agronomy

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

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Abstract: The management of downy mildew (*Plasmopara halstedii*) in sunflower, is heavily dependent on genetic resistance, whilst entomopathogenic fungi (EF) can reduce other sunflower diseases. In this work, we characterized *P. halstedii* from Spain and other countries collected in the past few years. Twenty-three races were identified (the most frequent in Spain being 310, 304, 705 and 715), with an increasing proportion of highly virulent races. Five isolates from countries other than Spain overcame the resistance in RHA-340. In addition, we assessed the efficacy of five EF against downy mildew and their effects on sunflower growth in axenic conditions. None of the entomopathogens reduced disease severity, nor did they have any effect on plant growth when applied together with *P. halstedii*. In contrast, three EF reduced some of the plant growth variables in the absence of the pathogen. Microbiological and molecular diagnostics suggest that the axenic system and the short experimental time used in this study did not favor the successful establishment of EF in the plants or their potential biocontrol effect. Our results show a shift in *P. halstedii* racial patterns and suggest that soil as a growth substrate and long infection times are needed for EF effectiveness against downy mildew.

Keywords: biological control; diseases of oil crops; downy mildew; endophytic fungi; entomopathogens; genetic resistance; integrated pest management; pathogen races; soilborne pathogens

Citation: Miranda-Fuentes, P.; García-Carneros, A.B.; Molinero-Ruiz, L. Updated Characterization of Races of *Plasmopara halstedii* and Entomopathogenic Fungi as Endophytes of Sunflower Plants in Axenic Culture. *Agronomy* **2021**, *11*, 268. https://doi.org/10.3390/agronomy11020268

Academic Editor: Vicente J. Febres
Received: 25 December 2020
Accepted: 28 January 2021
Published: 31 January 2021

1. Introduction

Downy mildew of sunflower (*Helianthus annuus* L.), caused by the obligate biotrophic oomycete *Plasmopara halstedii* Farl. Berl. and De Toni, is one of the most widespread diseases affecting this crop, and it is present in the five continents [1]. When *P. halstedii* zoospores emerge from dormant oospores present in the soil reaching seedling roots, primary, systemic infections of the host take place. Primary infections can cause damping-off or severe stunting. Infected plants manifest a pronounced chlorosis, which is restricted to areas bordering the main veins in the lower leaves, although they may cover the entire foliar surface in younger leaves [2]. When infected plants are exposed to high relative humidity and cool temperatures, the oomycete develops profuse cottony outgrowths from the underside of infected leaves, constituted by zoosporangia [3]. Sunflower downy mildew causes an average yield reduction of 3.5% worldwide. Crop losses of up to 100% are frequent when infections in the field are severe or highly localized [3,4].

The management of this pathogen in Europe is based on particular cultural practices, chemical pesticides (mainly, by seed treatments using the phenylamides metalaxyl, metalaxyl-M and mixtures of metalaxyl + mancozeb) [5,6], and the employment of genetically resistant sunflower hybrids [2]. Whereas cultural practices alone may not be effective enough to control the disease, reports of *P. halstedii* isolates becoming resistant to metalaxyl and metalaxyl-M [6,7] show that chemical control is not a sufficiently reliable tool against downy mildew in sunflower.

Genetic resistance is the most important control measure for this pathogen [2,8]. Nonetheless, the use of resistant sunflower hybrids is threatened by the continuous identification of new *P. halstedii* populations of increased virulence that overcome the genes for resistance in the crop [9,10]. Thus, information on the racial distribution of this pathogen is of crucial importance for its management by means of genetic resistance [2]. Although extensive research on the pathogenic characterization of *P. halstedii* was conducted in Spain between 1994 and 2006 [6,11], the evolution of races of *P. halstedii* in recent years has been largely unknown in this country, with the exception of a few studies [9].

On the other hand, biological control has triggered increasing interest in recent years, fostered by both researchers and agricultural policies worldwide, including the common agricultural policy in the European Union [12,13]. Amongst biocontrol agents, entomopathogenic fungi (EF), which are fungal species that are pathogenic to insects, have achieved great relevance in economic terms, with a plethora of EF-derived biopesticides being registered [13]. Several authors have reported the efficacy of EF as biocontrol agents against plant pathogens [14–19]. In sunflower, EF can have three different effects, the control of insect pests being the most classical one among them. Some EF species like *Beauveria bassiana* Bals. (Vuill), *Metarhizium brunneum* Petch and *M. anisopliae* (Metsch) are good alternatives for controlling sunflower banded moth *Cochylis hospes* Walsingham [20], grey corn weevil *Tanymecus dilaticollis* Gyllenhal [21], and wireworms *Agriotes* spp. [22]. Moreover, the promotion of sunflower growth and nutrition by some strains of *B. bassiana*, *M. brunneum* and *Isaria farinosa* (Holmsk.) has been reported [23]. Finally, we recently found that *B. bassiana* and *M. brunneum* not only inhibit the soilborne pathogenic fungi *Verticillium dahliae* Kleb. and *Cadophora helianthi* (L. Molinero-Ruiz, A. Martin-Sanz, C. Berlanas and D. Gramaje), but they also reduce Verticillium wilt in sunflowers. Moreover, this reduction in symptoms seems to be the consequence of a soil-mediated competition between *V. dahliae* and the EF [19]. Despite previous studies on the use of EF in sunflower, their efficacy against *P. halstedii* remains completely unexplored, and biological control of this pathogen has scarcely been addressed [24]. However, several endophytic strains of *B. bassiana* were applied against *Plasmopara viticola* (Berk. and Curtis) Berl. and De Toni, thereafter colonizing treated plants and eventually reducing symptoms of grapevine downy mildew [14,25].

Our study has focused on determining the effectiveness of both genetic resistance and biological control as sustainable methods for the management of sunflower downy mildew. Our objectives for this study were to: (i) conduct a survey to update the racial characterization of isolates of *P. halstedii* present in Europe, with particular emphasis on those from Spain; (ii) assess the efficacy of five strains of EF *M. brunneum* and *B. bassiana* against sunflower downy mildew; (iii) assess the effect of EF on the growth of sunflower plants; (iv) determine whether, or not, EF and *P. halstedii* simultaneously colonize inner tissues of sunflower.

