CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
Institute for Sustainable Agriculture
Córdoba (Spain)
IAS

BIOLOGICAL CONTROL AGENTS AGAINST SUNFLOWER PATHOGENS

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Junta de Andalucía
GOBIERNO DE ESPAÑA MINISTERIO DE CIENCIA E INNOVACIÓN

Outline

1. Current situation of the biological control of sunflower diseases.
2. Biological control by means of entomopathogenic fungi.
3. Biological control by means of soil inhabiting bacteria.
4. Conclusions.

1 Current situation of the biological control of sunflower diseases

Management of plant diseases

Agronomical, physical and cultural methods	Chemical control	Genetic resistance	Biological control
			

Biological control

The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be.

Biological control is one of the control strategies used in **Integrated Pest Management** or IPM (Directive 2009/128/EC, art. 12).

Major **uses** of biological control agents:

- Biological control of invertebrate pests using predators, parasitoids and pathogens.
- Biological control of weeds using herbivores and pathogens.
- Biological control of plant pathogens using antagonistic micro-organisms and induced plant resistance.

Eilenberg et al. 2001. BioControl 46: 387–400.

1 Current situation of the biological control of sunflower diseases

Microorganisms from the sunflower rhizosphere (*Pseudomonas* spp., *Xanthomonas* spp., *Agrobacterium* spp., *Bacillus* spp., *Azospirillum* spp. among others) have deserved attention because of their putative antagonistic effect and for being, at the same time, good colonizers of plant roots.

Some ***Pseudomonas* and *Bacillus*** were efficient at controlling the sunflower pathogens *Macrophomina phaseolina* and *Sclerotium rolfsii*. *Pseudomonas putida* showed reduction of sunflower downy mildew incidence in the greenhouse but it was no further assayed.

Some **plant extracts** have shown an *in vitro* detrimental effect against some soil-borne fungi affecting sunflower. This is the case of *Lycium europaeum* extracts, having chlorogenic acid as a major compound. They inhibited the micelial growth of *V. dahliae* and *S. sclerotiorum* as well as conidia and sclerotia formation.
Tej et al. 2018. Eur. J. Plant Pathol. https://doi.org/10.1007/s10658-018-1469-9

On dual culture, ***Trichoderma harzianum*** inhibited mycelial growth of the seed-borne pathogens of sunflower *Alternaria alternata*, *Bipolaris cynodontis*, *Fusarium culmorum* and *F. oxysporum*. Conidial germination of *F. culmorum* was also reduced.
Guclu and Ozer 2022. Lett. Appl. Bio. DOI: 10.1111/lam.13698

New approaches and options can still be explored

Outline

1 Current situation of the biological control of sunflower diseases.

2 Biological control by means of entomopathogenic fungi.

3 Biological control by means of soil inhabiting bacteria.

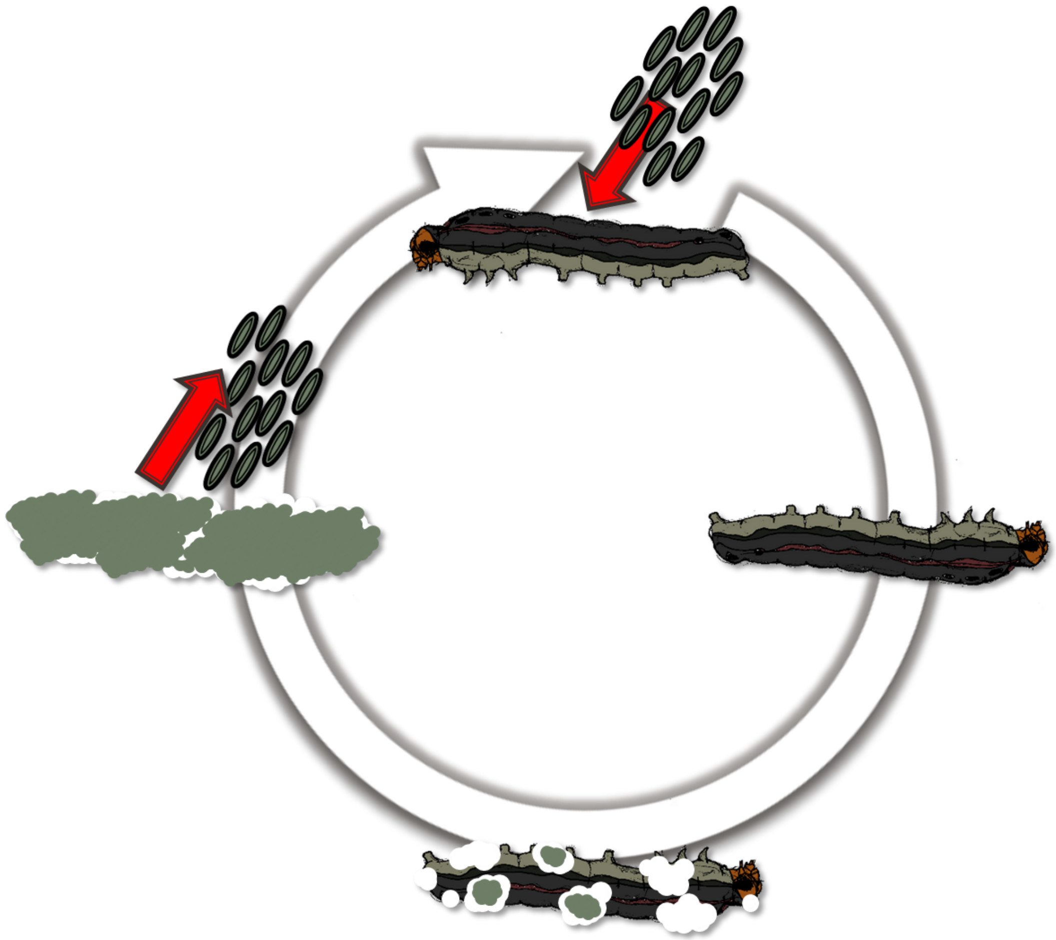
4 Conclusions.

Fungal entomopathogens

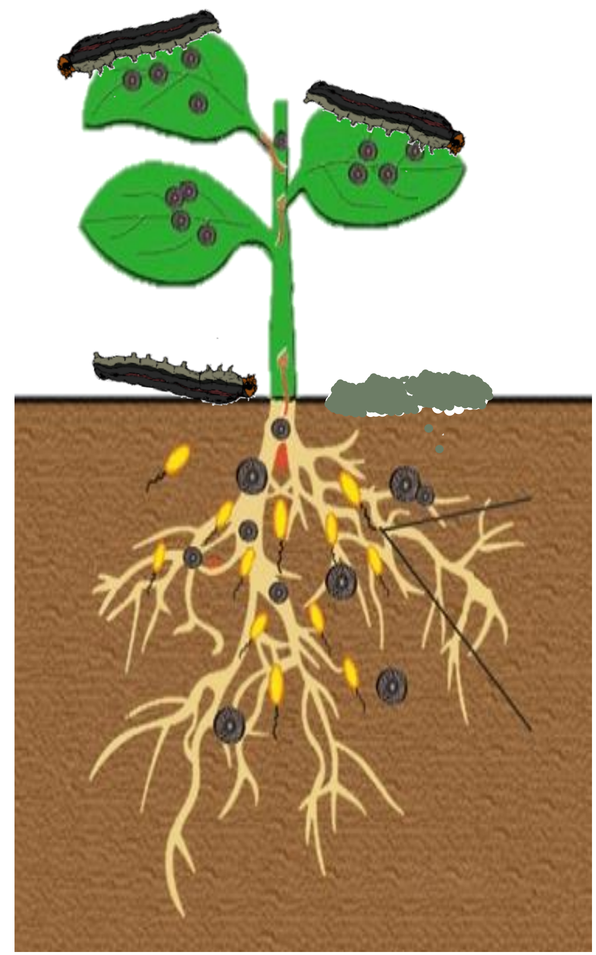
- Natural enemies of phytophagous insects. High efficacy.
- They are present in numerous environments.
- Very active commercial development.

