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Characterization and virulence of *Lecanicillium lecanii* against different aphid species

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Abstract Seven isolates of *Lecanicillium lecanii* (Zimmermann) Zare & Gams isolated in Spain from infected aphids were characterized using sequences of the Internal Transcribed Spacer (ITS) regions and also based on morphological and physiological characteristics. Four of these seven *L. lecanii* isolates were selected to assess their virulence against nymphs of *Myzus persicae* (Sulzer), *Nasonovia ribisnigri* (Mosley), *Macrosiphum euphorbiae* (Thomas) and *Aphis gossypii* Glover. Mortality (%), lethal concentration 50 (LC_{50}) and lethal time 50 (TC_{50}) were calculated. The analysis of the sequences of ITS region confirmed that the new isolates were clearly *Lecanicillium lecanii*. The set of isolates had similar radial growth (51.5–54.0 mm), except for ICAL1 (39 mm). The germination time 50 (GT_{50}) varied between 10.7 h (ICAL3) and 13 h (ICAL5). The isolate ICAL6 showed the highest value for conidial production (3.4×10^8 con ml^{-1}) and also produced the highest mortality for *M. persicae* (95%) and was more virulent than the commercial product Vertalec® (91.6%).

Keywords *Lecanicillium lecanii* · Hypocreales · Aphids · Biological control · Entomopathogenic fungi · Physiological host range

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Introduction

Entomopathogenic fungi species formerly within the genus *Verticillium* section Prostrata was recently reclassified using morphological characteristics and Internal Transcribed Spacer region (ITS) sequences, as genus *Lecanicillium* (Zare and Gams 2001). This DNA region is one of the most rDNA region used for phylogenetic analysis (Hillis and Dixon 1991; Arenal et al. 2000; Salazar et al. 1999). In this new classification *Lecanicillium lecanii* (Zimmermann) Zare & Gams is the type species and considered as a species complex, which includes *L. lecanii*, *Lecanicillium muscarium* (Petch) and *Lecanicillium longisporum* (Petch) (Zare and Gams 2001).

Nowadays, only *L. longisporum* and *L. muscarium* but not *L. lecanii* have been formulated and commercialized in Europe as the mycoinsecticides Vertalec® and Mycotal® to control aphids and whiteflies in protected crops, respectively.

Aphids are one of the most important insect pests affecting protected and open-grown crops, because they cause direct damage by feeding on crops and indirect damage as virus vectors. They are also distributed worldwide in most cash-crops. The most important aphid pests for ornamental and vegetable crops are the "melon or cotton aphid" *Aphis gossypii* (Glover), the "green peach aphid" *Myzus persicae* (Sulzer) and the "potato aphid" *Macrosiphum euphorbiae* (Thomas) (Blümel 2004). All these aphid species are extremely polyphagous and vectors of large number of plant viruses. Some other aphid species have a more specific relationship with their host plant, such as the "lettuce aphid", *Nasonovia ribisnigri* (Mosley). This aphid species colonizes the leaves inside the developing lettuce heads, reducing the efficacy of monitoring programs and insecticide treatments (Chaney and Wunderlich 2000).

In the last decades, biological control including the use of entomopathogenic fungi is an emerging strategy to control aphids, especially in high-value crops to substitute or complement traditional control based mainly on the use of traditional chemical insecticides. Entomopathogenic fungi play an important role in aphid biological control because aphids have morphological, biological and ecological characteristics that make them susceptible to be attacked by fungal pathogens, able to cause epizootics that reduce drastically aphid populations (Steinkraus 2006).

The species *L. lecanii* has been reported causing natural epizootics in aphid and mealybug populations in tropical and sub-tropical regions of the world (Shah and Pell 2003). In laboratory conditions many reports showed that *L. lecanii* isolates were more virulent compared to other genera belonging to the order Hypocreales (Ascomycota), as well as *Beauveria bassiana*, *Paecylomyces sp* and *Metharhizium anisopliae* against aphids species (Loureiro and Moino 2006; Åsman 2007). Also, differences in virulence on different aphid species were found among different isolates of *L. lecanii* (Van Hanh et al. 2007), which could be explained, probably, due to differences in their physiological characteristics (Jackson et al. 1985; Cortéz-Madrigal et al. 2003; Steinkraus 2006).

At this moment no commercial formulation is available based on *L. lecanii* in the market and the selection of fungal isolates for that purpose is the first step for the development of a new mycoinsecticide. Therefore, the aim of this work was to characterize different Spanish native isolates of *L. lecanii* obtained from different aphid species and to determine their virulence and physiological host range among the main aphid species pest in horticultural crops in Spain.

Materials and methods

Fungal isolates

Seven fungal isolates (named ICAL 1–7) were sampled and identified in our laboratory from several aphid species collected from aphid colonies maintained in the laboratory of the Centro de Ciencias Medioambientales (CCMA–CSIC) of Madrid (Spain) (Table 1). They were identified as *L. lecanii* by classical taxonomy following general and specific identification keys (Humber 1997; Zare and Gams 2004). Stocks of the isolates grown on Potato Dextrose Agar (PDA) media were conserved in 40% glycerol (v/v) at −20°C and were deposited in the Mycological Collection at the CCMA–CSIC (Madrid, Spain).

