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Purpureocillium lilacinum, potential agent for biological control of the leaf-cutting ant *Acromyrmex lundii*

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ABSTRACT

Many leaf-cutter ant species are well known pests in Latin America, including species of the genera *Acromyrmex* and *Atta*. An environmentally friendly strategy to reduce the number of leafcutter ants and avoid indiscriminate use of chemical pesticides is biological control. In this work we evaluated the effectiveness of a strain of the entomopathogen *Purpureocillium lilacinum*, against worker ants from six *Acromyrmex lundii* field colonies, after immersions in pure suspensions at a concentration of 1×10^6 conidia ml^{-1}. Survival of ants treated with *P. lilacinum* was significantly lower than that recorded in controls, and median lethal time (LT_{50}) was 6–7 days. *P. lilacinum* was responsible for 85.6% (80.6–89.7) of the mortality in inoculated ants, in which we found that the percentage of other entomopathogens that naturally infected ants decreased also, suggesting a good competitive capability of the fungus. Horizontal transmission to non-inoculated ants was also evidenced, given that 58.5% (41.9–64.2) of them died because of *P. lilacinum*. Moreover, we tested pathogenicity for three concentrations of this strain (1.0×10^4, 10^6 and 10^8 conidia ml^{-1}) and found a significantly faster mortality of ants and greater median percentage of infection at 10^8 conidia ml^{-1} of *P. lilacinum*. CL_{50} value was 2.8×10^5 conidia ml^{-1}. We thus propose the use of *P. lilacinum* as a biological control agent of leafcutter ants in crops and plantations.

1. Introduction

Leafcutter ants in the family Formicidae (tribe Attini), are a widely distributed group of insects, which are regarded as pests in tropical, subtropical and grassland regions of Latin America. To cultivate the symbiotic fungi from which they feed (Basidiomycota: Agaricales), these social insects generally use several plant species, and they can consume more plant biomass than any other animal group, including mammals (Hölldobler and Wilson, 1990). However, these ants are such an essential component of the ecosystems of which they are part, that complete eradication would negatively impact their functioning (Folgarait, 1998; Hölldobler and Wilson, 1990). Currently, chemical methods are used to control them, with products primarily based on sulfluramid and fipronil (Boaretto and Forti, 1997; De Coll and Ribero, 2003; Montoya-Lerma et al., 2012). However, these methods are highly inefficient for social organisms such as ants (Cherret, 1986; Della Lucia et al., 2013), and their deleterious effects to the ecosystems (Gunasekara et al., 2007; Houde et al., 2006) and to human health (Rauh et al., 2011; Tingle et al., 2003) are well documented. Biological control is an environmentally acceptable strategy that would reduce the number of these ants below the economic damage threshold.

Leaf-cutting ants can be expected to be susceptible to fungal diseases, because their social lifestyle may increase the risk of infection among nest-mates. However, few cases of fungal infections in nature are known (Schmid-Hempel, 1998). This is probably due to several reasons, such as: their cleaning behaviors, both of themselves (Hughes et al., 2002) and their food (Currie and Stuart, 2001); their mutualism with filamentous bacteria (Actinomycetes) that produce antibiotics and antifungal substances (Currie, 2001); the secretion of various bactericidal and fungicidal components by their glands (Fernández-Marín et al., 2006; Poulsen et al., 2002); and finally the removal from the nest of infected and dead individuals (Schmid-Hempel, 1998), probably due to the high transmission healthy ants can be exposed to (Hughes et al., 2002). However, all this care is not always sufficient since nests abandoned and/or killed by the presence of pathogenic fungi can be found (Authors, pers. obs.; Schmid-Hempel, 1998). This information allows us to assume that an inundative biological control strategy should exceed the threshold level of the colony's sanitation capability.