Mode of action of fungal entomopathogens

By contact



Endophytically



Verticillium wilt of sunflower (*Verticillium dahliae* Kleb.)

- Soil-borne fungus with more than 2000 host plant species.
- Microsclerotia (> 10 years).
- Interveinal chlorosis and yellowing of leaves, progressing upwards.
- Stem lesions.
- No active ingredients registered in the European Union.
- Control by means of genetic resistance.

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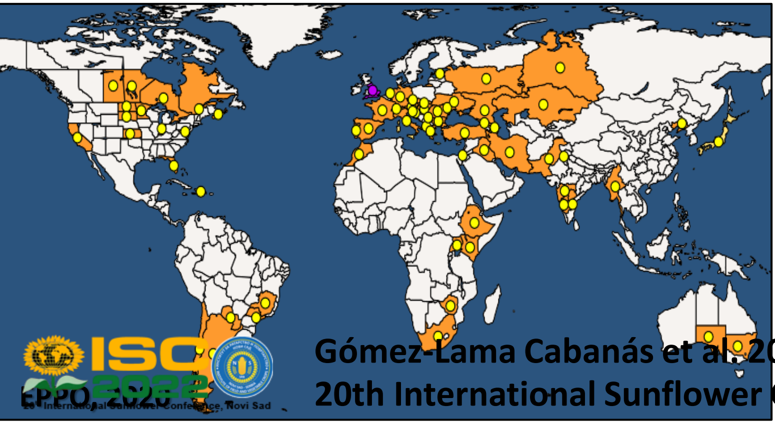
Sunflower downy mildew (*Plasmopara halstedii* Farl. Berl. and de Toni)

- One of the most widespread and important diseases worldwide.
- Obligate parasite with two types of infections:

Primary infections: resting oospores, affects seeds and seedlings (vertical transmision).

Secondary infections: zoospores infecting leaf tissues, low economic impact.

- Pre- and post-emergence damping-off, chlorosis bordering the main veins progressing downwards, stunting.
- Genetic resistance (more than 40 pathotypes) and chemical control (phenylamides, strobilurins, resistance-inducing chemicals, oxathiapiprolin).



Objectives

To determine whether strains of two different EF species *(Metarhizium brunneum* and *Beauveria bassiana*), could be suitable candidates as biological control agents against *V. dahliae.*

We carried out both *in vitro* experiments, in which the EF were dually plated against *V. dahliae*, and *in planta* experiments, in which we monitored the effect of the EF on the severity of Verticillium wilt symptoms in sunflowers in greenhouse. We also assessed the time lapse of EF in the substrate and performed a molecular detection of the fungi in the plants.

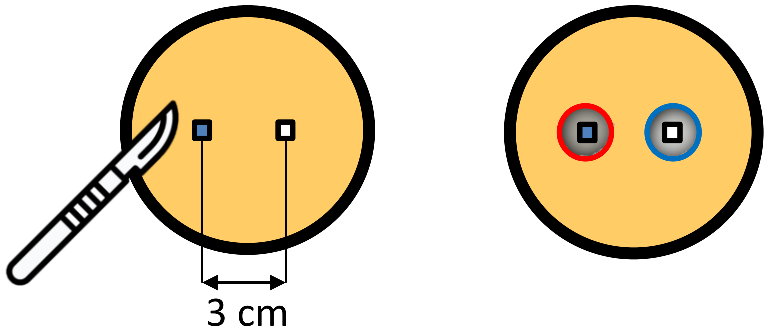
To determine the effectiveness of EF species (*Metarhizium brunneum* and *Beauveria bassiana*) as a method for the management of sunflower downy mildew.

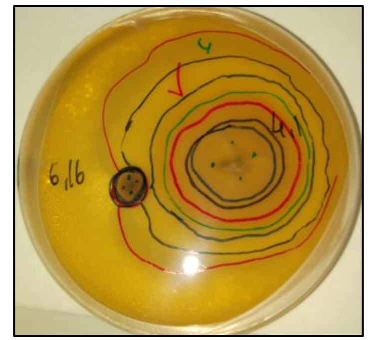
We assessed the effect of EF on downy mildew as well as on the growth of sunflowers in axenic culture. We determined whether, or not, EF and *P. halstedii* simultaneously colonize inner tissues of sunflower.

2 Biological control by means of entomopathogenic fungi: *V. dahliae*

- Entomopathogenic fungi: three strains of *M. brunneum* and two strains of *B. bassiana*.
- Six isolates of *V. dahliae*.

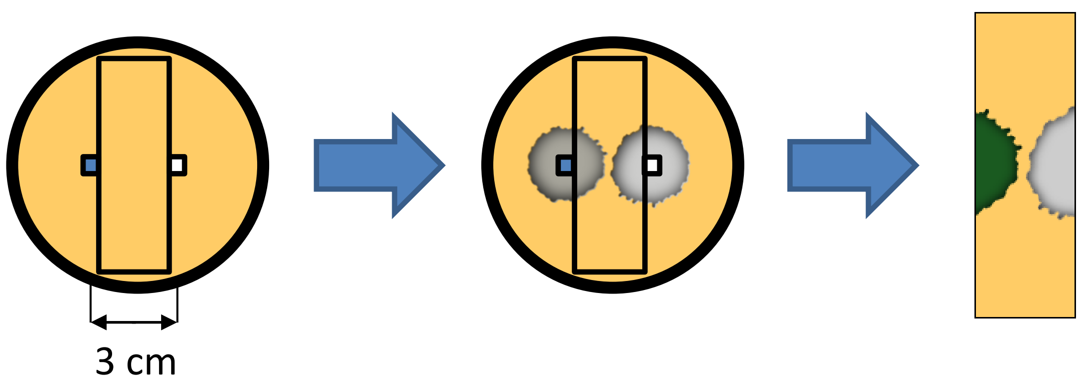
Dual cultures





$$\text{Inhibition of Micelial Growth (\%)} = 100 \times \frac{\text{Diam. colony Control} - \text{Diam. colony Treatment}}{\text{Diam. colony Control}}$$