2. Materials and Methods

2.1. Racial Characterization of Plasmopara halstedii

A total of 58 fields, in which sunflowers showed downy mildew symptoms, were sampled between 2011 and 2020 in Spain (49), France (3), Italy (3), Portugal (2), and Romania (1) (Table 1). Most of the samples were collected after unexpected disease outbreaks were observed. Sunflowers showing stunting and chlorosis in true leaves were collected from each field and taken to the laboratory. Samples consisting of tissue from more than one plant and collected in the same field location were treated as one. Following the methodology by our research group [6,9,26], infected leaves were excised, placed in humid chambers and incubated in darkness at 15 °C for 24–48 h until profuse sporulation occurred on the leaves. Then, leaves showing sporulation were immersed in sterile deionized water and vigorously shaken for 5 min until a zoosporangial suspension was obtained. The suspension was filtered through two layers of sterile gauze and homogenized by vortex.

The concentration of the suspension was adjusted to 4×10^4 zoospores mL^{-1} using a hemocytometer, specifically the Neubauer chamber (Blau Brand, Wertheim, Germany).

Table 1. List of sunflower samples collected in Europe, and included in this study, between 2011 and 2020.

Sample Number	Sample ID	N. of Plants	Collection Year	Sunflower Genotype	Geographic Origin. Location (Province). Country [a]
1	DM01-11	2	2011	Transol/2-4	Alameda del Obispo (Cordoba). SP
2	DM02-11	1	2011	Transol/18-2	Alameda del Obispo (Cordoba). SP
3	DM03-11	2	2011	102/SIg	Marchena (Seville). SP
4	DM04-11	3	2011	Bosfora	Fuentes de Andalucía (Seville). SP
5	DM05-11	3	2011	Transol	Fuentes de Andalucía (Seville). SP
6	DM01-13	1	2013	C1/DBa	Las Cabezas de San Juan (Seville). SP
7	DM02-13	2	2013	B2/DBa	Las Cabezas de San Juan (Seville). SP
8	1978-R1	2	2014	- [b]	Tomejil (Seville). SP
9	DB0	-	2014	-	Las Cabezas de San Juan (Seville). SP
10	LGB	1	2014	LG560440	Bornos (Cadiz). SP
11	1SM	3	2014	Experimental line	Sao Matias (Beja). PO
12	2SEP	2	2014	Experimental line	Serpa (Beja), PO
13	DM01-15	2	2015	-	Cerro Perea (Seville). SP
14	DM02-15	1	2015	-	Fuentes de Andalucía (Seville). SP
15	DM03-15	1	2015	-	Fuentes de Andalucía (Seville). SP
16	DM04-15	4	2015	-	Jerez de la Frontera (Cadiz). SP
17	DM01-16	2	2016	-	Fuentes de Andalucía (Seville). SP
18	DM02-16	-	2016	-	Alcalá del Río (Seville). SP
19	DM01-17	2	2017	LG5485	Montellano (Seville). SP
20	B-B190418	4	2018	Experimental line	Utrera (Seville). SP
21	B-S040518-1	4	2018	Experimental line	Alcalá del Río (Seville). SP
22	B-S040518-2	4	2018	Experimental line	Viso del Alcor (Seville). SP
23	B-S100518	4	2018	Experimental line	El Arahal (Seville). SP
24	B-B180518	4	2018	Experimental line	Cañada Rosal (Seville). SP
25	B-B210518-1	-	2018	Solnet	Espera (Cadiz). SP
26	B-B210518-2	-	2018	Solnet	Alcalá Guadaira (Seville). SP
27	B-B210518-3	-	2018	Bonasol	Utrera (Seville). SP
28	B-B220518	1	2018	Solnet	Huévar (Seville). SP
29	B-S230518	2	2018	Syedison	Palma del Río (Cordoba). SP
30	B-B290518-1	1	2018	Solnet	Trigueros (Seville). SP
31	B-B290518-2	1	2018	Solnet	El Trobal (Seville). SP
32	B-B300518	-	2018	Solnet	Paradas (Seville). SP
33	B-LG310518-1	3	2018	Experimental line	Las Cabezas de San Juan (Seville). SP
34	B-LG310518-2	3	2018	Experimental line	Lebrija (Seville). SP
35	B-LG310518-3	2	2018	Experimental line	Maribáñez (Seville). SP
36	B-F050618	1	2018	-	Escacena del Campo (Huelva). SP
37	B-E050618-1	2	2018	Artic	Paterna del Campo (Huelva). SP
38	B-E050618-2	1	2018	Artic-T	Paterna del Campo (Huelva). SP
39	B-P080618	2	2018	-	Tulcea, RO
40	B-B120618	-	2018	Bonasol	La Campana (Seville). SP
41	B-P130618-1	1	2018	64LE25	Cortona, IT
42	B-P130618-2	1	2018	64LE25-T	Cortona, IT
43	B-P130618-3	3	2018	XF16942	Cortona, IT
44	B-P130618-4	2	2018	P64LE99	Mauroux, FR
45	B-P130618-5	3	2018	P64LE25	Taybosc, FR
46	B-P130618-6	1	2018	P64LE25	Lectoure, FR
47	B-LG140618	2	2018	Experimental line	Écija (Seville). SP
48	B-S190618	2	2018	Experimental line	Olivares (Seville). SP
49	B-S280618	1	2018	-	Escacena del Campo (Huelva). SP
50	B-S230419 (SYT-1)	-	2019	M4	Villamanrique de la Condesa (Seville). SP
51	B-S080519-1 (2019HU01)	3	2019	-	Palma del Condado (Seville). SP
52	B-S080519-2 (2019-SE01)	3	2019	-	Gerena (Seville). SP
53	B-S230519-1 (GIB-1)	-	2019	M4	Villarrasa (Huelva). SP
54	B-S230519-2 (GIB-2)	-	2019	M4-T	Villarrasa (Huelva). SP
55	B-S190520-1	3	2020	M4	Fernán Núñez (Cordoba). SP
56	B-S190520-2	2	2020	M9	Pedro Abad (Cordoba). SP
57	B-S270520	1	2020	-	Andújar (Jaen). SP
58	Ph04-20	2	2020	M4	Calzada de Bureba (Burgos). SP

[a] SP = Spain, PO = Portugal, RO = Romania, IT = Italy, FR = France. [b] - = unknown.

