Evaluation of *Pseudomonas fluorescens* PICF7 as potential biocontrol agent against *Plasmopara halstedii* in sunflower

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ABSTRACT

- One of the most important biotic constraints for sunflower production is the soil-borne, obligate-parasitic oomycete *Plasmopara halstedii* Farl. Berl. & de Toni, which causes sunflower downy mildew (SDM). Control of SDM by means of biocontrol agents (BCAs) has been poorly investigated so far. Our previous studies have demonstrated that *Pseudomonas fluorescens* PICF7 is an effective BCA against Verticillium wilt of olive, a disease caused by the soil-borne pathogenic fungus *Verticillium dahliae* Kleb. Moreover, strain PICF7 was confirmed as endophyte in olive roots. Recently, colonization and persistence in *Arabidopsis thaliana* roots of strain PICF7 and one spontaneous rifampicin-resistant mutant derivative ($PICF7^{Rf}$) were assessed, thus confirming the ability of this strain to colonize different plant species. Considering these antecedents, we aimed in this study to determine whether *P. fluorescens* PICF7: (1) colonize and persist in sunflower roots; (2) promote growth of sunflower at early stages; and (3) control SDM.
- Hybrid seeds genetically susceptible to SDM were dipped in bacterial suspensions of PICF7 (aprox. 1 x 10^9 cfu/mL) or $PICF7^{Rf}$, (aprox. 1 x 10^9 cfu/mL) or in 10 mM $MgSO_4 \cdot 7H_2O$ (control treatment) at 23ºC, 150 rpm for 5h. Seeds of each treatment were germinated and subsequently immersed in a zoosporangial suspension of an isolate of *P. halstedii* collected in southern Spain in 2010 (2 x 10^4 zoosporangia/mL) prepared in water for 5-h at 23ºC, 150 rpm. Bioassays were then designed and conducted under controlled growth conditions. Ten pots per treatment were filled with perlite and five sunflower seeds were sown in each pot. Plants were grown at 23ºC, 70-100% relative humidity and a 12h photoperiod (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 18-25 days. Colonization and persistence of PICF7 and $PICF7^{Rf}$ in roots were assessed at different time points along the bioassays. Root macerates were diluted and sown in King's B (KB) agar (PICF7) or KB agar amended with rifampicin ($PICF7^{Rf}$). To assess growth promotion of sunflower seedlings upon bacteria inoculation, the dry-weight of the above-ground part of plants was determined at the end of the experiments. Disease incidence (percentage of diseased plants per pot) and symptoms were scored at 10 days after bacterial inoculation and at the end of the experiments.
- Results showed that strains PICF7 and $PICF7^{Rf}$ are able to colonize sunflower roots from the very first moment after seed bacterization (10^7-10^8 cfu/g) and persist until the end (about three weeks) of the experiments (10^7cfu/g). On the other hand, no significant differences of dry-weight were found between control and bacteria-inoculated plants. Plants inoculated with the pathogen displayed similar final disease incidences whether they were bacterized (52%, PICF7; 68%, $PICF7^{Rf}$) or not (64%).
- Although results showed that *Pseudomonas fluorescens* PICF7 and $PICF7^{Rf}$ are good colonizers of sunflower roots, neither increase of the above-ground biomass, nor control of SDM were observed under the tested experimental conditions. Root colonization ability by an enhanced green fluorescent protein (EGFP)-tagged derivative of strain PICF7 is currently being evaluated by confocal laser scanning microscopy (CLSM). The aim will be to determine whether the lack of SDM suppression could be explained by a failure of PICF7 to establish as an endophyte in sunflower root tissues.
- Biological control may offer an environmentally-friendly approach fitting criteria of modern sustainable agriculture and may be a complementary action to other disease control measures. This study is one of the first attempts to evaluate the potential biocontrol effectiveness of a beneficial, naturally-occurring bacteria originating from the same bioclimatic area as sunflower is cultivated.

Keywords: disease control - downy mildew - endophytic bacteria - plant-growth promotion - root colonization.

