

Potential of a *Cladosporium cladosporioides* strain for the control of *Tetranychus urticae* Koch (Acari: Tetranychidae) under laboratory conditions

Potencial de una cepa de *Cladosporium cladosporioides* para el control de *Tetranychus urticae* Koch (Acari: Tetranychidae) bajo condiciones de laboratorio

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ABSTRACT

The two spotted spider mite *Tetranychus urticae* is an important polyphagous pest worldwide. It is able to adapt to a wide variety of environments and has a high reproduction rate. In practice, farmers try to reduce losses by using synthetic acaricides. However, frequent and inadequate applications of acaricides have made this mite resistant to many active ingredients, creating the need to search for alternative control strategies. The aim of this research was to identify and to evaluate an indigenous strain of a fungus, which eliminated a *T. urticae* colony in a greenhouse. The isolated fungus was identified through a morphological and molecular characterization as *Cladosporium cladosporioides*. Thereafter, the mites were treated with five concentrations of *C. cladosporioides* conidia (2×10^4 , 2×10^5 , 2×10^6 , 2×10^7 , and 2×10^8 conidia ml⁻¹) and a positive control (commercial *Beauveria bassiana* strain, 1×10^6 conidia ml⁻¹). After 10 d, all treatments achieved at least 50% control; the concentrations 2×10^7 and 2×10^8 spores ml⁻¹ controlled 73.3% and 81.7%, respectively, surpassing the commercial strain slightly (72.3%). TL₅₀ ranged between 5 (2×10^8 spores ml⁻¹) and 8 (2×10^4 spores ml⁻¹) d, and LC₅₀ was 1.95×10^6 . The possible acaricidal effect of this strain on these mites is discussed.

Key words: entomopathogenic fungi, biological control of mites, *Beauveria bassiana*.

RESUMEN

El ácaro bimaclado *Tetranychus urticae* es una plaga polífaga importante a nivel mundial. Es capaz de adaptarse a una amplia variedad de entornos y tiene una alta tasa de reproducción. En la práctica, los agricultores intentan reducir las pérdidas utilizando acaricidas sintéticos. Sin embargo, las aplicaciones frecuentes e inadecuadas de acaricidas han hecho que este ácaro sea resistente a muchos ingredientes activos, lo que genera la necesidad de buscar estrategias de control alternativas. El objetivo de esta investigación fue identificar y evaluar una cepa nativa de un hongo, que erradicó una colonia de *T. urticae* en el invernadero. Se identificó el hongo aislado mediante caracterización morfológica y molecular como *Cladosporium cladosporioides*. Posteriormente, los ácaros se trataron con cinco concentraciones de conidias de *C. cladosporioides* (2×10^4 , 2×10^5 , 2×10^6 , 2×10^7 y 2×10^8 conidias ml⁻¹) y un control positivo (cepa comercial de *Beauveria bassiana*, 1×10^6 conidias ml⁻¹). Después de 10 d, todos los tratamientos lograron un control de al menos el 50%; las concentraciones de 2×10^7 y 2×10^8 esporas ml⁻¹ controlaron 73,3% y 81,7%, respectivamente, superando levemente la cepa comercial (72,3%). El TL₅₀ varió entre 5 (2×10^8 esporas ml⁻¹) y 8 (2×10^4 esporas ml⁻¹) d y la LC₅₀ fue de $1,95 \times 10^6$. Se discute el posible efecto acaricida de esta cepa para controlar ácaros.

Palabras clave: hongos entomopatógenos, control biológico de ácaros, *Beauveria bassiana*.