DNA amplification, sequencing and phylogenetic analysis

Fungal cultures were grown in PDA at 24°C for one week in darkness. Genomic fungal DNA was isolated with PowerSoil™ DNA Kit (MO BIO Laboratories, Inc., USA) following manufacturer's specifications. DNA was quantified by using NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA). Complete ITS region was amplified using the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) synthesized by MWG-BiotechAG (Spain). Polymerase chain reaction (PCR) was carried out in a 50 µl volume mixing the template DNA (2 ng μl^{-1}) with 1× PCR reaction buffer with 1.5 mM $MgCl_2$ (Roche Diagnostics Ltd.), 0.25% (v/v) Tween 20 (Merck), 5% Dimethyl Sulfoxide (v/v) (Merck), 0.25 mM of each dNTP

Table 1 *Lecanicillium lecanii* isolates used in this study

Isolate number	Original insect host	Host plant	Location	Date of isolation	GenBank accession number
ICAL1	*Brachycaudus helichrysi*	Chrysanthemum	Madrid	12-04-2004	FJ515765
ICAL2	*Brachycaudus helichrysi*	Chrysanthemum	Madrid	10-04-2006	FJ515766
ICAL3	*Macrosiphum euphorbiae*	Tomato	Madrid	24-03-2006	FJ515767
ICAL4	*Nasonovia ribisnigri*	Lettuce	Madrid	30-03-2006	FJ515768
ICAL5	*Brachycaudus helichrysi*	Chrysanthemum	Madrid	10-04-2006	FJ515769
ICAL6	*Myzus persicae*	Pepper	Madrid	10-04-2006	FJ515770
ICAL7	*Macrosiphum euphorbiae*	Tomato	Madrid	28-11-2005	FJ515771

(GE Healthcare, UK), 2 μM of each primer, 1.25 U Taq DNA Polymerase (Roche Diagnostics GmbH, Germany). A negative control was also included. Amplifications were carried out with a Perkin–Elmer Cetus 480 thermalcycler by using a 2.5 min denaturation step at 94°C followed by 40 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 57°C for 30 s and extension at 72°C for 1.5 min with a final extension at 72°C for 10 min. PCR products were checked by ethidium bromide-stained 1% agarose gel electrophoresis and purified with GFX^{TM} PCR DNA and Gel Band Purification kit (GE Healthcare, UK).

Double-stranded PCR products were sequenced directly as described by Moore et al. (1999) using the Taq dideoxy terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, USA), carrying out the reactions in an Applied Biosystems 373S DNA sequencer (Applied Biosystems, USA).

Forward and reverse sequences were compared and edited by using SeqEd^{TM} v1.0.3 (Applied Biosystems), and ITS consensus sequences were compared with the sequences placed in the GenBank database (National Center for Biotechnology Information) by using the BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information). Phylogenetically related sequences were aligned using the CLUSTAL X 1.81 software (Thompson et al. 1997), and ends of the alignments were trimmed using the Se-Al v2.0a11 Carbon software (Rambaut 1996). Phylogenetic analysis of aligned sequences was performed by using maximum-parsimony (MP) analysis with Heuristic Search algorithm implemented in PAUP* 4.0 b10 software (Phylogeny Using Parsimony Analysis), (Swofford 1998), with 1,000 replicates of random taxon addition and tree-bisection-reconnection (TBR) branch-swapping algorithm, and with a statistical analysis by Bootstrap (Felsenstein 1985) based on 1,000 resamplings. The trees were rooted with *Pandora neoaphidis* (AF543211) as outgroup for ITS phylogenetic analysis.

Morphological and physiological characterization

Radial growth, conidial size and production, and germination time 50 (GT_{50}) were evaluated to determinate morphological and physiological characteristics of the seven isolates of *L. lecanii*.

Radial growth of the isolates of *L. lecanii* was measured starting from seven-day-old cultures of each isolate grown on PDA media in darkness at 24°C. A sample of 5 mm diameter was taken with a sterile cutter from each isolate and then was inoculated in the center of a new Petri dish (9 cm Φ) containing the same culture media. The new Petri dishes—six replicates—were incubated in darkness at 24°C for 21 days following a completely randomized design.

Colonies of each fungal isolate obtained from the experiment described above were used to record the conidial production. For this purpose, conidia from a whole colony of each Petri dish were harvested with a sterile spatula and then introduced in 10 ml distilled water with 0.01% Tween 80 (v/v) and the suspension was homogenized on a vortex for 5 min. Conidia were quantified in a haemacytometer chamber and the number of conidia/ml was calculated using a conversion factor. Also, conidial size was measured on 100 conidia of each fungal isolate.

Germination tests were done starting from seven-days-old cultures grown on PDA medium. Conidia

from these cultures were harvested and homogenized as described above. Then, 100 µl aliquot was spread with a sterile Drigalsky loop in a Petri dish with PDA medium. The dishes were incubated in darkness at 24°C. The number of germinated conidia was recorded at 9 h and thereafter every 3 h up to 24 h after the experiment began. A conidium was considered germinated when the length of the germ tube was a half of the conidium length. Four replicates with 100 conidia for each isolate were performed at each evaluation time. To obtain the time when the 50% conidia were germinated (GT_{50}), median data of conidial germination at each time were fitted to a curvilinear line using SPSS v. 15 statistical package (SPSS 2003).