INTRODUCTION

One of the most important biotic constraints for sunflower (*Helianthus annuus* L.) production is the soil-borne obligate-parasitic oomycete *Plasmopara halstedii* Farl. Berl. & de Toni, which causes sunflower downy mildew (SDM). Over the last 30 years there has been considerable research on effective control

measures against the disease, such as the use of phenylamides and breeding for resistance (Gulya et al., 1997). Yet, SDM is a continuous threat to the crop due to the adaptability of the pathogen (Molinero-Ruiz et al., 2008). Several races of *P. halstedii*, which overcome genes of resistance incorporated into sunflower hybrids, as well as resistance of the oomycete to the phenylamide metalaxyl-M, have been reported in Spain (Molinero-Ruiz et al., 2002; 2008). Control of SDM by means of biocontrol agents (BCAs) has been poorly investigated so far. Biological control may offer an environmentally-friendly approach fitting criteria of modern sustainable agriculture and may be a complementary action to other disease control measures. Our previous studies have demonstrated that *Pseudomonas fluorescens* PICF7 (isolated from roots of nursery-propagated olives cv. Picual) is an effective BCA against Verticillium wilt of olive (Mercado-Blanco et al., 2004; Prieto et al., 2009), a disease caused by the soil-borne phytopathogenic fungus *Verticillium dahliae* Kleb. (López-Escudero and Mercado-Blanco, 2011) Moreover, strain PICF7 has been confirmed as a true endophyte of olive root tissues (Prieto and Mercado-Blanco, 2008; Prieto et al., 2011). Recently, colonization and persistence in *Arabidopsis thaliana* roots of strain PICF7 and one spontaneous rifampicin-resistant mutant derivative ($PICF7^{Rf}$) were assessed (Maldonado-González et al., 2012), thus confirming the ability of this strain to colonize different plant species. Considering these antecedents, we aimed in this study to determine whether *P. fluorescens* PICF7: (1) colonize and persist in sunflower roots; (2) promote growth of sunflower at early stages; and (3) control SDM.

MATERIALS AND METHODS

Preparation of bacterial inocula

Pseudomonas fluorescens PICF7 and $PICF7^{Rf}$ (Mercado-Blanco et al., 2004) were grown in King's medium B (KB) (King et al., 1954) agar or KB agar amended with rifampicin(50 µg/mL), respectively, during 24h at 25ºC. In order to prepare the inocula used for bacterization of sunflower seeds, PICF7 and $PICF7^{Rf}$ cells were recovered from the media by adding 10 mM $MgSO4{\cdot}7H_2O$ and scrapping bacterial biomass off using a sterile glass rod. Then, bacterial suspensions were centrifuged (4.500 rpm, 4 min.) and washed twice with 30 mL of 10 mM $MgSO4{\cdot}7H_2O$. Bacterial cell densities were spectrophotometrically (OD_{600}) adjusted, and the number of viable cells present in each inoculum (see below) was assessed by spreading serial dilutions of bacterial suspensions on appropriate solid media.

Sunflower seeds treatment: bacterization, seed germination and pathogen inoculation

Hybrid sunflower seeds genetically susceptible to SDM were surface disinfested by immersion in 20% sodium hypochlorite for 5 min., rinsed out three times with deionized water and air dried over sterile filter paper under sterile conditions. Disinfested seeds were then dipped in bacterial suspensions of PICF7 ($1.44\text{-}1.46 \times 10^9$ cfu/mL) or $PICF7^{Rf}$, ($6.3 \times 10^8 - 1.64 \times 10^9$ cfu/mL) (100 seeds each) or in 10 mM $MgSO_4{\cdot}7H_2O$ (control treatment) (150 seeds) at 23ºC, 150 rpm for 5h. Seeds were subsequently dried on sterile paper for 5 minutes. Afterwards, seeds of each treatment were germinated as previously described (Molinero-Ruiz et al., 2008). Briefly, one tray for each treatment was filled with perlite that was covered with two layers of watered paper. Bacterized seeds were placed between the layers under sterile conditions and incubated at 22-24ºC, 100% relative humidity (RH) for 36 h. Then, zoosporangia from cotyledons of sunflower plants previously infected by an isolate of *P. halstedii* collected in Huelva (Spain) were removed with deionized water. The suspension was filtered through two layers of sterile cheesecloth and the inoculum concentration was adjusted to 2×10^4 zoosporangia/mL. Bacterized and germinated seeds were immersed in the zoosporangial suspension or in water (control) at 23ºC and kept under shaking (150 rpm) for 5-h.

Bioassays design

Two different bioassays were designed and conducted under controlled growth conditions according to Molinero-Ruiz et al. (2008). Ten pots per treatment were filled with perlite and five sunflower seeds (treated as mentioned above) were sown in each pot. In experiment 1, plants were grown at 17-23ºC temperature range, whereas the temperature in experiment 2 was set at 23ºC. Both experiments were performed under a 12-h photoperiod (100 µE·m-2·s-1). Finally, two different RH were tested, 70 % and 100% (5 pots each).