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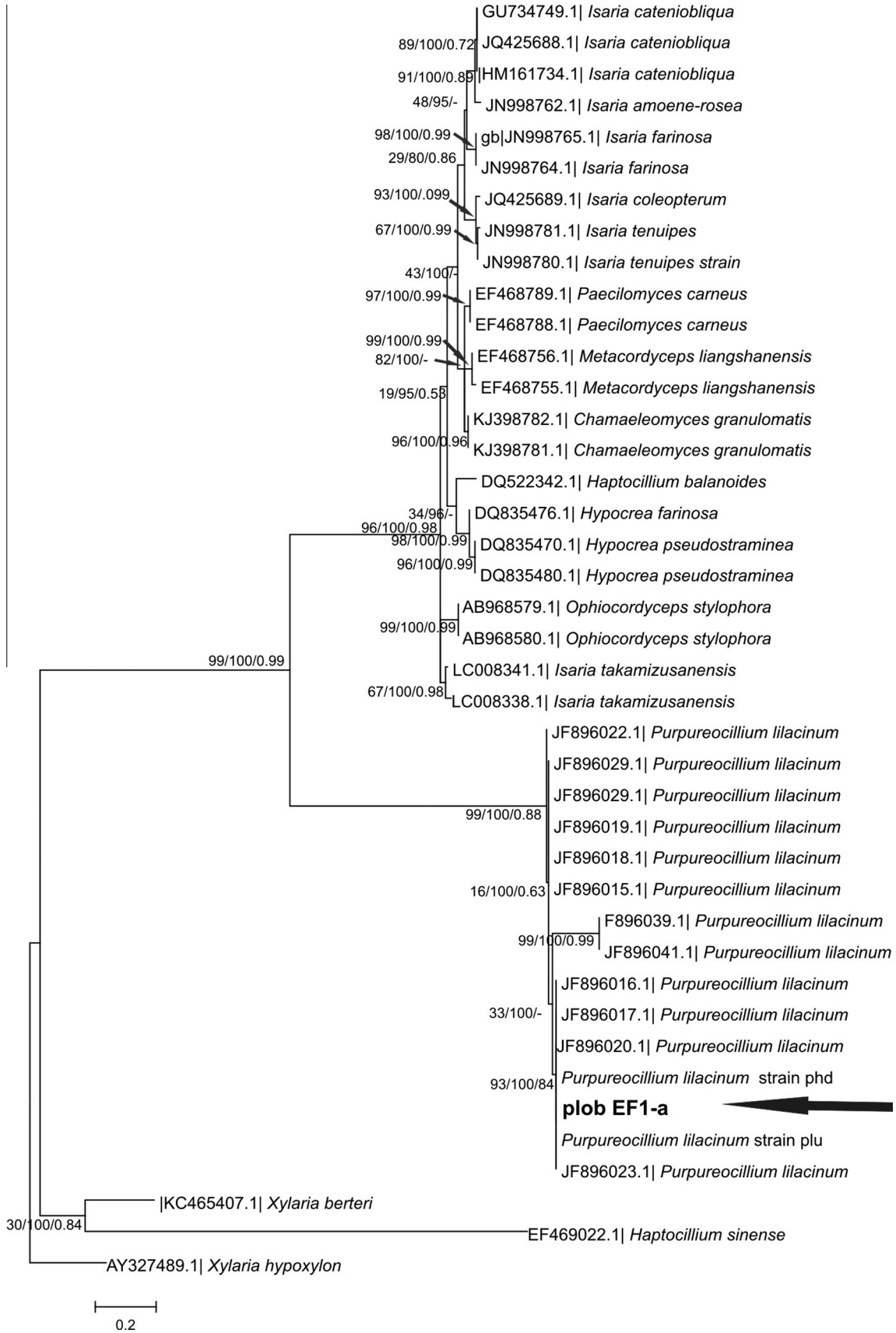

Fig. 1. Phylogram based on partial region of the EF-1α gene sequences for our isolate of *P. lilacinum* and other related species obtained from GenBank. Values of the bootstrap for maximum likelihood (ML) and maximum parsimony (MP) are shown, as well as probabilities for Bayesian inference (BI).

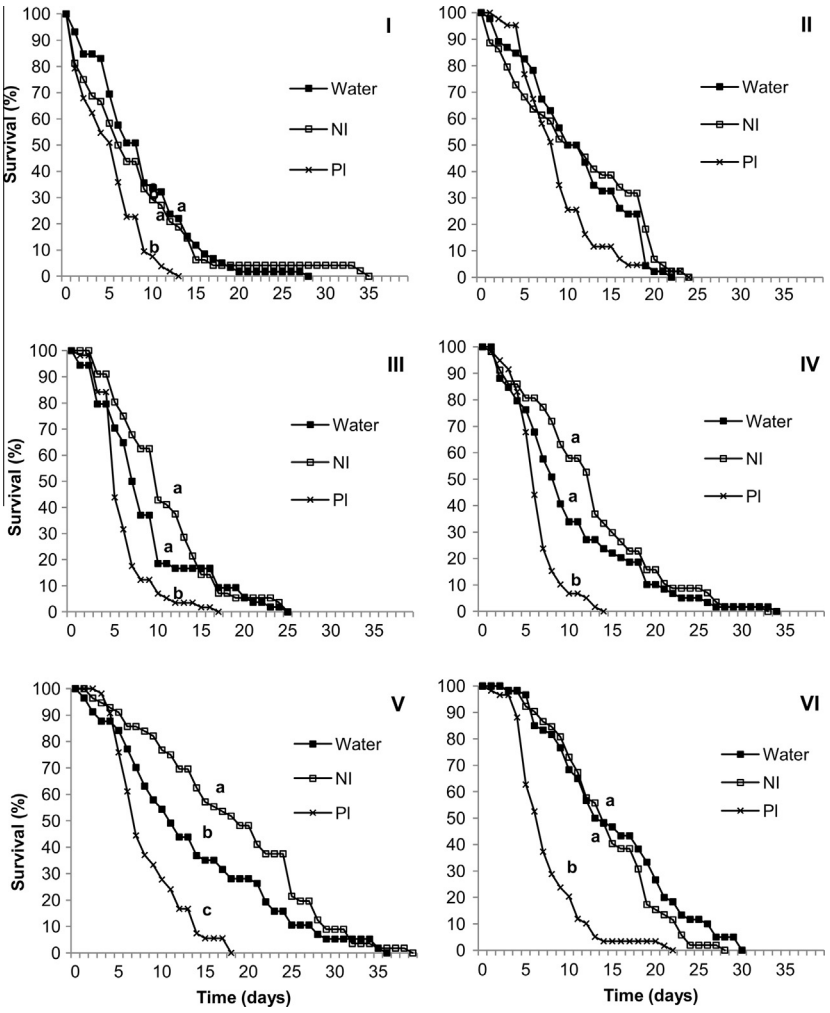

Fig. 2. Survival time (%) for each colony (I to VI) for ants treated with water, no immersion and with *P. lilacinum* (first assay). Survival distributions with the same letter are not significantly different (see text for *P*-values considered).