Virulence of selected *L. lecanii* isolates

Colonies of *M. euphorbiae, N. ribisnigri, A. gossypii* and *M. persicae* maintained in the laboratory of CCMA–CSIC of Madrid were used to determine the physiological host range of the selected group of isolates of *L. lecanii*. Aphid colonies were reared on its main host plant (*M. euphorbiae* and *N. ribisnigri* on lettuce, *M. persicae* on pepper, *A. gossypii* on melon) inside insect-proof cages in a growth chamber at a temperature of 23:18°C (day:night) and a photoperiod of 16:8 (L:D) h. Aphid species were synchronized before starting the bioassays to obtain nymphs of nearly equal age and weight.

Four of the *L. lecanii* fungal isolates with the best conidial production and germination time (ICAL1, ICAL3, ICAL4 and ICAL6) were selected from the previously described experiments to evaluate their virulence against *M. euphorbiae, N. ribisnigri, A. gossypii* and *M. persicae*. Fungal cultures of 10–15 days-old of the selected *L. lecanii* isolates were used to obtain a conidial suspension in 10 ml of distilled water with 0.01% Tween 80 (v/v). Spore concentration was estimated with a haemocytometer and adjusted to 10^5, 10^6, 10^7, 10^8, and 10^9 con ml^{-1}.

Bioassays were conducted in Petri dishes (5 cm Φ) containing a thin layer of 1.5% (w/v) water agar, and a detached leaf disk of lettuce, pepper or melon was placed on top. Fifteen second-instar nymphs of each aphid species were placed in the Petri dishes and then were sprayed with each isolate/conidia concentration. Four replicates were made from each conidia concentration and a non-treated control treatment was included, which was sprayed with sterile distilled water with 0.01% Tween 80 (v/v). A commercial formulation Vertalec® (Koppert) (*L. longisporum*) was included as standard. In this case, only the recommended dose by the manufacturer (2 g l^{-1}) was used.

After spraying, insects were incubated in controlled conditions of temperature (23:18°C, day:night) and photoperiod 14:10 (L:D). Mortality was recorded daily until ten days after fungal inoculation. Aphids were considered dead when they did not show movement, and then aphid cadavers were placed in a Petri dish (5 cm Φ) with a dampened filter paper and maintained at 20°C for 48 h to induce fungal sporulation. Mortality data were corrected using the Abbott's (1925) formula and then dose-mortality data were analyzed by PROBIT (Finney 1973) procedure using the SPSS statistical package (v.15.00) to calculate the median lethal concentration (LC_{50}) and the median lethal time (LT_{50}).

Results

Molecular characterization

ITS complete region from all ICAL isolates was a 500 bp sized fragment. *Torrubiella confragosa* which is the teleomorph of *L. lecanii* showed in BLAST analysis a high percentage of homology (99%) with different *L. lecanii* isolates. For MP analyses, the data matrix comprises 752 characters of which 419 were constant and 62 were variable parsimony-informative. In the MP analysis, 100 shortest trees of 416 steps with a consistency index (CI) of 0.938 and a retention index (RI) of 0.766 were found. The strict consensus with bootstrap values above 50% is indicated over respective branches (Fig. 1). In this figure, it can be observed that all ICAL isolates are related to the *Lecanicillium* genus. Among ICAL isolates it appears *T. confragosa* AM924163 with which they share 100% homology. The sequence distances among *T. confragosa* AM924163 and the seven ICAL isolates are of 0%, except for ICAL6 isolate, which is approximately 0.2%. This ICAL6 isolate seemed to be the most divergent.

The distances of *T. confragosa* IFO 8579 and *T. confragosa* NBRC 3231 with *L. lecanii* ICAL isolates varies from 0.2 to 0.4%, and the distances of these *T. confragosa* isolates with *L. longisporum* (CBS

