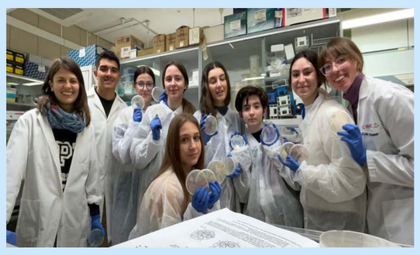
GENETIC MODIFICATION OF BACTERIA WITH FLUORESCENT PROTEINS TO ASSESS THEIR ANTAGONISTIC ABILITY AGAINST VASCULAR PATHOGENS IN MICROFLUIDIC CHAMBERS SIMULATING XYLEM BUNDLES



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INTRODUCTION

Xylella fastidiosa (Xf) is a gram-negative bacterium that causes disease in a wide range of host plants and is therefore a serious plant pathogen in terms of socio-economic impact.

On the other hand, *Verticillium dahliae* (Vd) is a soil-dwelling fungus causing a vascular disease known as verticillosis. Vd that can remain in the soil for up to 15 years.

Both pathogens Xf and Vd have characteristics in common such as no chemical (xylem/systemic) control measures, therefore early detection measures, the use of resistant or tolerant varieties or the development of new biocontrol tools are necessary. Our first hypothesis is that genetic modification can be carried out by bacterial conjugation of bacteria present in olive sap that have been selected as potential biological control agents of vascular pathogens, and introduce reporter genes coding for fluorescent proteins into them.

OBJETIVES

- Genetic modification of bacteria by conjugation using three types of plasmids previously inserted into E. coli (PBBR, RK2 and Tn7), tagged with fluorescent protein reporter genes (GFP-green, mScarlet-red, sYFP-yellow) and antibiotic resistance (tetracycline).
- Purification of the transconjugants and verification by molecular techniques of the correct insertion of the plasmid into the bacterial transconjugants.
- Fluorescence microscopy visualisation of transformed bacteria in microfluidic chambers to observe processes associated with colonisation (adhesion strength, motility, etc.) and competition, inhibition between two bacteria labelled with different colours.

MÉTHODS

1. FIRST SESSION

Presentation and theory on vascular pathogens: Verticillium dahliae and Xylella fastidiosa. Visit to the level 2 biological containment laboratory for Xylella fastidiosa. Inoculation of the culture medium of E.coli transformed with plasmids of interest in liquid medium. Extraction of plasmid DNA with commercial kit, DNA quantification and DNA quality check by agarose gel electrophoresis and nanodrop.

2. SECOND SESSION

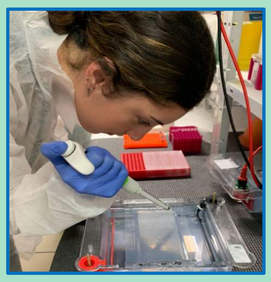
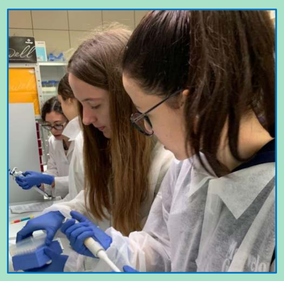
We performed bacterial conjugation with bacteria isolated from olive sap (BXOL: Bacteria from Olive Xylem) and E. coli strains carrying plasmids of interest.

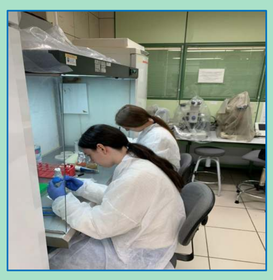
3. THIRD SESSION

Visualisation of the results of conjugation and performance of the passages for the purification of the transconjugant (transformed) bacteria. Carrying out PCR to verify the transconjugant for verification of the presence of the plasmids.

4. FOURTH SESSION

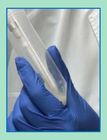
Theory on microfluidic chambers. We were taught how to set up a microfluidic chamber, and visualisation of bacteria transformed with different colours under a confocal microscope

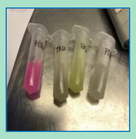



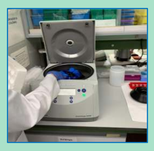
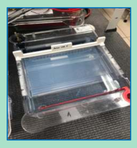
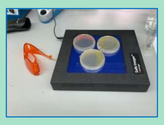
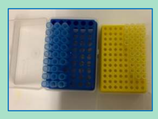


MATERIALS

- Falcon
- Planting handles
- Eppendorf
- Petri dishes
- Centrifugue
- Acarose gel
- Transilluminator
- Pipettes
- Pipette tips
- Nanodrop
- Aluminum foil
- Glass jars
- Microfluidic chamber
- Parafilm
- Lab Coats
- Plastic gloves
- Baker
- Elenmeyer
- Marker pen
- Fume cupboards
- Agitator
- Weighing scales
- Rack







CONCLUSIONS

1. E.coli, is an easy to transform bacterium, which can be used in conjugation.
2. The bacteria had a correct development and growth and acquired the fluorescent plasmids, conjugated by the E.coli bacteria.
3. The valid transconjugants were BXOL 162, which corresponds to the genus Variovorax, used as a control for conjugation with plasmids RK2 and PBBR, also BXOL 5 (genus Variovorax) conjugated with plasmids RK2 and PBBR, and finally conjugated BXOL 25 (Pseudomonas) with the RK2 plasmid.
4. The BXOL35 bacteria of the genus Firmicutes with the PBBR plasmid and the BXOL215 also of the genus Firmicutes with the plasmids Tn7, RK2 and PBBR are not considered valid transconjugants because the genetic profile of the BOX PCR is not the same as the wild-type genotype..

