find new plants and essential oils that presented antimicrobial activity in this way, in the case of plants having an infection by any of the bacteria that we have used, or against such strains of *E.coli* that causes urine infection. Substances to be able to cure them of a natural way because as we know there is a big problem worldwide with the overuse of antibiotics that must be solved because bacteria mutate creating resistance. As a plan for the future, this cannot be left at this point, that is, we must go deeper in the search for results with other oils and other bacteria.

In my opinion, I feel very fortunate that we have had the opportunity to carry out this project, with the materials and people with whom we have done it, I am very grateful that our teacher Antonio Quesada strives so much for our good and Manuel Espinosa that is essential.

Searching for antimicrobial agents in plants: potential activity of ethanolic extracts against plant pathogenic bacteria

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Summary

Plants produce a wide range of molecules with biological activity, including compounds with antimicrobial action. These are becoming more relevant due to the increase in antibiotic resistant microorganisms as possible treatments against them. This circumstance concerns to plant pathogenic bacteria as they can cause important losses in agriculture as well as environmental hazards.

In this research, we have studied the potential antimicrobial effect of twenty ethanolic extracts obtained from common plants, against some of the most important plant pathogenic bacteria: *Xanthomonas campestris*, *Pseudomonas syringae* and *Dickeya dadanti*. We have tested its activity against *Bacillus megaterium* and *Escherichia coli* as well.

Extracts obtained from *Eucaliptus globulus*, *Rosa sp* and *Punica granatum* peel were active against all the tested bacteria. The main antimicrobial activity was presented by pomegranate peel extracts. *Salvia officinalis* extract inhibited the growth of *B.megaterium* and *D. dadanti*. Weak activity was observed in *Sechium edule*, *Citrus limon* and *Stevia rebaudiana*, only active against one microorganism.

Our results suggest that plant extracts may be a good alternative to antibiotic treatments and remark the importance of considering their use as natural products with important ecological advantages to reduce infectious diseases in plants.

Keywords: Plant extracts, antimicrobial, *Pseudomonas syringae*, *Xanthomonas campestris*, *Dickeya dadanti*, *Bacillus megaterium*, *Escherichia coli*, disk diffusion method.

INTRODUCTION

Plants produce a wide range of molecules with biological activity that have been used with medicinal purposes since ancient times. These are secondary metabolites as polyphenols, alkaloids, tannins, terpenoids, flavonoids, etc. whose main advantages are that they are generally safe to human health, they do not have harmful effects on the environment and they are cheap. Plant metabolites with antimicrobial activity have received special interest in recent years [1]. The increase in pathogenic microorganisms resistant to antibiotics and the side effects of these products for the environment has led to look for new products as substitutes of antibiotics.

One of these applications has been the use of plant extracts to prevent food spoilage. The repeated use of antibiotics has resulted both in the accumulation of chemicals in food and feed chain and in unpleasant effects of those chemicals on human health. Many researchers have focused on the utilization of plant extracts as antimicrobial agents for food preservation. Zivković *et al*. [2] described the antimicrobial and antibiofilm potential of *Rosa canina* leaf extracts against *Pseudomonas aeruginosa* and *Salmonella typhimurium* and proposed its application for pharmaceutical and food inudstries. Al-Zoreky [3] described antimicrobial activity in pomegranate fruit peel extracts against food pathogens like *Listerya monocytogenes*, *Staphylococcus aureus*, *Yersinia enterocolitica* and *Salmonella enteritidis*. Bayoub *et al*. [4] reported antimicrobial activity of ethanolic extracts from different medicinal plants as *Thymus vulgaris*, *Rosmarinus officinalis* or *Rosa centifolia* against *Listeria monocytogenes* as well. In this context, plant extracts are potential antimicrobials for food preservation, considered nutritionally safe and easily biodegradable.

Plant extracts have been tested as well against human clinically important pathogens with positive results. Extracts from the root of *Salvia apiana* caused growth inhibition of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis* and *Candida albicans* [5]. Hydroalcoholic extracts of *Punica granatum* showed inhibitory activity against *Streptococcus mutans* and *Rothia dentocariosa*, two cariogenic bacteria [6].

Plants are attacked by pathogenic microbes as well, including bacteria. Although bacterial diseases can cause enormous losses, they are still poorly controlled with tradicional chemicals like copper [7]. Few studies have analyzed the use of plant antimicrobials against these pathogenic bacteria. *Allium sativum* extracts have shown activity in vitro against *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris* [8], and growth inhibition of some of these bacteria by extracts from several South African plant species has been described [9].

In this work we have studied the potential antimicrobial effects of twenty ethanolic extracts of plants, most of them collected from IES Zaidín Vergeles school garden, against three plant pathogenic bacteria: *Pseudomonas syringae*, *Xanthomonas campestris* and *Dickeya dadanti*. These are considered among the top ten plant pathogenic bacteria [10]. We have

also studied the inhibitory potential against *Bacillus megaterium* and *Escherichia coli*, two bacteria we usually work with in the laboratory.

MATERIALS AND METHODS

1. Plant material

Leaves of *Solanum tuberosum*, *Eryobotria japonica*, *Calendula sp*., *Tropaloeum maius, Narcissus sp*. *Sechium edule*, *Citrus limon*, *Kalanchoe sp*., *Physalis peruviana*, *Stevia rebaudiana*, *Eucaliptus globulus*, *Santolina sp*., *Aloe barbadensis*, *Rosa sp*., *Vicia faba*, *Borago officinalis*, *Allium cepa*, *Phyllostachis sp*. and *Salvia officinalis* and peels of *Punica granatum* were collected in Granada during February 2020. Many were collected at IES Zaidín Vergeles school garden and others were provided by the students. *Punica granatum* peels were also collected and used in this study.

2. Extraction of plant leaves

The leaves were dried at 50°C for 3-4 days. Pomegranate fruit were washed with running tap water and the separated from seeds. The dried leaves and pomegranate peel were cut into small pieces and 2g were soaked in 10 ml of 70% ethanol and then crushed with the help of mortar and pestle. The mixture was filtered through Whatman's filter paper No.1 and kept at ambient temperature for 48 h. The extracts were stored at -5°C in bottles until use.

3. Microorganisms

The microorganisms used in this study were selected for being frequent plant pathogens [10]. Therefore, the following gram-negative bacteria were used: *Xanthomonas campestris, Pseudomonas syringae* and *Dickeya dadanti*. Other bacteria used in the determination of the plants antimicrobial activity were the gram-negative *Escherichia coli* and gram-positive *Bacillus megaterium;* these bacterias were selected as we have used them in previous studies. All the microorganisms were provided by the Estación Experimental del Zaidín (CSIC).

4. Antibacterial activity evaluation: Agar disk diffusion method

Twenty mililiters of TSA (triptone soya agar) medium were poured into sterile Petri dishes as a basal medium. 5 ml of semisolid medium (triptone soya broth + agar 0,6%) at 42ºC were inoculated with 100 microliters of liquid culture (TSB or LB medium) of the bacteria to be tested and spread on the basal solid medium.

The antimicrobial activity was evaluated by the agar disk diffusion method. Sterile Whatman filter paper disks (6 mm diameter) were soaked into plant extracts, let dry on filter paper to remove the excess of the product and deposited them on the plates prepared as we have indicated before. Estimation of inhibitory activity has been made measuring the total area of the inhibition halo and subtracting the surface of the filter paper disks.

RESULTS

Twenty extracts from different plants have been used in our experiments and only seven showed antimicrobial activity (Table 1). Three of them were active only against one species of microorganism. *S. edule* against *X. campestris*; *C. limon* inhibited the growth of *E. coli* and *S. rebaudiana* showed activity against *D. dadanti*. In all these cases, the activity wasn't very important, with small inhibition halos.