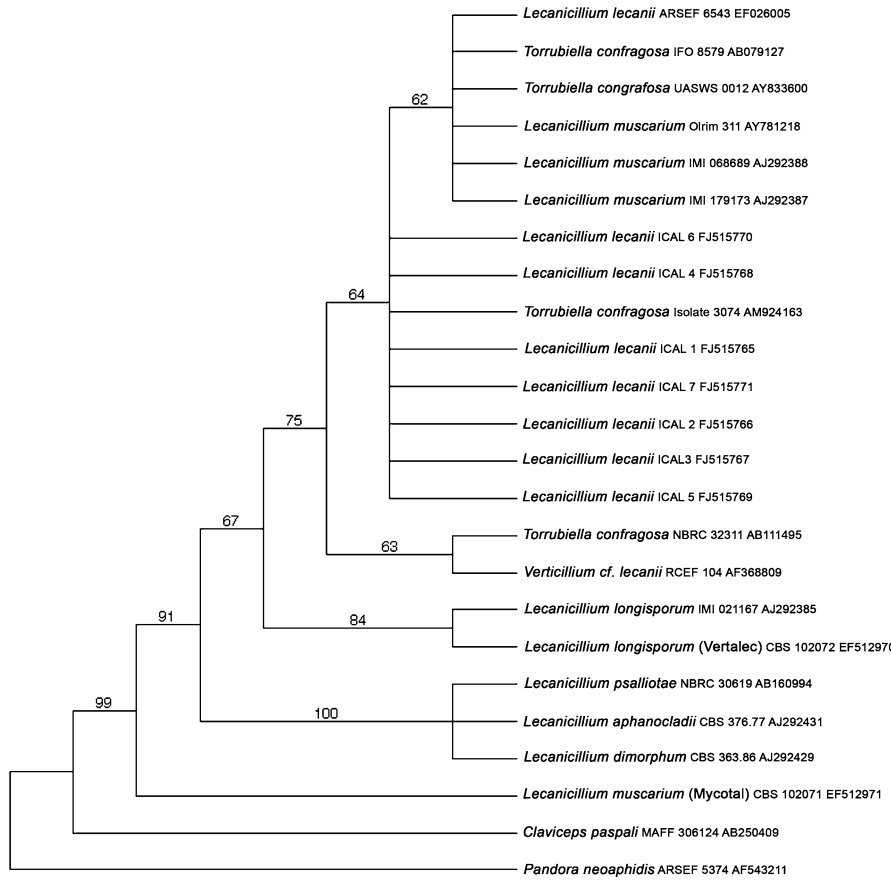

Fig. 1 Strict consensus tree obtained by maximum-parsimony recovered using the sequences of ITS and 5, 8 rDNA regions of different isolates belonging to Order Hypocreales. The outgroup is *Pandora neoaphidis* ARSEF 5374. The support of each branch is indicated by bootstrap percentage calculated for 1,000 resamplings when >50%

102072 and IMI 021167) are close to 2%, whereas with *L. muscarium* (Olrim 311, IMI 068689, IMI 179173 and CBS 102071) it reaches 7%. But possibly, due to the close relationship among these three species, ambiguities could appear in their phylogenetic analysis. Isolates identified as *L. muscarium* may appear as *T. confragosa* or *L. lecanii* isolates, and likewise *L. muscarium* isolates may appear in two different clades.

Morphological and physiological characterization

All of the ICAL isolate colonies were yellowish white with deep yellow reverse, the conidia had a typically short-ellipsoidal shape and sizes of the conidia are represented under Table 2. All ICAL conidia measures were homogeneous in size and shape and varied between (2.27 × 1.0) and (2.77 × 1.0) µm.

ICAL1 isolate obtained from *B. helichrysi* showed the lowest radial growth (39 mm) among all of the *L. lecanii* isolates studied, including ICAL2 and ICAL5 obtained from the same insect and host plant, Chrysanthemum (*Chrysanthemum indicum*). The remaining isolates had similar radial growth, and varied between 51.0 and 54.0 mm for ICAL6 and ICAL4, respectively (Table 2).

The highest value for conidial production was observed in the ICAL6 isolate—3.4×10^8 con ml^{-1}—isolated from *M. persicae*, and the lowest production was obtained with the ICAL5 (1.7×10^7 con ml^{-1}) isolated from *B. helichrysi*.

All of the *L. lecanii* isolates achieved 90% or more of germination after 24 h of incubation at 24°C. Germination rates fitted a polynomial third order equations and showed high R^2 values (>0.989, $P < 0.001$) for all isolates (Table 2). Considering all isolates, the GT_{50} varied between 10.7 h for ICAL3 and 13 h for ICAL5. The isolates ICAL1, ICAL4 and ICAL6 showed similar GT_{50} values ($\approx$11 h). However, ICAL2 and ICAL7 form a group of isolates showing intermediate values of GT_{50} (around 12 h) (Table 2).

Table 2 Physiological parameters of the *Lecanicillium lecanii* isolates when grown on potato dextrose agar (PDA) medium in controlled conditions

Isolates number	Radial growth[a] (mm)	Conidial production[b] (conidia ml^{-1})	Germination time 50 (GT_{50})[c] (h)	Conidial size[d] (μm)
ICAL1	39.0 ± 0.52	8.0 ± 2.93 × 10^7	11.2 ($y = -343.13 + 65.18x - 3.30x^2 + 0.05x^3$; $R^2 = 0.977$, $P < 0.001$)	2.37 ± 0.23 × 1 ± 0.01
ICAL2	52.3 ± 0.42	6.2 ± 1.32 × 10^7	12.3 ($y = -196.89 + 33.97x - 1.34x^2 + 0.02x^3$; $R^2 = 0.989$, $P < 0.001$)	2.49 ± 0.13 × 1 ± 0.02
ICAL3	53.3 ± 0.56	8.2 ± 2.13 × 10^7	10.7 ($y = -261.89 + 49.22x - 2.27x^2 + 0.03x^3$; $R^2 = 0.994$, $P < 0.001$)	2.56 ± 0.29 × 1 ± 0.02
ICAL4	54.0 ± 0.68	9.8 ± 2.22 × 10^7	11.1 ($y = -357.35 + 70.53x + 3.80x^2 + 0.07x^3$; $R^2 = 0.991$, $P < 0.001$)	2.46 ± 0.19 × 1 ± 0.02
ICAL5	52.3 ± 1.14	1.7 ± 5.59 × 10^7	13.0 ($y = -133.66 + 19.61x - 0.42x^2 + 0.005x^3$; $R^2 = 0.991$, $P < 0.001$)	2.27 ± 0.27 × 1 ± 0.01
ICAL6	51.5 ± 0.80	3.4 ± 4.70 × 10^8	11.6 ($y = -147.07 + 27.12x - 100x^2 + 0.01x^3$; $R^2 = 0.989$, $P < 0.001$)	2.77 ± 0.26 × 1 ± 0.02
ICAL7	52.5 ± 1.17	2.7 ± 1.53 × 10^7	12.0 ($y = -101.79 + 15.25x - 0.23x^2 + 0.002x^3$; $R^2 = 0.997$, $P < 0.001$)	2.38 ± 0.18 × 1 ± 0.01

