Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i1.3392

Artículo

# Identification of five strains of *Simplicillium* associated with pustules of coffee rust in Costa Rica

Gerardo García-Nevarez[1]
Leslie Carnero-Avilés[1§]
Eduardo Hidalgo-Jaminson[2]

1 Campo Experimental Delicias-INIFAP. Carretera Delicias-Rosales km 2, Delicias, Chihuahua. CP. 33000. (garcia.gerardo@inifap.gob.mx).

2 Centro Internacional de Agricultura y Biociencias. Turrialba, Cartago, Costa Rica. CP. 30501. (e.hidalgo@cabi.org).

Autora para correspondencia: carnero.leslie@inifap.gob.mx.

## Abstract

Coffee rust (*Hemileia vastatrix*) is one of the most economically important diseases in coffee crops due to the production losses of up to 50% that it causes. In addition to the conventional chemical control of this disease, several natural enemies, such as mycoparasitic fungi, regulate its incidence and severity. The study aimed to identify and characterize five strains of fungi associated with the natural control of *H. vastatrix* isolated from the coffee-growing area of Turrialba, Cartago, Costa Rica. Five strains of *Simplicillium* were identified by molecular analysis; measurements of macroscopic characteristics, microscopic characteristics, radial growth, and conidia production were made. According to the analysis in the NCBI database, the five strains matched the species *Simplicillium lanosoniveum*. Overall, macroscopic characteristics were similar between the isolates; the most notable difference was the coloration of the culture medium. Strains that showed a color change from white to brown had lower radial growth. The arrangement of the phialides, the shape and size of the conidia, and the microscopic features were generally consistent with the genus *Simplicillium*. Conidia size and production were statistically different between strains ($p< 0.0001$). In addition, the strains with the largest conidia recorded the lowest values of conidia production. These results provide the first report that characterizes strains of the genus *Simplicillium* associated with *H. vastatrix* in Costa Rica.

## Palabras clave:

cultural characterization, natural biological control, physiological characterization.

DOI: https://doi.org/10.29312/remexca.v16i1.3392

## Introduction

Coffee crops face multiple diseases, with coffee rust (*Hemileia vastatrix*) being one of the most economically significant. This disease causes premature leaf to drop and branch drying, which can result in a loss of more than 50% of the crop and even the death of plants (Aristizábal and Johnson, 2022).

The most devastating rust outbreak in Central America occurred during the 2012-2013 cycle, with estimated losses exceeding 499 million dollars, leading several countries to declare a national emergency (Promecafé, 2013; IICA, 2013). The usual control methods for this disease include the exclusive use of conventional fungicides or copper-based protectors; nevertheless, these have effects that can affect the microbial composition of the soil, altering the functions and biochemical processes that occur in it, which also caused plants to be more susceptible to diseases (Meena *et al*., 2020). Alternatively, sustainable management techniques can be implemented through the use of natural enemies of *H. vastatrix* associated with coffee rust (García-Nevárez *et al*., 2019).

Previously, the mycoparasitic fungus *Lecanicillium lecanii* (Zimmerman) (Guharay *et al*., 2001) was reported as a biological controller of *H. vastatrix* (Santiago-Elena *et al*., 2020). This mycoparasite attacks when rust is active (within the living tissue of the host) by introducing its germ tube into cell membranes to later produce metabolites that inhibit germination, affect the morphology of uredospores, and cause the death of the fungus (Moricca and Ragazzi, 2008).

Due to the above, when isolating parasitic fungi from rust, it is assumed that the isolated fungus is *Lecanicillium* (*Verticillium*), without further evidence to support its identification. This assumption can be risky, especially when new research protocols are initiated. The inclusion of new isolated fungi associated with the natural control of pests or diseases requires correct identification before establishing experiments focused on a biological control program and it is essential for the production of quality biopesticides (Safavi, 2010).

This process must be carried out by specialized taxonomists by identifying morphological characteristics, such as the shape, size, and germination speed of the conidia, as well as the appearance of the colony (shape, elevation, surface appearance, and color) (Hajek and Leger, 1994) and requires the use of molecular markers for their proper identification. Molecular identification by amplification of the Internal Transcribed Spacer (ITS) offers fast and reliable results for most fungal taxa, which is why it is currently considered one of the main diagnostic tools (Lücking *et al*., 2020).