Extractos vegetales	*Bacillus megaterium*	*Escherichia coli*	*Xanthomonas campestris*	*Pseudomonas syringae*	*Dickeya dadantii*
Patata (*Solanum tuberosum*)	-	-	-	-	-
Nispero (*Eryobotria japonica*)	-	-	-	-	-
Caléndula (*Calendula sp.*)	-	-	-	-	-
Capuchina (*Tropaloeum maius*)	-	-	-	-	-
Narciso (*Narcissus sp.*)	-	-	-	-	-
Chayote (*Sechium edule*)	-	-	22,0	-	-
Limonero (*Citrus limon*)	-	10,2	-	-	-
Kalanchoe (*Kalanchoe sp*)	-	-	-	-	-
Uvilla (*Physalis peruviana*)	-	-	-	-	-
Estevia (*Stevia rebaudiana*)	-	-	-	-	22,0
Eucalipto (*Eucaliptus globulus*)	84,8	50,3	125,7	84,8	50,3
Santolina (*Santolina sp.*)	-	-	-	-	-
Aloe (*Aloe barbadensis*)	-	-	-	-	-
Rosal (*Rosa sp.*)	35,3	22,0	84,8	84,8	10,2
Haba (*Vicia faba*)	-	-	-	-	-
Borraja (*Borago officinalis*)	-	-	-	-	-
Cebolla (hojas) (*Allium cepa*)	-	-	-	-	-
Bambú (*Phyllostachis sp.*)	-	-	-	-	-
Granada (*Punica granatum*)	172,8	84,8	172,8	125,7	172,8
Salvia (*Salvia officinalis*)	50,3	-	-	22,0	-

Table 1. Antimicrobial activity of ethanolic extracts of selected plants against *B. megaterium*, *E. coli* and plant pathogenic bacteria *X. campetris*, *P. syringae* and *D. dadanti*.

Salvia officinalis had activity against two bacteria. It clearly inhibited the growth of *B. megaterium* and showed a weaker activity against *P. syringae*.

Three extracts, those obtained from leaves of *E. globulus*, *Rosa sp* and peel of *Punica granatum* had inhibitory activity against all the tested bacteria (Figures 1, 2 and 3). The biggest inhibition halos were observed with *P. granatum* peel extract, mainly against *B. megaterium*, *X. campestris* and *D. dadanti*.

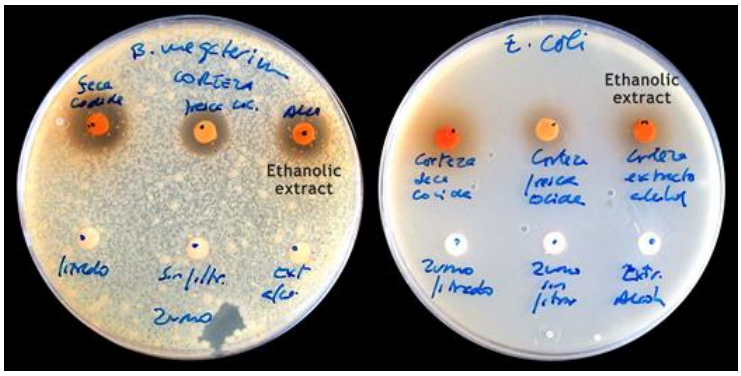

Figure 1. Antimicrobial activity of *Punica granatum* extracts against *B. megaterium* and *E. coli*. Pomegranate juice did not show inhibitory activity against those two strains.

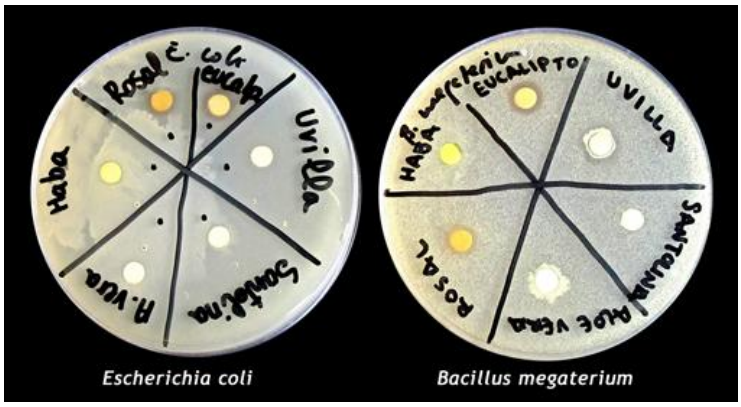

Figure 2. Antimicrobial activity of *Rosa sp* and *E. globulus* extracts against *E coli* and *B. megaterium*.

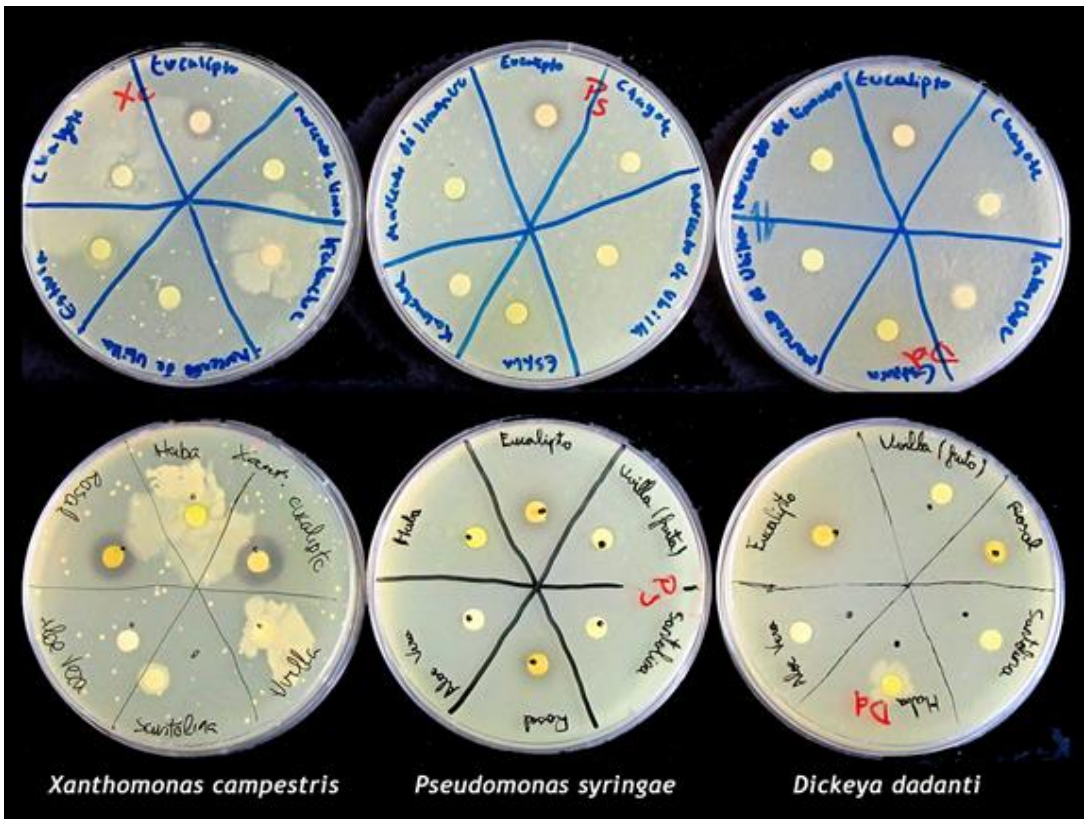

Figure 3. Antimicrobial activity of *E. globulus* and *Rosa sp*. extracts against plant pathogenic bacteria.

DISCUSSION

Plants produce a great range of molecules known as secondary metabolites that can be sources of medicinal agents. In recent years, special interest has received the study of some of these compounds as an alternative to antibiotics for several reasons. On one side, as a consequence of the excessive use of antibiotics there has been an increase in resistant strains, with a high risk of transmitting such resistances to other microorganisms [11]; on the other side, and specially in treatments against plants pathogens in crops, by the undesirable effects that those compounds can have in the environment, as they can result in toxic residues [12].