[a] Values in this column represent the mean radial growth ± SE from six replicates

[b] Values in this column represent the mean conidial production ± SE from six replicates

[c] Conidial germination rates were fitted to a polynomial third order equation

[d] Values of conidial size represent the mean length ± SE × mean width ± SE measured on 100 conidia

Virulence of selected *L. lecanii* isolates

All of the four selected *L. lecanii* isolates (ICAL1, ICAL3, ICAL4 and ICAL6) showed pathogenic activity against the second-nymphal instar of *M. persicae*, *N. ribisnigri*, *M. euphorbiae* and *A. gossypii*, with different levels of virulence among aphid species. Also, mortality of *L. lecanii* isolates are dose-dependent and mortality obtained with Vertalec® was higher compared with the mortality achieve by the selected *L. lecanii* isolates at the highest dose of 1×10^9 con ml^{-1} tested (Fig. 2). However, Vertalec® started to produce aphid mortality between six and seven days after treatment while ICAL isolates tested produced mortality four days after treatment (data no shown).

Mortality of *M. persicae* at the dose of 1×10^9 con ml^{-1} ranged from 56.6% (ICAL4) to 95% (ICAL6). ICAL6 isolate was more virulent than Vertalec®, which produced a mortality of 91.5%, ten days after treatment (Fig. 2). For *M. persicae*, ICAL6 isolate obtained from the same aphid species, showed the lowest LC_{50} (1.05×10^7 con ml^{-1}) and LT_{50} (3.1 d) (Tables 3 and 4). ICAL4 isolate obtained from *N. ribisnigri*, was the less virulent to *M. persicae*, showing in consequence the highest LC_{50} (3.65×10^8 con ml^{-1}) (Table 3). The isolate that showed the highest lethal time for *M. persicae* was ICAL3, which was isolated from *M. euphorbiae* (Table 4).

ICAL4 and ICAL6 isolates followed the same trend when tested against *M. euphorbiae*, achieving 63.1 and 88.3% mortality at the dose of 1×10^9 con ml^{-1} ten days after treatment, respectively (Fig. 2). For *M. euphorbiae* the commercial Vertalec® at the recommended dose of 2 g l^{-1} was more virulent than all of our new ICAL isolates of *L. lecanii*. ICAL6 isolate was the most virulent for *M. euphorbiae* compared with the remaining isolates, showing the lowest LC_{50} and LT_{50} values (Table 3 and 4).

The highest mortality (90%) was observed for the ICAL4 against *N. ribisnigri*. The ICAL4 isolate was originally obtained from an individual of this same aphid species. Mortality obtained with ICAL4 was similar than that obtained with Vertalec® (91.5%) (Fig. 2). ICAL1 isolate obtained from *B. helichrysi* showed the lowest LC_{50} (6.90×10^7 con ml^{-1}) against *N. ribisnigri* (Table 3).

ICAL3 was the most virulent isolate for *A. gossypii*, including Vertalec®, achieving a mortality