The study aimed to perform a cultural and physiological characterization and a molecular identification of fungal strains associated with *H. vastatrix* pustules in the coffee-growing area of Turrialba, Costa Rica.

## Materials and methods

The study included five fungal strains from the coffee-growing area of Turrialba, Cartago, Costa Rica in the localities of San Juan Norte (SJ), Jabillos (JV), Aquiáres (AQ), Santa Rosa (SR), and CATIE (EC), where the coffee trees were located between 600 and 950 masl. The samples were coffee leaves with advanced lesions of rust and fungi growing on them (Figure 1).

Revista Mexicana de Ciencias Agrícolas
DOI: https://doi.org/10.29312/remexca.v16i1.3392

**Figure 1. Coffee leaf with rust pustules and *Simplicillium* growing on them.**

Strain isolation. It was performed by seeding the inoculum in plates with potato dextrose agar (PDA) medium; this inoculum was obtained from the pustules that presented a cottony growth on them.

Macroscopic characterization. Monosporic isolates of 10 colonies per strain were performed by dilutions ($10^{-4}$, $10^{-5}$ and $10^{-6}$); these were seeded and incubated at 25-28 °C for 15 days and then the color (Munsell Color Company 1988), the shape, elevation and striation formed were recorded according to the criteria proposed by Fox (1999).

## Molecular identification

The genetic material was extracted according to the protocol described by Kuske *et al*. (1998), followed by an amplification of the ITS region using the primers $ITS_1$ (5'-TCCGTAGGTGAACCTGCGG-3') and $ITS_4$ (5'-TCCTCCGCTTATTGATATGC-3') by polymerase chain reaction (PCR); the product obtained from the amplification was visualized in a 1% agarose gel for subsequent sequencing. The consensus of the sequences was carried out with the BioEdit program and they were compared with those reported in the National Center for Biotechnology Information (NCBI) database using the Blast tool.

## Microscopic characterization

The sample size necessary to measure the size of the conidia was determined by measuring groups with different numbers of conidia and calculating the standard deviation (SD) in each case. The sample size that would guarantee a stable SD was selected (Cortez *et al*., 2003). Based on these results, measurements were made in five replications of monosporic colonies of each strain. In each replication, four groups of conidia were measured, observing 35 conidia per group, using an Omax ToupView® x 86 camera mounted on a pre-calibrated compound microscope.

## Radial growth and conidia production

The serial dilution technique was used to isolate each monosporic strain and culture them in PDA in Petri dishes. After 10 days of incubation at 25-28 °C, the average radial diameter was measured with a Truper® Caldi-6 MP digital vernier. To assess conidia production, one $cm^2$ of each strain was

DOI: https://doi.org/10.29312/remexca.v16i1.3392

suspended in 10 mL of sterile distilled water with 0.1% Tween 80%, subjected to an ultrasonic bath for three minutes, and stirred in a vortex for one minute. The conidia were counted with a Neubauer chamber. Radial growth was measured and conidia were counted in 10 colonies per strain.

### Statistical analysis

The experiment was conducted using a completely randomized design with 10 replications of each colony to assess radial growth and five to assess conidia size. The analysis was performed using general linear and mixed models with the InfoStat statistical package. For the variables of number and size of conidia, the DGC mean separation test ($p$= 0.05) was used in order to eliminate the lack of the transitive characteristic between means and facilitate the discussion of the results. For the radial growth variable, the LSD test was performed ($p \leq$ 0.05).

## Results

Macroscopic characterization. The most noticeable difference between strains was the color change at the bottom of the Petri dish. The JV and SR strains exhibited an olive yellow coloration, whereas EC, AQ and SJ presented yellow, white, and pale yellow coloration, respectively. The white coloration of the mycelium was consistent across all strains, as were the circular shape and raised edge. Only AQ showed abundant striae in the culture medium during growth (Table 1), which could sometimes be seen from the top of the colony (Figure 2).

