Genetic modification of bacteria with fluorescent proteins to evaluate their antagonistic capacity against vascular pathogens in microfluidic chambers simulating xylem bundles

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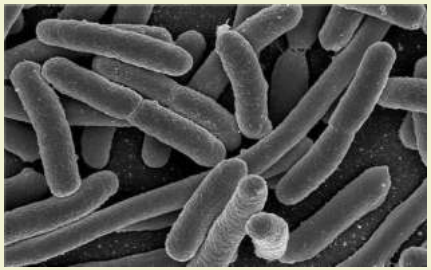
Índex:

1. Introduction:

Xylella fastidiosa:

- Bacteria
- More than 600 species are affected
- Transmitted by insects
- Olive quick decline syndrome, citrus variegated chlorosis, Pierce's disease in grapevines...



Verticillium dahliae:

- Fungus
- More than 300 species are affected
- In the soil
- Verticillium wilt

1. Introduction:

Both pathogens:

- Confined growth in their xylem: genetic and pathogenic variability in their population.
- They can travel great lengths with infected plant material.
- No current control measures against them. Early detection methods and the evolution of new biocontrol methods are very important and necessary.

1. Introduction:

- First hypothesis:

  Genetic modification by bacterial conjugation of bacteria from the olive tree sap and introduce reporter genes encoding for fluorescent proteins into them.

- Second hypothesis:

  Genetic modification will allow the visualization of mixtures of the different colored bacteria using a microfluidic chamber (simulates vascular bundles of a plant's xylem) to monitor their growth and their potential microbial antagonism.

2. Objectives:

1. Inoculation in culture medium of *E. coli* transformed with plasmids

1. Bacterial conjugation with bacteria isolated from olive sap and *E. coli* strains carrying plasmids (PBBR, RK2 y Tn7), tagged with fluorescent protein reporter genes (GFP-green, mScarlet-red ,sYFP-yellow) and antibiotic resistance (tetracycline)

1. PCR verification of the transconjugants and the correct insertion of the plasmids

1. Assembly of a microfluidic chamber, visualization of transformed bacteria with different colors in a chamber

3. Theoretical foundations:

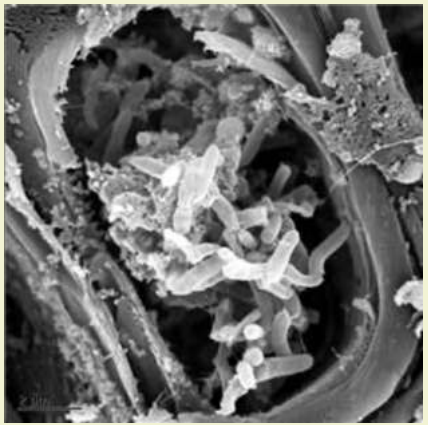
- Quarantine plagues (dangerous plagues):

  Plagues from protected areas are quarantined plagues. Only certain areas of the territory will be protected.

- Quality plagues (doesn't require eradication):

  The new rules include measures to deal with pests from non-EU countries. They also propose to extend, simplify and harmonize the existing plant passport regime. These will be necessary for transfers between professional operators, but not for sales to non-professional users.

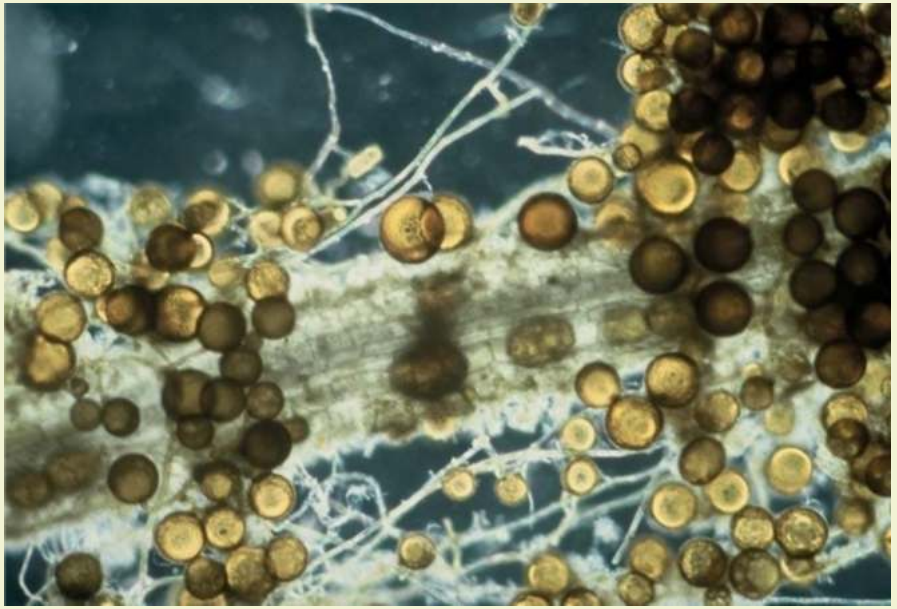
U.C. Berkeley
Vascular bundles of plant xylem