The racial characterization of the *P. halstedii* isolates was conducted by calculating the coded virulence formula (CVF) using the method described by Gulya et al. [27]. The CVF is a 3-digit number based on the resistant/susceptible reaction of three sets of three sunflower lines each (differentials) to the inoculation with *P. halstedii* zoospore suspensions. The sets are as follows: set 1 (HA-304; RHA-265; RHA-274), set 2 (PMI3; PM17; 803-1) and set 3 (HAR-4; QHP1; HA-335) [27]. Within each set, resistant reactions have a value of 0 and susceptible reactions have values of 1 (for differential 1), 2 (for differential 2) or 4 (for differential 3) [11,27]. The addition of these values for each set results in the 3-digit CVF. In

addition to the nine differentials, isolates of *P. halstedii* were also inoculated into the inbred line RHA-340, which carries a highly effective genetic resistance against *P. halstedii* [28]. Seeds of each of the sunflower genotypes were germinated prior to inoculation with *P. halstedii* according to the methodology followed by our group [19]. Briefly, a thin layer of perlite was put into Petri plates and covered with sterile filter paper previously moistened with sterile deionized water. Seeds were disinfested by a 5-min immersion in 10% sodium hypochlorite and three subsequent rinses in sterile deionized water and then they were air-dried in a sterile laminar flow cabinet for 5 min and placed into the plates. The seeds were incubated in darkness at 24 °C for 48 h until 2–8 mm long- radicles emerged. Then, pericarps and seminal membranes were removed to prevent contamination. For each differential and isolate, two replicates of 10 seedlings each were immersed in 20 mL of suspensions of 4×10^4 sporangia mL^{-1} for 4 h at 18 °C in darkness. Each replicate was transplanted into one 0.7-l pot filled with sterile perlite. Plants were incubated in a growth chamber at 20 °C and a 12-h photoperiod for 12 days, until expansion of the first pair of true leaves. Then, they were incubated for 48 h at 100% relative humidity under the same conditions of temperature and light to induce pathogen sporulation. The reaction of each differential line to each isolate of *P. halstedii* was assessed on the 14th day after inoculation. It was recorded as being susceptible if profuse sporulation appeared on cotyledons and/or true leaves or as resistant if no sporulation occurred [6]. The inoculation tests were performed twice, or even a third time if any of the reactions were not clear.

2.2. Effect of Entomopathogenic Fungi on Sunflower Downy Mildew and on the Growth of Sunflower

The effect of five EF strains against *P. halstedii* and on sunflower was studied in two experiments that were conducted under controlled conditions. In the first one, the effect of entomopathogens on the development of symptoms of downy mildew in sunflowers was assessed. In the second experiment, we analyzed whether entomopathogenic fungi have a particular effect on the development of sunflower plants in the absence of *P. halstedii*.

2.2.1. Biological Materials: Plasmopara halstedii and Entomopathogenic Fungi

According to the results of the experiments described in 2.1, the isolate 1SM of *P. halstedii* was used (Table 1). This isolate was characterized as race 304 (Table 2) and routinely maintained by subsequent inoculations of seedlings of the inbred line HA-304, which is susceptible to all races of the pathogen.

In addition, the five EF strains included in this study were EAMa 01/58-Su, EAMb 09/01-Su and EAMb 01/158-Su (*M. brunneum*), and EABb 01/33-Su and EABb 04/01-Tip (*B. bassiana*). They were selected based on their efficacy against other sunflower pathogens [19] and they are deposited in the Entomopathogenic Fungi Collection (CRAF) at the University of Cordoba (Spain) and the Spanish Collection of Culture Types (CECT) at the University of Valencia (Spain) [23]. More information on their origin and other traits of interest is available in Miranda-Fuentes et al. [19].

Table 2. Racial characterization of isolates of *Plasmopara halstedii* collected in Europe from year 2011 to 2020.

Sample ID	Sample Number	Isolate Reference Number [1]	Race [2]	Reaction of RHA-340 Line [3]	Sample ID	Sample Number	Isolate Reference Number	Race	Reaction of RHA-340 Line
DM01-11	1	Ph01-11	100	R	B-B290518-1	30	Ph11-18	110	R
DM02-11	2	Ph02-11	310	R	B-B290518-2	31	Ph12-18	310	R
DM03-11	3	Ph03-11	310	R	B-B300518	32	Ph13-18	114	R
DM04-11	4	Ph04-11	710	R	B-LG310518-1	33	Ph14-18	315	R
DM05-11	5	Ph05-11	710	R	B-LG310518-2	34	Ph15-18	311	R
DM01-13	6	Ph01-13	304	R	B-LG310518-3	35	Ph16-18	711	R
DM02-13	7	Ph02-13	304	R	B-F050618	36	Ph17-18	304	R
1978-R1	8	1978-R1	710	R	B-E050618-1	37	Ph18-18	312	R

Table 2. *Cont.*

Sample ID	Sample Number	Isolate Reference Number [1]	Race [2]	Reaction of RHA-340 Line [3]	Sample ID	Sample Number	Isolate Reference Number	Race	Reaction of RHA-340 Line
DB0	9	DB0	700	R	B-E050618-2	38	Ph19-18	312	R
LGB	10	LGB	713	R	B-P080618	39	Ph20-18 *	705	R
1SM	11	1SM * [4]	304	R	B-B120618	40	Ph21-18	711	R
2SEP	12	2SEP *	700	R	B-P130618-1	41	Ph22-18 *	301	R
DM01-15	13	Ph01-15	310	R	B-P130618-2	42	Ph23-18 *	715	S
DM02-15	14	Ph02-15	310	R	B-P130618-3	43	Ph24-18 *	715	S
DM03-15	15	Ph03-15	310	R	B-P130618-4	44	Ph25-18 *	715	S
DM04-15	16	Ph04-15	314	R	B-P130618-5	45	Ph26-18 *	715	S
DM01-16	17	Ph01-16	705	R	B-P130618-6	46	Ph27-18 *	715	S
DM02-16	18	Ph02-16	704	R	B-LG140618	47	Ph28-18	114	R
DM01-17	19	Ph01-17	317	R	B-S190618	48	Ph29-18	714	R
B-B190418	20	Ph01-18	305	R	B-S280618	49	Ph33-18	304	R
B-S040518-1	21	Ph02-18	715	R	B-S230419 (SYT-1)	50	Ph01-19	705	R
B-S040518-2	22	Ph03-18	714	R	B-S080519-1 (2019HU01)	51	Ph02-19	715	R
B-S100518	23	Ph04-18	315	R	B-S080519-2 (2019-SE01)	52	Ph03-19	715	R
B-B180518	24	Ph05-18	311	R	B-S230519-1 (GIB-1)	53	Ph06-19	505	R
B-B210518-1	25	Ph06-18	311	R	B-S230519-2 (GIB-2)	54	Ph07-19	705	R
B-B210518-2	26	Ph07-18	712	R	B-S190520-1	55	Ph01-20	304	R
B-B210518-3	27	Ph08-18	313	R	B-S190520-2	56	Ph02-20	714	R
B-B220518	28	Ph09-18	313	R	B-S270520	57	Ph03-20	715	R
B-S230518	29	Ph10-18	315	R	Ph04-20	58	Ph04-20	705	R