Introduction

The efficient control of spider mites has been reported for invertebrate pathogenic fungi (van der Geest *et al.*, 2000). The entomopathogenic fungi *Beauveria bassiana* (Bals.-Criv.) Vuill., *Hirsutella thompsonii* Fischer and *Metarhizium anisopliae* (Metschn.) Sorokin were observed to be promising controls for *T. urticae* (Jeyarani *et al.*, 2011). Invertebrate pathogenic fungi are efficient control agents against mites and other arthropods and are increasingly involved in pesticide resistance management in combination

with sublethal doses of acaricides (Amjad *et al.*, 2012). Moreover, they can be applied in combined strategies, for example, along with the predatory mites *Phytoseiulus longipes* (Amjad *et al.*, 2012) or *Neoseiulus californicus* (Oliveira *et al.*, 2013).

The breakdown of the *T. urticae* colony in our greenhouse as a result of an entomopathogenic fungal outbreak, apparently from the genus *Cladosporium*, sparked interest in its identification and evaluation of its potential as a biological control agent for this mite. This fungus has been widely

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studied against *T. urticae* (Eken and Hayat, 2009). These authors tested 13 strains of *C. cladosporioides* in a lab bioassay, and the total mortality percentage varied from 50.95 to 74.76% for *T. urticae*, with a LT_{50} range between 2.34-3.9 d. These results led the authors to the conclusion that this fungus is a promising candidate for the biological control of *T. urticae*. Since no studies on *Cladosporium* spp. versus mites are known to have been conducted in Colombia so far, the aim of this study was to test the pathogenicity of these Colombian strains against *T. urticae*.

Materials and methods

Studies were conducted in the Laboratory of Entomology, Faculty of Agricultural Sciences, at the Universidad Nacional de Colombia, Bogota. Deceased mites (*T. urticae*) were collected from bean plants (*Phaseolus vulgaris*, variety Cerinza) established in a greenhouse (mean temperature $22^{\circ}\text{C} \pm 10^{\circ}\text{C}$ measured using a TFA Indoor Digital Thermo Hygrometer during the entire assay, $80\% \pm 3\%$ relative humidity and 12:12 h light:darkness). *P. vulgaris* in the vegetative stage (three weeks after germination) served as host plants for the mite colony; the plants were irrigated every three days. In order to avoid the presence of any infested mites, healthy mites were obtained from the Center of Biosystems of the Universidad Tadeo Lozano in Chia (Cundinamarca, Colombia) and from Flores de Tenjo Ltda. (Tenjo, Cundinamarca, Colombia).

Strain isolation and identification

The fungal strain was isolated from dead mites in the colony in the entomology greenhouse of the Faculty of Agricultural Sciences, where an apparently epizootic event occurred in 2010. The fungus was cultured in Potato Dextrose Agar (PDA) medium and kept it in an incubator at 25°C . This procedure was repeated until monoconidial cultures were obtained.

The fungus was identified by considering its morphological and genetic characteristics. The morphological part was described using a culture grown on a solid PDA medium following the key developed by Bensch *et al.* (2012). For the molecular identification of the fungus, genomic DNA was isolated as follows: (i) to a 2 ml Eppendorf tube containing 1000 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% Sodium Dodecyl Sulfate, SDS) and 425-600 mm sized acid-washed glass beads (Sigma-Aldrich, St. Louis, USA), a small lump of fresh mycelia was added by using a sterile toothpick. After a quick vortex mix, the Eppendorf tube was left at room temperature for 15 min and centrifuged at 10,000 g for 10 min at 4°C . (ii) The supernatant was

transferred to a 1.5 ml Eppendorf tube, and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added; the sample was vortexed and centrifuged at 10,000 g for 10 min at 4°C ; this step was repeated using an equal volume of chloroform-isoamyl alcohol (24:1). (iii) After transferring the supernatant to a new 1.5 ml Eppendorf tube, an equal volume of isopropyl alcohol was added and briefly mixed by inversion and stored at -20°C for at least for 1 h. The sample was centrifuged at 10,000 g for 10 min and the supernatant was discarded. (iv) The resultant DNA pellet was washed in 300 μl of 70% ethanol two times, and the supernatant was discarded each time after the pellet was centrifuged at 10,000 g for 5 min at 4°C . The DNA pellet was air dried and dissolved in 50 μl of Tris-EDTA and incubated with RNAase (10 $\mu\text{g}/\text{ml}$) at 37°C for 30 min. Different dilutions (1:1 and 1:5) of the purified DNA were used in 50 μl of the PCR mixture. The internal transcribed spacer region (ITS) from the rRNA was amplified using the ITS 1-4 primers, following the conditions described by Avellaneda-Torres *et al.* (2014). The obtained sequences were analyzed using the software Genius, version PRO 5.1.5., and compared with sequences in the NCBI GenBank using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990; Benson *et al.*, 2005).