3. Theoretical foundations:

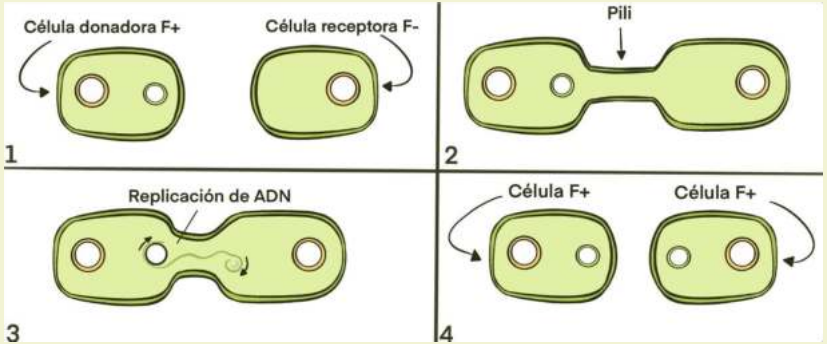
Healthy plants live in association and interact with a variety of microorganisms (plant microbiome).

Some components of the plant-associated microbiome can be harnessed as biocontrol agents that provide protection against plant pathogens.



3. Theoretical foundations:

- Bacterial conjugation:



The cell presents the F plasmid, which contains the genetic information to form pili. The cell presenting the plasmid is called F+; the cell that does not contain it is called F-.

F- → recipient

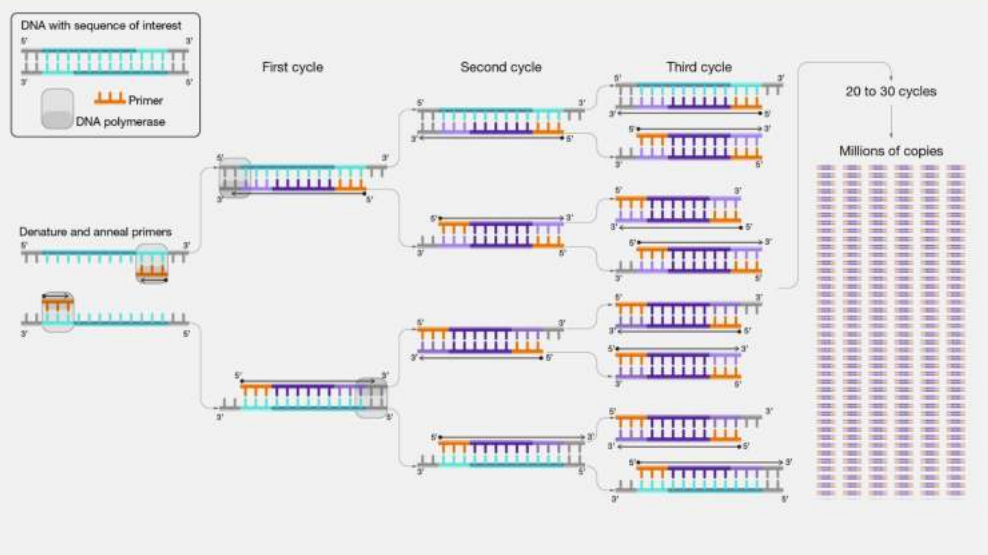
F+ → information donor

- Microorganism cultures:

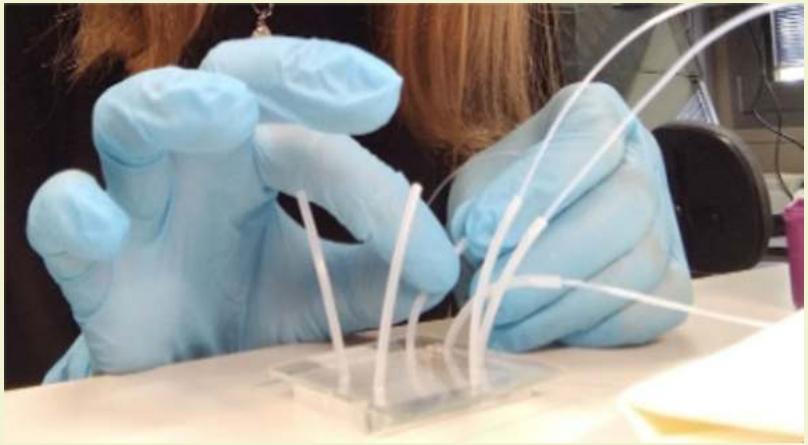
In our research we have used the depletion technique for cultivation.

3. Theoretical foundations:

- PCR: it’s a fast and very accurate way to diagnose certain infectious diseases and genetic changes.



- Microfluidic chambers: they simulate ecological niches at micrometer scales creating a controllable microenvironment.



4. Materials and methods:

- Microorganisms that were used:

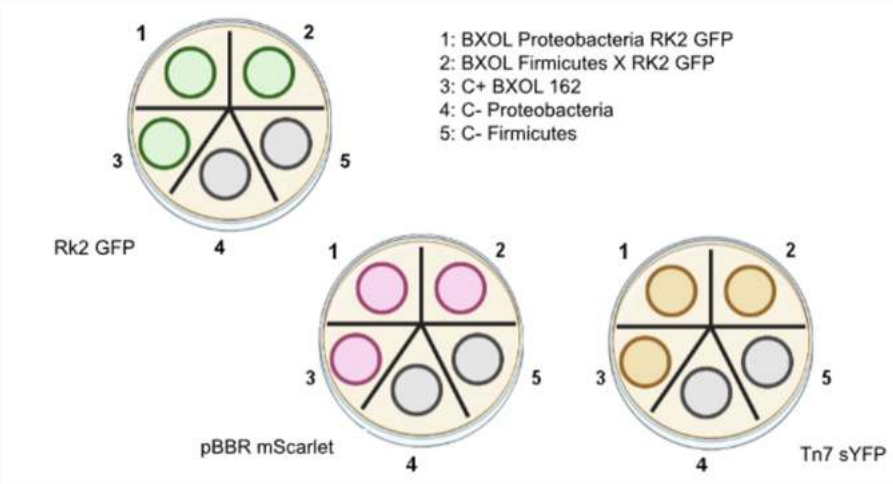
BXOL	Genotype	Niche	Species	Phylum
5	Picual	Sap	*Variovorax*	Proteobacteria
125	Wild olive tree	Sap	*Methylobacterium*	Proteobacteria
162	Picual	Stem	*Variovorax*	Proteobacteria
182	Arbequina	Stem	*Bacillus*	Firmicutes
200	Wild olive tree	Stem	*Curtobacterium*	Actinobacteria
215	Wild olive tree	Stem	*Bacillus*	Firmicutes
225	Wild olive tree	Stem	*Microbacterium*	Actinobacteria
XOL25		Sap	*Pseudomonas*	Proteobacteria
XOL31		Sap	*Methylobacterium*	Proteobacteria
XOL35		Sap	*Bacillus*	Firmicutes
XOL43		Sap	*Methylobacterium*	Proteobacteria

Plasmids	Characteristics
pBBr	It's not inserted into the genome, it produces a high number of copies and it can be toxic.
RK2	It's not inserted into the genome, it produces a low number of copies and it's more stable.
Tn7	*Region att* is well conserved in almost all bacteria, is more stable and requires a transposase.