Colonization and persistence of strain PICF7 and $PICF7^{Rf}$ in sunflower roots were assessed at different time points in experiment 1 (two days after bacteria inoculation [t2], t14 and t25). In experiment 2, only population of PICF7 was determined (see below) at t2 and t18. For that purpose, two-three root macerates were serially diluted with 10 mM $MgSO4{\cdot}7H_2O$ and sown in KB agar (PICF7) or KB agar

amended with rifampicin (PICF7^{Rf}). Plates were grown for 48 h at 25ºC. Additionally, two-three roots in experiment 2 were examined before and after pathogen treatment to check whether introduced PICF7 population could have been affected (diluted or removed) after *P. halstedii* inoculation. All root systems tested proceed from plants grown at 70% RH.

To evaluate growth promotion of sunflower seedlings upon *P. fluorescens* PICF7 and PICF7^{Rf} inoculation, the above-ground part of plants from each pot were removed at t25 (experiment 1) and t18 (experiment 2), placed inside an envelope and oven-dried at 70ºC during 72-h. Dry-weights were then determined.

To assess whether PICF7 could be a potential effective BCA against *P. halstedii*, disease incidence was scored at the end of the experiments according to the percentage of plants showing sporulation and/or chlorosis per pot.

Statistical analyses

The incidence of SDM and dry-weight of the plants were subjected to analysis of variance ($\alpha = 0.05$).

RESULTS

Colonization and persistence of P. fluorescens *PICF7 in sunflower roots*

Experiment 1 confirmed that PICF7 and its Rf-resistant derivative were able to colonize and persist on/in roots of sunflower along the experiment (Table 1). Besides, no differences in the dynamics of introduced populations were found between both strains. Considering this result, PICF7^{Rf} was not included in the second experiment. On the other hand, results from experiment 2 showed that the treatment of sunflower seedlings with *P. halstedii* (or water) did not significantly affect the size of bacterial populations already present in bacterized seeds. In fact, after pathogen (or water) inoculation an increase of the population of *P. fluorescens* PICF7 was observed (Table 1).

Table 1. Population sizes of *Pseudomonas fluorescens* PICF7 and PICF7^{Rf} recovered from root tissues of sunflower grown at 70% RH along the two experiments

	Bacterial population log(10) g^{-1} fresh root				
	Experiment 1			Experiment 2	
Time (days)	PICF7	PICF7^{Rf}	Time (days)	PICF7	
2	8.9 ± 0.15	8.6 ± 0.32	2	7.3 ± 0.56	(8.2 ± 0.21)* (8.8 ± 0.14)**
14	7.5 ± 0.21	7.5 ± 0.07			
25	7.4 ± 0.26	7.2 ± 0.10	18	7.8 ± 0.28	

Means values are represented with standard deviation (SD).* Population after *Plasmopara halstedii* inoculation;**population after deionized water treatment (5h, 150 rpm at 23ºC). Data are means of 2-3 1 g fresh root samples.

Evaluating of growth promotion of sunflower seedlings by P. fluorescens *PICF7*

Growth stimulation of above-ground parts of plants originated from bacterized seeds was not observed at the end of the experiments (Table 2). As expected, plants inoculated with *P. halstedii,* regardless they were previously bacterized or not, showed lower weight in comparison to non-inoculated plants. Regarding the influence of RH conditions in plant growth results showed that plants inoculated with the pathogen and cultivated at 100% RH yielded lower aerial biomass than those ones grown at 70% (experiment 1).

Table 2. Dry-weight determination of above-ground biomass of sunflower seedlings after different treatments and RH conditions

	Dry weight (g)			
Treatment	Control		*P. halstedii*	
	70% RH	100% RH	70% RH	100% RH
Experiment 1				

Control	0.50 ± 0.05 a	0.46 ± 0.04 a	0.37 ± 0.03 b	0.28 ± 0.02 c
P. fluorescens				
PICF7	0.50 ± 0.04 a	0.50 ± 0.07 a	0.34 ± 0.05 b	0.28 ± 0.04 c
PICF7^{Rf}	0.49 ± 0.05 a	0.49 ± 0.04 a	0.39 ± 0.04 b	0.24 ± 0.05 c
Experiment 2				
Control	0.56 ± 0.06 a	0.54 ± 0.03 a	0.37 ± 0.03 b	0.33 ± 0.06 b
P. fluorescens				
PICF7	0.50 ± 0.07 a	0.55 ± 0.02 a	0.34 ± 0.03 b	0.35 ± 0.05 b

Mean values ± SD followed by the same letter are not significantly different according to Tukey test (P= 0.05) for each experiment.