Preparation of inoculum

The inoculum was produced using pure fungus cultures grown on PDA in sealed Petri dishes and incubated at 25°C . The spore solutions were prepared by adding 10 ml of distilled and sterile water and 0.1% Tween 20 on the pure culture and scraping the medium with a sterilized needle. This solution was passed through a cloth, and the number of conidia per ml was counted in a Neubauer chamber under a light microscope.

Five different concentrations were prepared from a 2×10^8 conidia ml^{-1} stock suspension, which were diluted in water at 2×10^4 , 2×10^5 , 2×10^6 , and 2×10^7 conidia ml^{-1} for immediate application in the bioassay.

Bioassays under controlled conditions

Humid cotton was placed in a Petri dish and was covered with an upside-down rose leaf (variety Charlotte grafted on the Natal Briar rootstock). Then, 20 mites were placed on each leaf (experimental unit). Every experimental unit was repeated five times. The fungus was applied using a mobile sprayer located in the weed greenhouse of the Faculty of Agricultural Sciences. First, the sprayer was calibrated as follows: 1 kg/cm^2 pressure, 0.48 m s^{-1} speed and 0.57 l min^{-1} as volume. After spraying the Petri dishes,

they were placed in an incubator (25°C, 80 % RH, 12:12 h light:darkness) for 10 d.

The seven treatments were conducted as follows: T1: negative control (water and Tween 20 0.05 %), T2: positive control applying the commercial product Bassianil[®], with *Beauveria bassiana* as the active ingredient (Perkins Ltd., Palmira, Colombia) at a concentration of 1×10^6 conidia ml^{-1} . Based on previous experiments and the study by Saranya *et al.* (2010), six conidia concentrations were selected; the treatments (fungus concentrations = conidia ml^{-1}) were T3: 2×10^4 , T4: 2×10^5 , T5: 2×10^6 , T6: 2×10^7 , T7: 2×10^8 . The entire experiment was repeated three times over time. The mites were counted for 10 d, starting on the first day after application, and the accumulated percentage of dead individuals was calculated.

Statistical analysis

Abbott's equation (1925) was used in order to correct the mortality data. The effects of treatments on mite mortality were compared using the general linear model procedure (PROC GLM, SAS Institute 2013); significant differences between the treatments were identified with Tukey's test HSD ($P < 0.05$). The treatments were arranged using a completely randomized design.

Results and discussion

Strain identification

The isolated strain taken from the PDA cultured fungus presented a brownish dark color surface, a black color reverse; the plain surface was haired and occasionally turned to dust. At the microscopic level, we identified hyphae septate, branched and dark (data not shown). These results are corroborated by the observations of Bensch *et al.* (2012), who described the surface ornamentation of conidia in the *C. cladosporioides* complex as quite variable, ranging from smooth, or almost so, to irregularly verruculose-rugose, verrucose or rough-walled in some species.

The extracted DNA was visualized with agarose gel electrophoresis (Fig. 1A), and the size of the successfully amplified sequence, using ITS1-4 primers, was about 513 base pairs (Fig. 1B). After the sequencing and DNA data comparison using BLAST search, highly homologous sequences were obtained with GeneBank samples in NCBI. This indicated that the microorganism corresponds to the fungus of the division Ascomycota, subphylum Pezizomycotina, order Capnodiales, family Cladosporiaceae, genus *Cladosporium*,

and species *Cladosporioides*. Its coverage was 100%, the E-value was 0.0, and the identity was 100% (accession number LN808903.1).