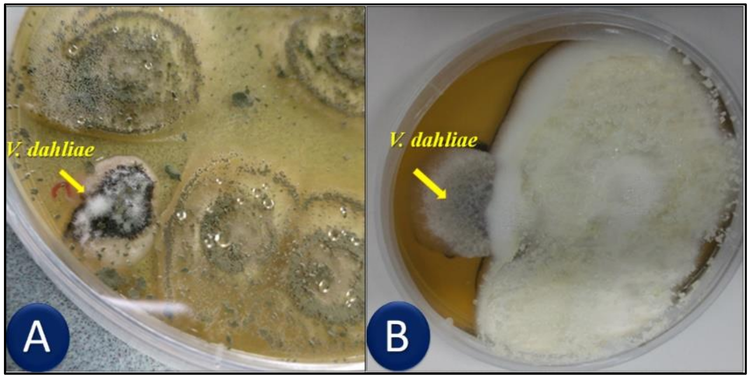
Slides under the microscope



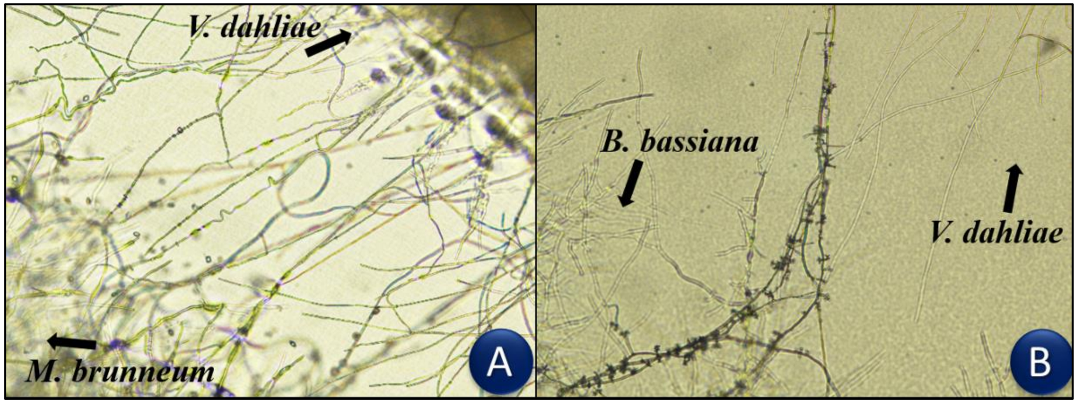
Results

IMG: 8.3 – 63.5% (EF and *V. dahliae* isolate)

Most efficient (EABb 01/33-Su) and less efficient (EAMb 01/158-Su)



Antagonism: **micelial overgrowth** and **antibiosis**.



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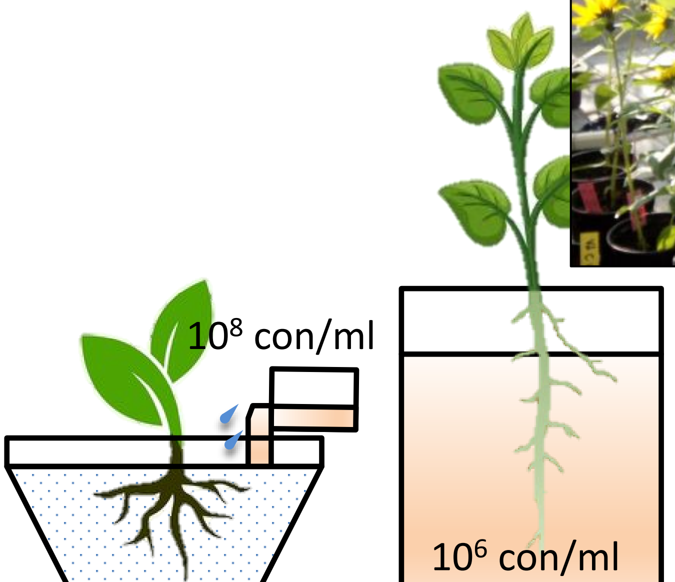
20th International Sunflower Conference, Novi Sad, Serbia, June 20 – 23, 2022

Experiment under greenhouse conditions

- Entomopathogenic fungi: two strains of *M. brunneum* and two strains of *B. bassiana*.
- One isolate of *V. dahliae* (VdS0113) and one susceptible genotype (RHA821)



EF, 4 DAS

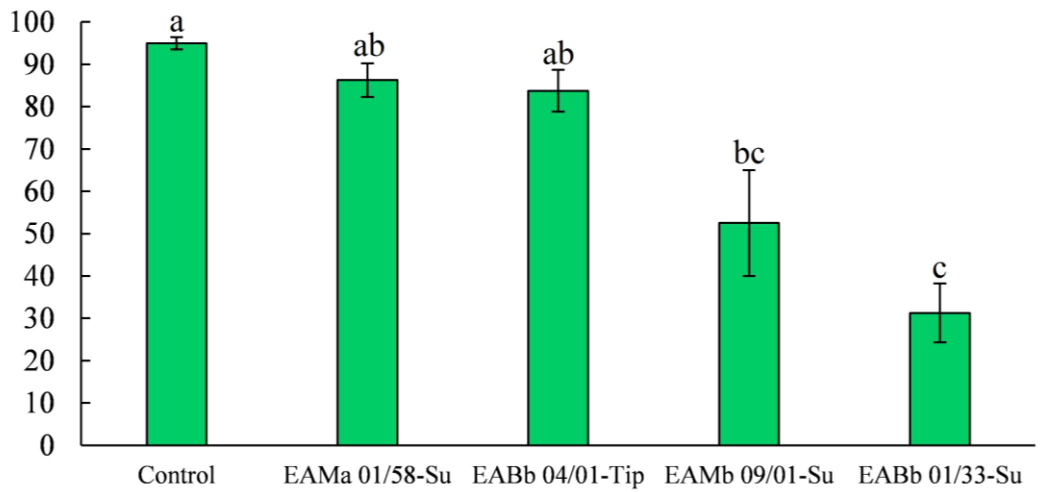
Vd, 34 DAS

Greenhouse, 34 – 64 DAS

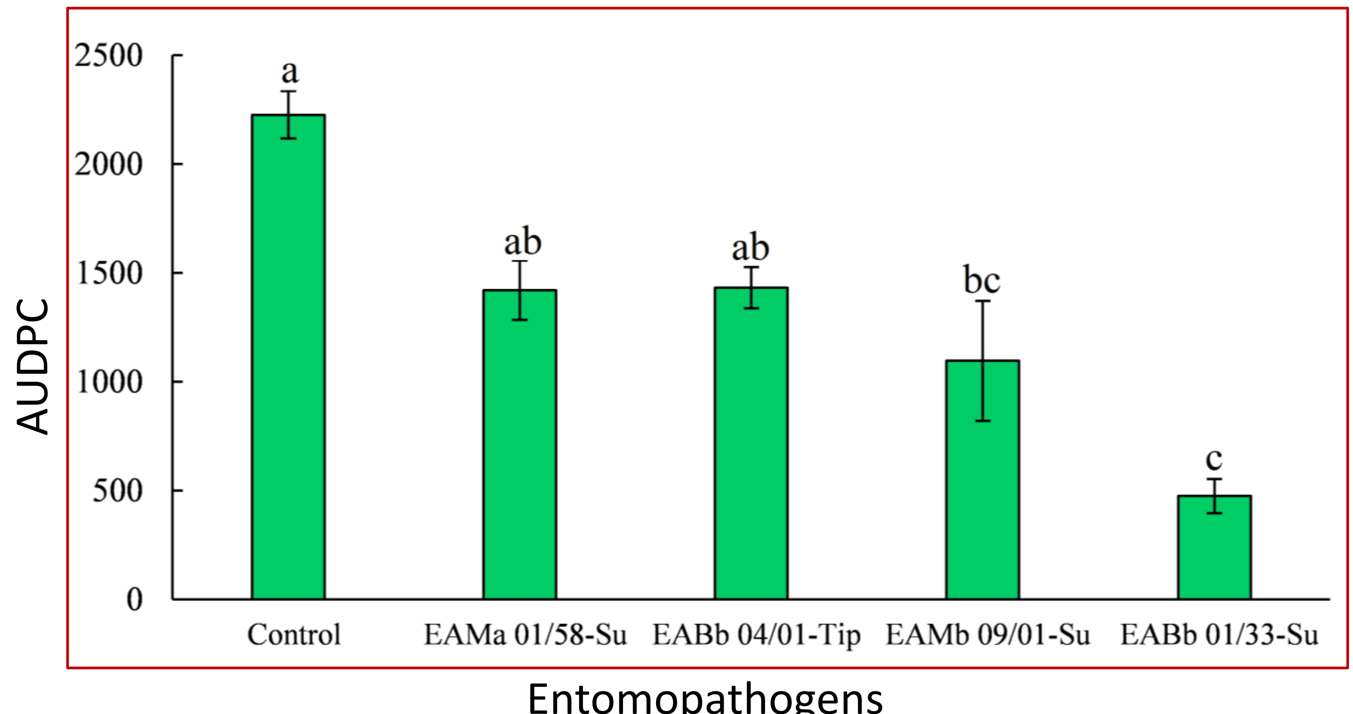
- Weekly disease severity and AUDPC.
- Weekly assessment of EF populations in the soil.