Assessment of the potential biocontrol of P. fluorescens *PICF7 against* P. halstedii

Number of diseased plants per pot after inoculation with *P. halstedii* pathogen or water (control) evidenced that neither PICF7 nor PICF7^{Rf} significant reduced the development of SDM under the tested conditions. Thus, means values of disease incidence at the end of the experiments (Final DI) is shown in Table 3.

Table 3. Final disease incidence (DI) (%)

	Final DI (%)			
	Experiment 1		Experiment 2	
	70% RH	100% RH	70% RH	100% RH
Control	75	64	64	76
PICF7	70	80	52	68
PICF7^{Rf}	85	88		

Mean values of disease incidence at the end of the experiments. The final time for experiments 1 and 2 were t25 and t18 respectively.

DISCUSSION

Colonization and persistence of BCAs in the rhizosphere of the host plant is a fundamental prerequisite for effective biological control (Mercado-Blanco and Bakker, 2007). In this present study, we have demonstrated that *P. fluorescens* PICF7 can colonize and persist in roots of sunflower, as has also been shown for other plants species (Mercado-Blanco et al., 2004; Maldonado-González et al., 2012; Prieto and Mercado-Blanco, *unpublished results*). Strain PICF7 could be recovered from root macerates until the end of experiments here conducted (t25, in experiment 1, and t18, in experiment 2), although population decreased approximately one order of magnitude during bioassays. Despite strain PICF7 displayed good colonization ability in sunflower roots, results showed that PICF7 neither controlled SDM nor promoted growth of plants under the experimental conditions used. On the other hand, aerial parts of plants inoculated with the *P. haltedii* and cultivated at 100% RH showed lower weight than those ones grown at 70% in experiment 1.Therefore, the influence of RH in promote growth in sunflower must be more deeply investigated in future studies. The absence of SDM biocontrol by strain PICF7 is in contrast to the effective biocontrol that this strain displayed against *V. dahliae* in nursery-propagated olive plants (Mercado-Blanco et al., 2004; Prieto et al., 2009) and in *A. thaliana* (Maldonado-González et al., 2012).

Lack of effective SDM control by PICF7 could be due to different causes. On the one hand, strain PICF7 could not be an effective BCA against this oomycete in general, or not being able to control this pathogen when infecting this particular plant host. It is known that a given tripartite plant-pathogen-BCA interaction depends on a broad range of (a)biotic factors highly interdependent and finely modulated. For example, the BCA *P. fluorescens* CHAO produces 2,4-Diacetylphloroglucinol (Phl), an antibiotic effective against diverse phytopathogenic fungi. However, some of them (e.g., *Fusarium oxysporum*ATCC 16417 and ATCC 7808, *F. solani* ATCC 38136, *F. napiforme* M-3563, etc) are able to produce fusaric acid which interferes with Phl synthesis (Haas and Défago, 2005). It can be speculated that *P. halstedii* may counteract potential antibiosis mechanisms deployed by *P. fluorescens* PICF7. On

the other hand, the methodological approach here used could have influenced the biocontrol performance of strain PICF7. For instance, disease pressure could have been excessive in our experimental conditions. Early symptoms were scored 8 days after pathogen inoculation and disease development might have taken place too fast to allow the effective deployment of potential biocontrol traits such as siderophore production and/or antibiosis (Mercado-Blanco et al., 2004; Prieto et al., 2009). Likewise, the way in which the pathogen or PICF7 were introduced into the system under study might also have a decisive influence. For instance, in the olive/*V. dahliae* pathosystem, the pathogen (as conidia suspension) was mixed with soil (Mercado-Blanco et al., 2004), or applied by dipping the roots in the *A. thaliana*/*V. dahliae* interaction (Maldonado-González et al., 2012). Meanwhile, strain PICF7 was applied here to the seeds and not to the soil (*A. thaliana*/*V. dahliae* pathosystem) or to the roots (olive/*V. dahliae* interaction). Furthermore, initial density at which the BCA was applied could also have an important influence (Djavaheri, 2007). Finally, another explanation could be the failure of strain PICF7 to establish as an endophyte in sunflower root tissues. Indeed, Prieto et al. (2009) have suggested that effective suppression of Verticillum wilt epidemics in olive plants seems to require the early establishment of PICF7 both at the surface and the interior of olive root tissues, prior to root colonization by *V. dahliae*.

Whether PICF7 is able to colonize sunflower inner root tissues or not remains to be elucidated. Ongoing experiments using an EGFP-tagged derivative of strain PICF7 and CLSM (Prieto and Mercado-Blanco, 2008) will shed light on this issue. The present research provides the first attempt in the use of BCAs for control of SDM in an environmentally-friendly manner.

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