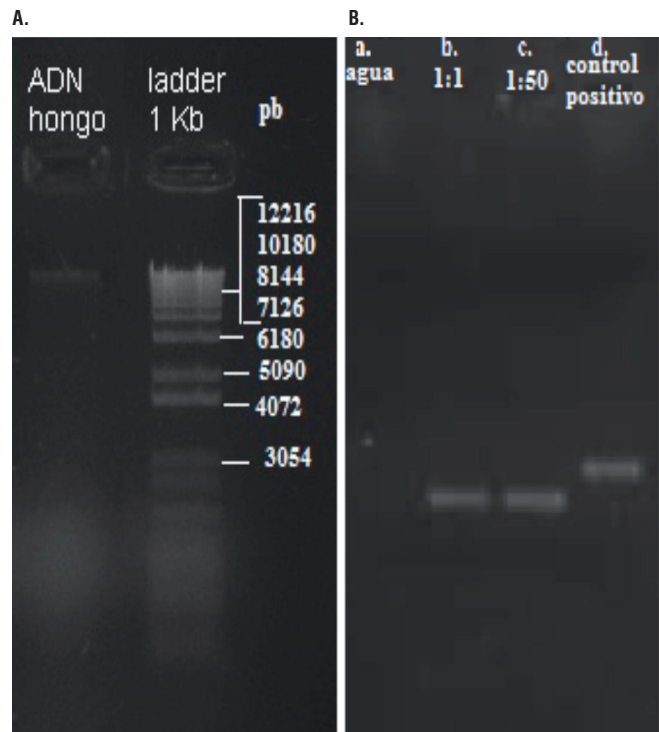


FIGURE 1. Agarose gel analysis of DNA extracted from a fungus strain (A) and Gel electrophoresis of amplicons products with the primers ITS1-4 of the rRNA gene on purified genomic DNA (B). Amplicons (513 bp) from 1:1 (b) and 1:50 (c) ADN dilutions; NC: negative control; no DNA (a). PC: positive control (d). MM: 1 Kb DNA molecular weight marker; DNA lambda (Thermo).

Cladosporium spp. These species are ubiquitous, saprobic, dematiaceous fungi and have been associated with human and animal opportunistic infections (Sandoval-Denis *et al.*, 2015). Their entomopathogenic potential has been described since the early eighties, when their pathogenicity to insects was recorded (Samways and Grech, 1986). Nowadays, *Cladosporium* spp. are considered effective biological control agents against insects and mites at the laboratory level (Sosa-Gomez *et al.*, 2010; Bahar *et al.*, 2011). Nevertheless, among the arthropod-associated ascomycete fungi, uncertainties remain about the extent to which species in ubiquitous genera, such as *Cladosporium*, *Aspergillus*, *Penicillium*, and *Fusarium*, are pathogenic to arthropods or might be opportunistic secondary pathogens or saprotrophs that colonize available cadavers (Samways and Grech, 1986). Thus, histopathologic studies are necessary in order to prove whether this organism is the causal agent of an arthropod's death.

Entomopathogenic activity of *Cladosporium cladosporioides*

It was observed that the dead mites presented a brown or dark green color resulting from the attack of the fungus. When the fungus entered an advanced stage of development, the color turned black. Table 1 shows significant differences between the different conidia concentrations for all days (Tukey's test, $P < 0.05$). The concentration 2×10^4 conidia ml^{-1} achieved moderate control of the mites, 62%. The highest mortality (81.7%) was obtained with 2×10^8 conidia ml^{-1} , suggesting that this fungus might be a promising candidate for the control of mites and should be evaluated under field conditions.