[1] An individual code was assigned to each isolate of *Plasmopara halstedii*, representing a population collected in a single sunflower field. [2] Race was determined according to the coded virulence formula (CVF) described by Gulya et al. [26], each of the three digits corresponding to the response of three sets of three sunflower differentials to inoculation with a *P. halstedii* isolate. The first differential within each set is coded with the number 1, the second differential with 2 and the third one with 4 if a susceptible reaction occurs, whereas resistant reactions are given a value of 0. The sum of all reactions in each set is obtained and its value assigned to each of the three digits of the formula. [3] Reactions were quantified as resistant (R) or susceptible (S). [4] * Asterisks show isolates from countries other than Spain.

2.2.2. Effect of Entomopathogenic Fungi on Sunflower Downy Mildew

Seeds of HA-304 were germinated under controlled conditions as described in 2.1 for 48 h until they had 2–8-mm long radicles, then their pericarps and seminal membranes were removed. The seedlings were sown in sterile glass tubes (one seedling per tube) each containing 40 mL of Hoagland and Knop's culture medium (components per liter of deionized water: agar (7.1 g), $Ca(NO_3)_2 \cdot 4H_2O$ (0.95 g), KNO_3 (0.61 g), $MgSO_4 \cdot 7H_2O$ (0.49 g), $NH_4H_2PO_4$ (0.12 g), $C_6H_5FeO_7$ (0.02 g), $MnSO_4 \cdot H_2O$ (2.273 mg), $ZnSO_4 \cdot 7H_2O$ (0.5 mg), $CuSO_4 \cdot 5H_2O$ (0.025 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.025 mg), H_2SO_4 98% (0.5 µL); pH adjusted to 5.7 using NaOH).

Once the 48-h-old seedlings had been sown on the medium, they were treated with the EF and, thereafter, inoculated with *P. halstedii*. In the case of EF, conidial suspensions were prepared according to Miranda-Fuentes et al. [19]: firstly, the five EF strains were plated by streaking a loop of conidial suspensions onto potato dextrose agar (PDA) plates (BD, New Jersey, USA), and incubated at 24 °C in darkness for 7 days. Then, the mycelium of each strain was recovered using a sterile Drigalski spatula and suspended in 5 mL of sterile deionized water. The suspensions were filtered through sterile gauze to separate the mycelium and homogenized by vortex. The conidia concentration of the suspension was adjusted to 10^7 conidia mL^{-1}. The seedlings in the tubes were treated with 5 µL of EF conidial suspensions and inoculated with the same volume of zoosporangial suspension of *P. halstedii* 24 h later in sterile conditions. Zoosporangial suspensions were prepared as described in 2.1, with the concentration adjusted to 4×10^4 zoospores mL^{-1}. Control plants were inoculated with *P. halstedii* as described 24 h after being treated with 5 µL of sterile deionized water instead of EF.

In order to allow both a higher humidity and gas exchange, the tubes were sealed with laboratory film into which an 8-mm hole was perforated. The experimental unit consisted of one seedling, and four replications were established for each treatment following a completely randomized design. Seedlings were incubated in a growth chamber at 24 °C with a photoperiod of 14 h of light for 14 days. Then, the severity of downy mildew in each

plant was assessed by using a percentage scale from 0% (no symptoms) to 100% (cottony fungal growth completely covering the cotyledons and first true pair of leaves and evident in the base of the stem). Additionally, we measured the height, length of root system and dry weight of both the shoot and root system of each plant at the end of the experiment. Dry weights were recorded once plants were dried at 60 °C for 96 h, right after the 14 days of incubation.

2.2.3. Effect of Entomopathogenic Fungi on Sunflower Growth

In order to assess the possible effects of treatments with EF on the sunflowers' growth in the absence of *P. halstedii*, an additional experiment was devised. Forty-eight-hour-old seedlings of the same inbred line used in the experiments described in 2.2.2 (HA-304) were sown on Hoagland and Knop's culture medium as described above. Immediately afterwards, they were treated with the five EF strains. Specifically, plants were treated with 5 µL of conidial suspensions of each fungal strain, previously adjusted to 10^7 conidia mL^{-1}. Control plants were treated with 5 µL of sterile deionized water. The experimental design and the number of replications were similar to those described in 2.2.2. Plants were incubated for 14 days. At the end of the experiment, they were uprooted and the height, length of root system and dry weight of both the shoot and the root system of each plant was recorded. Although their height and root length were assessed immediately after the plants had been uprooted, dry weights were recorded once plants had been dried at 60 °C for 96 h, right after the end of the experiment.

2.2.4. Microbiological and Molecular Detection

The colonization of the plants by the five EF was assessed at the end of the experiments, i.e., 14 days after treatment with EF. The plants from each treatment were processed as follows: one 8-mm leaf disc was cut from the first pair of true leaves of each plant using a sterile puncher. One 1-cm fragment of roots and a similar fragment of the stem were also excised using a scalpel. Leaf discs and root and stem fragments were disinfested by a 5-min immersion in 1% NaClO and two rinses in sterile deionized water, then air-dried and plated onto Sabouraud dextrose agar + chloramphenicol (0.5 g L^{-1}) (Scharlab, S. L., Spain). The plates were sealed with laboratory film and incubated at 24 °C in darkness for 15 days and, during this time, the presence or absence of fungal growth of *M. brunneum* or *B. bassiana* in each fragment was assessed daily. Colonization was expressed as the percentage of plated samples showing EF growth.

