Paecilomyces niveus Stolk & Samson, 1971 (Ascomycota: Thermoascaceae) as a pathogen of *Nasonovia ribisnigri* (Mosley, 1841) (Hemiptera, Aphididae) in Brazil

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Abstract

Nasonovia ribisnigri is a key pest of lettuce (*Lactuca sativa* L.) in Brazil that requires alternative control methods to synthetic pesticides. We report, for the first time, the occurrence of *Paecilomyces niveus* as an entomopathogen of the aphid *Nasonovia ribisnigri* in Pinhais, Paraná, Brazil. Samples of mummified aphids were collected from lettuce crops. The fungus *P. niveus* (PaePR) was isolated from the insect bodies and identified by macro and micromorphology. The species was confirmed by sequencing Internal Transcribed Spacer (ITS) rDNA. We obtained a sequence of 528 bp (accession number HQ441751), which aligned with *Byssochlamys nivea* strains (100% identities). In a bioassay, 120 h after inoculation of *N. ribisnigri* with pathogenic *P. niveus* had an average mortality of 74%. The presence of *P. niveus* as a natural pathogen of *N. ribisnigri* in Brazil suggests that it may be possible to employ *P. niveus* to minimize the use of chemical insecticides.

Keywords: Lactuca sativa, Aphididae, entomopathogenic fungi, biological control.

Registro de *Paecilomyces niveus* Stolk & Samson, 1971 (Ascomycota: Thermoascaceae) como patógeno de *Nasonovia ribisnigri* (Mosley, 1841) (Hemiptera, Aphididae) no Brasil

Resumo

Nasonovia ribisnigri é uma praga-chave do cultivo de alface (*Lactuca sativa* L.), exigindo métodos alternativos ao controle químico. Este trabalho registrou pela primeira vez, a ocorrência de *Paecilomyces niveus* como agente entomopatogenico do afídeo *N. ribisnigri* em Pinhais, Paraná, Brasil. Amostras de afídeos mumificados foram coletadas em plantas de alface. O fungo *P. niveus* (PaePR) foi isolado do corpo dos insetos e identificado por macro e micromorfologia e, confirmado por sequenciamento da região ITS do DNA ribossomal. A sequencia parcial de 528 bp (número de acesso HQ441751) apresentou alinhamento com 100% de identidade com sequencias de raças de *Byssochlamys nivea*. No bioensaio de patogenicidade *P. niveus* apresentou uma mortalidade média de *N. ribisnigri* de 74% até 120 horas da inoculação. O registro da presença de *P. niveus* como um patógeno natural de *N. ribisnigri* no Brasil sugere o potencial de utilização para minimizar o uso de inseticidas.

Palavras-chave: Lactuca sativa, Aphididae, fungo entomopatogênico, controle biológico.

1. Introduction

Aphids (Hemiptera: Aphididae) are key pests of lettuce (*Lactuca sativa* L.) (Díaz et al., 2010). *Nasonovia ribisnigri* (Mosley) is one of the most important aphid parasites of lettuce (Reinink and Dieleman, 1993; Asman, 2007). *N. ribisnigri* is found throughout the lettuce head, even in the developing leaves at the center, whereas other

species prefer external leaves (Liu and McCreight, 2006; Scorsetti et al., 2010). Chemical pesticides are the primary means of aphid control (Barber et al., 1999; Dedryver et al., 2010). However, *N. ribisnigri* is resistant to some insecticides (Martin et al., 1996; Rufingier et al., 1997; Barber et al., 1999; Dedryver et al., 2010). One alternative to the use of insecticides to reduce the occurrence of this pest is integrated pest management involving the application of entomopathogenic fungi (Dorschner et al., 1991; Zaki, 1998; Steinkraus, 2006; Asman, 2007; Díaz et al., 2010; Scorsetti et al., 2010, 2012; Skinner et al., 2012). Fungal pathogens are the most important pathogens of aphids and epizootics are frequently observed that often rapidly reduce aphid populations (Steinkraus, 2006). Chemical insecticides and biological control by entomopathogenic fungi may be used alternatingly or simultaneously, if compatibility or synergism between them is identified (Anhalt et al., 2010).

Several Ascomycota genera, such as *Beauveria*, *Lecanicillium* and *Isaria* also infect aphids (Humber et al., 2011). There is very little available information about the control of aphids using entomopathogenic fungi in Brazil. New fungal strains are important for *N. ribisnigri* control, and may lead to improvements in lettuce production.