These results surpassed the efficacy of the commercial *B. bassiana* strain (72.3 %). The negative control (only water + Tween) showed a mortality of 20.7%, which was similar to the results obtained by Chandler *et al.* (2005). They hypothesized that a high mortality in the control might be associated with an interaction between fungus and non-fungus mortality factors in a treatment bioassay; for instance, lesions caused by transferring the mites to the experiment unit, a short life cycle and a non-uniform age of the mites.

Table 1 gives evidence for the progressive activity of the fungus: the concentration $2 \times 10^8 \text{ ml}^{-1}$ caused a 50% mortality after 5 d, $2 \times 10^7 \text{ ml}^{-1}$ after six, $2 \times 10^6 \text{ ml}^{-1}$ after seven and the treatments with the lowest concentration ($2 \times 10^5 \text{ ml}^{-1}$ and $2 \times 10^4 \text{ ml}^{-1}$) caused 50% mortality after 8 d. A similar mortality rate was reported by Gatarayih (2009), who observed that *B. bassiana* caused a lethal time, LT_{50} , between 5.5 and 8.9 d. Unfortunately, it is impossible to compare our results with other reports because of different experimental

designs of bioassays with different fungi and arthropods (Chandler *et al.*, 2005; Bahar *et al.*, 2011). However, other criteria for fungi selection should be considered, such as a high conidia production and high final mortality rates (Chandler *et al.*, 2005; Bahar *et al.*, 2011). Lower conidia concentrations (2×10^4 and 2×10^5 conidia ml^{-1}) are able to reduce mite populations. However, higher concentrations (2×10^6 , 2×10^7 and 2×10^8 conidia ml^{-1}) definitely shorten the lethal period, which was corroborated by our experiments.

A mean lethal concentration LC_{50} of 1.95×10^6 conidia ml^{-1} was found. This concentration was low compared with the LC_{50} of other fungi, such as *B. bassiana* (8.65×10^7 conidia ml^{-1}), *Hirsutella thompsoni* (1.06×10^8 conidia ml^{-1}), and *M. anisopliae* (2.43×10^7 conidia ml^{-1}), on *T. urticae* (Chandler *et al.*, 2005). Our results indicate that the fungus showed a good control potential towards mites. It is possible that the fungus also acted through toxic bioactive metabolites, increasing the effect with a typical mycoparasitic infection (Bensaci *et al.*, 2015). Samways and Grech (1986) stated that *Cladosporium oxysporum* is capable of inducing major decreases in the aphids *Toxoptera cictriculus* and *T. erytrae*. Moreover, Shaker *et al.* (2019) provided support for this hypothesis through the isolation of two major compounds from *C. cladosporioides*, which were identified as 3-phenyl propanoic acid and 3-(4 β -hydroxy-6-pyranonyl)-5-isopropylpyrrolidin-2-one. These compounds caused 100% mortality for the aphid *Aphis gossypii*.

The toxic properties and degree of their toxicity vary depending on the administration, chemical structure and concentration (Piecková and Jesenská, 1999). Since *Cladosporium* spp. form tiny conidia in high amounts (Piecková and Jesenská, 1999), it can be concluded that this fungus is

TABLE 1. Percentage of corrected mortality (Abbott, 1925) of *Tetranychus urticae* after applications of *Cladosporium cladosporioides* over 10 days.