2

Symptoms at the end of the experiment



Two strains of EF significantly reduced symptoms of verticillium in sunflowers.



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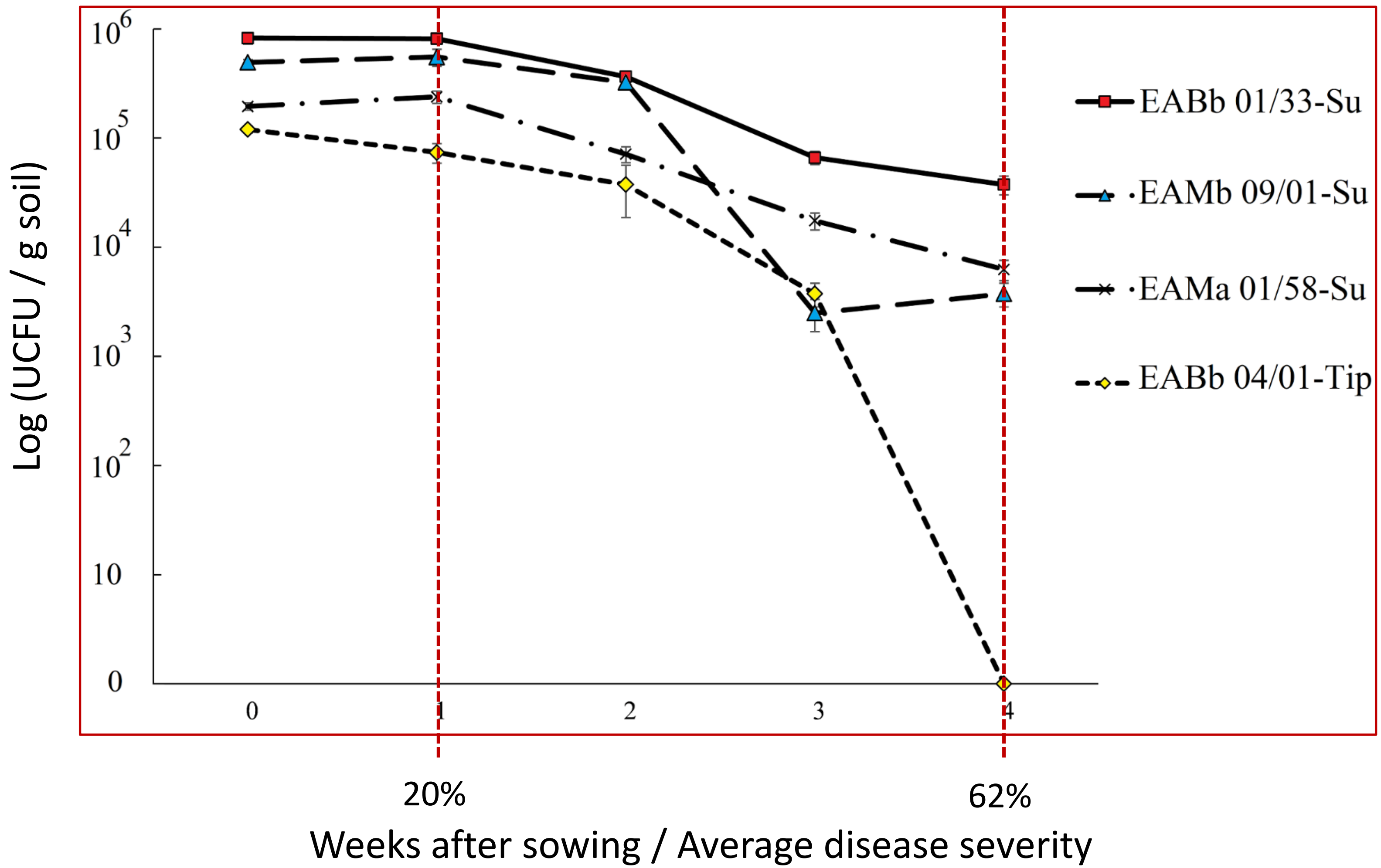
2 Populations of EF in the soil of sunflowers inoculated with *V. dahliae*



The EF were able to efficiently persist in the soil.

Molecular detection of EF and *V. dahliae* inside the plants

Amplification of the region consisting of the 5.8S ribosomal DNA and internal transcribed spacers 1 and 2 using the primer set ITS5/ITS4.

When the sunflowers were inoculated with *V. dahliae*, we detected the pathogen, but not the entomopathogen, by molecular methods.

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DOI: 10.1111/ppa.13230

ORIGINAL ARTICLE

Plant Pathology
WILEY

Evidence of soil-located competition as the cause of the reduction of sunflower verticillium wilt by entomopathogenic fungi

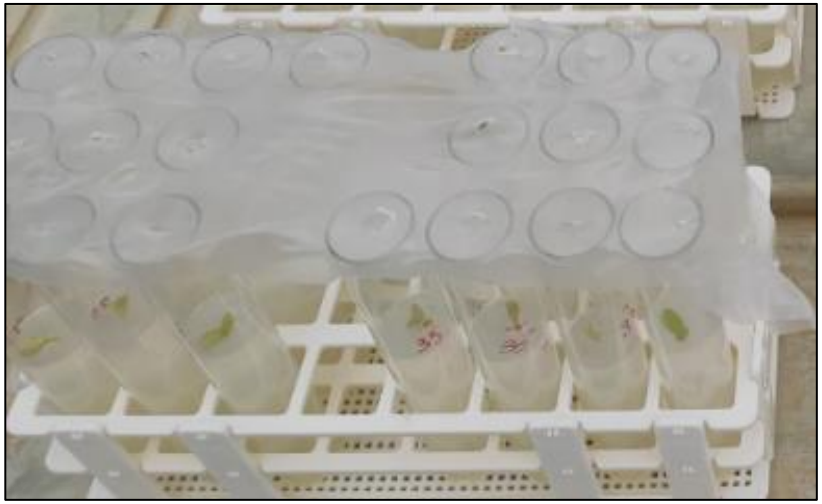
Pedro Miranda-Fuentes[1,2] | Ana Belén García-Carneros[1] | Ana María Montilla-Carmona[1] | Leire Molinero-Ruiz[1]

The protection that the EF *B. bassiana* and *M. brunneum* confer against verticillium wilt might not be plant-located, but is more likely to be the consequence of their competition with *V. dahliae* in the soil.