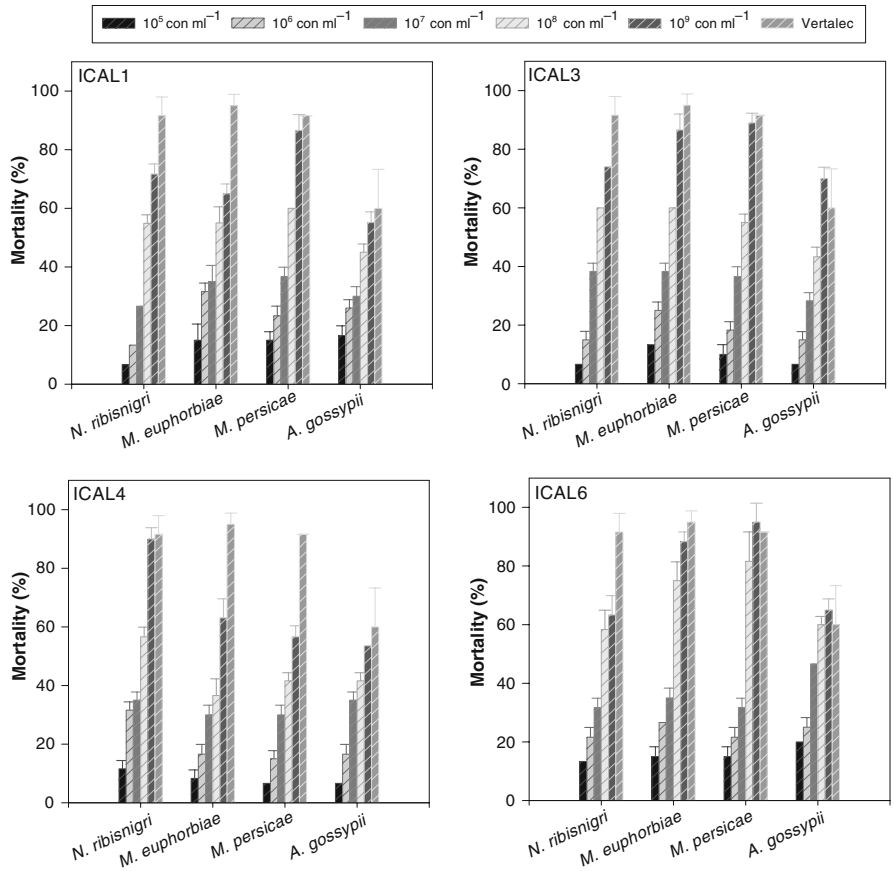

Fig. 2 Percentage + SE of mortality of *Myzus persicae*, *Nasonovia ribisnigri*, *Macrosiphum euphorbiae* and *Aphis gossypii* exposed to different doses of the selected isolates of *Lecanicillium lecanii* and to Vertalec® (*Lecanicillium longisporum*) at the concentration recommended by the manufacturer (2 g l^{-1}) ten days after incubation

of 65% at the dose of 1×10^9 con ml^{-1}, ten days after treatment (Fig. 2). For ICAL3, the LC_{50} was estimated in 1.33×10^8 con ml^{-1} and a LT_{50} of 6.7 d (5.7–10.5 d) (Tables 3 and 4). However, the lowest LT_{50} value (4.9) was obtained with ICAL6, suggesting that this isolate acts faster than the rest of the selected *L. lecanii* isolates.

Discussion

Torrubiella confragosa is the teleomorph of *L. lecanii*. *Torrubiella confragosa* NBRC 32311 and *T. confragosa* IFO 8579 isolates share 99–100% of homology with ICAL isolates suggesting that the isolates described in the present work belong to the species *L. lecanii* (Zare and Gams 2008).

Despite that ITS region has been used widely as a phylogenetic marker (Hillis and Dixon 1991; Arenal et al. 2000; Salazar et al. 1999), it can be noticed in our study some ambiguities in the position of some isolates. These kinds of ambiguities have already been reported by some authors such as Kouvelis et al. (2008). This uncertainty observed when using the ITS region might be due to the fact that very close-related species in some cases are difficult to be handled based only in one gene sequence. This suggests that it might be necessary to combine ITS data with some other phylogenetic studies based on other genes sequence data in order to have a more accurate classification. The same ambiguity can be observed in the analysis of the partial SSU rDNA (Zare and Gams 2008), where *L. lecanii* and *L. muscarium* species appear in the same clade close to *L. psalliotae* in the MP analysis. The *L. lecanii* isolates used by Zare and Gams (2008) in their study were included in our analysis. Likewise as observed in Fig. 1, the Mycotal isolate (CBS102071), re-identified as *L. muscarium* (Zare and Gams 2001), turns out to be moved from the rest of *L. lecanii* and *L. muscarium* isolates to another branch. This confirms the information published by Kouvelis et al. (2008), since isolates identified

Table 3 Median lethal concentration (LC_{50}) of the different isolates of *Lecanicillium lecanii* against second-instar nymph of *Myzus persicae*, *Nasonovia ribisnigri*, *Macrosiphum euphorbiae* and *Aphis gossypii* ten days after exposure to different spore concentrations

Insect target	Isolate	Slope ± SE [log10 (dose)]	LC_{50}[a]	95% CL[b]	χ^{2}[c]	df[d]	*P*-value[f]
Myzus persicae	ICAL1	0.526 ± 0.061	2.14×10^{7}	$1.08–5.0 \times 10^{7}$	5.641	18	0.001
	ICAL3	0.596 ± 0.065	2.92×10^{7}	$1.57–6.0 \times 10^{7}$	6.19	18	0.001
	ICAL4	0.406 ± 0.062	3.65×10^{8}	$1.29–2.0 \times 10^{9}$	1.12	18	0.001
	ICAL6	0.696 ± 0.068	1.05×10^{7}	$0.60–2.0 \times 10^{7}$	19.9	18	0.001
Nasonovia ribisnigri	ICAL1	0.551 ± 0.065	8.60×10^{7}	$4.29 \times 10^{7}–2.0 \times 10^{8}$	1.41	18	0.001
	ICAL3	0.539 ± 0.064	6.90×10^{7}	$3.43 \times 10^{7}–2.0 \times 10^{8}$	1.71	18	0.001
	ICAL4	0.536 ± 0.062	1.93×10^{7}	$0.98–4.0 \times 10^{7}$	9.68	18	0.001
	ICAL6	0.546 ± 0.062	2.78×10^{7}	$1.42–6.0 \times 10^{7}$	7.22	18	0.001
Macrosiphum euphorbiae	ICAL1	0.341 ± 0.056	6.35×10^{7}	$2.28 \times 10^{7}–3.0 \times 10^{8}$	4.72	18	0.001
	ICAL3	0.534 ± 0.061	2.05×10^{7}	$1.04–6.0 \times 10^{7}$	3.89	18	0.001
	ICAL4	0.413 ± 0.060	1.9×10^{8}	$7.66 \times 10^{7}–8.0 \times 10^{8}$	2.80	18	0.001
	ICAL6	0.579 ± 0.063	1.26×10^{7}	$0.67–2.0 \times 10^{7}$	7.68	18	0.001
Aphis gossypii	ICAL1	0.275 ± 0.056	3.78×10^{8}	$8.89 \times 10^{8}–5.0 \times 10^{9}$	1.84	18	0.001
	ICAL3	0.497 ± 0.064	1.33×10^{8}	$6.07–4.0 \times 10^{8}$	1.97	18	0.001
	ICAL4	0.373 ± 0.060	3.55×10^{8}	$1.17 \times 10^{8}–2.0 \times 10^{9}$	3.08	18	0.001
	ICAL6	0.322 ± 0.057	3.30×10^{8}	$9.92 \times 10^{7}–3 \times 10^{8}$	1.59	18	0.001