We have screened twenty common plants, most of them collected from our school garden in order to assess the existence of antimicrobial activity against three of the most important plant pathogenic bacteria and two control microorganisms routinely used in the laboratory, *B. megaterium* and *E. coli*. Seven of the ethanolic extracts showed antimicrobial activity. Three of them only affect one bacterial strain. *Salvia officinalis* extracts inhibited the growth of *E. coli* and only one of the three pathogenic plant bacteria, *P. syringae*, with low activity. Antimicrobial activity of *S. officinalis* against *E. coli* has been recently described [13]. Extracts obtained from three plants were able to inhibit the growth of all the tested bacteria. These were *E. globulus*, *Rosa sp*. and *P. granatum*. Baştaş [14] described antimicrobial activity of extracts of *E. globulus* and *Rosa canina* against *P. syringae* pv tomato and proposed that some plant extracts may be used to combat plant diseases like the bacterial speck on tomato.

The extracts with the strongest antimicrobial activity were those obtained from pomegranate peel. The most sensitive bacteria were *X. campestris* and *D. dadanti* among the pathogenic ones and *B. megaterium*. Several studies have revealed an important antimicrobial activity in pomegranate peel extracts. In relation to plant pathogenic bacteria, it has been reported that *P. granatum* peel extracts were effective against some plant pathogenic bacteria, like *Ralstonia solanacearum*, *Xanthomonas gardnier* and *Erwinia carotovorum* [15]. These authors propose that the antimicrobial activity may be related to the presence of polyphenols and tannins, especifically gallagic acid and punicalagin.

Plant extracts have been widely used to prevent food spoilage [2,3,4] or as a treatment against some human pathogens [5,6] but little research has been made about their use against plant pathogenic bacteria. Up to now, the control of bacterial diseases in plants has been mainly achieved by the use of antibiotics and copper compounds that can be toxic for living beings and the environment. Our study suggests that plant extracts are a good alternative to those treatments and remark the importance of considering their use to reduce disease incidence in plants. In conclusion, further research must be done to find new natural compounds against plant pathogenic bacteria.

Acknowledgements

We would like to express our thanks to Antonio Quesada Ramos and Manuel Espinosa Urgel for their supervision, support and encouragement. Without their guidance and never-ending patience, this project would not have been possible. We are also thankful to the Estación

Experimental del Zaidín (CSIC) for giving us the opportunity to work on this interesting project. Finally we are also grateful to Sonia Anaya Ordóñez, who showed us the importance of the research and development of new antibiotics.

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MY OWN IDEAS

Pablo Delgado Alaminos

This project has turned out to be quite important for me because I have been involved almost as much as possible preparing some extracts and oils and especially discussing the results. I have learned a lot about the activity that plants have against bacteria and it seemed very didactic to us that we prepared the extracts and oils of the plants ourselves, but the best thing of all was learning to interpret the results of our experiments, directly observing the Petri dishes.

In general, the discussion of the results has been the most complicated thing, because for example in some dishes we couldn't see if there was activity or not due to the halo that wrapped the extract or the oil was very small, this has been the most complicated thing, especially at the beginning. But other activities such as preparing extracts, dishes or measuring inhibition halos have been simpler activities.

These experiments that we have carried out, although they do not seem as professionals as the experiments of a laboratory are also very important because there is very little information about some similar experiments that we have done, so hopefully we could discover something.

What I propose to do in the next year is to continue with this project, that is to say, as we already know which plants are the most active (thyme, savory, eucalyptus, rose bush and pomegranate) we can continue studying them, testing them against other bacteria or against other types of pathogens. Another project that could be done in the future would be to test the resistance of bacteria against certain chemicals to try to find a new substance that can be used to disinfect, for example.

About the fact that we have been able to do all these experiments with the help of experts like the doctor Manuel Espinosa from EEZ, it seems to me a unique opportunity, due to we can learn much more, they have also provided us with materials and they have guided us in our project. The truth is that we are quite lucky and I hope that the next courses can also have this opportunity.

Being able to carry out these projects in an institute means that there is also a practical part and that it is not all theoretical. This is how we learn better because we can see with our own eyes how the antimicrobial agents of plants act on bacteria. It can even motivate us to work on this because we have been able to see how a true researcher works and we have been able to appreciate how interesting is this, because at any moment something can be discovered.

Manal Mansour

This project has made me realize the importance of developing new antibiotics to fight infections. Antimicrobial resistance is a growing problem globally and the overuse of them is the main factor, it is necessary to raise awareness and promote the appropriate use of antibiotics.

Projects like this give us, students, opportunity to learn and experience about topics that we don't learn through books.

Alejandro Ortiz Minaeva

At first, I'll say that this project means a lot to me, truthfully I think that I have learned so many things about what is the procedure that all the projects have to got. I have felt like a real researcher when I was doing and studying the experiments made during in the project… A very good feeling, honestly.

We have been for 5-6 months doing experiments, getting some information about what we're facing (through internet, from our own old articles and even with meetings of researchers and other people that came in order to help and answer our questions), analysing results of each experiment, planning future experiments and the most important, having fun and learning about the scientific research.

In my personal opinion I have to say that this 'school projects' are not as common as we all thought that they are in the scholar program; moreover, I think that we are the only ones that we can say that we're are doing a research project in our highschool (at least of Granada), and that makes it very special for us, the students.

And the most exciting and motivating part is the fact that we know that we can find a new plant with antimicrobial activity that no one have investigate before. The feeling of being helping the research community makes us feel like true scientists working, it makes us feel to be part of something bigger than a simple activity of a regular anatomy class.

I really hope that this kind of projects could be included in all the highschool programs because I think that every student has the right of feel like a true scientist. After a few years we can make it happen, hopefully.

And I want to propose some ideas of future projects like: study the coronavirus and how do they affect, or the virus in general, to the living beings and how we can fight against them. I really appreciate all what our teacher have given us.

Aarón Villoslada Calvo

We have spend the last 9 months of hard work, of hours looking for good plants to investigate them, hearing concentrated to the teacher speaking about what was that project and how we will work in it, with talks of amazing scientific professionals speaking about pathogenic bacteria and all the ways to fight them. All of this was... just fantastic.

I learned hundred of things about plants, the form of bacteria, about procedures of research and science. And how to solve some problems in the laboratory, as we had to face that some of our plates were contaminated.

Working as a team is always important for the development of a person. It is also important to learn theoretical concepts; all of them are important to carry out our project. But I think that if I had to choose what I liked the most, it would be to see that nothing is what we believe, and that some plants, which I have known since I was a young boy, which I have played with and despised, can become an antidote for a disease or to attack a bacteria.

This project that we have carried out, could help other researchers to reduce the search for possible plants from which the cure of an infectious disease can be obtained, of the infinite ones that exist, and I think that this is also something that I loved. Doing in this research, we could be part of something very big; we may not become famous for it, but to think that we have been able to help on a professional scale is something that fills me with pride and satisfaction.

In this project we have focused mainly on discovering substances with antimicrobial activity. But we have left the research about antibiofilm action of our extracts. Probably in the next project we could focus on that question, how to destroy biofilms ant thus make the most resistant bacteria weaker.

These types of projects fascinate me, because they bring us closer to science and teach us the most real and unfiltered part of it. This not only helps us to learn more about scientific topics, but also to know if we want to dedicate our lives to it, or to be simple amateurs in love with the wonders of science.