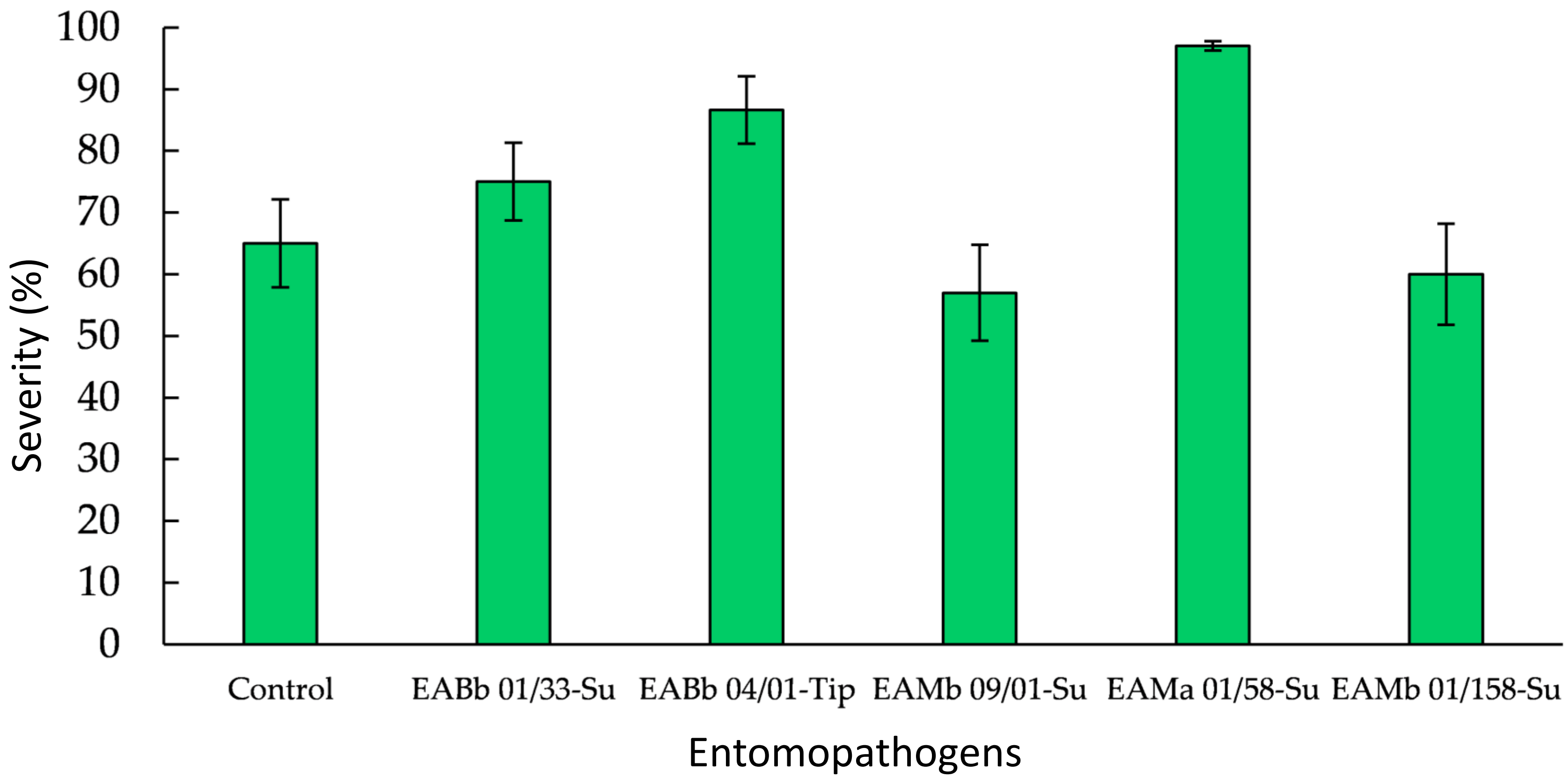
2 Biological control by means of entomopathogenic fungi: *P. halstedii*

- Entomopathogenic fungi: three strains of *M. brunneum* and two strains of *B. bassiana*.
- One isolate of *P. halstedii* (1SM, race 304) and one susceptible genotype (HA304)

Germinated seeds individually sown in Hoagland and Knop's culture medium.
Treatment with EF (10^7 con./ml, 5µl).
Inoculation with *P. halstedii* 24 h later (4×10^4 zoospores/ml, 5µl).



Effect of EF on downy mildew



No significant differences found for:

- Downy mildew severity (0% no symptoms, to 100% cottony growth completely covering the cotyledons and first true pair of leaves and evident in the base of the stem).
- Shoot height and root length
- Dry weight of shoots and roots.

Effect of EF on the growth of sunflower

- Entomopathogenic fungi: three strains of *M. brunneum* and two strains of *B. bassiana*.

Germinated seeds individually sown in Hoagland and Knop's culture medium. Treatment with EF (10^7 con./ml, 5µl).

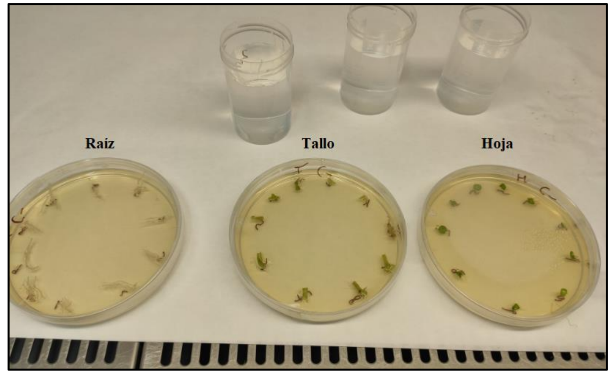
EF	Shoot height (cm)	Root length (cm)	Dry weight of shoot (g)	Dry weight of roots (g)
Control	14.7 ± 0.8 a	12.3 ± 0.9 a	1.2 ± 0.1 a	0.4 ± 0.0 a
EABb 01/33-Su	**9.8 ± 1.5 b**	**9.2 ± 1.2 c**	**0.7 ± 0.1 b**	**0.2 ± 0.0 c**
EABb 04/01-Tip	13.0 ± 0.9 a	11.1 ± 0.5 abc	0.9 ± 0.1 a	**0.3 ± 0.0 bc**
EABb 09/01-Su	15.4 ± 0.7 a	**9.9 ± 0.6 bc**	1.2 ± 0.1 a	0.3 ± 0.1 abc
EAMa 01/58-Su	15.1 ± 0.7 a	11.6 ± 0.3 ab	1.1 ± 0.1 a	0.3 ± 0.0 abc
EAMb 01/158-Su	14.8 ± 0.7 a	11.2 ± 0.5 abc	1.1 ± 0.1 a	0.3 ± 0.0 ab

Some of the EF reduced some of the growth variables of sunflower.