Moreover, at the end of the experiments, molecular diagnostic analyses were conducted following the methodology of Miranda-Fuentes et al. [19]. First, all of the plants from both experiments were surface sterilized and lyophilized. Then, total genomic DNA from each plant was extracted and purified using the i-genomic Plant DNA Extraction NucleoSpin® Plant II (Macherey-Nagel GmbH & Co. KG, Valencienner Str. 11, 52355 Düren, Germany) kit following the manufacturer's instructions. Both the quality and concentration of the purified DNA were determined with a fluorometer (Qubit 3.0, Invitrogen) and the DNA samples were adjusted to a final concentration of 10 ng μL^{-1}. In the *P. halstedii*–EF experiment, the identity of *P. halstedii* was confirmed by amplification of the nuclear DNA coding for the large ribosomal unit (28S rDNA) using LR0R and LR6-O primers [29]. The presence of EF as endophytes in sunflower tissues was confirmed by amplification of the region consisting of the 5.8S ribosomal DNA and internal transcribed spacers 1 and 2 using the primer set ITS5/ITS4 [30]. Optimized PCR assays were carried out in a final volume of 25 µL containing 0.4 µM of each primer, 800 µM dNTPs, 2.5 µL 10 × PCR buffer (800 mM Tris-HCl, pH 8.3–8.4 at 25 °C, 0.2% Tween 20 *w/v*), 0.75 U Horse-Power™ Taq DNA polymerase (Canvax Biotech), 2.5 mM $MgCl_2$ and 20 ng DNA. The following profile was used for the amplifications of the ITS region: 3 min initial denaturation at 95 °C; 30 cycles of 30 s denaturation at 56 °C, 2 min of annealing at 72 °C and 30 s extension at 95 °C; and a final extension step of 10 min at 72 °C. The profile for the amplification of the *P. halstedii* specific region was as follows: 3 min initial denaturation at 95 °C; 30 cycles of 1 min at 94 °C

denaturation, 45 s at 50 °C annealing, extension for 1 min at 72 °C; a final extension step of 7 min at 72 °C. As positive DNA controls, we used mycelial DNA samples of the five EF grown on PDA, of isolate 1SM of *P. halstedii*, and of sunflower. Sterile deionized water was used as a negative amplification control. Polymerase chain reactions were conducted using a T1 thermocycler (Whatman Biometra). Lastly, amplification products were separated by horizontal electrophoresis in 3.5% agarose gels containing 0.05 µL mL^{-1} GoodView™ nucleic acid stain (SBS Genetech Co., Ltd., Room 202, Building 2, No.1 Shangdi 4th Street 100085 Beijing, China) and visualized over a UV light source. A 100–2000 bp BrightMAX™ DNA ladder (Canvax Biotech) was included in the electrophoresis.

2.2.5. Data Analysis

All of the experiments were conducted twice. In each of them sunflower seed from different lots and different biological replicates of the five EF strains and 1SM isolate (in the EF–*P. halstedii*) were used. In each of the two replicate experiments, since the interaction between replicates when the experiments were combined was not significant [31], the data were pooled and analyzed using Statistix® 10 (Analytical Software, Tallahassee, FL, USA). Percentage severity of downy mildew was transformed using the arcsine transformation, $Y = arcsine\sqrt{\frac{severity}{100}}$ and subjected to Analysis of Variance (ANOVA). In the case of the length and the dry weight of the shoot and the root system, the data were not transformed. Prior to the analysis, the data were checked for ANOVA's requirements: homogeneity of variances (Brown and Forsythe test), normality (Shapiro-Wilk test), and independence of residues (graphical test). Means from different treatments were compared using Fisher's Least significant difference (LSD) test ($\alpha = 0.05$) when significant differences were obtained.

3. Results

3.1. Racial Characterization of Plasmopara halstedii

The nine differential lines inoculated with all of the isolates showed different patterns of resistant/susceptible reactions, thus allowing the racial characterization of all of the *P. halstedii* isolates (Table 2).

A total of 22 races were identified in Spain between 2011 and 2020. The most frequent races in the country were 310 (12.2% of the isolates), 304 (10.2%), 705 (8.2%), 715 (8.2%), and races 311, 315, 710 and 714 (6.1% each), whereas only one (2.0%) or two (4.1%) isolates of the rest of the races were found.

We identified highly virulent races of *P. halstedii* in Spain throughout the duration of the study since, as previously mentioned, the isolates mostly came from samples collected in fields where unexpected downy mildew outbreaks had occurred. Twenty-four of the isolates overcame the Pl_1 resistance gene in RHA-265, showing a 3 as the first CVF digit. Additionally, 20 isolates overcame both Pl_1 in RHA-265 and Pl_2 in RHA-274, and therefore showed a 7 as the first digit. Lastly, four of the isolates were able to only infect the susceptible line HA-304, thus having a 1 as first digit, and one isolate had a 5, since it was controlled by Pl_1 (RHA-265), but not by Pl_2 (RHA-274). Differentials PM17 and 803-1 were resistant to all of the isolates and therefore their second digit was assigned a 0 (14 isolates, to which PMI3 was resistant) or a 1 (35 isolates, to which PMI3 was susceptible). The third digit of the CVF was the most diverse one. The three differentials of the third set were resistant to 12 isolates, which gave a 0. Three isolates had a 3, due to the susceptibility of a HAR-4 and QHP1, and 14 isolates had a 4, since only HA-335 was susceptible in the third set. Finally, 13 of the isolates were assigned a 5, since they overcame the resistance of both HAR-4 and HA-335.

With regard to the eight isolates of *P. halstedii* obtained from countries other than Spain, almost all of them were of highly virulent races. The two isolates collected in Portugal were of races 304 and 700. The only isolate from Romania was of race 705. The three isolates from France belonged to race 715. Two of the isolates from Italy belonged to race 715, whereas the other one was of race 301. Furthermore, this race was not found in any of the isolates collected in Spain (Table 2). In this group, we identified the highest virulence

of *P. halstedii*. With the exception of 1SM and Ph22-18, all of the remaining isolates were able to overcome Pl_1 (RHA-265), Pl_2 (RHA-274), Pl_{PMI13} (PMI3), and Pl_6 (HA-335), thus having a 7 and a 5 in the first and third digits, respectively. Importantly, the five isolates characterized as race 715 (two from Italy and three from France) were also able to infect differential RHA-340, previously considered resistant to all races. These results have been published recently [26].

3.2. Effect of Entomopathogenic Fungi on Sunflower Downy Mildew

The severity of downy mildew caused by isolate 1SM in the susceptible line HA-304 varied between 57% (strain EAMb 09/01-Su) and 97% (EAMa 01/58-Su), but no significant differences were observed when compared to the severity in the control plants inoculated only with *P. halstedii* (65%) (Figure 1).