P. lilacinum (Ascomycota: Hypocreales) (formerly *Paecilomyces lilacinus*) is known primarily as a pathogen of nematodes (Mendoza et al., 2004; Roumpos, 2005), though it has also been found as a natural pathogen of *Triatoma infestans*, the vector of Chagas disease (Marti et al., 2006), and it has been proven effective for the control of the white fly *Trialeurodes vaporariorum*, the cotton aphid (*Aphis gossypii*) and the red mite (*Tetranychus urticae*) (Fiedler and Sosnowska, 2007). Moreover, it has been found as a part of the entomopathogenic fungi diversity associated to the red fire ant *Solenopsis invicta* (Authors, pers. obs.) and the pathogenicity of several strains has been demonstrated on these ants (Liu et al., 2010). In the leafcutter ants *Atta capiguara* and *A. laevigata*, *P. lilacinum* has been recorded as a pathogen of winged females (Rodrigues et al., 2009). To our knowledge *P. lilacinum* has never been recorded as being found on workers of leafcutter ants.

The present work aimed to determine whether *P. lilacinum* could be used to control *A. lundii*, a species we selected as a test subject because of its great relevance as a pest and its wide distribution in Argentina (Bonetto, 1959; Kusnezov, 1978).

We evaluated, for the first time, the pathogenicity of *P. lilacinum* toward *A. lundii*, under laboratory conditions, with individual immersion and with *post-mortem* disinfection, in order to reach the maximum possible virulence; and we assessed the horizontal transmission from inoculated ants to non-inoculated ones. We then examined ant mortality in response to three different doses of this fungus, in more dense groups immersed together and without *post-mortem* disinfection, with the goal of establishing the necessary dose which allows to obtain the greatest mortality in a more realistic situation.

2. Materials and methods

2.1. Collection of ants

A. lundii worker ants were collected from the foraging trails of six field colonies at different sites in Buenos Aires province, Argentina (GPS coordinates: 34°52′2″S, 58°4′26″W; 34°51′17″S, 58°04′29″W; 34°53′02″S, 58°01′03″W; 34°52′38″S, 58°00′59″W; 34°54′34″S, 57°56′10″W and 34°48′53″S, 58°10′27″W, colony number I to VI, respectively), between November 2009 and April 2010 for the first bioassay (Section 2.3), and between November 2010 and January 2011 for the second bioassay (Section 2.4). From each colony, 300 worker ants were collected for the first assay, and 500 for the second. In all cases, after collection ants were taken to the laboratory and kept in a room at 25.9 ± 1.4 °C for 2–3 days in containers with water and 20% g ml^{-1} sugar solution *ad libitum*. After that time, those ants still alive were used for the assays.

2.2. Isolation and molecular identification of P. lilacinum

P. lilacinum was isolated from the fungus garden of an *Acromyrmex lobicornis* colony collected in Mercedes, Corrientes,

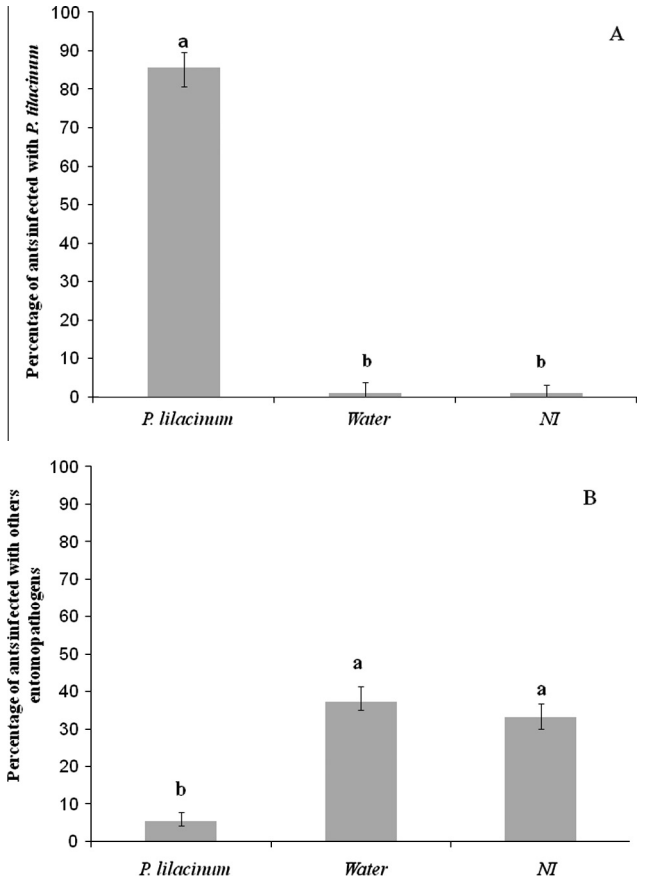

Fig. 3. Percentage of ants for the first assay (groups of 5 ants) infected with *P. lilacinum* (A) or other entomopathogens (B) (median and quartiles) for each treatment (*P. lilacinum*, water and no immersion). Bars with the same letter are not significantly different ($P < 0.017$).