2

Microbiological detection of EF inside the plant

Isolation of five EF on Sabouraud Dextrose Agar.
Root, shoot and leaf tissue.



Entomopathogen	Root (%)	Shoot (%)	Leaf (%)
Control	0.0	0.0	0.0
EABb 01/33-Su	87.5	0.0	0.0
EABb 04/01-Tip	30.0	20.0	10.0
EABb 09/01-Su	20.0	22.2	0.0
EAMa 01/58-Su	20.0	0.0	0.0
EAMb 01/158-Su	20.0	30.0	20.0

None of the EF strains was recovered from plants inoculated with *P. halstedii.*

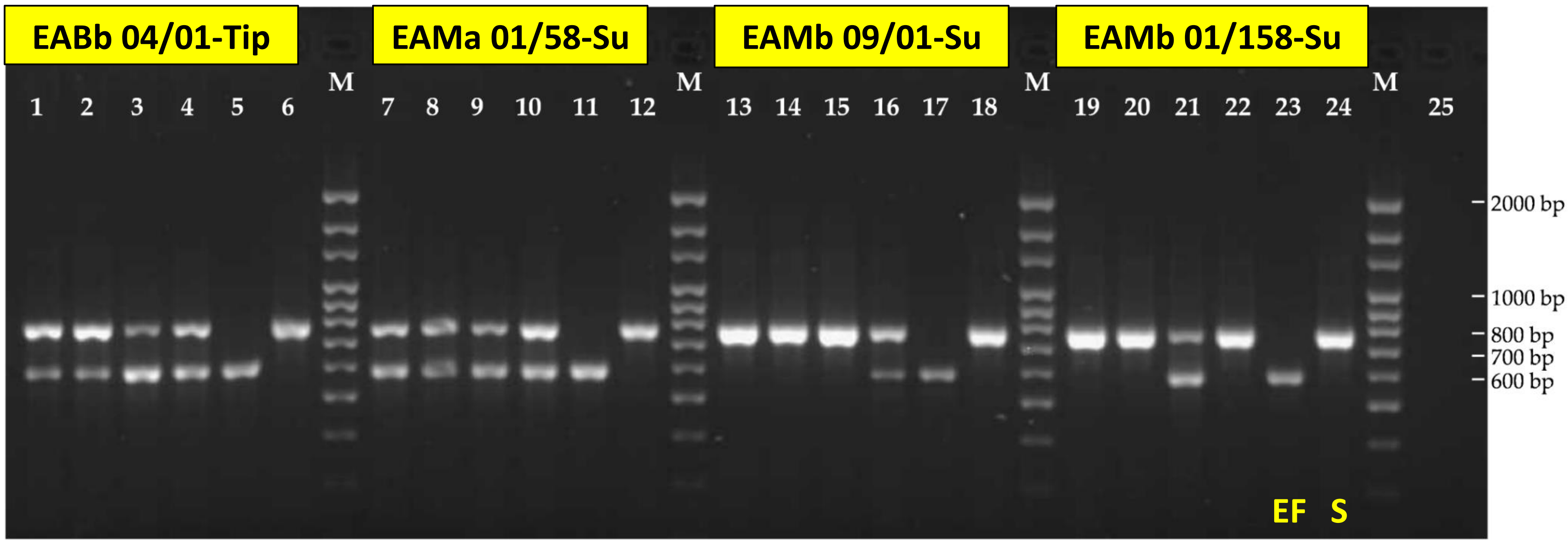
The five EF strains were isolated from non- inoculated sunflowers, although with percentages of isolation that varied depending on plant part and EF strain.

2

Molecular detection of EF inside the plant

EF: amplification of the region consisting of the 5.8S ribosomal DNA and internal transcribed spacers 1 and 2 using the primer set ITS5/ITS4.

P. halstedii: amplification of the nuclear DNA coding for the large ribosomal unit (28S rDNA) using LR0R and LR6-O primers.



DNA from EF was only amplified in plant samples treated with EF but not inoculated with *P. halstedii*.

agronomy

MDPI

Article

Updated Characterization of Races of *Plasmopara halstedii* and Entomopathogenic Fungi as Endophytes of Sunflower Plants in Axenic Culture

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† These authors contributed equally to this work.

The **obligate nature** of *P. halstedii* infections and the **strong dependence** of *Metarhizium* and *Beauveria* spp. **on soil ecology and characteristics**, seem to condition the bioactivity of EF against downy mildew. This is highly dependent on environmental conditions involving the experimental soil system.

Outline

1. Current situation of the biological control of sunflower diseases.
2. Biological control by means of entomopathogenic fungi.
3. Biological control by means of soil inhabiting bacteria.
4. Conclusions.

3 Biological control by means of soil inhabiting bacteria

The biological control by **soil-inhabiting microbes** has proven to be a successful strategy **against soil-borne pathogens**. It has been widely implemented in the management of Fusarium and Verticillium diseases.

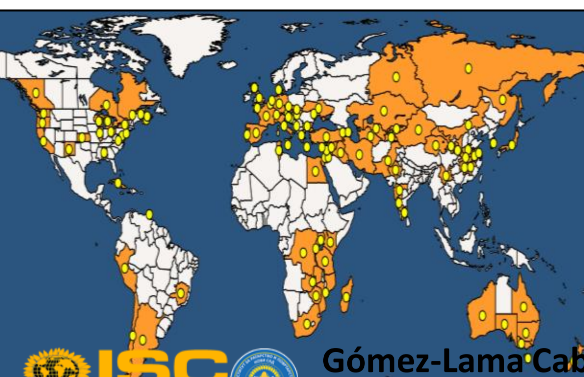
The most common microbial **genera** with effective biocontrol activity against this type of diseases are *Pseudomonas*, *Bacillus*, *Streptomyces*, *Trichoderma*, non-pathogenic *Fusarium oxysporum*, and some arbuscular mycorrhizal fungi.
Bubici et al. 2019. Front. Microbiol. 10:1290. doi: 10.3389/fmicb.2019.01290

Our group identified biocontrol agents against **Verticillium wilt of olive tree** and **Fusarium wilt of banana**. Most of these antagonists originated from the olive tree or the banana **rhizosphere soil or roots**, as native constituents of the root endophytome.
Köberl et al. 2017. Sci. Rep. 7:45318. doi: 10.1038/srep45318
Gómez-Lama Cabanás et al. 2018. Front. Microbiol. 9:277. doi: 10.3389/fmicb.2018.00277

Pseudomonas simiae PICF7, a highly versatile beneficial endophytic rhizobacteria isolated from olive roots, shows **biocontrol and plant-growth promoting abilities**. Moreover, it displays significant biocontrol of Verticillium wilt of olive and Fusarium wilt of banana.
Gómez-Lama Cabanás et al. 2021. J. Fungi 7:194. doi: 10.3390/jof7030194

Verticillium wilt of sunflower (*Verticillium dahliae* Kleb.)

- Soil-borne fungus with more than 2000 host plant species.
- Microsclerotia (> 10 years).
- Interveinal chlorosis and yellowing of leaves, progressing upwards.
- Stem lesions.
- No active ingredients registered in the European Union.
- Control by means of genetic resistance.



Gómez-Lama Cabanás et al. 2022

Sunflower broomrape (*Orobanche cumana* Wallr.)

➢ Disease caused by a root holoparasitic plant that depends enterely on sunflower to complete its life cycle and reduces dramatically the crop yield.