4. Materials and methods:

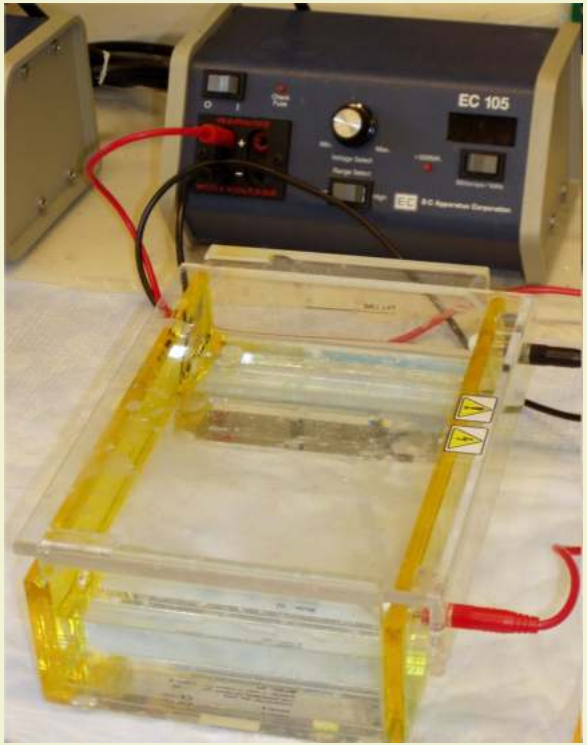
- Bacterial conjugation:

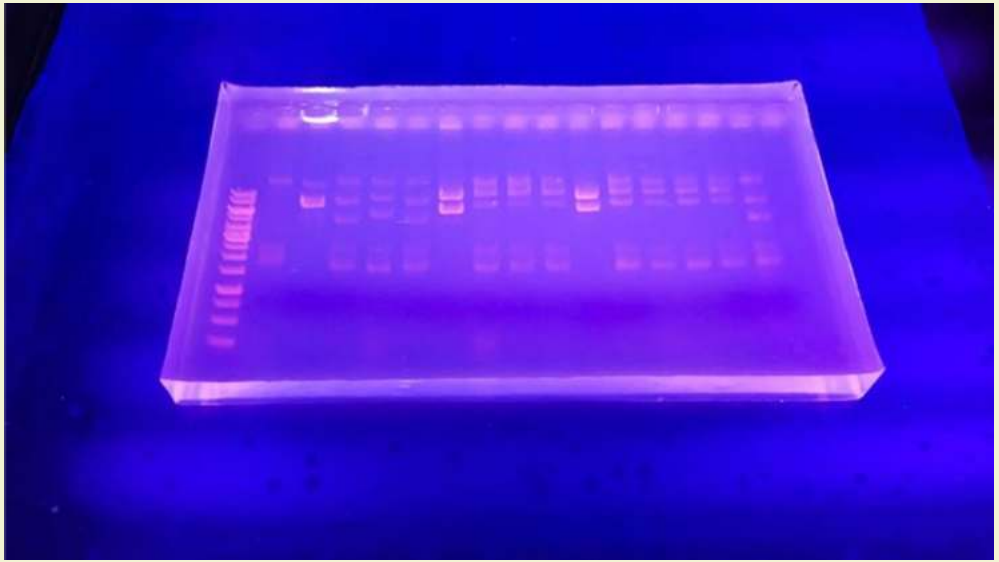
	pBBR mScarlet	RK2 GFP	Tn7 sYFP	pTNS3	BXOL
pBBR mScarlet	300uL				400uL
RK2 GFP		300uL			400uL
Tn7 sYFP			300uL	300uL	400 uL



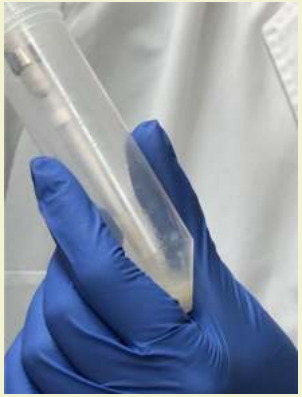
4. Materials and methods:

- Electrophoresis: A technique used in laboratories to separate DNA, RNA or proteins according to their size and electrical charge.





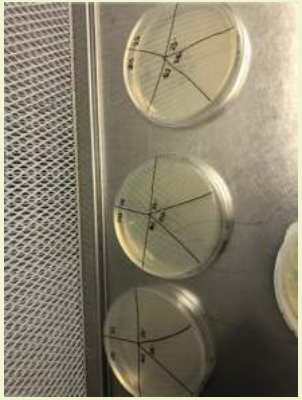
4. Materials and methods:



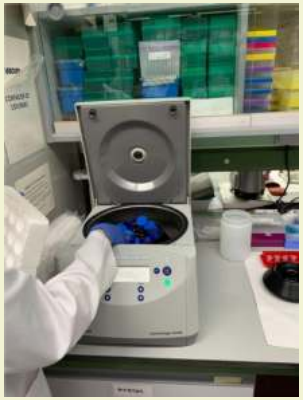
Falcon tube

Inoculation loop

Eppendorf

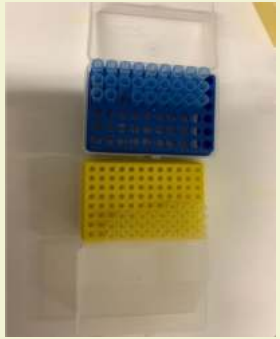


Petri dish



Centrifuge

Transilluminator and special glasses



Pipette tips

Aluminum foil

Parafilm

Pipettes

Lab coats

4. Materials and methods:

Gloves

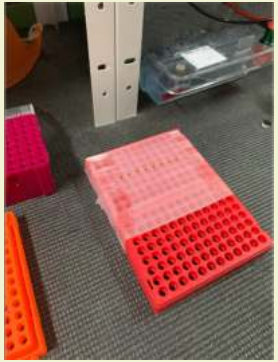
Beaker

Erlenmeyer flask

Fume cupboard

Agitator

Laboratory scale and spatula



Racks

PCR machine

Nanodrop

4. Materials and methods:

- Session 1: Presentation and theoretical concepts. Visit to the level 2 containment laboratory. Inoculation of the culture medium of *E.coli*. Extraction, quantification and quality check of plasmid DNA.

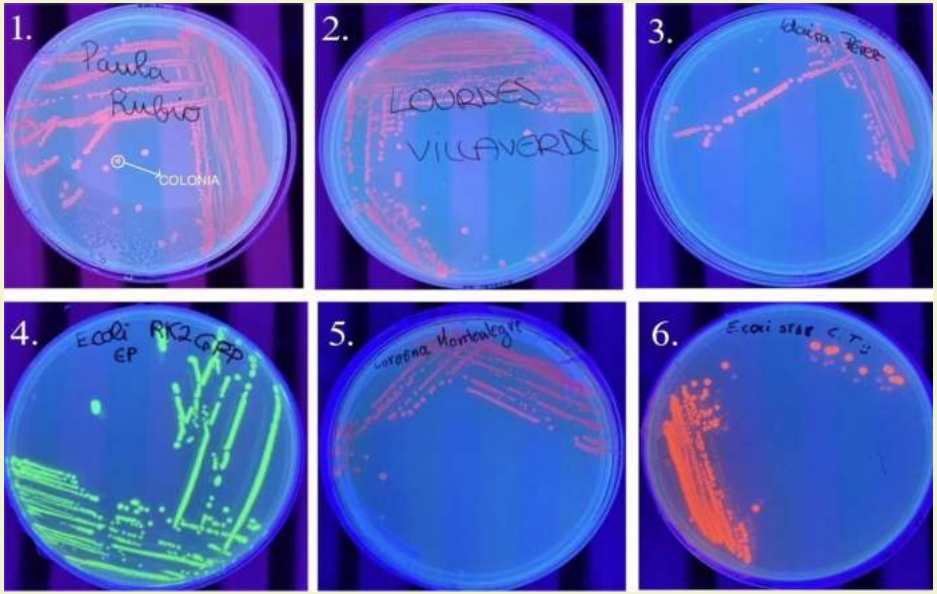
- Session 2: Bacterial conjugation process with bacteria from olive tree sap and E.coli strains with plasmids of interest.

- Session 3: Visualization of the results of the conjugation and the performance of a PCR to verify the presence of both the trancojugated and the plasmids.

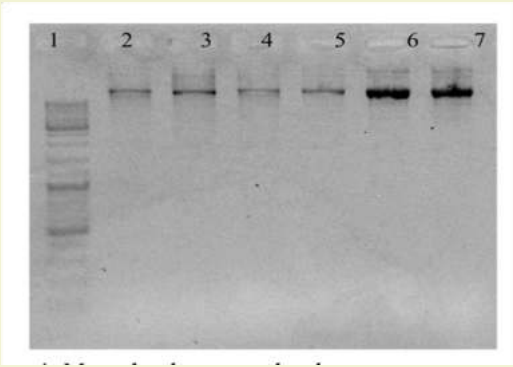
- Session 4: Theory on microfluidic chambers, microfluidic chip assembly and the visualization of the different colored bacteria under a confocal microscope.