Nuño Gutiérrez Martín

For me, this project has been an alternative, another way of learning alternative to a conventional class with paper, book and pen. These projects are unique experiences, and experiences that you have to be proud to participate in this, since we were taken to the "scientific world" with fun things that we have learned by solving difficulties such as; the lack of budget, more homemade methods, hours limited to our student position or the most important, leave the experiments in our laboratory and have to continue our project from home, with methods limited telematically by COVID-19. Despite all these difficulties I think we have been up to this project, providing plant species with easy daily access, studing its potential antimicrobial properties against bacteria from different families as Escherichia coli or plant pathogens, and looking for the production of natural antibiotics for this important problem of superbacteria, bacteria resistant to several antibiotics. Concept that we have learned with talks, studying our bacteria and the methods of making essential oils that help us to train without book, that is why these projects or study route are and should be more important in other subjects, since they form and give us things like publications in magazines to use in our future.

Another project for other students in other years should be the study of insects, since insects and plants are practically linked and using our project could study the insects related to the plants that we have studied with antimicrobial activity and see if there is a relationship between this activity and its fauna of insects.

Mario Agustin Navarro García

This project has been very satisfactory to do because it is the first time that the things we have learned in the institute serve for something, not on a personal level, which were already useful, but served to help many other people.

With this project I have learned to carry out what I have studied and to collaborate with other classmates. It has also helped me learn about how to solve problems. My biggest difficulty has been facing a project like this for the first time, but in the end I have learned and I think I am capable of carrying out more projects.

I think the work has been very important because it teaches us what in the future we will be asked to do. And we will be ready.

For the future it would be interesting to study the functioning of stem cells, but I really do not care because all the work will help us. Also thank the high school for bringing us professional researchers to help us with the project. Without them we would have not learn the same.

Microbiome associated to the olive (*Olea europaea* L.) pollen

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Summary

Pollen-associated microbiomes are becoming a point of interest in research due to their potential involvement in maintaining good gut health of insects incorporating pollen as part of their diet. Moreover, the modulating effects of pollen microbiota on the development of allergic symptoms in humans has been described for several allergenic species. Our aim was to assess the presence of microorganisms on the surface of the olive tree pollen from two cultivars, and also in the pollen of some other species as a comparison. We managed to set the conditions for culture and preliminary morphological characterization of bacterial strains in this material. All pollens assayed displayed a rich and variate composition in bacteria with multiple morphological shapes and sizes under microscopical observation. We also identified a relevant content in yeasts associated to this pollen. Future studies are fully planned to proceed with molecular identification of this microbiota, and to assess its effects on olive pollen allergenicity.

Keywords: allergy, *Artemisia*, bacteria, insects, *Lilium*, microbiome, *Olea europaea*, pollen, yeast

INTRODUCTION

Pollen grains are likely to represent a unique habitat for microbia, as they contain intrincate, highly structured and specialized pollen walls, with the outer layer (exine) usually containing open spaces (orbicules) which are in most cases filled and/or covered by a complex mix of components named pollen coat. As an extremely large variety of sizes, shapes, patterns and exine textures are displayed by pollen grains, and multiple types of pollen coats have been described as well, we can straightforwardly assume that pollen-associated microbiomes must be also extremely changing between pollen species.

Some indirect clues regarding the composition of pollen microbiome was obtained after the analysis of the microbiota associated to bees [1,2,3,4,5]. These studies have shown that the gut microbial community of insects is quite broad, and in some extent different from pollens.

Few studies to date have focused into specifically analyzing the composition of microbiomes in pollen grains. Some of these studies have been able to identify up to 12 bacterial and 33 fungal genera in eight different pollen species [6,7]. In 2016, Obersteiner *et al.* [8] also identified the microbiome associated to timothy grass and birch tree pollens. Many of these studies also aimed to determine whether the microbial diversity associated to the pollen was somehow able to correlate with the presence of pollution and/or the allergenicity of some of these pollen [8,9,10,11,12].

The first aim of the present work was to analyze and compare the colonizing microbes on the allergenic pollen of the olive tree (*Olea europaea* L.), which is broadly studied by the hosting research group. Also, the analysis was extended to the pollen grain from a species widely used to study plant reproductive biology: *Lilium longiflorum*, which is noticeably different from the olive pollen in terms of size and characteristics of the pollen coat [13] and to a highly allergenic pollen like *Artemisia*.

MATERIAL AND METHODS

1. Pollen collection

Mature pollen samples from 2 different olive (*Olea europaea* L.) cultivars: 'Picual' (sample #1) and 'Arbequina' (sample #2) were obtained from genotyped olive trees at two different locations in Andalusia (Spain): Granada (sample #1; GPS coordinates: S 37°11' 17.5'', W 3° 36' 24.1'') and Armilla (sample #2; S 37° 8' 27.6'', W 3° 37' 6.6''). Pollen samples were collected in paper bags by vigorously shaking the olive inflorescences, sieved through 150 and 50 µm mesh nylon filters to remove debris, and then stored at -20 °C and at room temperature until use.

Eastern lily (*Lilium longiflorum* Thunb. cv. 'White Heaven') were purchased at a local market. Flower buds were left to open at 25 °C, 60% humidity, and a 12h photoperiod under

natural irradiation. Pollen grains were collected from the anthers by brushing, dried for 2 days and stored at -20°C and at room temperature until use.

Artemisia vulgaris pollen was kindly provided by Inmunal SAU (Alcalá de Henares, Madrid). This pollen was subjected to long-term storage at -20 °C.

2. Isolation of bacteria from pollen

Fifty milligrams of each pollen sample ['Picual' frozen (-20ºC); 'Picual' room temperature (RT); 'Arbequina' frozen (-20ºC); 'Arbequina' (RT); Eastern Lily Frozen (-20ºC); Eastern Lily (RT) and Artemisia frozen (-20 °C)] were mixed each with 5 ml of shaking solution (0.05% Tween 80 and 0.18% $Na_4P_2O_7$ [14]) and agitated for 30 min. This was followed by serial dilutions with 0.02% Tween 80 + 0.085% NaCl to 2.5x 5x and 10x dilutions. Different volumes (100 µL, 500 µL, 1000 µL) of each dilution were plated in triplicate onto:

a) 1:10 diluted LB medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany),

b) 1:10 LB medium with cycloheximide (50 mg/mL dissolved in H_2O).

c) 1:10 LB medium with ampicillin (100 mg/mL dissolved in H_2O), kanamycin (50 mg/mL dissolved in H_2O,) tetracycline (10 mg/mL dissolved in H_2O) and cycloheximide (50 mg/mL dissolved in H_2O).

The plates were incubated aerobically for five days at 25°C. Colony counts were determined for each one of the three of LB plates. Morphologically different colonies were selected and subcultured from single colonies for pure culture isolation.

Each clearly identified single colony was grown independently in liquid LB medium (1:10 no-agar) to confirm purity by microscopic observation of the cellular morphology. For conservation and further analysis, pure cultures were grown until an optical density of 0.8 to 1.2 at 600 nm (OD_{600}) was reached, and glycerol was added to 20%. Glycerol STABs were stored at −80°C.

3. Microscopical observations.

Microscopical observations of the cultures were performed in a Nikon Eclipse Ti-U microscope (Nikon, Tokyo, Japan) equipped with Differential Interferential Contrast (DIC) optics and a DFK72AUCO2 camera (ImagingSourceJenoptik, Bremen, Germany).

RESULTS AND DISCUSSION

1.Preliminary culture and assessment of the microbial load of pollen samples.

Culture of LB plates alone or in the presence of cycloheximide and antibiotics + cycloheximide produced a differential number of colonies when the olive pollen isolate

obtained from the 'Picual' cultivar and maintained at RT was assayed (Figure 1).

Overall, lower dilutions assayed produced a huge growth on the plates, which made impossible colony counting (not shown), whereas dilutions 10x produced a differential, countable growth of colonies, as shown in Figure 1.