Argentina in November 2008. It was inoculated on Potato Dextrose Agar (PDA) supplemented with antibiotics (penicillin, 100 U ml^{-1}; streptomycin, 100 μg ml^{-1}). After obtaining a pure culture, conidia were collected and stored in glycerol 20% at −80 °C until using them. Morphological examination was made using the *Purpureocillium* species description given by Luangsa-ard et al. (2011) and Perdomo et al. (2013). Average viability (Lacey and Brooks, 1997) was greater than 95% in all cases before the start of the bioassays.

For molecular identification, genomic DNA was extracted from mycelia of *P. lilacinum* grown on PDA for 7 days, following a modified version of the CTAB method used by Augustin et al. (2013). For phylogenetic analysis we used the primers EF1-5R (5′-GTGAT ACCACGCTCACGCTC-3′) and EF-3F (5′-CACGTCGACTCCGGCAAGT C-3′) which amplified a partial region of the nuclear elongation factor-1α (EF-1α) gene, spanning a total of 352 nucleotides. PCR amplifications were performed using a Veriti 96-well Thermal Cycler (Applied Biosystem®) in a total volume of 50 μl containing: 1× buffer reaction; 2 mM of Mg^{+2}; 25 ng of DNA; 0.4 μM of each primers; and 1 U of Taq polymerase. PCR conditions were as follow: 2 min of denaturation at 95 °C, followed by 40 cycles consisting of 30 s at 95 °C, 60 s at 52 °C and 90 s at 72 °C, and finally 5 min of extension at 72 °C. PCR products were analyzed by agarose gel electrophoresis using GelRed™ (Biotium) for DNA visualization, using UV transillumination. The bands of interest were excised from gel, and they were purified and sequenced by Macrogen Corporation. All sequences were aligned using ClustalW algorism and were edited manually using MEGA 5 (Tamura et al., 2011).

Table 1
Percentages of ants naturally infected with *P. lilacinum* or other entomopathogens, with external fungi or without fungi for both types of controls discriminated by colony, for the first assay.

Treatment: water	I	II	III	IV	V	VI
P. lilacinum	6.8	4.3	1.9	0.0	0.0	0.0
Other entomopathogens	42.4	34.8	58.5	36.2	19.3	38.3
External fungi	35.6	47.8	30.2	44.8	35.1	30.0
Without fungi	15.3	13.0	9.4	19.0	45.6	31.7
Treatment: NI (no immersion)	I	II	III	IV	V	VI
P. lilacinum	0.0	9.1	0.0	3.5	1.8	0.0
Other entomopathogens	37.5	29.5	83.9	31.6	14.5	34.6
External fungi	45.8	29.5	10.7	49.1	47.3	38.5
Without fungi	16.7	31.8	5.4	15.8	36.4	26.9

Phylogenies were reconstructed using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI). All sequences used in phylogenetic inferences were obtained from GenBank. We named each sequence with the GenBank access number followed by the species name.

2.3. Virulence and horizontal transmission assays

To assess the potential pathogenicity of *P. lilacinum*, we collected ants from the field as described in Section 2.1. We used 60 ants for each treatment, from each of the six colonies. Conidia suspensions of *P. lilacinum* were obtained from 15-days-old cultures in PDA with antibiotics (penicillin, 100 U ml^{-1}; streptomycin, 100 μg ml^{-1}), which were maintained at 25.9 ± 1.4 °C and 52.8 ± 11.2% relative humidity. We prepared suspensions at a concentration of 1×10^6 conidia ml^{-1}, by harvesting conidia from the surface culture with a 0.01% Tween 80 solution, and we quantified the conidia using a Neubauer haemocytometer. Each ant was individually immersed for 10 s in a microtube containing 100 μl of the suspension. We established two controls: ants immersed in a 0.01% Tween 80 solution (hereafter: water) and ants with no immersion (hereafter: NI). After treatments, we placed 5 ants in Petri dishes (9 cm diameter) with sterile filter paper, water and 20% sugar solution *ad libitum*. In all treatments, we added 2 non-inoculated ants, which whose abdomens were painted with non toxic red paint marker (Markal®), with the goal of determining horizontal transmission. We removed dead ants daily, until all individuals were dead. After death, ants were washed individually in series of 30-s immersions, first in a 5% sodium hypochlorite solution and then twice in sterile distilled water, in order to remove other spores from the cuticle (Lacey and Brooks, 1997); afterward, each ant was transferred to an individual sterile humid chamber. We observed them daily to score the presence of entomopathogenic fungi (*P. lilacinum* or others) on ant bodies, considered as such if they appeared from intersegmental membranes and/or the joints of the legs and antennae. Other fungal growth was considered external (non-entomopathogenic).