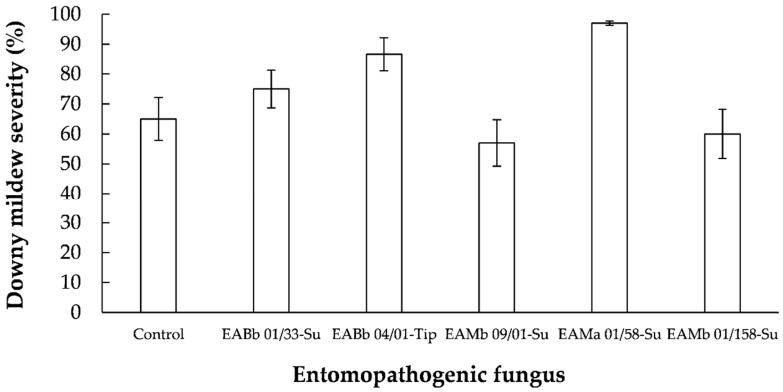

Figure 1. Downy mildew severity (%) in sunflower plants inoculated with *Plasmopara halstedii* and treated with five entomopathogenic fungi. Control plants were inoculated with *P. halstedii* and treated with water. Vertical bars represent the standard error of the mean of eight replications. The severity of downy mildew in each plant was assessed by using a percentage scale from 0% (no symptoms) to 100% (cottony fungal growth completely covering the cotyledons and first pair of leaves, and evident in the base of the stem).

Additionally, the EF did not significantly influence sunflower plants in either shoot height, root length or dry weight of shoots and roots. Plant height and root length ranged from 7.3 (EABb 01/33-Su and EABb 04/01-Tip) and 6.9 cm (EAMb 01/158-Su), respectively, to 10.2 (EAMa 01/58-Su) and 9.3 cm (EAMb 09/01-Su). Dry weight of shoots varied between 0.55 g (EABb 01/33-Su) and 0.84 g (EAMb 09/01-Su). Dry weight of roots ranged from 0.13 g (EAMb 01/158-Su) to 0.21 g (EAMb 09/01-Su) (Table 3.2).

The inoculation with *P. halstedii* seemed to interfere with plant colonization by EF, as none of the strains consistently recovered from the stems, particularly the leaves of the plants in the presence of the oomycete. Similarly, the EF could not be diagnosed using PCR in any of the plants from the treatments with both EF and *P. halstedii*.

Table 3. Shoot height (cm), root length (cm), and shoot and root dry weight (g) of young sunflower plants treated with five entomopathogenic fungi and inoculated with isolate 1SM of *Plasmopara halstedii*. Seedlings were treated with 5 µL of conidial suspensions (10^7 conidia mL^{-1}) of each entomopathogenic fungus and, 24 h later, inoculated with the same volume of a zoospore suspension (4×10^4 zoospores mL^{-1}) of *P. halstedii*. The controls were treated with 5 µL of sterile deionized water instead of entomopathogenic suspensions, followed by inoculation with *P. halstedii* 24 h later. Treatment and control plants were grown on Hoagland and Knop's culture medium in a growth chamber at 24 °C with a photoperiod of 14 h of light for 14 days.

Entomopathogenic Fungus (EF)	Shoot Height [1] (cm)	Root Length (cm)	Shoot Dry Weight [2] (g)	Root Dry Weight (g)
Control	9.06 ± 1.55 [3]	9.11 ± 0.86	0.62 ± 0.15	0.16 ± 0.05
EABb 01/33-Su	7.33 ± 1.26	8.78 ± 0.67	0.55 ± 0.12	0.15 ± 0.03
EABb 04/01-Tip	7.33 ± 1.44	9.20 ± 1.03	0.58 ± 0.16	0.18 ± 0.04
EABb 09/01-Su	9.83 ± 1.70	9.30 ± 0.67	0.84 ± 0.19	0.21 ± 0.04
EAMa 01/58-Su	10.23 ± 1.99	7.73 ± 0.86	0.58 ± 0.15	0.15 ± 0.03
EAMb 01/158-Su	8.69 ± 1.84	6.91 ± 0.86	0.58 ± 0.16	0.13 ± 0.04

[1] Lengths were assessed 14 days after treatment. [2] For dry weight evaluation, plants were dried at 60 °C for 96 h 14 days after treatment. [3] Mean value of eight replications ± standard error.

3.3. Effect of Entomopathogenic Fungi on Sunflower Growth

Plants were significantly affected by the treatments with EF in the four variables estimated: shoot height ($p = 0.0011$), root length ($p = 0.0424$), shoot dry weight ($p = 0.0007$) and root dry weight ($p = 0.0408$).

The strain EABb 01/33-Su caused the lowest values in the four variables of plant growth. Moreover, it was the only treatment significantly different from the others both in height and in dry weight of the shoot, whereas strains EAMb 09/01-Su and EABb 04/01-Tip were significantly different from the other treatments in both length and weight of root system, respectively (Table 4). The height of the plants and length of their roots ranged from 9.83 and 9.17 cm (EABb 01/33-Su), respectively, to 14.61 (averaged across all of the treatments except EABb 01/33-Su) and 11.57 cm (averaged across the Control, EABb 04/01-Tip, EAMa 01/58-Su and EAMb 01/158-Su), respectively. Dry weight of shoots of the plants varied between 0.67 g (EABb 01/33-Su) and 1.11 g (average for all of the remaining treatments). Dry weight of roots ranged from 0.24 g (EABb 01/33-Su) to 0.39 g (Control) (Table 4).

The recovery percentages of the five EF when plants were only treated with the EF are shown in Table 5. The five EF strains were isolated from the sunflower plants, although with percentages of isolation that varied between plant part and EF strain. Moreover, strains EABb 04/01-Tip and EAMb 01/158-Su recovered from all parts of the plant (Table 5). The molecular analyses showed that strains EABb 04/01-Tip and EAMa 01/58-Su successfully colonized the inside of the stems and were able to persist at least until the end of the experiment (14 days), since diagnostic bands of the EF were observed in samples of the four replications (individual plants). These EF-specific lower molecular weight bands were simultaneously visible with the 750 bp from the DNA of sunflower (Figure 2). In the case of strains of *Metarhizium* EAMb 09/01-Su and EAMb 01/158-Su, the diagnostic band was only observed in one of the four replicates (Figure 2). Finally, no endophytic ability was detected for the *B. bassiana* strain EABb 01/33-Su, since the band diagnosing this species was not observed in any of the plants.