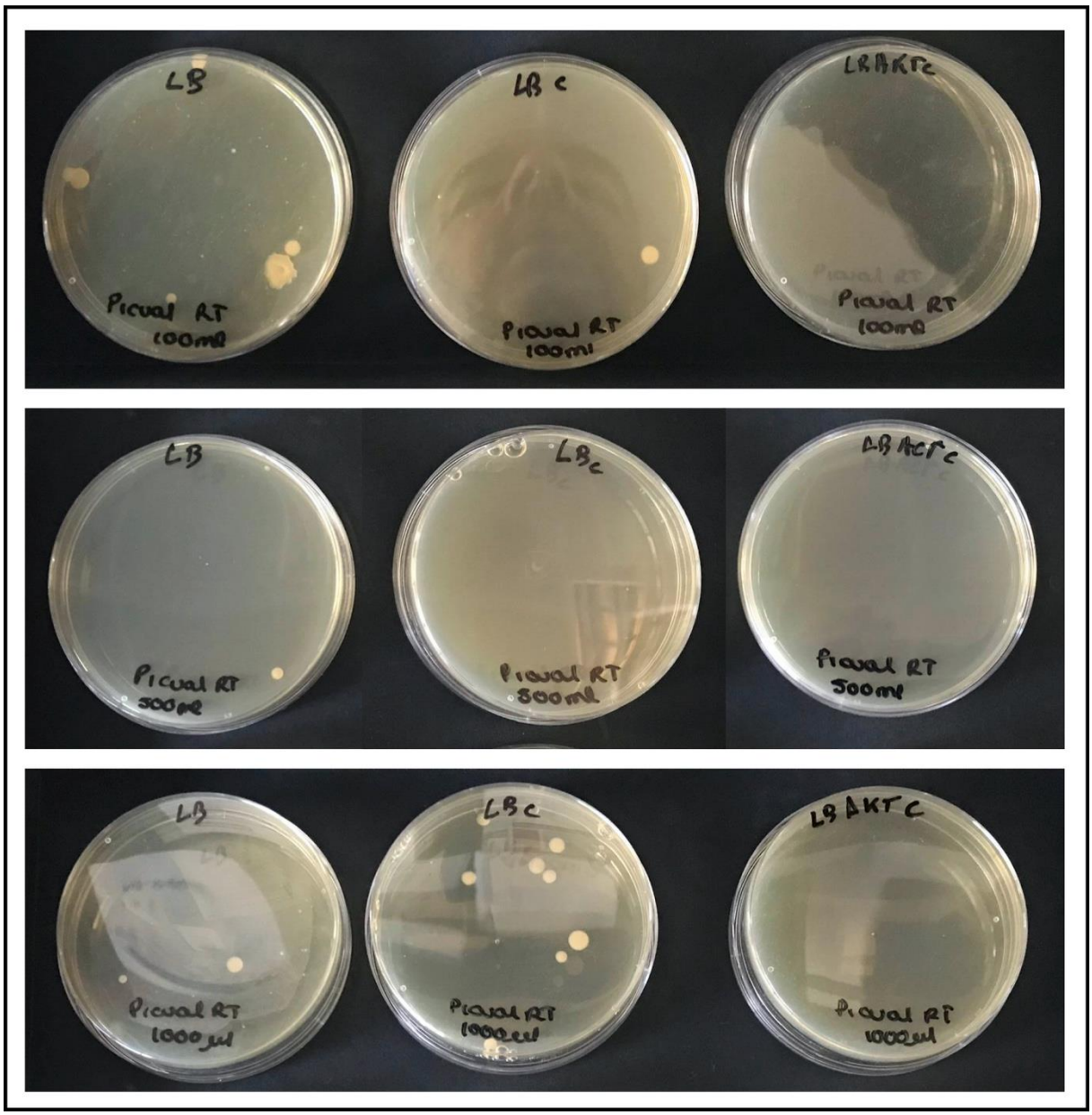

Figure 1. Colonies resulting from the incubation of plates with 100 µl (upper row), 500 µl (central row) and 1000 µl (lower row) of olive 'Picual' pollen maintained at RT isolate (diluted 1:10) on LB medium alone (left column), or with the addition of cycloheximide (central column, LBc) or antibiotics + cycloheximide (right column AKTC).

As a rule of thumb, growth of colonies on LB plates alone was higher than in the LBc or LBAKTc plates, showing that treatment with either cycloheximide and antibiotics +

cycloheximide was able to prevent the growth of most fungi and bacteria+fungi, respectively. The results also show that the olive pollen of the 'Picual' cultivar contained a significant number of bacteria able to form colonies. A similar result was obtained when the 'Arbequina' cultivar of olive was used as the source of pollen (not shown).

The use of pollen stored at -20ºC instead of RT did not significantly alter the results obtained, and a relevant number of colonies was also obtained when the pollen of both olive cultivars was assayed (Figure 2).

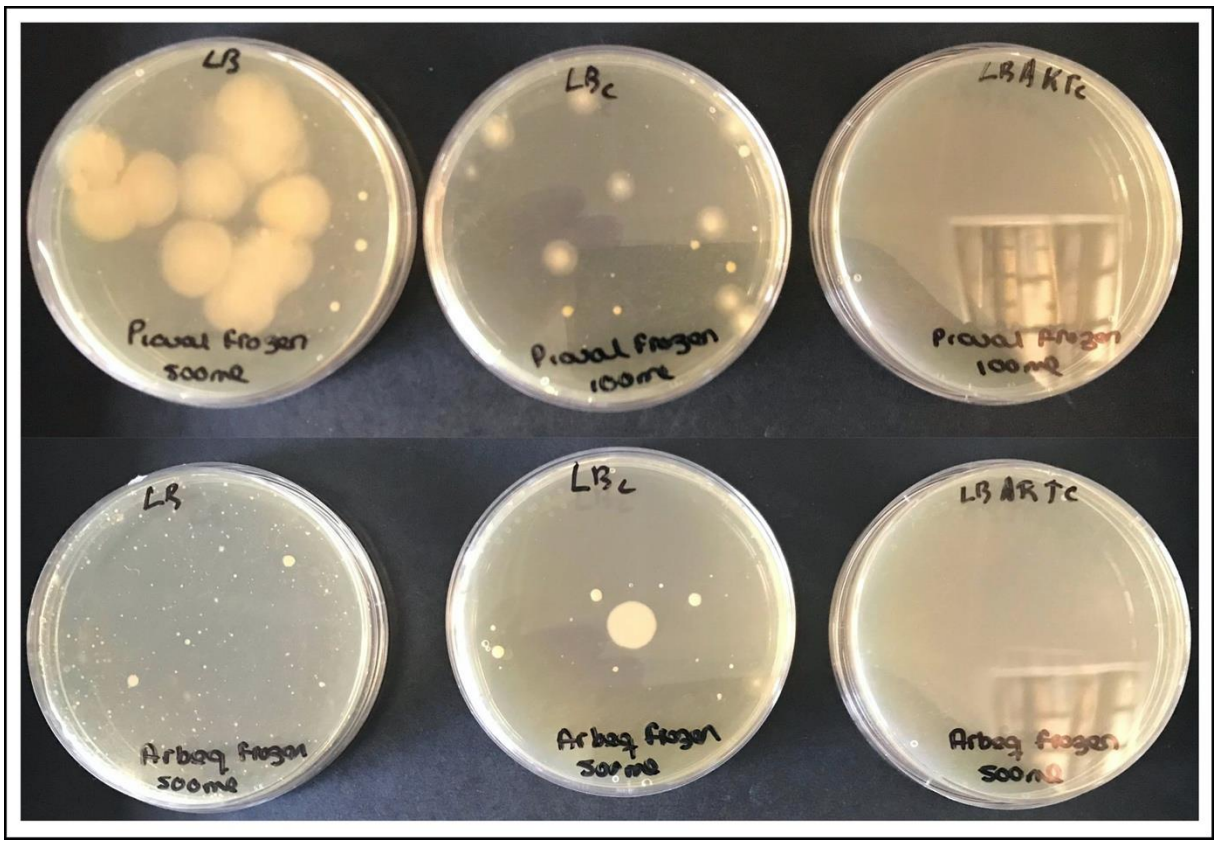

Figure 2. Colonies resulting from the incubation of plates with 500 µl of the olive 'Picual' pollen (upper raw) or 'Arbequina' pollen (bottom raw) maintained at -20ºC isolates (diluted 1:10) on LB medium alone (left column), or with the addition of cycloheximide (central column, LBc) or antibiotics + cycloheximide (right column, AKTC).