**Table 1. Colony morphology of fungal strains associated with pustules of *Hemileia vastatrix*.**

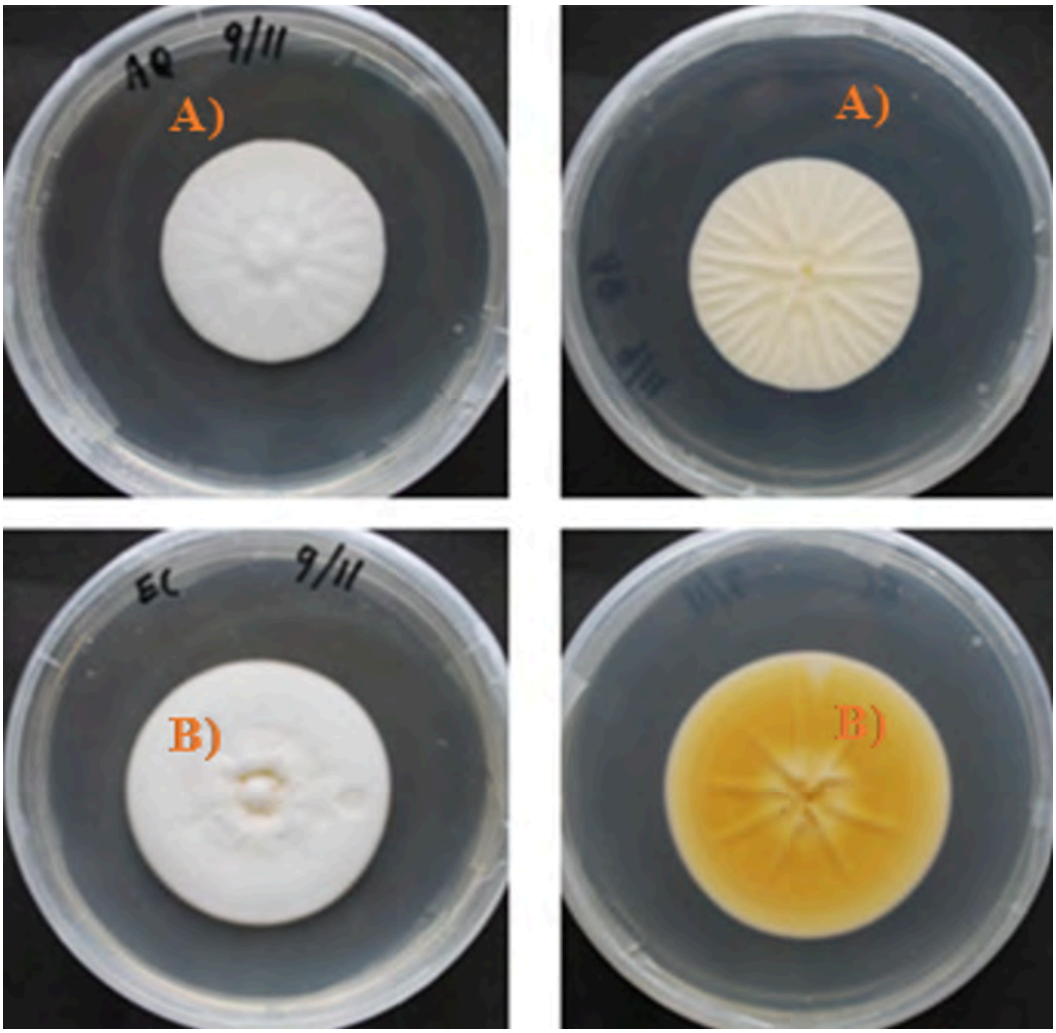
| Strain | Color front/back | Munsell notation | Shape | Edge | Relief | Striae formation |
|---|---|---|---|---|---|---|
| EC | White/yellow | 2.5Y 7/8 | Circular | Complete | Raised | Low |
| AQ | White/white | 5Y 8/2 | Circular | Complete | Raised | Abundant |
| JV | White/olive yellow | 2.5Y 6/6 | Circular | Complete | Raised | Low |
| SR | White/olive yellow | 2.5Y 6/6 | Circular | Complete | Raised | Low |
| SJ | White/pale yellow | 2.5Y 8/4 | Circular | Complete | Raised | Low |

SR= Santa Rosa strain; AQ= Aquiáres; EC= CATIE; JV= Jabillos; SJ= San Juan Norte.

Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i1.3392

**Figure 2. Monosporic colonies of strains of A) Aquiáres and B) CATIE. Front and back views.**

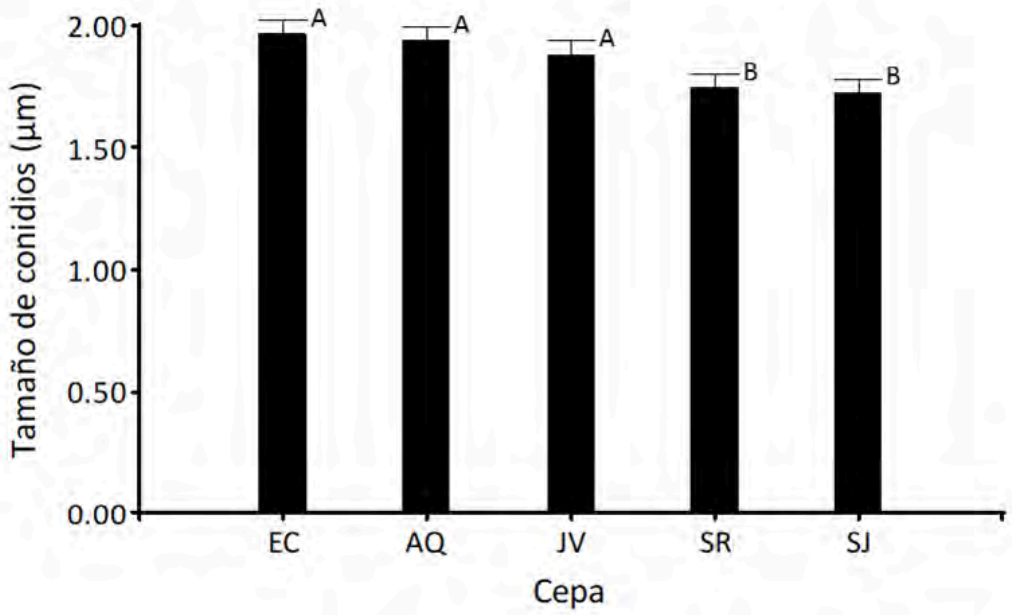



The size of the conidia was significantly different between strains ($p \leq 0.003$). The EC, AQ, and JV strains, which had a size of 1.97, 1.94 and 1.88 µm, respectively, were statistically the same (DGC= 0.05), but with a significant difference compared to SR (1.75 µm) and SJ (1.73 µm) (Figure 3).

Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i1.3392

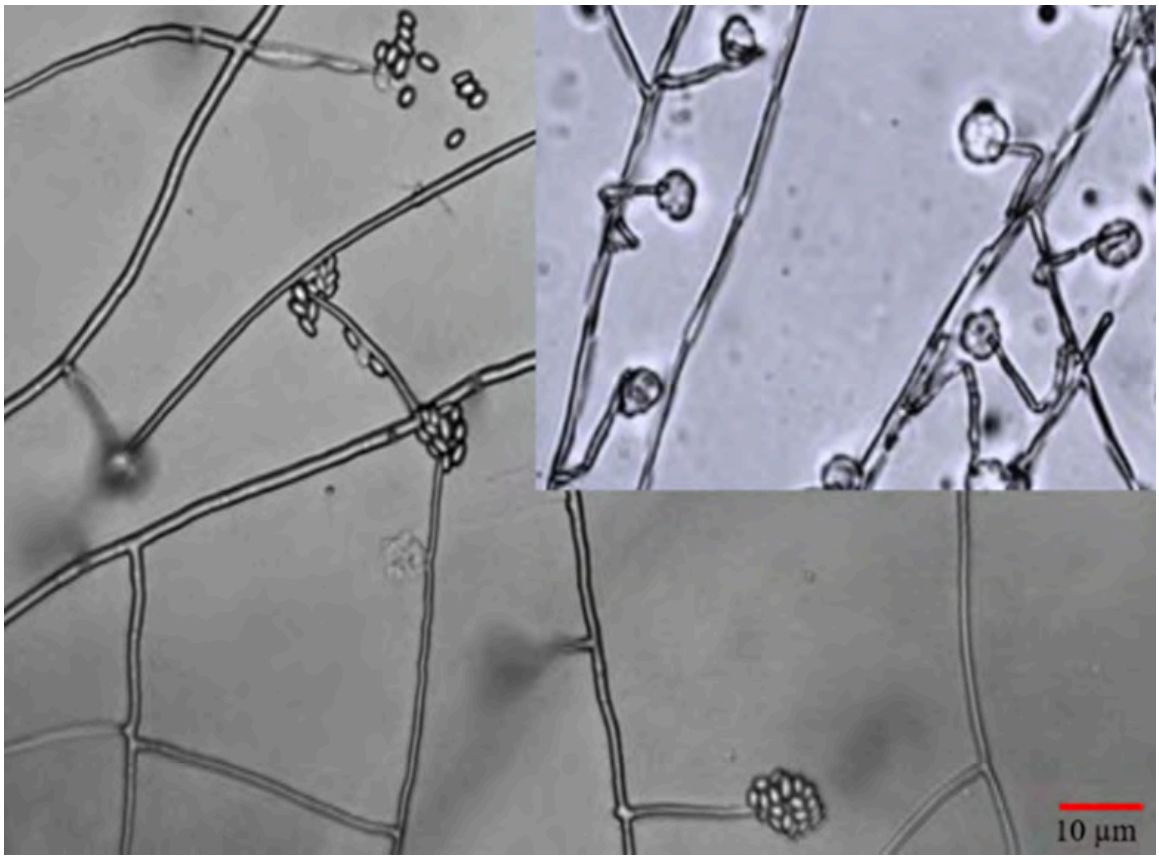
**Figure. 3. Conidia size (µm) of *Simplicillium* sp. strains. Means with the same letter are statistically equal ($p \geq 0.05$) (DGC= 0.05). EC= CATIE strain; AQ= Aquiáres; JV= Jabillos; SR= Santa Rosa; SJ= San Juan Norte.**




Microscopic characterization. The division of the genus *Verticillium* by Zare and Gams (2004) is based on systematic studies, separating taxa formed by solitary phialides from the genus *Lecanicillium* and placing them within the genus *Simplicillium*. From this perspective, the microscopic characteristics of the evaluated strains coincide with those of the latter genus. Oval conidia (1.7 to 1.97 µm) were also observed within globose heads that were suspended from solitary and sometimes prostrate phialides (Figure 4).

Revista Mexicana de Ciencias Agrícolas
DOI: https://doi.org/10.29312/remexca.v16i1.3392