We report, for the first time, the occurrence of *P. niveus* as entomopathogen of the aphid *N. ribisnigri* in Pinhais, Paraná, Brazil.

2. Material and Methods

2.1. Collection, isolation, and identification of filamentous fungi

During the monitoring of the entomofauna in a commercial lettuce crop in Pinhais Country, Paraná, Brazil (25º 25' S and 49º 08' W, 930 m), dead specimens of *N. ribisnigri* with fungal mycelia growth on the body surface were found. The climate in the area was temperate according to Köppen as Cfb, (Peel et al., 2007), the average temperature was 21.2 ± 5 °C, and the relative humidity was 84 ± 10%.

Twenty mummified aphid specimens were collected during the fall. To promote fungal development and sporulation and to confirm that fungal infection was the cause of aphid death, specimens were placed on moist filter paper inside plastic Petri dishes, and then incubated for 7 days at 25 ± 1 °C under a 16:8 h light:dark photoperiod, and 60 ± 10% relative humidity (RH) Fungal mycelia grown on the surface of insect bodies and cultured for 7 days on potato dextrose agar medium (PDA) at 28 °C ± 1 °C.

Preliminary fungal identification was carried out by examining the macro and microscopic features of the colonies after slide culturing on PDA at 28 ± 1 °C (Hoog and Guarro, 2000).

Molecular identification of the fungus was performed by sequencing internal transcribed spacer (ITS) rDNA. Purified DNA was obtained as described in Gerrits van den Ende and Hoog (1999). The rDNA regions ITS1, 58 S, and ITS2 were sequenced using ITS5 and ITS4 primers (White Junior et al., 1990). Sequencing was performed in an automated sequencer (ABI 3700, Applied biosystems, Foster City, CA, USA). Sequences were edited and aligned using the Staden sequence analysis package v1.6.0 (Staden, 1996). Sequence analysis was performed using the sequence alignment software BLASTn, which was run against the NCBI's database (National Center for Biotechnology Information website).

Phylogenetic analysis was performed using Mega 5.1 software (Tamura et al., 2007) and applying the neighbor-joining method (Saitou and Nei, 1987), and Jukes-Cantor correct distance model (Jukes and Cantor, 1969).

2.2. Pathogenicity bioassays

Koch's postulates were used to determine the link between *P. niveus* and *N. ribisnigri*. In order to produce an inoculum, a suspension of the isolate was grown on PDA for 7 days at 28 °C ± 1 °C in Petri dishes and then incubated for an additional seven days at 25 °C ± 1 °C, a 16:8 h light:dark photoperiod under 70 ± 10% RH. The concentration of conidia in the filtrate was estimated using an improved Neubauer brightline hemocytometer (Reichart) under a Leitz Dialux 20 EB light microscope (400x). Suspensions were diluted to a final concentration of 1×10^8 conidia mL^{-1}.

A total of 100 third-instar *N. ribisnigri* nymphs were randomly assigned to a fungal treatment group and untreated control group. For each treatment, a 50-mm diameter leaf disc was cut out of a healthy lettuce plant. For the fungal treatment group, the leaf disc was dipped with 2 µL of conidial suspension using a micropipette. The leaf discs were then fed to aphids in Petri dishes containing filter paper moistened with sterile distilled water. Each dish contained 10 aphids. The aphids were then transferred to an environmentally controlled room (25 ± 1 °C, 16:8 h light: dark photoperiod with 70 ± 10% RH) and evaluated every 2 days for 10 days. Dead insects were collected and immersed in 70% ethanol for surface sterilization and were then transferred to individual Petri dishes containing moist filter paper and incubated for 7 days at 25 ± 1 °C under a 16:8 h light:dark photoperiod and 60 ± 10% RH.

2.3. Statistical analysis

Mortality data were corrected using the Abbott Formula (Abbott, 1925) and percentage values were arcsine transformed ($\sqrt{(x/100)}$). Mean mortality data (fungus and water) were compared using the Scott-Knott test as implemented in Sisvar 5.3 software (Ferreira, 2010). Results were considered significant at the 0.05 level.

3. Results

Paecilomyces niveus (PaePR) was the only fungus recovered from *N. ribisnigri* collected from lettuce. After sequencing the PCR amplicon of the ITS regions of the fungal rDNA we obtain a sequence of 528 bp (Table 1).

Table 1. Fungal strains used for phylogenetic analysis of *Paecilomyces niveus* isolated from the aphid *Nasonovia ribisnigri*.