Table 4. Height (cm), length of root system (cm), and dry weight (g) of shoot and root system of sunflower young plants treated with five strains of entomopathogenic fungi. Seedlings were treated with 5 µL of conidial suspensions (or 5 µL of sterile deionized water (Control)) and grown on Hoagland and Knop's culture medium in a growth chamber at 24 °C with a photoperiod of 14 h of light for 14 days.

Entomopathogenic Fungus (EF)	Shoot Height [1] (cm)	Root Length (cm)	Shoot Dry Weight [2] (g)	Root Weight (g)
Control	14.72 ± 0.83 [3] a	12.33 ± 0.93 a	1.15 ± 0.06 a	0.39 ± 0.03 a
EABb 01/33-Su	9.83 ± 1.54 b	9.17 ± 1.16 c	0.67 ± 0.09 b	0.24 ± 0.03 c
EABb 04/01-Tip	13.03 ± 0.94 a	11.10 ± 0.49 abc	0.98 ± 0.09 a	0.28 ± 0.02 bc
EABb 09/01-Su	15.44 ± 0.73 a	9.99 ± 0.62 bc	1.20 ± 0.13 a	0.32 ± 0.05 abc
EAMa 01/58-Su	15.07 ± 0.70 a	11.64 ± 0.29 ab	1.09 ± 0.07 a	0.32 ± 0.04 abc
EAMb 01/158-Su	14.80 ± 0.74 a	11.22 ± 0.49 abc	1.13 ± 0.05 a	0.33 ± 0.02 ab

[1] Lengths were assessed 14 days after treatment. [2] For dry weight evaluation, plants were dried at 60 °C for 96 h 14 days after treatment. [3] Mean value ± standard error. Data were analyzed separately for each of the four variables. In each column, means followed by different letters are significantly different according to Fisher's Least Significant Difference test ($p = 0.05$).

Table 5. Isolation of five endophytic entomopathogenic fungi (EF) on Sabouraud dextrose agar from roots, stem, and leaves of sunflower young plants treated with the aforementioned EF or with sterile deionized water (Control). Seedlings were treated and grown on Hoagland and Knop's culture medium in a growth chamber at 24 °C, with a photoperiod of 14 h of light for 14 days before tissue samples were plated for fungal isolation. From each plant, one sample each of roots, stem, and leaves was plated.

Entomopathogenic Fungus	Roots	Stem	Leaves
Control	0.0 [1]	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	22.22	0.0
EAMa 01/58-Su	20.0	0.0	0.0
EAMb 01/158-Su	20.0	30.0	20.0

[1] Isolation was expressed as the percentage (%) of plated tissue samples that showed EF growth.

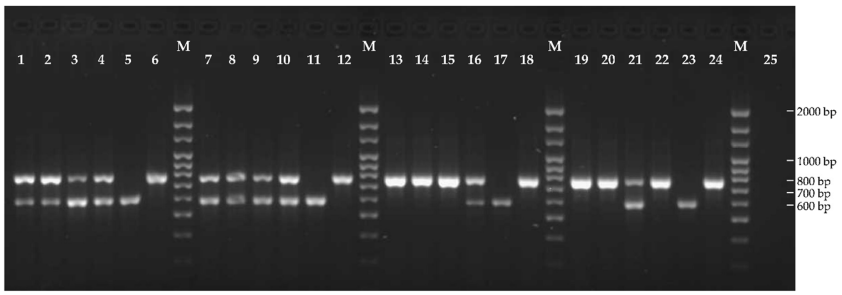

Figure 2. Bands from PCR amplification of the ribosomal DNA region (5.8S rDNA and internal transcribed spacers 1 and 2) with the universal primer set ITS5 and ITS4 from individual sunflower plants only treated with entomopathogenic fungi (EF). The higher molecular weight band is sunflower-specific, and the lower molecular weight band is EF-specific. M, 100–2000 bp BrightMAX^{TM} DNA ladder (Canvax Biotech); Lanes 1–4, DNA from four individual plants of sunflower breeding line HA-304 treated with *Beauveria bassiana* strain EABb 04/01-Tip; Lane 5, DNA of EABb 04/01-Tip; Lane 6, DNA of sunflower HA-304; Lanes 7–10, DNA from four individual plants of sunflower breeding line HA-304 treated with *Metarhizium brunneum* strain EAMa 01/58-Su; Lane 11, DNA of EAMa 01/58-Su; Lane 12, DNA of sunflower HA-304; Lanes 13–16; DNA from four individual plants of sunflower breeding line HA-304 treated with *M. brunneum* strain EAMb 09/01-Su; Lane 17, DNA of EAMb 09/01-Su; Lane 18, DNA of sunflower HA-304; Lanes 19–22, DNA from four individual plants of sunflower breeding line HA-304 treated with *M. brunneum* strain EAMb 01/158-Su; Lane 23, DNA of EAMb 01/158-Su; Lane 24, DNA of sunflower HA-304; Lane 25, negative control of PCR amplification.

4. Discussion

At least 42 races of *P. halstedii* have been identified around the world [32]. What is of interest in our study is that it shows that over 50% of those races, i.e., 22, have been found in Spain between 2011 and 2020, which constitutes an important increase in races compared to previous studies [6,11], and reveals that the pathogen has evolved in terms of both diversity and pathogenicity. In fact, Viranyi et al. [33] suggested that the increasing numbers of reports of new races indicated that *P. halstedii* shows evolution for virulence according to selection pressure from its host. The large number of races identified in Spain in this study demonstrates that racial profiles of downy mildew are shifting, as suggested by recent studies [2,9]. Among all of the isolates sampled in the country in this work, race 310, which was the most common one between 1994 and 2006 [6,11], was frequently identified. Currently, race 310 is less frequent, since it was found only in 12.2% of the isolates sampled between 2011 and 2020. Moreover, this race was scarcely found from 2015 on, when several other new races, e.g., 311 and 705, had a significant presence. In fact, in this study we describe for the first time the presence of 13 races of *P. halstedii* in Spain: 304, 305, 311, 313, 314, 315, 317, 704, 705, 712, 713, 714, and 715. Notwithstanding the limited number of sunflower isolates from countries other than Spain, we found that races 304 and 700 are present in Portugal, whereas race 705 is present in Romania, race 301 is present in Italy, and race 715 is present in Italy and France. To the best of our knowledge, and although the four races were described in other European countries [32,33], none of them had been reported in any of the four countries before [33,34].