**Figure 4. Solitary phialides and conidia of *Simplicillium* sp. (CATIE strain).**

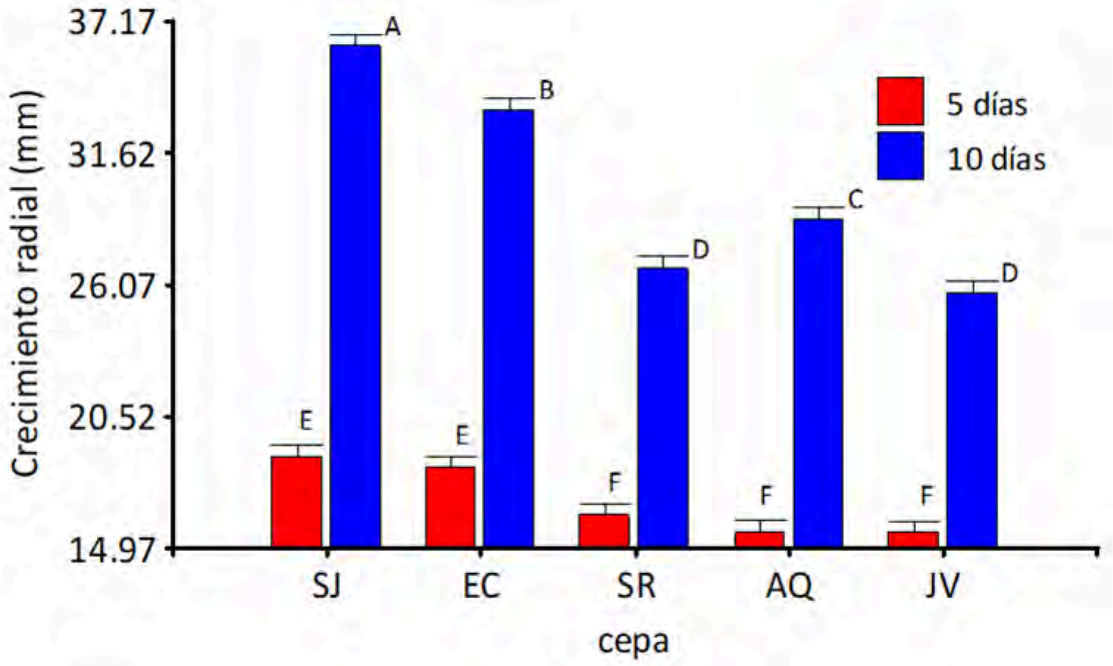



Radial growth and conidia production. There was a highly significant difference between the strains with respect to radial growth after five and ten days of growth in PDA ($p< 0.0001$). The strains that showed the highest growth in the five days were SJ and EC with 18.9 and 18.39 mm, respectively, whereas SR, AQ and JV showed the lowest values with 16.44, 15.67 and 15.65 mm, respectively, and did not differ statistically from each other ($p\geq 0.05$). After 10 days, the highest growth values were obtained by the SJ (36.1 mm) and EC (33.5 mm) strains, followed by AQ (28.8 mm), whereas SR (26.8 mm) and JV (25.7 mm) showed the lowest values. These results indicate a high variability within the same genus (Figure 5).

Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i1.3392

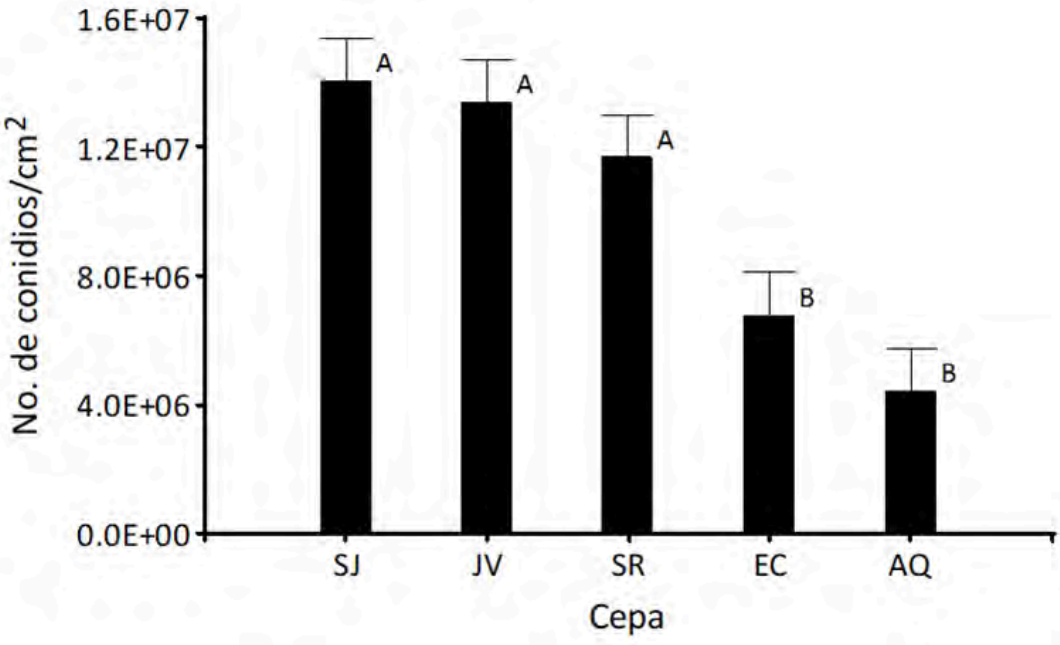
**Figure 5. Radial growth (mm) of *Simplicillium* sp. strains. Means with the same letter do not differ statistically (LSD= 0.05). SJ= San Juan Norte strain; EC= CATIE; SR= Santa Rosa; AQ= Aquiáres; JV= Jabillos.**




Conidia production varied significantly between strains ($p$< 0.0001). The SJ, JV, and SR strains showed higher production with values of $1.4\times10^{7}$, $1.3\times10^{7}$ and $1.2\times10^{7}$ cm$^{-2}$, whereas the SR and SJ strains presented the smallest conidia size (1.75 and 1.73 µm). On the contrary, it was recorded that the EC and AQ strains had larger structures (1.97 and 1.94 µm, respectively); however, they produced significantly less; EC produced $6.8\times10^{6}$ conidia cm$^{-2}$ and AQ $4.4\times10^{6}$ conidia cm$^{-2}$, values that did not differ statistically from each other ($p\geq$ 0.05) (Figure 6).