- Online sessions: Working together on the documents that had been shared previously. They were shared through google drive therefore everybody could work together after following the teachers and investigators instructions.

5. Results:



Petri dishes with *E.coli* genetically modified with fluorescent proteins and seeded by depletion.

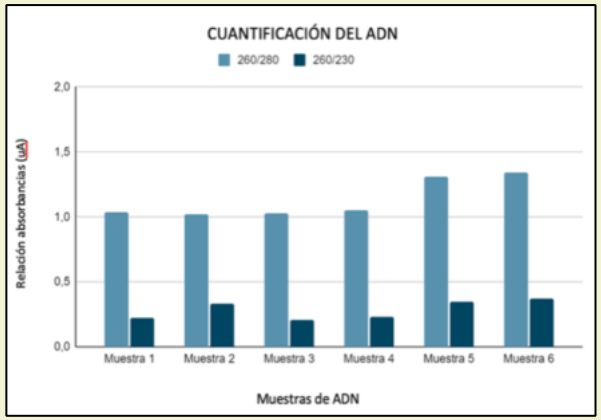


Results of agarose gel electrophoresis of plasmid DNA extracted with a commercial kit from a culture medium of *E. coli* transformed with plasmids of interest.

5. Results:

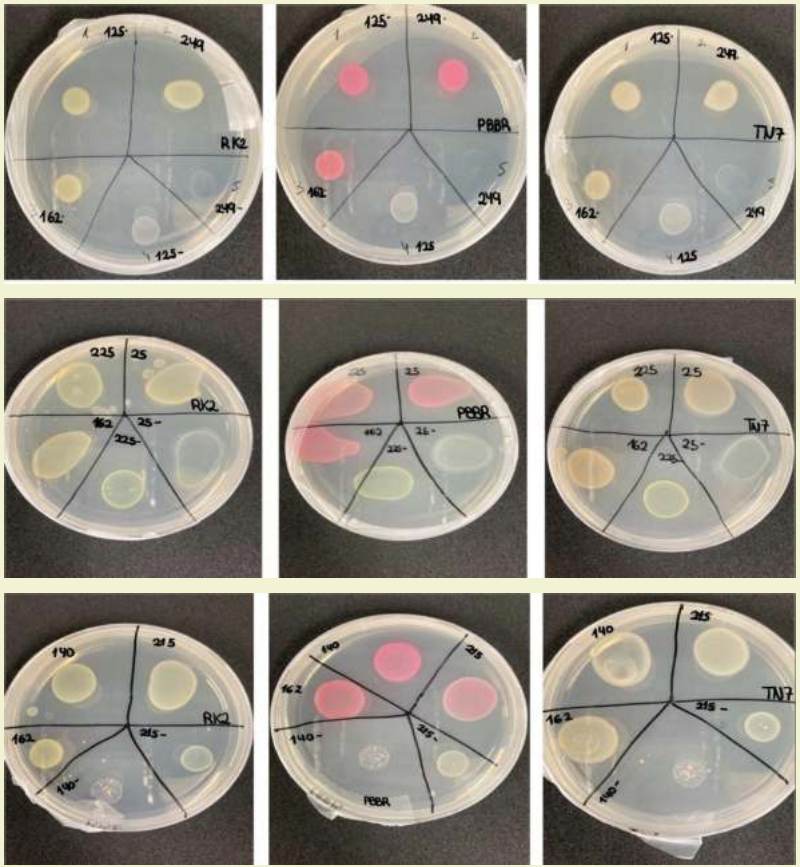
Sample ID	ng/µl	260/280	260/230
Muestra 1	68,89	1,04	0,22
Muestra 2	119,28	1,022	0,33
Muestra 3	66,57	1,03	0,21
Muestra 4	68,24	1,05	0,23
Muestra 5	85,24	1,31	0,35
Muestra 6	86,41	1,34	0,37

Nanodrop quantification of plasmid DNA extracted from a culture medium of *E.coli* transformed with plasmids of interest



Nanodrop quantification of plasmid DNA extracted from a culture medium of *E.coli* transformed with plasmids of interest.