The pollen from a different species like *Artemisia vulgaris* also allowed the identification of colonies in a similar way than olive pollen (Figure 3). However, *Lilium* pollen failed in a first trial to generate bacterial growth (not shown), as the pollen used corresponded to pollen stored over very long period at RT (over three years). A new pollen collection from fresh lily plants allowed bacterial growth as well (Figure 4).

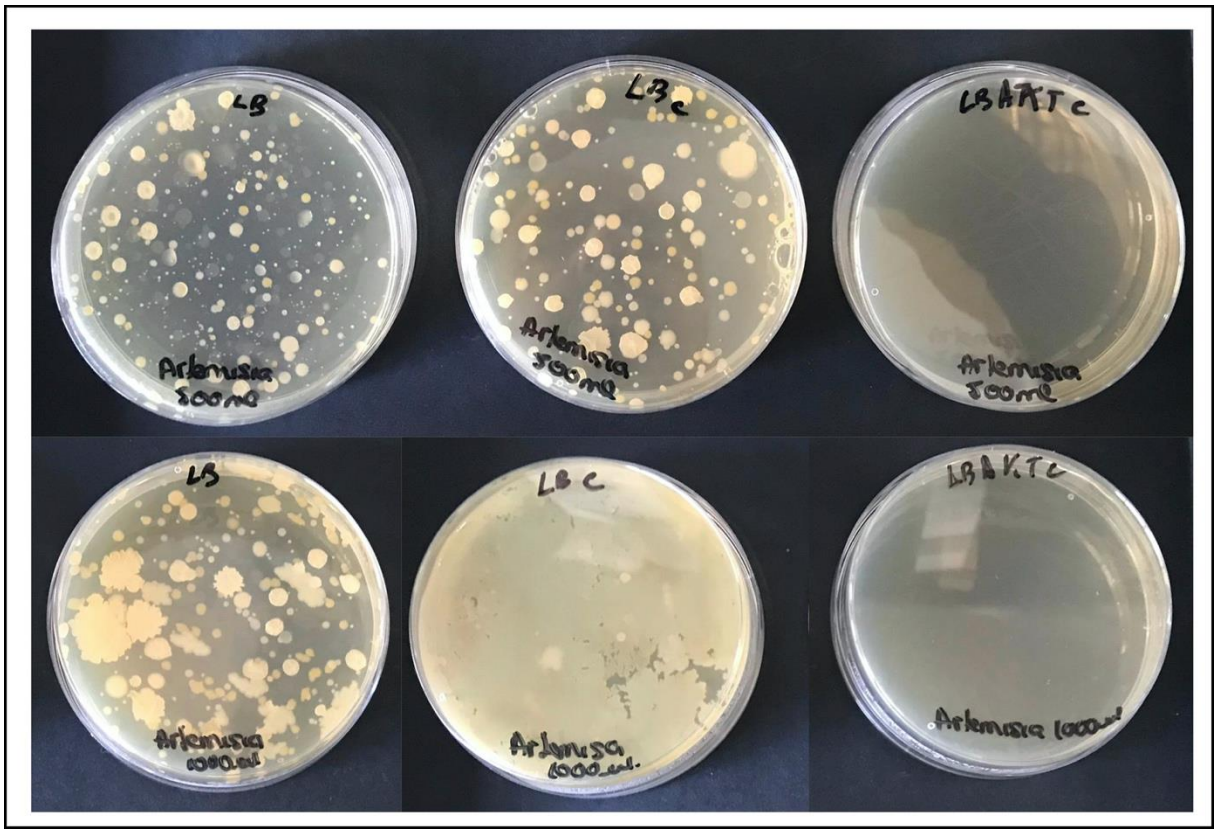

Figure 3. Colonies resulting from the incubation of plates with 500 µl (upper row) and 1000 µl (lower row) of *Artemisia vulgaris* pollen maintained at -20°C isolate (diluted 1:10) on LB medium alone (left column), or with the addition of cycloheximide (central column, LBc) or antibiotics + cycloheximide (right column, AKTC).

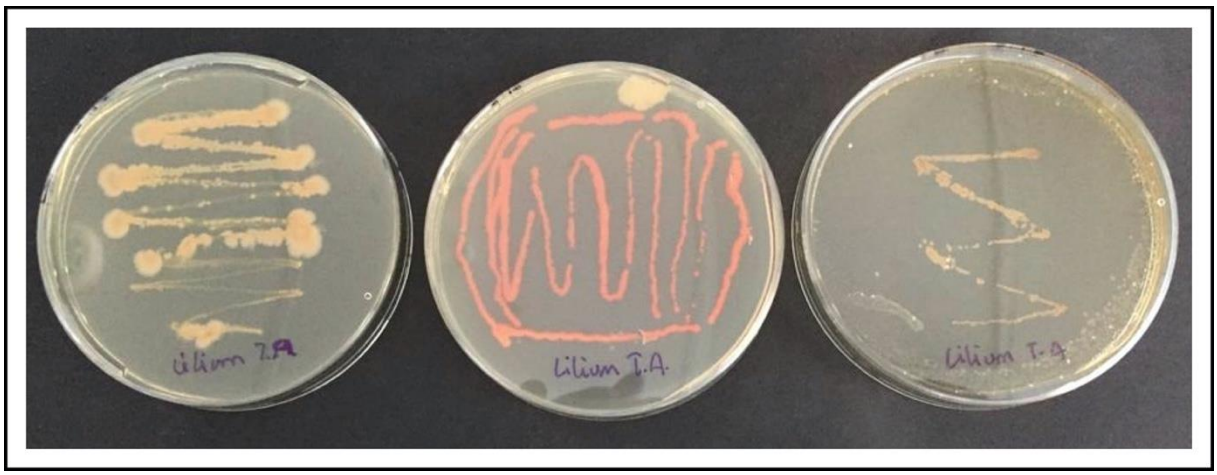

Figure 4. Colonies resulting from the incubation of plates with 500 µl of lily pollen maintained at -RT isolate (diluted 1:10) on LB medium alone

2. Isolation of representative colonies.

Once the presence of a significant number of microorganisms over the surface of the pollen grains was detected, the STABs generated were seeded again on new plates by streaking with a platinum inoculation loop in order to generate isolated colonies. A large number of isolated colonies were obtained after culture, which were numbered (Figure 5) to proceed with microscopical identification as well as with future molecular identifications. Only well isolated, representative colonies were selected. Colonies likely representing fungal growth were omitted. Such experiments were carried out with olive pollen isolates from the 'Picual' cultivar only.