DAA	Control	2×10^4	2×10^5	2×10^6	2×10^7	2×10^8	<i>B. bassiana</i> 2×10^6	F _{value}	P _{value}
1	1.3 ± 0.75 c	6.7 ± 4.6 b	9.0 ± 0.9 b	14.3 ± 1.5 a	14.3 ± 1.9 a	16.7 ± 3.3 a	6.7 ± 3.1 b	21.5	<0.0001
2	2.3 ± 0.9 d	11.7 ± 2.6 c	14.3 ± 1.9 bc	20.0 ± 3.1 ab	23.7 ± 6.1 a	27.0 ± 3.6 a	14.7 ± 4.3 bc	26.0	<0.0001
3	3.7 ± 0.75 e	16.0 ± 3.0 d	20.3 ± 4.3 cd	27.0 ± 5.2 abc	32.3 ± 7.5 ab	35.0 ± 3.1 a	24.0 ± 3.5 bcd	29.6	<0.0001
4	5.0 ± 1.2 d	22.0 ± 3.2 c	27.3 ± 4.9 bc	32.7 ± 6.5 abc	40.7 ± 10.9 a	42.3 ± 2.5 a	34.3 ± 7.0 ab	22.7	<0.0001
5	7.7 ± 1.5 e	28.7 ± 5.7 d	32.3 ± 5.1 cd	37.3 ± 6.9 bcd	47.3 ± 12.4 ab	51.7 ± 3.3 a	44.3 ± 8.9 abc	21.6	<0.0001
6	9.0 ± 0.9 c	36.0 ± 6.3 b	42.3 ± 8.0 ab	44.3 ± 6.0 ab	54.0 ± 13.0 a	57.3 ± 6.8 a	53.0 ± 6.5 a	23.9	<0.0001
7	11.3 ± 1.8 c	43.7 ± 8.3 b	48.3 ± 8.7 ab	52.0 ± 6.6 ab	60.0 ± 10.5 a	62.3 ± 5.4 a	58.3 ± 4.9 a	30.2	<0.0001
8	14.3 ± 1.9 c	51.7 ± 10.1 b	54.0 ± 9.5 b	59.0 ± 4.8 ab	63.7 ± 8.4 ab	69.0 ± 6.9 a	65.0 ± 5.4 ab	32.5	<0.0001
9	19.0 ± 3.8 c	59.0 ± 8.5 b	57.7 ± 8.2 b	63.0 ± 5.7 ab	68.0 ± 7.8 ab	75.0 ± 8.3 a	68.0 ± 6.5 ab	32.9	<0.0001
10	20.7 ± 4.9 c	62.0 ± 9.8 b	63.0 ± 7.3 b	70.3 ± 5.4 ab	73.3 ± 7.0 ab	81.7 ± 6.1 a	72.3 ± 6.0 ab	42.4	<0.0001

DAA: Days after application. *Means followed by the same letter are not significantly different (Tukey HSD, $P \leq 0.05$).

an efficient candidate for the biological control of arthropods. Singh *et al.* (2016) described the potential of toxins produced by *Cladosporium velox* for exhibiting activities of Alpha glucosidase inhibitors, affecting the digestion and moulting of *Spodoptera litura*.

It was observed that the LT₅₀ of the mites oscillated between seven and nine days when treated with *Cladosporium* and *B. bassiana*, which was significantly different from the control. The lowest LC₅₀ (1.11x10⁶ conidia ml⁻¹) occurred on d 10. The rate of mortality decreased during the entire observation period, whereas the standard deviation increased. Despite the fact that the differences between the commercial strains and our isolate were not statistically significant because of the high mortality in the control. The results suggest that our tested strain may be a promising candidate for biological control. Another experiment under controlled conditions and with higher conidia concentrations verified under semi-controlled and field conditions may give further evidence of the efficacy of this strain as a control agent of mites. Moreover, further studies on secondary metabolites of this strain and their toxicity to tetranychid mites might create options for biological control strategies against this pest. For example, Shaker *et al.* (2019) successfully extracted ethyl acetate from *C. cladosporioides*, leading to 100% control of aphids.

Acknowledgments

This research was financially supported by the Faculty of Agricultural Sciences of the Universidad Nacional de Colombia, Bogota. We would like to thank Sandra Esperanza Melo, Aquiles Enrique Darghan and Diana Zabala for their statistical support. Special thanks to Rogerio Biaggioni Lopes for his constructive comments on the manuscript. We are highly indebted to the anonymous reviewers for their extraordinary helpful comments on an earlier version of this manuscript.

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