5. Results:

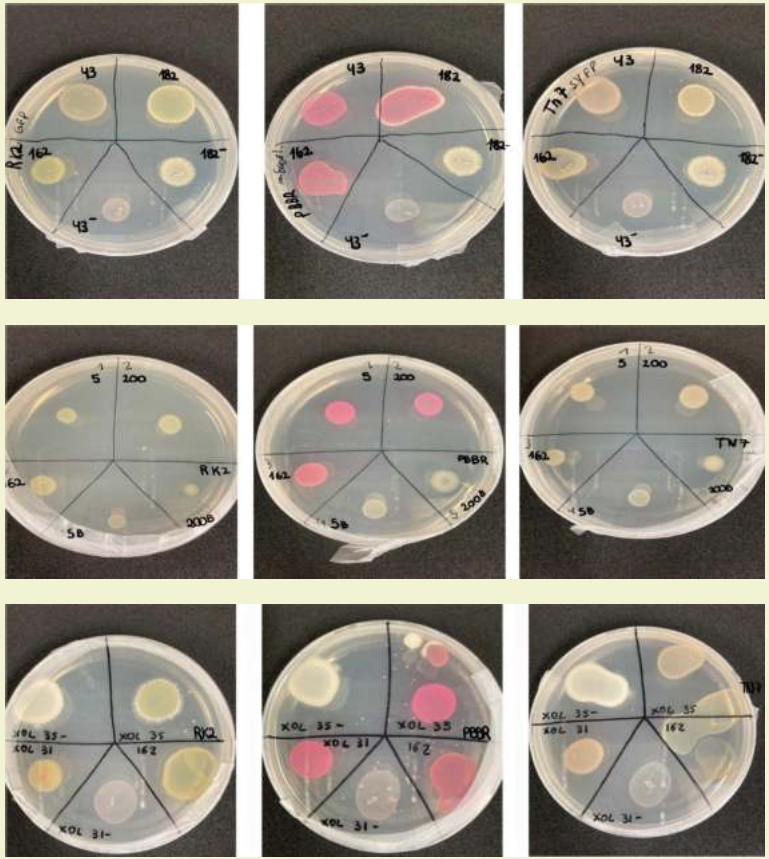


Petri dishes with **Methylobacterium (125) and Bacillus (249)** conjugated with three different plasmids (RK2-GFP, pBBR-mScarlet and Tn7-sYFP), and also **unconjugated as a negative control**. In addition, **Variovorax (162)** as a control was also conjugated with all plasmids.

Microbacterium (225) and Bacillus (249)

Methylobacterium (BXOL140) and Bacillus (215)

5. Results:



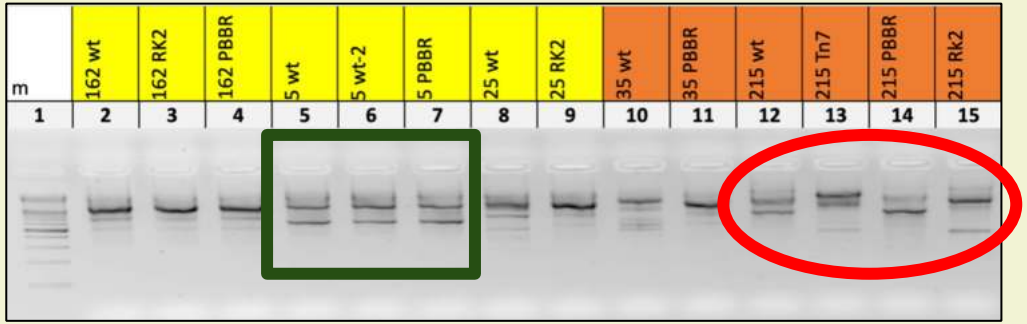
Mecthylobacterium (43) and Bacillus (182)

Variovorax (5) and Curtobacterium (200)

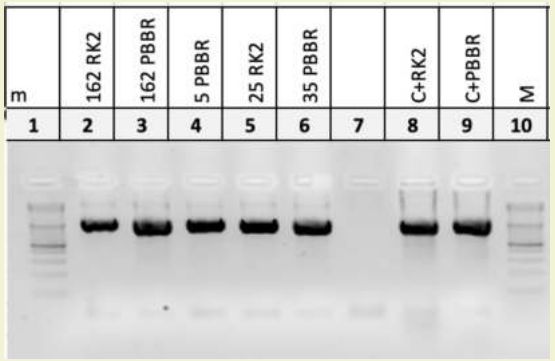
Bacillus (35) and Methylobacterium (31)

5. Results:

Depletion passages with fluorescent bacteria after conjugation.