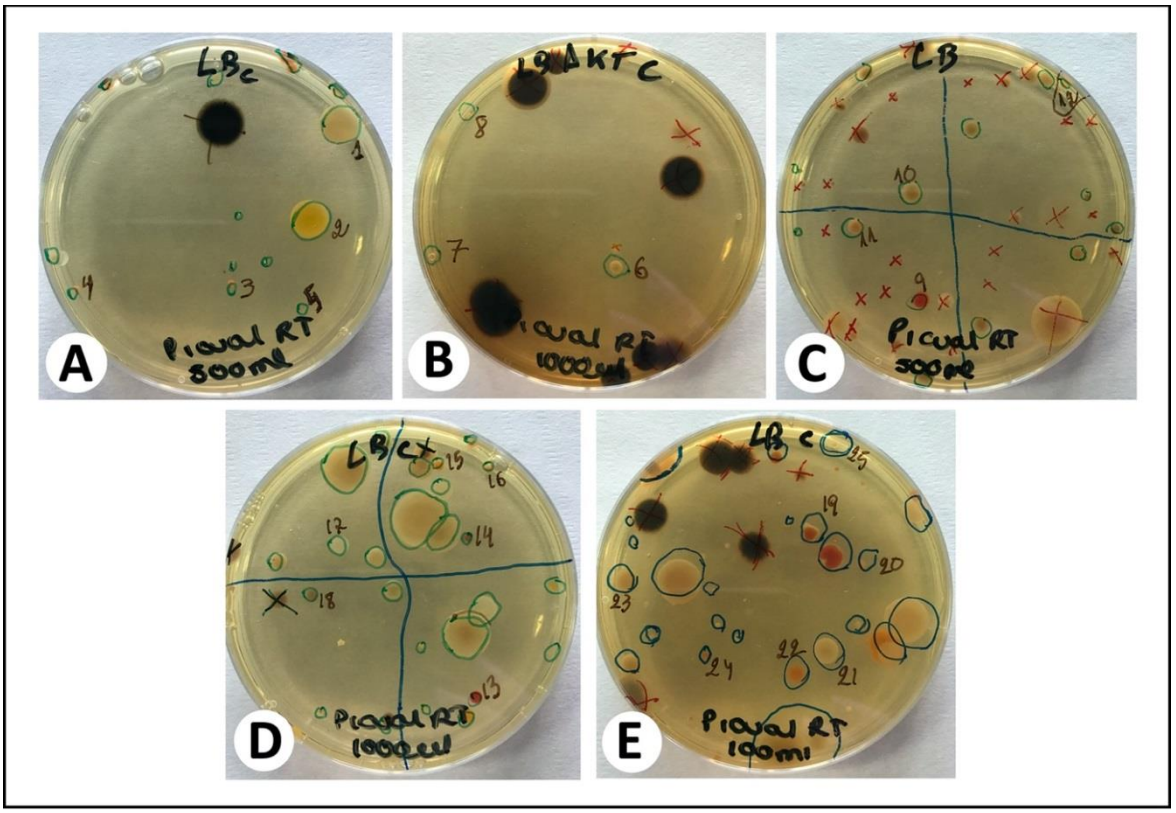

Figure 5. Colonies resulting from the incubation of plates seeded with isolates from olive pollen of the 'Picual' cultivar on LB medium alone or with the addition of cycloheximide (LBc) or antibiotics + cycloheximide (AKTC). Well-isolated colonies were numbered 1-25.

3. Morphological variability of microorganisms isolated from pollen.

Isolated colonies were resuspended in LB medium and used for microscopical observation using Nomarsky optics as detailed. Figure 6 shows the morphological characteristics of the colonies isolated from the olive pollen of the 'Picual' cultivar. Pictures show a broad panel of shapes and sizes, including coccus, short and long bacillus, and filamentous bacteria. Noticeably, many of the colonies were assigned to yeasts also with a variety of sizes and shapes (Figure 7). Although the majority of the colonies only present a single type of microorganism, some pictures (i.e. Fig 7B) reveal the presence of at least two coincidental microorganisms, which indicates that further isolation would be necessary.

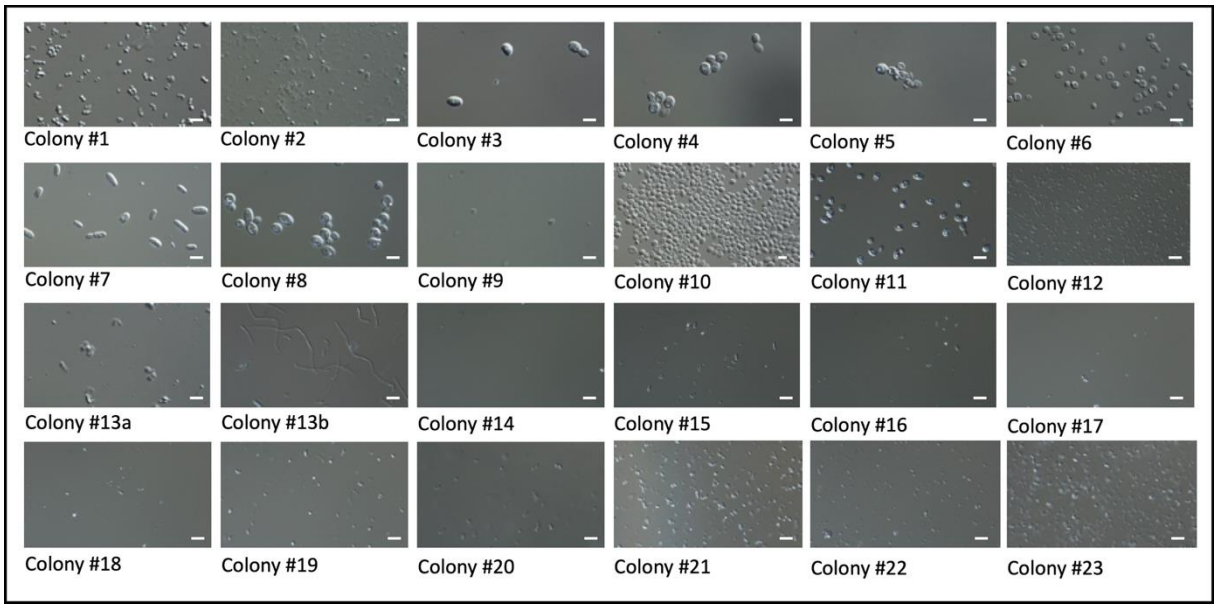

Figure 6. Variability of microorganisms analyzed by light microscopy (DIC) observation concerning individual colonies isolated from olive pollen of the 'Picual' cultivar. Bar: 10 µm.