RESULTS

E.coli was grown on culture medium using the depletion technique and incubated in a culture chamber at an optimal temperature for growth to develop.

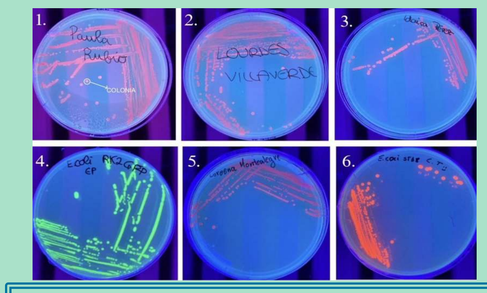
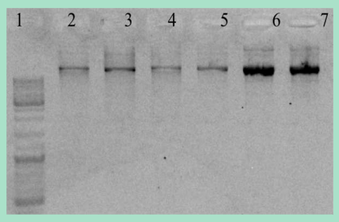


Figure 1. E.coli *genetically modified with fluorescent proteins and seeded by depletion.*



The extracted DNA was of good quality as defined bands were observed in the different samples and at an adequate concentration.

Figure 2. Results of agarose gel electrophoresis of plasmid DNA extracted with a commercial kit from E.coli culture medium transformed with plasmids of interest.
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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

Table 1. *Collection of BXOL bacteria (Bacteria of the Olive Xylem) used for the conjugation experiment.*

SAMPLES	ng/µl	260/280	260/230
Sample 1	68,89	1,04	0,22
Sample 2	119,28	1,022	0,33
Sample3	66,57	1,03	0,21
Sample 4	68,24	1,05	0,23
Sample 5	85,24	1,31	0,35
Sample 6	86,41	1,34	0,37

Table 2. *Nanodrop quantification of plasmid DNA extracted from* E.coli *culture medium transformed with plasmids of interest.*

Once the *E. coli* bacteria were inoculated and cultured with the fluorescent plasmids and the BXOL bacteria for conjugation, they were seeded in petri dishes and the plates with the conjugation droplets were obtained.

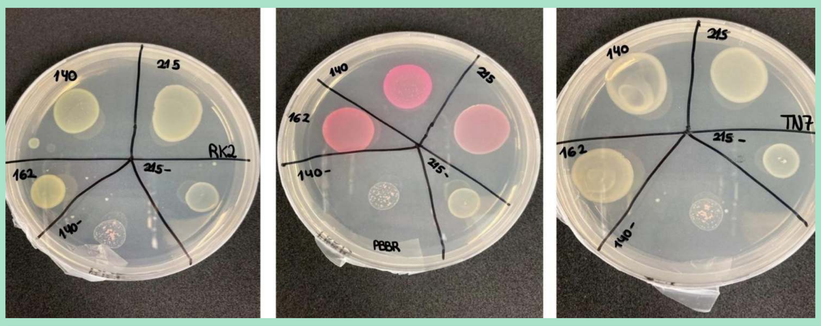

Figure 3. *Petri dishes with* Methylobacterium *(BXOL140) and* Bacillus *(215) conjugated with three different plasmids (RK2-GFP, pBBR-mScarlet and Tn7-sYFP), and also unconjugated as negative control. In addition,* Varioborax *(162) as a control was also conjugated with all plasmids.*

Transconjugants that fluoresced and grew on tetracycline medium (bacteria 162,5,25,35 and 215) (Figure 5) were analysed by PCR to determine whether the transconjugant that was growing was the same as the one that started the experiment or whether further purification steps were required, so BOX PCR was performed.

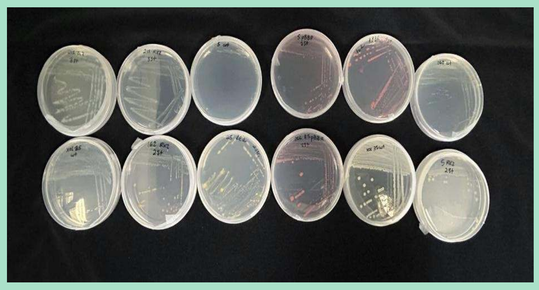

Figure 5. *Depletion passages with fluorescent bacteria after conjugation*

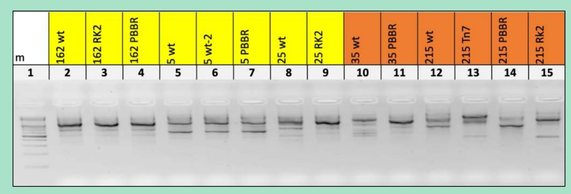

Figure 4. *PCR results. Yellow colour, confirmed transconjugated bacteria that are the same as the wild-type strain.*

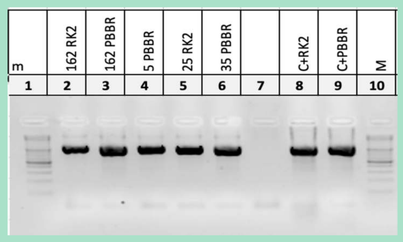

Figure 6. *Results of PCR plasmid insertion*

In the gel, Figure 4, it can be seen that the profiles of **transconjugants 162, 5 and 25** (in yellow) correspond to the wild-type genotype. However, transconjugants 35 and 215 (in orange) do not have the same profiles as the wild type.

To check the correct insertion of the plasmid, a second PCR was performed (Figure 6).

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