2.4. Dose dependence assay

To estimate dose dependence and obtain LD_{50} (the dose resulting in mortality of 50% of the sample), we used 100 ants for each treatment, from each of the same six colonies which were collected as described in Section 2.1. We prepared conidia suspension of *P. lilacinum* at concentrations of 1×10^4, 10^6 and 10^8 conidia ml^{-1} in 0.01% Tween 80 solution. Ants were submerged together for 10 s in 20 ml of conidia suspension. Control ants were immersed in 0.01% Tween 80 solution. Then, all ants were placed together in a sterile container (14.5 cm long, 14.5 cm wide and 6 cm high), sealed with aluminum foil and closed with a lid (no openings),

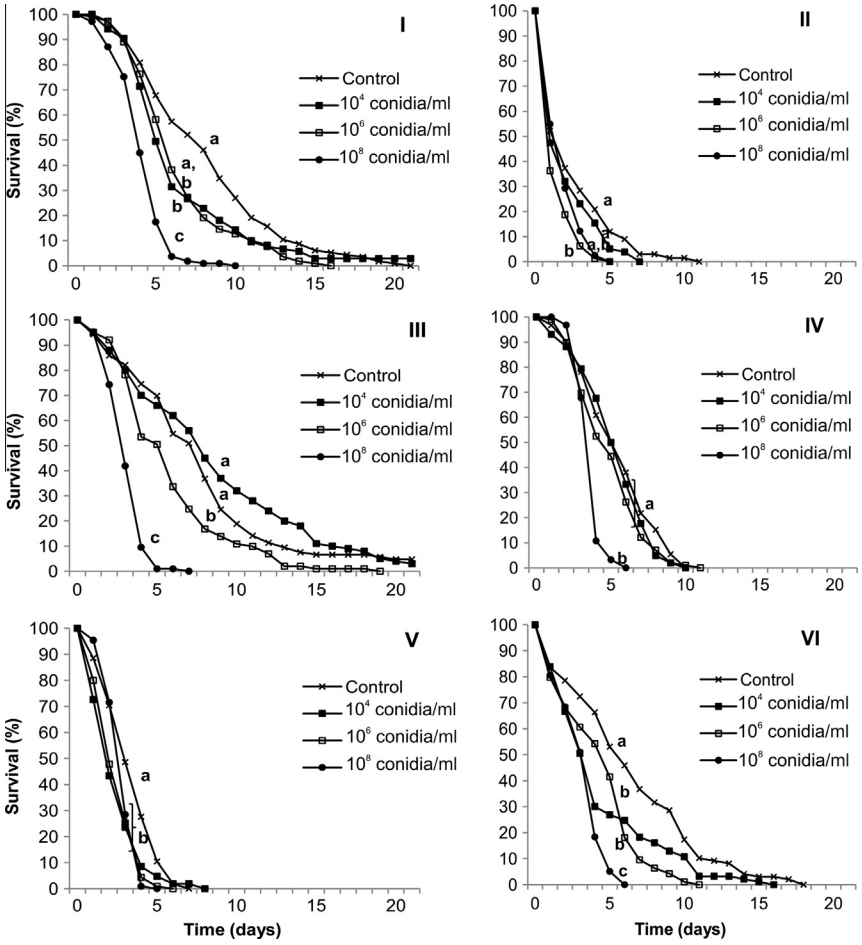

Fig. 4. Survival time (%) discriminated by colony (I to VI) after being treated with different concentrations of conidial suspension of *P. lilacinum* (second assay). Survival distributions with the same letter are not significantly different applying the Bonferroni correction whenever necessary (see text for the *P*-values considered).

Table 2
Percentages of ants naturally infected with *P. lilacinum* or other entomopathogens, with external fungi or without fungi for controls, discriminated by colony, for the second assay.

Treatment: water	I	II	III	IV	V	VI
P. lilacinum	0.0	0.0	0.0	0.0	0.0	0.0
Other entomopathogens	80.9	52.5	14.9	27.2	31.4	73.5
External fungi	0.0	69.3	69.3	58.7	43.8	20.4
Without fungi	19.1	1.5	15.8	14.1	24.8	6.1

with sterile filter paper on the bottom, water and 20% sugar solution *ad libitum*. The mortality of ants was daily monitored for 21 days. Dead ants were not washed in order to simulate a real situation. Each ant was directly transferred to an individual sterile humid chamber; and checked daily for the presence of *P. lilacinum*, other entomopathogens, and external fungi.

2.5. Statistical analysis

Survival distributions of treatments by colonies and of colonies for each treatment were compared with a survival analysis using the Kaplan–Meier method, and we considered the Mantel test to obtain a probabilistic value (SYSTAT 13 ©, 2009). We compared the distributions among multiple groups, and then we carried out pair-wise comparisons whenever required, adjusting the *P* value with the Bonferroni correction. Since, even after transformations, data did not conform to the normal distribution, we used the non parametric Kruskal–Wallis test (for multiple groups) and Mann–Whitney test (for pair-wise comparisons) (SYSTAT 13 ©, 2009) to compare LT_{50} (lethal time, i.e., the time required to kill 50% of the ants) and *P. lilacinum* percentages of infection among and between colonies and treatments. All contrast analyzes were adjusted by the Bonferroni correction to maintain $\alpha = 0.05$. Probit analysis was used to obtain LD_{50} (lethal dose, i.e., the dose required to kill 50% of the ants) (Finney, 1971). For the latter, cumulative mortalities on the 5th day (day at which mortalities were lower than 100%) of each colony were corrected by Abbott's formula: (% treatment mortality − % control mortality)/(100 − % control mortality). (Abbott, 1925).