[a] LC_{50} values are expressed in conidia ml^{-1}

[b] 95% Confidential limits

[c] Pearson χ^{2}

[d] Degrees of freedom

[f] *P* values represent the probability of the slope

morphologically as *L. lecanii* (with typically short-ellipsoidal shaped conidia and homogeneous sizes and a white yellowish colony coloration and deep yellow reverse) fit well with *L. lecanii* but not with *L. muscarium* (which have heterogeneous shape and size of conidia and uncolored colony reverse). *Lecanicillium lecanii* ARSEF 6543, with homologies from 100% to 99% with *T. confragosa* isolates (IFO 8579 and UASWS 0012), appear close to some *L. muscarium* isolates, whereas the Mycotal isolate, identified as *L. muscarium*, appears in another different branch far from the rest (Fig. 1). Also Sugimoto et al. (2003) have found some similar ambiguities.

All seems to indicate that the ITS region alone can fail to resolve some phylogenetic relationships, and is insufficient to place some isolates in their appropriate *Lecanicillium* branches in phylogenetic studies. It will be desirable to start a deeper phylogenetic study of this genus based on new sequences from different genes. This will allow a clear understanding of the positioning of some isolates. The combination of our morphological and molecular data, strongly suggest that ICAL isolates are *L. lecanii*, which in addition pointed the need to combine both types of information, since the exclusive use of morphological or molecular data alone can lead to confusion.

Also, as described by Jackson et al. (1985), morphological characteristics of fungal isolates of *L. lecanii* could be associated with both physiological and pathogenic characteristics. We considered all these characteristics in the selection procedure of *L. lecanii* isolates used in our study. Our results showed similar radial growth among *L. lecanii* isolates when grown on PDA at 24°C, except for the ICAL1 isolate which grows 23.6–27.7% less than the other set of isolates, including those obtained from *B. brassicae*. No relationship was found between radial growth and conidial production among ICAL isolates obtained from *B. helichrysi*. The ICAL5 showed the lowest conidial production and GT_{50}; and, therefore, this isolate was omitted and only ICAL1 was selected for the virulence studies.

In the same way, ICAL4 isolate showed the highest radial growth, but ICAL6 isolate achieved

Table 4 Median lethal time (LT_{50}) of the different isolates of *Lecanicillium lecanii* against second-instar nymph of *Myzus persicae*, *Nasonovia ribisnigri*, *Macrosiphum euphorbiae* and *Aphis gossypii* ten days after exposure to different spore concentrations

Insect target	Isolate	Slope ± SE [log10 (dose)]	LT_{50}^{a}	95% CL[b]	χ^{2c}	df[d]	*P*-value[f]
Myzus persicae	ICAL1	2.922 ± 0.595	4.1	3.6–5.0	0.576	14	0.001
	ICAL3	4.452 ± 0.820	5.3	4.8–6.1	4.776	14	0.001
	ICAL4	4.285 ± 0.907	4.6	5.8–8.6	1.596	14	0.001
	ICAL6	4.105 ± 0.607	3.1	2.8–3.4	4.678	14	0.001
Nasonovia ribisnigri	ICAL1	2.756 ± 0.597	4.3	3.7–5.6	2.456	14	0.001
	ICAL3	3.406 ± 0.793	5.7	5.0–7.4	1.614	14	0.001
	ICAL4	3.080 ± 0.609	4.4	3.8–5.3	1.587	14	0.001
	ICAL6	3.636 ± 0.808	3.1	2.7–3.6	1.840	14	0.001
Macrosiphum euphorbiae	ICAL1	3.439 ± 0.781	5.5	4.9–6.8	3.562	14	0.001
	ICAL3	5.820 ± 1.158	5.0	4.6–5.9	0.705	14	0.001
	ICAL4	4.224 ± 0.882	6.3	5.6–8.1	2.277	14	0.001
	ICAL6	3.750 ± 0.598	3.2	2.9–3.6	2.511	14	0.001
Aphis gossypii	ICAL1	5.030 ± 1.022	6.8	6.2–8.2	1.589	14	0.001
	ICAL3	3.206 ± 0.826	6.7	5.7–10.5	1.454	14	0.001
	ICAL4	4.528 ± 0.934	6.6	5.8–8.6	0.937	14	0.001
	ICAL6	3.908 ± 0.780	4.9	4.5–5.6	1.066	14	0.001

[a] LT_{50} values of lethal time 50 are expressed in days

[b] 95% Confidential limits

[c] Pearson χ^2

[d] Degrees of freedom of the slope

[f] *P* values represent the probability of the slope

the highest conidial production (3.4×10^8 con ml^{-1}), suggesting that this isolate could be a good candidate for mass production.