DOI: https://doi.org/10.29312/remexca.v16i1.3392

**Figure. 6. Production of conidia of *Simplicillium* sp. strains. Means with the same letter are statistically equal (DGC= 0.05). SJ= San Juan Norte strain; JV= Jabillos; SR= Santa Rosa; EC= CATIE; AQ= Aquiáres.**




Molecular identification. The EC, AQ, SR, SJ, and JV strains isolated and analyzed in the NCBI database matched the species *Simplicillium lanosoniveum*. The sequences for each identified strain were selected to be registered in the NCBI GenBank database (Table 2).

**Table 2. Species identified and access number generated in the NCBI based on similarity percentages.**

| Strain | Organisms | No. of access of NCBI |
|---|---|---|
| CATIE (EC) | *Simplicillium lanosoniveum* | OQ325474 |
| Aquiáres (AQ) | *Simplicillium lanosoniveum* | OQ325475 |
| Santa Rosa (SR) | *Simplicillium lanosoniveum* | OQ325476 |
| San Juan Norte (SJ) | *Simplicillium lanosoniveum* | OQ325477 |
| Jabillos (JV) | *Simplicillium lanosoniveum* | OQ325478 |

## Discussion

The results of this study contrast with other publications involving the natural biological control (NBC) of *H. vastatrix* in the same region of Costa Rica. This difference is mainly due to previous publications pointing to the fungus *L. lecanii* as the main natural enemy of coffee rust (Vandermeer *et al*., 2009; Jackson *et al*., 2012); in contrast, in the present research, the strains analyzed corresponded to *Simplicillium lanosoniveum*. The incorrect determination of the natural controller of *H. vastatrix* may be caused by the similar appearance of the fungus while parasitizing rust; both *Simplicillium* and *Lecanicillium* form a white cottony coating. In addition, these genera share morphological characteristics, which often leads to incorrect classification (Lim *et al*., 2014).

The cultural characteristic with the greatest variation between strains was the color of the colonies seen from the bottom of the Petri dish. One explanation for this could be the formation of resistance

DOI: https://doi.org/10.29312/remexca.v16i1.3392

structures submerged in the culture medium; an aspect described by Inderbitzin *et al*. (2011) in *Verticillium* species. After 15 days of growth, it was observed that the JV and SR strains tend to form resistance structures in less time since the color of the submerged mycelium changed from white to olive yellow (2.5Y 6/6 on the Munsell scale) during incubation; similar observations were recorded by Cortez *et al*. (2003) on *Lecanicillium* isolates.

The tendency of the strains to create resistance structures in a shorter time may be a consequence of lower radial growth, as observed in our results that show that the JV and SR strains presented the lowest growth values (Figure 5). In addition, color changes in the culture medium may be associated with the strain's ability to produce secondary metabolites (VanderMolen *et al*., 2013).

Since information on the ability of *Simplicillium* to produce conidia is very limited at this time, comparisons can be made with related taxa such as *Verticillium* or *Lecanicillium*. James *et al*. (2016) found a high diversity of fungi by molecular analysis in coffee leaf discs infected by rust. Based on comparisons of leaf discs without the presence of rust, the authors assumed that there were 15 possible mycoparasitic fungi.

Nonetheless, no effectiveness tests were conducted to examine whether these fungi actually had any degree of control over *H. vastatrix*. In a study conducted in coffee-growing areas of Veracruz, Mexico, Gómez *et al*. (2017) isolated mycoparasitic fungi from rust pustules and found a fungus of the genus *Simplicillium* with high biological control properties. In a recent study by Si *et al*. (2022) they recorded *S. lanosoniveum* as a new hyperparasite of the wheat rust *Puccinia graminis*, despite being a different crop, this research also confirms that this fungus is associated with the biological control of rusts.