3. Results

3.1. Identification of P. lilacinum

Morphological identification coincided with the description of *P. lilacinum* given in Luangsa-ard et al. (2011). Our phylogenetic analysis of the EF-1α gene region was congruent with the taxonomy of this group, showing the same relationship among species as reported in previous works (Luangsa-ard et al., 2011; Perdomo et al., 2013). The phylogeny obtained placed our isolate (plob EF1-a) within the clade of *P. lilacinum* (Fig. 1).

3.2. Virulence and horizontal transmission assays

Survival distributions of the *A. lundii* workers treated with an individual immersion differed significantly among colonies for each treatment ($\chi^2 = 18.582$, $P = 0.002$ for NI; $\chi^2 = 39.436$, $P = 0.000$ for water; and $\chi^2 = 39.436$, $P = 0.000$ for ants treated with *P. lilacinum*). Therefore, we had to analyze each colony separately. Survival time of ants treated with *P. lilacinum* was significantly lower than that recorded in controls for all cases, except colony II (Fig. 2). Survival between controls (water and NI) did not differ for five of six colonies ($P > 0.025$), except for colony V in which ants treated with water showed greater mortality than NI ($\chi^2 = 5.712$, $P = 0.017$, $\alpha = 0.025$). Nonetheless, in colony V, ant survival for water treatment was significantly higher than for those treated with *P. lilacinum* ($\chi^2 = 15.708$, $P = 0.000$, $\alpha = 0.016$) (Fig. 2).

Median (and quartiles) LT_{50} for ants treated with *P. lilacinum* were 6.5 (6.0–7.0) days, 10.0 (9.0–11.8) days for ants treated with water and 11.0 (10.0–12.8) days for NI. Median LT_{50} of all treatments differed significantly ($H = 8.715$, df = 2, $P < 0.05$), but for controls did not differ from each other ($U = 13.5$, df = 1, $P > 0.025$), with survival being higher than in ants treated with *P. lilacinum*.

Ants treated with *P. lilacinum* showed percentages of infection higher than 69.8% in all colonies, with a median of 85.6% (Fig. 3A). On the other hand, the percentage of other naturally occurring entomopathogens decreased in ants treated with *P. lilacinum*, compared to control ants (Fig. 3B).

Although the percentages of other fungi found in controls were not the same (Table 1), the statistical comparison among the six colonies showed that they were not significantly different (for *P. lilacinum*: $U = 15.000$, df = 1, $P = 0.592$; for other entomopathogens: $U = 24.000$, df = 1, $P = 0.337$; for external fungi: $U = 16.000$, df = 1, $P = 0.749$; for without fungi: $U = 16.000$, df = 1, $P = 0.749$). In most controls we found high percentages of entomopathogens, and even a small percentage of *P. lilacinum*. These other entomopathogens were mainly *B. bassiana* and species in the genera *Aspergillus* and *Fusarium*. Colony III exhibited the highest level of infection with other entomopathogens in NI treatment (83.9%). External fungi percentages were high (median 35.4% and 42.2% for ants treated with water and NI, respectively), even when ants had been disinfected after their death.

We confirmed a horizontal transmission from inoculated ants to non-inoculated ones. A median of 58.5% (41.9–64.2) of these ants died because of *P. lilacinum* infection, which was statistically higher than controls ($U = 0.0$; df = 1, $P = 0.000$). On the other hand, we were able to confirm that the painting did not affect ant survivorship, because there were no significant differences between the mortality of painted ants and those without painting in the control treatments (water and NI) (in both cases $P > 0.05$).

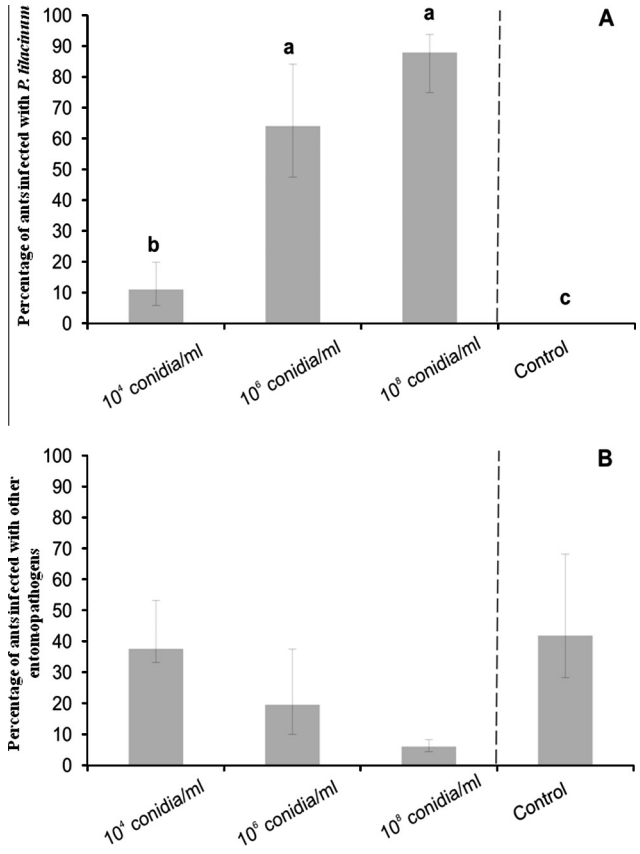

Fig. 5. Percentages of ants for the second assay (groups of 100 ants) infected with *P. lilacinum* (A) or other entomopathogens (B) (median and quartiles), for each conidia concentration of *P. lilacinum*. In A, bars with the same letter are not significantly different ($P > 0.008$). In B, no differences were found ($P > 0.05$).