➢ Initial development of symptoms around flowering of sunflower. Symptoms have a similar appearance to those of water stress.

➢ Sunflower broomrape is mainly controlled by means of genetic resistance. New races are identified.

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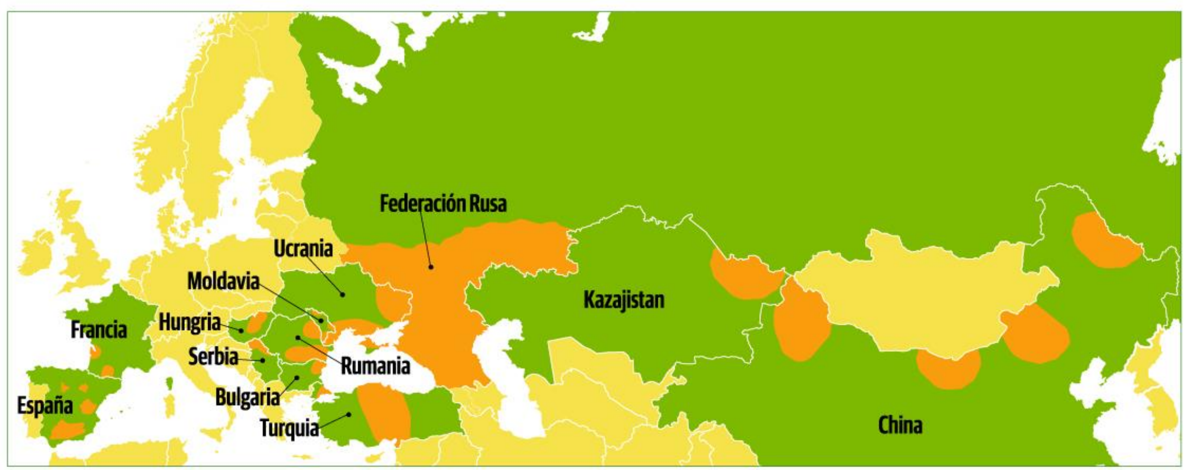

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3 Biological control by means of soil inhabiting bacteria

Objectives:

Evaluating the *in vitro* antagonism of a collection of soil inhabiting bacteria with good biocontrol effect on wilts of olive tree or banana, against *V. dahliae* from sunflower.

Evaluating, under greenhouse conditions, a collection of soil inhabiting bacteria, with known biocontrol effect of wilts of olive tree or banana on:
- The infection of sunflower by *O. cumana*.
- The growth of sunflower.

Regarding sunflower diseases, **preliminary results** suggested that bacterization of sunflower seeds with *Pseudomonas* spp. from olive roots can promote plant growth as well as result in significant reductions of the number of broomrape nodules.
Gómez-Lama Cabanás et al. 2018. Abstracts of the International Symposium on Confection Sunflower Technology and Production, p.41. Wu Yuan County, Inner Mongolia (China), August 8-10, 2018.

Bacterial strains from plant rhizosphere used in this work

Reference	Taxa	Host of isolation	Previous works	Experiment-pathogen
IAS-B-364	*Pseudomonas chlororaphis*	Banana	Gómez-Lama Cabanás et al. 2021	Plates-Vd
IAS-B-931	*Pseudomonas chlororaphis subsp. aurantiaca*	Banana	Gómez-Lama Cabanás et al. 2021	Plates-Vd
IAS-B-944	*Pseudomonas chlororaphis subp. aureofaciens*	Banana	Gómez-Lama Cabanás et al. 2021	Plates-Vd, greenhouse-Oc
IAS-B-1013	*Pseudomonas chlororaphis*	Banana	Gómez-Lama Cabanás et al. 2021	Plates-Vd
IAS-B-1054	*Pseudomonas* sp.	Banana	Gómez-Lama Cabanás et al. 2021	Plates-Vd, greenhouse-Oc
PIC25	*Pseudomonas* sp.	Olive	Gómez-Lama et al. 2018a	Plates-Vd, greenhouse-Oc
PIC28	*Bacillus* spp.	Olive	Gómez-Lama Cabanás et al. 2018b	Plates-Vd, greenhouse-Oc
PIC105	*Pseudomonas indica*	Olive	Gómez-Lama Cabanás et al. 2018a	Plates-Vd, greenhouse-Oc
PIC141	*Pseudomonas* sp.	Olive	Gómez-Lama Cabanás et al. 2018a	Greenhouse-Oc
PIC167	*Paenibacillus terrae*	Olive	Gómez-Lama Cabanás et al. 2018b	Greenhouse-Oc
PICF4	*Pseudomonas fluorescens*	Olive	Mercado-Blanco et al. 2004	Greenhouse-Oc
PICF6	*Pseudomonas* sp.	Olive	Mercado-Blanco et al. 2004	Plates-Vd, greenhouse-Oc
PICF7	*Pseudomonas simiae*	Olive	Mercado-Blanco et al. 2004	Plates-Vd, greenhouse-Oc

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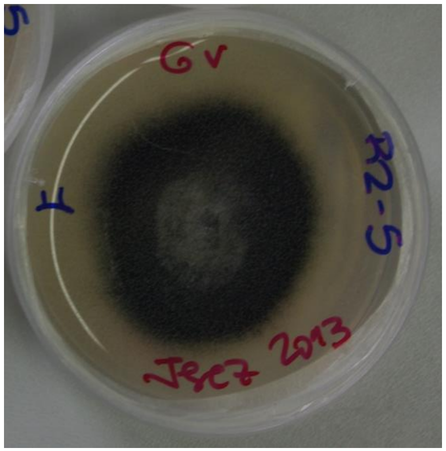
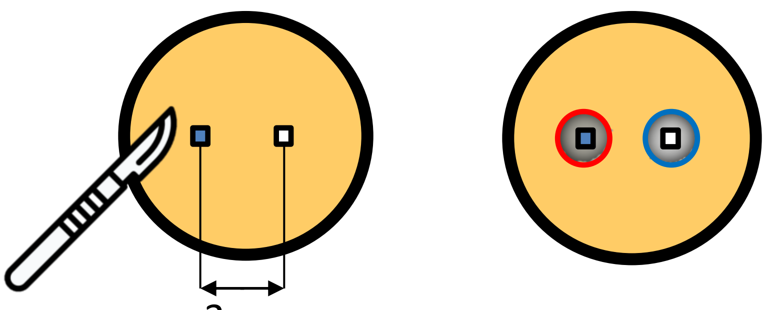
Biological control by means of soil inhabiting bacteria: *V. dahliae*

Dual cultures



Reference	Year of collection	Geographical location	Host	Previous works
Vd01-13	2013	Cadiz (Andalusia, Spain)	Sunflower	Martin-Sanz et al. 2018. Front. Plant Sci. 9: 288
Vd09-16	2016	Kastamonu (Ahmetbey, Turkey)	Sunflower	Martin-Sanz et al. 2018. Front. Plant Sci. 9: 288

- Ten bacterial strains



Nutrient Agar (NA)
Potato Dextrose Agar (PDA)

$$\text{Inhibition of Micelial Growth (\%)} = 100 \times \frac{\text{Diam. colony Control} - \text{Diam. colony Treatment}}{\text{Diam. colony Control}}$$