Name	Gene Bank	Source	Origin	Size sequence (bp)
Paecilomyces niveus	HQ441751	*Nasonovia ribisnigri*	Brazil	528
Byssochlamys nivea	FJ389938	Oat grain	Ukraine	569
Byssochlamys nivea	FJ389936	Pasteurized fruit juice	Switzerland	566
Byssochlamys nivea	FJ389935	Milk of cow	USA	566
Byssochlamys nivea	DQ464363	Surface of mechanical grape harvester	USA	536
Byssochlamys nivea	FJ389936	Pasteurized fruit juice	Switzerland	569
Byssochlamys fulva	FJ389943	Fruit juice	Switzerland	569
Byssochlamys fulva	FJ389940.1	Bottled fruit	UK	566
Byssochlamys fulva	FJ389941	Unknown source	-	566
Byssochlamys fulva	DQ459372	Vineyard soil	-	547
Paecilomyces saturatus	FJ389951	Acetic acid	Brazil	567
Byssochlamys laguncullariae	FJ389946	Unknown source	France	567
Byssochlamys laguncullariae	FJ389945	Pasteurized strawberries	Netherlands	567
Paecilomyces saturatus	FJ389950	Unknown source	Japan	568
Paecilomyces formosus	FJ389920	*Annona squamosa*	Brazil	569
Paecilomyces formosus	FJ389921	Soil	Thailand	570
Paecilomyces variotii	FJ895878	Soil	Brazil	610
Paecilomyces variotii	AY753331	Soil	Thailand	551
Byssochlamys zollerniae	FJ389933	Wood of *Zollernia ilicifolia* and *Protium heptaphyllum*	Brazil	564
Paecilomyces sinensis	EU272527.	*Espeletia* sp.	Colombia	627
Paecilomyces divaricatus	FJ389932.	Pectin	Mexico	560

Comparison of the obtained sequence to others in the database suggested that the isolate was from *Byssochlamys nivea* (FJ389938 with 100% of similarity), which is the teleomorphic phase of *Paecilomyces niveus*. The obtained sequence had 94-100% similarity to sequences from 21 strains of *Paecilomyces* sp., and these were used for phylogenetic analysis (Figure 1). The sequence was aligned and submitted to Genbank (accession number HQ441751).

A tree based on rDNA ITS sequencing was built using a neighbor-joining method and applying Jukes-Cantor correct distance model with 1000 bootstrap inferences, as implemented in Mega 4.0.2. Two major groups with high bootstrap values were obtained. The isolate PaePR was clustered with its teleomorph, *B. nivea.*

Pathogenicity assays showed significant difference between two treatments (F-value = 110.162 p-value <0.001). *N. ribisnigri* showed a mean mortality of 74% 120 hours after inoculation with *P. niveus.* Control aphids remained asymptomatic and had a mortality of 12%. The pathogen was recovered from the insect body surface and the identitity of the pathogen was confirmed as *P. niveus* using morphological and molecular techniques (Figure 2).

The isolate PaePR is deposited in the Mycological Collection LabMicro- Laboratório de Microbiologia e Biologia Molecular, Departamento de Patologia Básica, Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, PR, Brazil.

4. Discussion

The genus *Paecilomyces* is a mitosporic fungus that has a wide natural distribution and several entomopathogenic species (Alves, 1998; Steinkraus, 2006; Zimmermann, 2008; Sun and Liu, 2008). Although this entomopathogen infects several pests (Alves, 1998), this is the first record of its attack on *N. ribisnigri*. Hypocreales (Ascomycota) can be important for reducing aphid populations (Steinkraus, 2006).

According to Samson et al. (2009) the genus *Byssochlamys* is morphologically well defined and characterized by almost naked ascomata in which croziers and globose asci are formed with ellipsoidal ascospores. All *Byssochlamys* species have a *Paecilomyces* anamorph that belongs to the *Paecilomyces* sect.

The isolate was initially characterized as *Paecilomyces* sp. by macro- and micro-morphological analysis. ITS sequencing and phylogenetic analysis indicated that the isolate (PaePR) obtained in this study was clustered with its teleomorph, *B. nivea.* Samson et al. (2009) verified by phylogenetic analyses that the genus *Byssochlamys* includes nine species, five of which form teleomorphs, i.e., *B. fulva*, *B. laguncullariae*, *B. nivea*, *B. spectabilis* and *B. zollerniae*, whereas four are asexual, namely *P. brunneolus*, *P. divaricatus*, *P. formosus*, and *P. saturatus*.