3.3. Dose dependence assay

Survival distributions of *A. lundii* differed significantly among colonies for each treatment ($\chi^2 = 225.454$, $P = 0.000$ for control; $\chi^2 = 260.258$, $P = 0.000$ for ants treated with 10^4 conidia ml^{-1}; $\chi^2 = 364.379$, $P = 0.000$ for ants treated with 10^6 conidia ml^{-1}; and $\chi^2 = 185.219$, $P = 0.000$ for ants treated with 10^8 conidia ml^{-1}).

In general, survival times of ants treated with the highest concentration was significantly lower than those treated with other concentrations (Fig. 4). When all treatments were pooled (three concentration of *P. lilacinum* + control), we found significant differences among them in survival time of ants ($P < 0.05$); this was also the case when comparing concentrations of *P. lilacinum* ($P < 0.025$), with the exception of colony V, in which survival of ants treated with any concentration did not differ statistically ($\chi^2 = 4.131$, $P = 0.127$, $\alpha = 0.025$). Therefore, we made contrasts for the other five colonies ($\alpha = 0.008$). We found in most colonies that the lowest concentration was not statistically different to the control, whereas the highest concentration always resulted in survival times statistically lower than control (Fig. 4).

Median (and quartiles) LT_{50} were 4.0 (2.3–5.0) days for ants treated with 10^4 conidia ml^{-1} of *P. lilacinum*, 5.0 (2.8–5.8) days for those treated with 10^6 conidia ml^{-1}, and 3.5 (3.0–4.0) days for ants treated with 10^8 conidia ml^{-1}, and 5.5 (3.5–7.5) days for control. LT_{50} did not differ statistically among treatments ($H = 2.544$, df = 3, $P = 0.467$), but differences among colonies became smaller at the highest concentration.

The value of CL_{50} was 2.8×10^5 conidia ml^{-1} (obtained from the following regression line: $y = 0.515x + 2.1967$, $R^2 = 0.6532$), using the cumulative mortalities on the 5th day, corrected by Abbott's formula (38%, 28% and 96% for 10^4, 10^6 and 10^8 conidia ml^{-1}, respectively).

Ants inoculated with the highest concentration of *P. lilacinum* showed levels of infection higher than 71.6% in most colonies (except in colony VI, in which it was 39.8%), with a median of 88.0% (Fig. 5A). Ants treated with 10^6 conidia ml^{-1} had a lower median (64.1%), although not statistically different from the value obtained at the highest concentration. On the other hand, we found that ants treated with 10^4 conidia ml^{-1} exhibited a very low median value (11.1%), with a maximum of 44.9%.

Regarding the occurrence of other entomopathogens, we also observed a trend in their percentages to decrease as the concentration of *P. lilacinum* increased, although we found no statistically significant differences ($H = 6.0$, df = 2, $P > 0.025$) (Fig. 5B).

Contrary to our findings in the previous assay, we did not find *P. lilacinum* naturally infecting control ants (Table 2). We found high percentages of entomopathogens, particularly in colonies I and VI (80.9% and 73.5% respectively). In colony I, the largest number corresponded to *Aspergillus* sp. infections (85 infected ants out of 115 sampled), while in colony VI most of the infections occurred with *Aspergillus* sp. and species of *Fusarium* (37 and 43 infected ants out of 98 sampled, respectively). We recorded high percentages of external fungi in most colonies (except in colony I and VI). External fungi appeared at a higher percentage (median of 51.3%) than in the previous assay, probably because no disinfection was carried out in this instance.

4. Discussion

This work represents the first report of *Purpureocillium lilacinum* infecting worker ants in the *Acromyrmex* genus; moreover, it shows its high virulence as an entomopathogen of *A. lundii*.

Our results lead us to propose that *P. lilacinum* could be a good potential biological control agent of leaf-cutting ants, with a great capacity to infect ants regardless of their natural entomopathogen loads. The *P. lilacinum* treatment showed that the percentage of infection by the other entomopathogens found on these field worker ants was reduced and replaced by *P. lilacinum* infection in inoculated ants, suggesting a good competition capacity of the inoculated fungus.

We found a significantly lower survival of ants treated with *P. lilacinum* compared to controls, which was related to *P. lilacinum* in 85.6% (80.6–89.7) of the cases; we also recorded an LT_{50} of 6–7 days at a concentration of 1×10^6 conidia ml^{-1}. These results are similar to those obtained in other pathogenicity tests at the same concentration with other commonly used entomopathogens against leaf-cutting ants, such as *Beauveria bassiana* or *Metarhizium anisopliae*. For example, an LT_{50} of 5 days was shown for the same concentration of *B. bassiana* over *Atta sexdens sexdens* soldiers, although with a lower effect of these strains (41.7% percentage of infection) (Loureiro and Monteiro, 2005), and an LT_{50} of 6 days in *Atta bisphaerica* workers (Ribeiro et al., 2012). Similar results were obtained for two isolates of *M. anisopliae* on *A. sexdens sexdens*, with LT_{50} between 3.8 and 6.0 days, but with 45.1% percentage of recuperation (Loureiro and Monteiro, 2005).