3

Results

Reference	Taxa	Inhibition of *Verticillium dahliae* (%) Vd01-13 NA	Vd01-13 PDA	Vd09-16 NA	Vd09-16 PDA
Control		0.00 A	0.00 A	0.00 A	0.00 A
IAS-B-364	***Pseudomonas chlororaphis***	**83.55 E**	**88.12 E**	**87.82 G**	65.79 F
IAS-B-944	***Pseudomonas chlororaphis***	**83.75 E**	**88.05 E**	**78.91 F**	27.14 D
IAS-B-931	***Pseudomonas chlororaphis subsp. aurantiaca***	**84.25 E**	35.50 B	**79.97 F**	12.54 B
PIC28	***Bacillus* sp.**	55.73 B	33.15 B	63.11 E	20.82 CD
PICF6	***Pseudomonas* sp.**	**78.47 E**	40.33 C	33.28 B	17.07 BC
PICF7	***Pseudomonas simiae***	27.82 B	**88.68 E**	41.04 C	23.84 CD
IAS-B-1054	***Pseudomonas* sp.**	**83.65 E**	-----	**87.91 G**	-----
IAS-B-1013	***Pseudomonas chlororaphis***	**83.15 E**	-----	**87.20 G**	-----
PIC25	***Pseudomonas* sp.**	50.85 D	53.80 D	53.93 D	22.46 CD
PIC105	***Pseudomonas indica***	40.48 C	56.01 D	58.96 DE	42.45 E

➢ Significant differences of inhibition between *V. dahliae* isolates: independent analyses.

➢ Five bacterial strains showed interesting results (inhibition >70%) against both isolates. Two additional strains inhibited the growth of one isolate.

3 Biological control by means of soil inhabiting bacteria: *O. cumana*

- Ten strains (*Bacillus*, *Paenibacillus*, *Pseudomonas*)
- One population of *O. cumana* and one susceptible genotype of sunflower (B117)
- Greenhouse (± 25ºC) for 5 weeks

Results

Control of broomrape

Reference	Taxa	Height	N. nodules*	Weight	
				Root	Aboveground parts
Control ($MgSO_4$)		16.79 A	5.71 AB	0.72 A	3.44 ABCD
IAS-B-944	*Pseudomonas chlororaphis subp. aureofaciens*	**20.20 BC**	5.14 AB	**1.98 FGH**	3.75 ABC
PIC28	*Bacillus* sp.	**17.89 BC**	8.00 A	**2.26 H**	4.05 A
PIC167	*Paenibacillus terrae*	17.76 AB	6.00 AB	**1.40 BCD**	2.96 D
PICF4	*Pseudomonas fluorescens*	**22.13 C**	**1.29 C**	**1.56 DEF**	3.51 ABCD
PICF6	*Pseudomonas* sp.	**20.53 BC**	7.00 AB	**2.04 GH**	3.87 AB
PICF7	*Pseudomonas simiae*	19.49 ABC	2.86 BC	**1.63 DEFG**	3.09 CD
IAS-B-1054	*Pseudomonas* sp.	19.00 AB	2.86 BC	**1.28 BC**	3.24 BCD
PIC25	*Pseudomonas* sp.	17.86 AB	3.14 BC	**1.56 DEF**	3.33 ABCD
PIC105	*Pseudomonas indica*	**20.20 BC**	**0.71 C**	**1.32 BC**	2.88 D
PIC141	*Pseudomonas* sp.	16.79 A	7.71 A	**1.76 EFG**	2.96 D

- All the bacteria significantly increased the weight of roots. Some of them also caused less nodules.
- One *Bacillus* and four *Pseudomonas* were associated to taller plants.

Results

Growth promotion

Reference	Taxa	Height	Weight	
			Root	Aboveground parts
Control ($MgSO_4$)		20.36 A	0.76 A	3.27
IAS-B-944	*Pseudomonas chlororaphis subp. aureofaciens*	19.03 A	**1.45 BC**	2.93
PIC28	*Bacillus* sp.	18.79 A	**1.76 C**	3.52
PIC167	*Paenibacillus terrae*	18.57 A	1.13 AB	2.86
PICF4	*Pseudomonas fluorescens*	20.43 A	**1.49 BC**	3.21
PICF6	*Pseudomonas* sp.	21.74 A	**1.66 C**	3.64
PICF7	*Pseudomonas simiae*	19.93 A	**1.74 C**	3.26
IAS-B-1054	*Pseudomonas* sp.	20.00 A	**1.65 C**	2.99
PIC25	*Pseudomonas* sp.	14.79 B	**1.51 BC**	2.99
PIC105	*Pseudomonas indica*	20.00 A	**1.51 BC**	3.15
PIC141	*Pseudomonas* sp.	20.29 A	**1.44 BC**	3.52

- All except one of the bacteria significantly increased the weight of roots. There were no statistical differences of weight of shoots and leaves of the plants.
- One *Pseudomonas* was associated to shorter plants.

4 Conclusions

2

The protection that *B. bassiana* and *M. brunneum* confer against verticillium wilt might not be plant-located, but is more likely to be the consequence of their competition with *V. dahliae* in the soil.

The inefficacy of entomopathogens against *P. halstedii* in our works might be related to environmental conditions of the experimental soil system. This is not unexpected, since *P. halstedii* is an obligate parasite that shelters into the plant and *Metarhizium* and *Beauveria* spp. are soil-dependent and affected by soil ecology and characteristics.

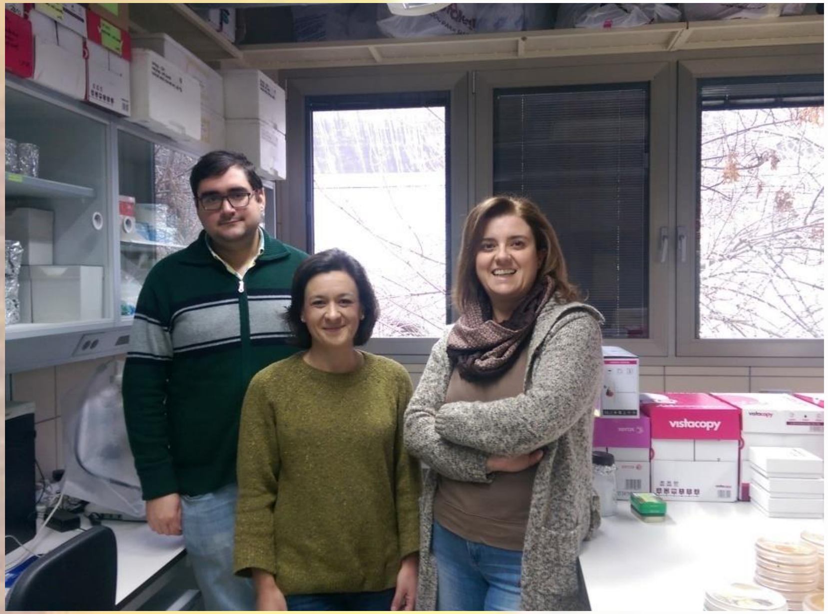
3

Although the inhibition of *V. dahliae* by soil-inhabiting bacteria depended on the fungal isolate, five strains showed promising results. Future research will address the study of their efficacy under greenhouse conditions.

The bacteria were associated to increases of root weight of sunflower irrespective of the presence of *O. cumana*. In inoculated plants, some of the tested strains were associated to low number of nodules.

Thank you for your attention!!

Lab. Diseases of Field Crops



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