Particularly interesting is the identification of a number of races overcoming the Pl_6 resistance gene present in HA-335 and noted with a 4, 5, 6 or 7 as the last digit. According to our results for Spain, race 304 of *P. halstedii* existed in the country in as early as 2013. Similarly, the new race 705 had already been recovered in 2016 [9]. Overall, 55% of the isolates characterized in this study (32 out of 58 isolates) were capable of infecting the resistant line HA-335. This was the case with several isolates from Spain and one (race 304) from Portugal, but also with all of the isolates from Romania (race 705), Italy (race 715), and France (race 715). In addition, all those recovered in Spain in 2019 and 2020 were also pathogenic to HA-335 (races 304, 505, 705, 714 and 715). Of notice, sunflower RHA-340 is resistant to isolates of race 715 from Spain whereas it is susceptible to isolates of race 715 from France and Italy. This shows that isolates having the same code (i.e., 715) can lead to different reactions in some genotypes that are widely used in sunflower breeding for resistance to downy mildew (i.e., RHA-340) and suggests the need of updating the nine differentials that were internationally agreed upon more than 20 years ago to be utilized for race identification of *P. halstedii*. In any case, our results agree with those of other authors from the Czech Republic and Hungary [32,35,36] and confirm that the previously suggested widening of distribution of highly virulent *P. halstedii* races [2] is a reality in Europe.

When we assessed the effect of five EF strains against downy mildew in sunflower in an axenic culture, none of them exerted a significant disease reduction effect. In a previous study in which we challenged *V. dahliae* and *C. helianthi* of sunflower with the same five EF and under in vitro conditions (plates with culture medium), we found that strains EABb 01/33-Su (*B. bassiana*) and EAMb 09/01-Tip (*M. brunneum*) were able to inhibit the mycelial growth of both sunflower pathogens [19]. Furthermore, these strains significantly reduced Verticillium wilt severity in sunflowers grown in the greenhouse for 9 weeks [19]. Several studies proved that strains of the EF *B. bassiana* significantly reduced downy mildew incidence and severity in grapevine after leaf sprays [14,25]. Whereas none of the five EF strains had an effect on sunflower growth when combined with *P. halstedii*, some of them reduced some of the growth parameters when applied alone. Similarly, short-term lower vegetative growth was reported in sorghum [23] and wheat plants [37,38] 2 to 4 weeks after being treated with two of the strains used in this study (*B. bassiana* EABb 04/01-Tip and *M. brunneum* EAMa 01/58-Su). Those authors suggested that detrimental effects of the EF on plants could be caused by direct competition between plant and EF for nutrients [23], as well

as by fungal detraction of carbon from photosynthesis [37]. Furthermore, treatments with EF may cause stresses, thus jeopardizing the performance of crops [37–40]. However, most of the aforementioned stresses are produced in early stages of plant development and their effects disappear at the end of the plant cycle [23,37,38,41], causing higher yields [37,38] or more pronounced vegetative growth [23,41]. Protection against plant pathogens can even be conferred in late stages of plant growth [19]. Since our experiments were carried out in an in vitro axenic culture, within a short time-lapse (14 days) and with only 24 h elapsing between the EF application and inoculation with *P. halstedii*, these conditions prevented the EF from fully expressing their potential. Our previous study reported the efficacy of EF against Verticillium wilt following their application to the soil one month before inoculation with *V. dahliae* [19]. Moreover, these EF strains are highly adapted to the soil environment [42]. The effect of the EF included in this study against sunflower downy mildew could probably be significant under experimental conditions that favor their survival and competence in the growth substrate, and these should be explored in future investigations.

Regarding the endophytic behavior of the EF used in this study, *B. bassiana* EABb 04/01-Tip and *M. brunneum* EAMa 01/58-Su were able to consistently colonize the sunflower plants. It is worthwhile mentioning that we worked under axenic conditions and that the colonization of young plants by EF was assessed 14 days after treatment. The endophytic property of these EF strains was previously reported in other plant species and systems, such as sorghum [23,41], melon [43], and wheat [37,38]. In the case of sunflower, plant colonization by these strains has already been reported through both microbiological [23] and molecular [19] approaches. Most studies focus on transient colonization of plants by EF after foliar spraying [43,44], but there is strong evidence of long-term endophytic colonization following soil treatment [19,23]. However, colonization patterns tend to be erratic and inconsistent, especially when assessed through the re-isolation of the EF from plant tissue [14,25]. Both colonization and endophytic behavior are largely dependent on the inoculation procedures, the host plant and the fungal species [23,25,37,38,41,44].

This study provides information on two relevant topics, viz. the racial distribution of *P. halstedii* in Spain and other European countries in recent years and the role of EF as endophytic colonizers of sunflower and possible future biological control agents against downy mildew. Regarding the first topic, information on the races of *P. halstedii* is an important decision-making tool for farmers, who have the option of choosing the hybrids they grow, based on their genetic resistance; and for sunflower breeders, who will find baseline information for their breeding programs. With respect to the second topic, EF could have a great potential for the integrated management of sunflower diseases, as they have shown significant effects against *V. dahliae* and *C. helianthi* [19]. This research on their efficacy against downy mildew is a first step in the biological control of the disease. Future research should elucidate whether some of the tested strains are effective against downy mildew under environmental conditions involving a soil system that favors their bioactivity. This issue appears to be a vital one due to the obligate nature of *P. halstedii* infections and to the strong dependence of *Metarhizium* and *Beauveria* species on soil ecology and characteristics.

Author Contributions: Conceptualization, L.M.-R. and P.M.-F.; methodology, P.M.-F. and A.B.G.-C.; formal analysis, P.M.-F. and L.M.-R.; resources, L.M.-R.; data curation, P.M.-F., A.B.G.-C., and L.M.-R.; writing—original draft preparation, P.M.-F. and L.M.-R.; writing—review and editing, P.M.-F., A.B.G.-C. and L.M.-R.; supervision, L.M.-R. All authors have read and agreed to the published version of the manuscript.

Funding: Spanish National Research Council project PIE201940E058 to L.M.-R., Spanish Ministry of Economy and Competitiveness project AGL2016-80483-R to E. Quesada-Moraga and Spanish Ministry of Education, Culture and Sports, grant number FPU16/03983 to P.M.-F.

Data Availability Statement: The data presented in this study are available by request from the corresponding author.

Acknowledgments: We thank E. Quesada-Moraga for providing the entomopathogenic fungi used in this work. Pedro Miranda-Fuentes is a PhD student from the Spanish Ministry of Education, Culture and Sports (grant number FPU16/03983).

Conflicts of Interest: The authors declare no conflict of interest.

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