Insect death occurs through a dependent relationship in the amount of conidia (Butt and Goettel, 2000) and, in fact, we found a significantly faster mortality of ants and a greater median percentage of infection at a higher concentration of *P. lilacinum* conidia, allowing us to consider a dose of 1×10^8 conidia ml^{-1} as the best. However, we found that the amount of conidia left on the ant body is three orders of magnitude lower than that inoculated, and still there was no saturation (unpublished data), so greater concentrations could still be used.

Furthermore, we confirmed that *P. lilacinum* could be horizontally transmitted to other non-inoculated ants. Conidia of *P. lilacinum* could be transferred by direct contact between ants and/or from the "contaminated" containers in which they were maintained. We found a percentage of infection of 58.5%, which was two times higher than the percentage obtained in groups of 5 *A. echinator*'s ants exposed to *M. anisopliae* (Hughes et al., 2002).

We report for the first time the presence and abundance of several entomopathogens in *A. lundii* workers from field colonies. Previously, only *Aspergillus flavus* was known to be an occasional pathogen of leaf-cutting ants, particularly of *A. echinator* (Schmid-Hempel, 1998; Hughes and Boosma, 2004), whereas in another study with *A. echinator* and *A. octospinosus* ants, none of the foragers or nest workers collected was found to be infected by entomopathogenic fungi (Hughes et al., 2004). Only for *A. bisphaerica* it was found an abundance and diversity similar to that reported here (Ribeiro et al., 2012). We found that the diversity of such pathogens varied among colonies, as well as for the same colony from year to year. This was particularly evident with natural *P. lilacinum* loads, because it was found in control groups of the first assay in five of six colonies, whereas it disappeared in the second. This could be the result of the fungus not being able to survive or persist in the same place from one year to the next, although another possible explanation is that the ants became "immunized" due to the first exposition.

Most studies focusing on entomopathogenic infection used ants which had been kept in the laboratory (Hughes et al., 2002; López et al., 1999; Mattoso et al., 2011; Poulsen et al., 2006). This may well represent a situation of lower natural entomopathogen loads. We believe that this type of studies is rather risky because, in nature, ants are always exposed to pathogens. In fact, this could be one of the reasons why field tests do not reproduce laboratory results. Furthermore, the entomopathogens naturally carried by the ants were responsible for the high mortality found in controls. In addition, most studies use a low number of samples from field colonies or laboratory-maintained colonies (Hughes et al., 2002; Hughes and Boosma, 2004; López et al., 1999; Mattoso et al., 2011; Poulsen et al., 2006; Ribeiro et al., 2012). However, we purposely used ants brought from several colonies from the field, which resulted in differences in survival among colonies, contrary to what has been previously reported. Differences in colony survival could be related to several reasons such as ecological variability among sampled sites, size, age, sampling time and immunological status of each colony and, obviously, the presence or absence of other pathogens. Despite colony differences, we found that survivorship of ants treated with *P. lilacinum* was lower than controls in all colonies tested, lending robustness to our results.

When we compared the second assay with the first, in which ants were maintained in smaller groups (5 instead of 100 ants), the percentage of infection was lower for denser groups in ants inoculated with the same concentration (1×10^6 conidia ml^{-1}), reaching a median of 64.1% (in groups of 100 ants) compared to 85.6% (in groups of 5). This could be a consequence of the behavioral and chemical defenses of ants, and/or their immunological defenses. Firstly, in the leaf-cutting ant *A. echinator*, for example, the importance of three defense mechanisms against *M. anisopliae* has been shown: allogrooming, self-grooming and the secretion of chemicals with antibiotic properties from metapleural glands; it has also been shown that increasing the amount of individuals leads to a reduction in mortality and percentage of infection, probably because they clean each other more (Hughes et al., 2002). In our case, allogrooming was observed more frequently in *P. lilacinum*-treated ants than in controls, although it was not quantified. Thus, this could be a reason for the reduction on the percentage of infection in more dense groups. Secondly, we have hypothesized a better immunological status of the more dense groups because these ants had been previously exposed (in nature) to this fungus (see above).

However, in the second assay the LT_{50} for controls was half of that found in the first assay (median 5.5 days versus 11.0 days, respectively). This result was surprising because it was expected that the coexistence of more ants would result in higher survival, given that they are social insects and the largest number of ants would turn into action the group defense mechanisms (Hughes et al., 2002). The fact that the average load of other entomopathogens across colonies in the second assay was higher (46.7%) than that of the first one (38.2%) was translated in a faster mortality.

Finally, we observed that following the traditional methodology (Lacey and Brooks, 1997), post-mortem cuticle disinfection, was

not very effective, because the percentages of external fungi found in ant dead bodies were high. When no disinfection was carried out (second assay), this percentage increased only in 15%.

Nonetheless, considering that some isolations of this fungus have been found to be pathogenic to immunocompromised and even immunocompetent humans (Khan et al., 2012; Saghrouni et al., 2013), caution should be advised when using it in household gardens, although it should not be a problem for crops or plantations, especially if formulated in such a way that its specificity and retrieval is increased.

5. Conclusions

We propose, for the first time, *P. lilacinum* as a good potential biological control agent for leaf-cutting ants because of the great reduction on ant survival, high percentages of infection, horizontal transmission to other ants and great capacity to infect ants despite natural entomopathogen loads.

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