This is the first report of *P. niveus* as a parasite of *N. ribisnigri* in Brazil. This study provides data for future research into the use of fungal isolates for the

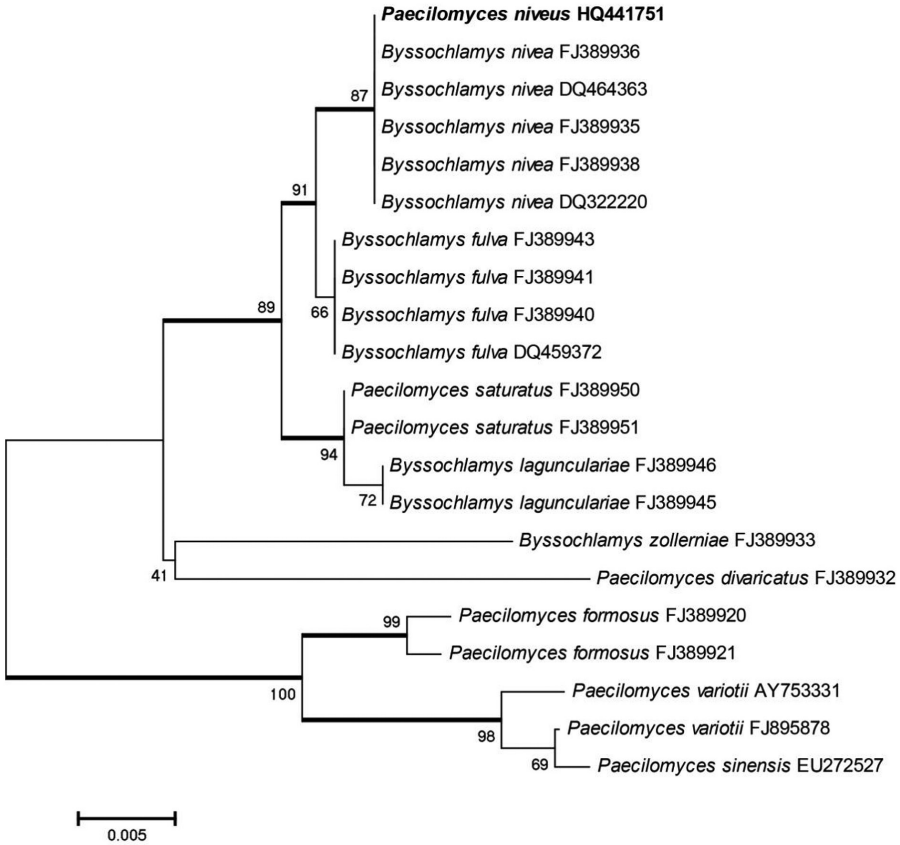

Figure 1. Phylogenetic tree of *Paecilomyces* and *Byssochlamys* species based on ITS sequences. The tree was constructed using a neighbor-joining method, as implemented in MEGA 4.0.2. Bold branches indicate bootstrap values > 80 from 100 resampled datasets. The strain in bold, *Paecilomyces niveus*, was isolated as part of this study. The sequence for this strain is available in GenBank, accession No. HQ441751.

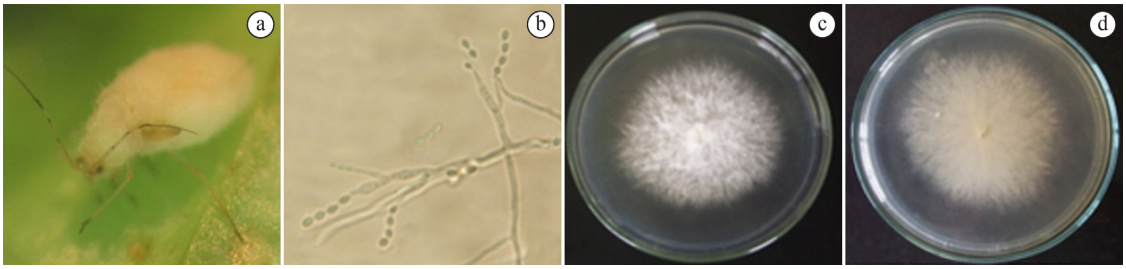

Figure 2. *Nasonovia ribisnigri* body covered by *Paecilomyces niveus* mycelia (a); Micromorphology of *P. niveus* mycelia using optical microscopy (40X) showing conidiophores and conidia (b); *P. niveus* isolated from *N. ribisnigri* in PDA medium, macromorphology (colony reverse) (c); macromorphology (colony obverse) (d).

biological control of aphids and additional diversity data of entomopathogenic fungal diseases.

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