PCR results. Yellow color, confirmed transconjugated bacteria to be the same as the wild type strain



PCR results to detect plasmid insertion

6. Conclusions:

1. *E.coli* is an easy-to-transform bacterium, which can be used in conjugation.

1. The bacteria developed and grew well and acquired the fluorescent plasmids conjugated by the *E.coli* bacteria.

1. The valid transconjugants were BXOL 162 corresponding to the genus *Variovorax,* used as a control for conjugation with the RK2 and PBBR plasmids, also BXOL 5 (genus *Variovorax*) conjugated with the RK2 and PBBR plasmids and finally BXOL 25 (*Pseudomonas*) conjugated with the RK2 plasmid.

1. The bacteria BXOL35 from the genus *Firmicutes* with the plasmid PBBR and BXOL215 also from the genus Firmicutes with the plasmids Tn7, RK2 and PBBR are not considered as valid transconjugants because the genetic profile of the BOX PCR is not the same as the wild-type genotype.

8. Acknowledgements:

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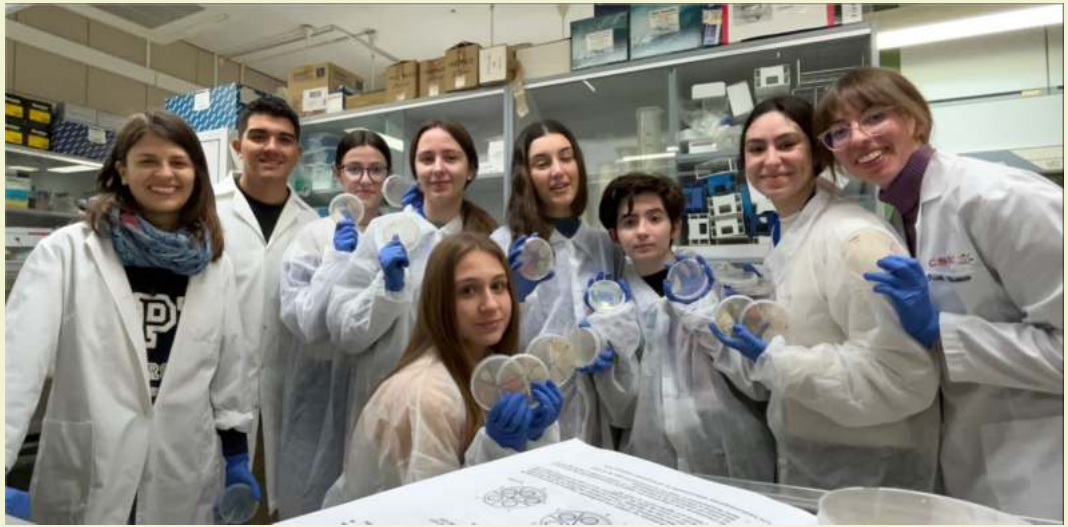
Thank you so much everyone.



9. Bibliography

·Velasco Amo, P.M (2022): Recent research accomplished on early detection of *Xylella fastidiosa* outbreaks in the Mediterranean Basin. ResearchGate. (link)

·Portillo G. (2017). Verticillium. JardineriaOn. (link)

·Consejo Europeo de la Unión Europea. Fitosanidad. (link)

·Olga Martín, Bernardo González, María Josefina Poupin. From Microbial Dynamics to Functionality in the Rhizosphere: A Systematic Review of the Opportunities With Synthetic Microbial Communities. Frontiers. (link)

·Pruebas de PCR. MedlinePlus. (Link)

·Mike Smith (2023). ELECTROPHORESIS. National human genome research institute. (Link)

·Leonardo De La Fuente, Emilie Montanes, Yizhi Meng, Yaxin Li, Thomas J. Burr, H. C. Hoch, Mingming Wu. Assessing Adhesion Forces of Type I and Type IV Pili of *Xylella fastidiosa* Bacteria by Use of a Microfluidic Flow Chamber. ASM Journal. (link)

·Velasco Amo, P.M. ¿Científica o paparazzi? elDiario. (link)

·Luis Flores. Laboratorio de Microfluidos y BioMEMS. UTEC. (link)
·Sesión 3, fallos en la conjugación bacteriana (link)

THANK YOU FOR YOUR ATTENTION!