In this study, germination speed varied among isolates between 10.7 and 13 h, which are similar to those obtained by Cortéz-Madrigal et al. (2003) for a group of isolates of *L. lecanii* characterized to control *Toxoptera aurantii* in cocoa. We considered this parameter as one of the most important in the selection procedure because it is responsible for the fast completion of the infection process enhancing the possibilities of the fungi to be used as a myco-insecticide to control aphid pests (Steinkraus 2006).

A positive relationship has been found between the speed of germination and the virulence of the ICAL3 isolate infecting *A. gossypii*, as was found by Yokomi and Gottwald (1988) for some *L. lecanii* isolates. However, ICAL6 which required 1 h more than ICAL3 to achieve the 50% of germination was the most virulent against *M. persicae* and *M. euphorbiae* and killed all aphid species faster than the rest of the isolates tested. Also, the whole set of our ICAL isolates killed all of the aphid species tested faster than Vertalec® (*L. longisporum*), being a great advantage for aphid control, as these insects are r-strategists and increase their population size in a very short period of time.

The LT_{50} found for *M. persicae* treated with ICAL6 (3.1 days) was similar to that obtained with *L. lecanii* (JAB 02) isolate against the same aphid species feeding on pepper (Loureiro and Moino 2006) and was one day faster compared with the two *L. lecanii* strains that cause 100% mortality of *M. persicae* (Van Hanh et al. 2007). Also, ICAL6 at the highest dose (1×10^9 con ml^{-1}) resulted 3.5% more virulent for *M. persicae* than Vertalec® (2 g l^{-1}). Furthermore, Åsman (2007) found that three species of *Lecanicillium sp.* were more pathogenic to the lettuce aphid than Vertalec®. In addition, *N. ribisnigri* was more susceptible to Vertalec® under laboratory than under greenhouse conditions, compared to *M. euphorbiae* and *M. persicae* (Fournier and Brodeur 2000). For *N. ribisnigri*, the lowest LC_{50} was obtained with ICAL4 which indicates the affinity

of this fungal isolate for the original insect host where it was isolated. This suggests the existence of specific proteases of *L. lecanii* against the host aphid cuticle, as was observed by Bye and Charnley (2008).

Estimates of mortality and LC_{50} values for *A. gossypii* exposed to all of the isolates tested were higher than those obtained for the three other aphid species, indicating that *A. gossypii* was the less susceptible aphid species to ICAL isolates among the four aphid species tested. However, our results differ with those obtained by Van Hanh et al. (2007) who concluded that *A. gossypii* is more sensitive to fungal infection than *M. persicae*. These differences could be explained by the trichomes present on the melon leaf surface that may affect conidial germination or survival. Also the composition of the melon plant could affect the virulence of the *L. lecanii* isolates, as was observed for *B. bassiana* isolates against nymphs of the whitefly *Bemisia tabaci* (Santiago-Álvarez et al. 2006), but this hypothesis needs further investigation. From our results, ICAL3 obtained from *M. euphorbiae* showed the highest virulence, even higher than Vertalec® against *A. gossypii* with a maximun mortality of 65%.

Considering all of the characteristics of the new *L. lecanii* isolates, we considered that our ICAL6 is the most promising isolate for developing a new biopesticide for aphid control. In addition, further studies related to its efficacy to control plant pathogens such as phytoparasitic nematodes or phytopathogenic fungi should be continued.

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Dr. Beatriz Diaz is a researcher in entomology devoted to study biology and ecology of Lepidoptera and aphids, physical barriers to control insect vectors and currently biological control using entomopathogenic fungi.

Dr. Monike Oggerin is involved in Molecular Microbial Ecology and Phylogenetics studies from several environments. On particular interest is the relationship between fungi and plants and the role of fungi in extreme environments. Actually she is part of Molecular biology of extremophylic microorganisms group at the Applied Microbiology Unit (lead by Dr. R. Amils) at CBM-UAM.

Dr. Lopez Lastra Previous related studies on entomopathogenic fungi and host interactions had been developed by Dr. Lopez Lastra and her leading group in Argentina.

Dr. Rubio The work conducted by Dr. Rubio was orientated to develop molecular phylogeny in the identification of fungi using DNA sequences, and one of the first one to apply such techniques to the identification of the complex of species belonging to the genus *Rhizoctonia*.

Dr. Alberto Fereres has more than 25 years of experience in biology and ecology of aphids, aphid-virus interactions, insect probing and feeding behavior, host plant resistance to insects and cultural control of insect vectors of plant disease. This work was carried out at the Instituto de Ciencias Agrarias (ICA-CCMA), Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.