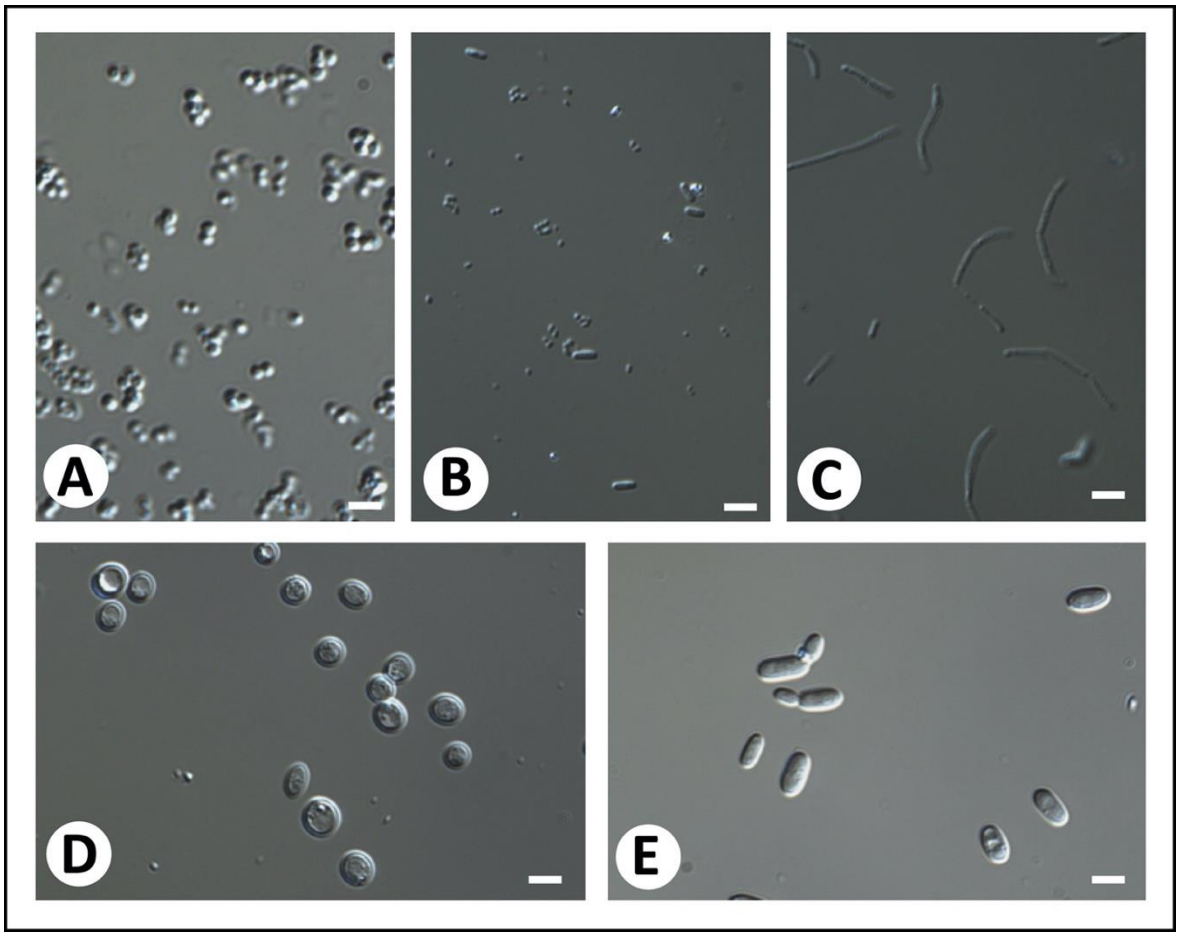

Figure 7. Variability of microorganisms analyzed by light microscopy (DIC) observation concerning individual colonies isolated from olive pollen of the 'Picual' cultivar. Large magnification of several representative colonies. A: coccus; B: coccus+short bacillus; C: long bacillus; D and E: likely two morphologically different types of yeast. Bar: 10 µm.

4. Future Perspectives.

This is a preliminary report, which allowed us to picture the presence of multiple microorganisms on the surface of all the pollen grains we assayed. Due to the short time and number of sessions we managed to perform, the research was shortly developed. First, all experiments should be subjected to biological and technical replications. Also, quantification and statistical analysis of data should be implemented in order to reach relevant conclusions.

The research was planned to include the use of DNA sequencing strategies to precisely identify the microorganisms present on the pollen grains. For this purpose, we designed a number of PCR amplification experiments by using the following well-defined oligonucleotides, amplifying a fragment of c.a. 1400 bp covering the majority of the 16S gene [15] and further bioinformatic analysis:

16f27: AGAGTTTGATCMTGGCTCAG

16r1488: CGGTTACCTTGTTAGGACTTCACC

The project also included an approach to the analysis of the putative ability of the pollen microbiome to modulate the elicitation of symptoms in allergic patients. For this purpose, we proposed challenging of atopic/non-atopic patients blood with: a) whole pollen extracts, and b) crude bacterial isolates from pollen and bacterial cultures extracts under *ex vivo* challenging assays and then the assessment of the expression of induced allergenicity biomarkers (iNOS, histamine, IL-8, IL-10, IL-13…), by using the methods described by several publications of the hosting group (i.e. refence [16]).

Acknowledgements

In spite of the short lifespan of the work because of the sudden interruption of the project by the appearance in our lives of SARS-CoV-2 pandemic, we had the opportunity of tasting a sip of the scientific work carried out in a proper laboratory, the preparation of a scientific report and the use of sophisticated equipment. We would like to thank our institute, the Zaidín Experimental Station and particularly our teachers José Luis Galindo (IES Blas Infante) and Juana Fuentes (IES Cartuja) the opportunity of making science and their support and advice, respectively. We would like to specially thank Dr. Manuel Espinosa (EEZ-CSIC) his assistance and permanent support, particularly in those microbiological aspects which are not primary techniques in the guest laboratory. We apologize for non-canonical protocols followed here, as we all authors have been learning altogether in this less-familiar field for us.

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MY OWN IDEAS

Andrea Rodríguez Muñoz. IES Cartuja.

La oportunidad de poder asistir a la experiencia del proyecto PIIISA fue muy especial, ya que me permitió descubrir un mundo nuevo que todavía no conocía ni sabía que evolucionaba de esa forma tan impresionante. Para la carrera que yo quiero estudiar, farmacia, tiene una parte dedicada a lo que estuvimos experimentando y me resulta curioso haber estado aprendiendo algo que me podría ayudar. Por último, me interesaría mucho a qué nivel han evolucionado hasta ahora y ver los resultados de nuestro gran trabajo, ya que la última vez me llamó mucho la atención la forma en la que se encontraban las colonias y lo mucho que habían avanzado los cultivos.

Isabel María Requena Linares. IES Blas Infante.

"My perceptions and impressions are that I expected working in a group and a lab to be less enjoyable. However, to be honest, it has made me more interested in science because now I can see it is a program with infinite learning possibilities."

Gloria Ehigie Omowa. IES Cartuja.

Bueno en mi perspectiva sobre el proyecto PIIISA de la microbiota del olivo, he aprendido bastante de ello. Además de el tema del proyecto que es bastante interesante, me ha gustado la manera en la que hemos llevado la investigación. El uso del material del laboratorio, como obteníamos muestras, las comparábamos y las estudiábamos en un microscopio electrónico. Además que la dinámica del grupo era muy agradable, así que en conclusión, desearía volver a este tipo de proyecto de nuevo y aunque no lo hayamos terminado me ha aportado muchas nuevas experiencias que atesorar.

Sandra Morales López, IES Cartuja.

En estos días de trabajo en el laboratorio, nos pudieron mostrar como es el trabajo de investigación. Nos demostraron que no todo es fácil y que no todo siempre sale como queremos. El trabajo en grupo fue una de las mejores partes, ya que nos repartimos el trabajo y no era tan duro. Pudimos ver las bacterias que se esconden en el polen de olivo y eso fue sorprendente. Nos permitieron utilizar objetos del laboratorio, siendo la primera vez que estábamos cerca de esos materiales y pudimos ver las dificultades que tiene trabajar con ellos. Otra cosa que fue estupenda fue ver los laboratorios en sí. Ver maquinarias específicas. Gracias a esta experiencia, he podido ver que realmente me gustaría trabajar en investigación en un futuro, aunque quizá no por esa rama de las ciencias. También fue genial ver los cultivos de bacterias que obtuvimos, comparar los resultados obtenidos a partir de cultivos bacterianos del polen natural y congelado del olivo y otras especies vegetales (lirios), ordenar las placas, hacer el recuento de colonias y seleccionar aquellas necesarias y útiles y rechazar las contaminadas, etc.

Miriam Piquero Pardo. IES Cartuja.

Esta experiencia para mi ha sido muy reconfortante, ya que he podido ampliar mis conocimientos sobre la ciencia. Aunque haya sido más corta de lo esperado he aprendido una gran cantidad de cosas. Nuestro proyecto se trataba de la microbiota del olivo, en el hemos recolectado colonias bacterianas y reconocido estas mismas. En mi opinión gracias a estos proyectos podemos aprender de una forma más práctica y divertida. Y la verdad me

gustaría seguir participando en este proyecto aunque en la situación actual que nos encontramos no podemos.

Paula Ruiz. IES Blas Infante.

El proyecto me ha parecido muy interesante y considero que he aprendido mucho. Gracias a él me he interesado más por hacer algo relacionado con la ciencia en el futuro.

High School Students for Agricultural Science Research