## Conclusions

The results obtained provide the first characterization of strains of *Simplicillium lanosoniveum* associated with *H. vastatrix* pustules in Costa Rica.

## Bibliography


1 Aristizábal, L. F. and Johnson, M. A. 2022. Monitoring coffee leaf rust (*Hemileia vastatrix*) on commercial coffee farms in Hawaii: early insights from the first year of disease incursion. Agronomy. 12(5):1134-1149. https://doi.org/10.3390/agronomy12051134.

2 Cortez, M. H.; Alatorre, R. R.; Mora, A. G.; Bravo, M. H.; Ortiz, C. F. and Aceves, N. L. A. 2003. Characterization of multisporic and monosporic isolates of *Lecanicillium* (=*Verticillium*) *lecanii* for the management of *Toxoptera aurantii* in cocoa. BioControl. 48(3):312-334.

3 Fox, D. 1999. Microbiology. *In:* a laboratory manual. 5th Ed. Sherman. California, United States of America. 28 p. ISBN: 0-8053-7646-1.

4 García-Nevárez, G. and Hidalgo-Jaminson, E. 2019. Efficacy of indigenous and commercial *Simplicillium* and *Lecanicillium* strains for controlling *Hemileia vastatrix.* Revista Mexicana de Fitopatología. 37(2):237-250. https://doi.org/10.18781/r.mex.fit.1810-4.

5 Gómez, D. C. I.; Pérez, P. E.; Escamilla, P. E.; Martínez, B. M.; Carrion, V. G. L. L. y Hernández, L. T. I. 2017. Selección *in vitro* de micoparásitos con potencial de control biológico sobre roya del café (*Hemileia vastatrix*). Revista Mexicana de Fitopatología. 36(1):172-183. https://doi.org/10.18781/r.mex.fit.1708-1.

6 Guharay, F.; Monterroso, D. y Staver, C. 2001. El diseño y manejo de la sombra para la supresión de plagas en cafetales de América Central. Agroforestería en las Américas. 8(29):22-29.

7 Hajek, A. E. and Leger, R. J. 1994. Interactions between fungal pathogenesis and insect hosts. Annual Review of Entomology. 39(1):293-322.

DOI: https://doi.org/10.29312/remexca.v16i1.3392


8 Inderbitzin, P.; Bostock, R. M.; Davis, R. M.; Usami, T.; Platt, H. W. and Subbarao K. V. 2011. Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. Plos One. 6(12):e28341.

9 Jackson, D. W.; Zemenick, K. and Huerta, G. 2012. Occurrence in the soil and dispersal of *Lecanicillium lecanii*, a fungal pathogen of the green coffee scale (*Coccus viridis*) and coffee rust (*Hemileia vastatrix*). Tropical and Subtropical Agroecosystems. 15(2):389-401.

10 James, T. Y.; Marino, J. A.; Perfecto, I. and Vandermeer, J. 2016. Identification of putative coffee rust mycoparasites via single-molecule DNA sequencing of infected pustules. Applied and Environmental Microbiology. 82(2):631-639. https://doi.org/10.1128/AEM.02639-15.

11 Kuske, C. R.; Banton, K. L.; Adorada, D. L.; Stark, P. C.; Gil, K. K. and Jackson, J. P. 1998. Small-scale DNA sample preparation method for filed PCR detection of microbial cells and spores in soil. Applied and Enviromental Microbiology. 64(7):2463-2472. https://doi.org/10.1128/AEM.64.7.2463-2472.1998.

12 Lim, S. Y.; Lee, S.; Kong, H. G. and Lee, J. 2014. Entomopathogenicity of *Simplicillium lanosoniveum* isolated in Korea. Mycobiology. 42(4):317-321. https://doi.org/10.5941/MYCO.2014.42.4.317.

13 Lücking, R.; Aime, M. C.; Robbertse, B.; Miller, A. N.; Ariyawansa, H. A.; Aoki, T.; Cardinali, G.; Crous, P. W.; Druzhinina, I. S.; Geiser, D. M.; Hawksworth, D. L.; Hyde, K. D.; Irinyi, L.; Jeewon, R.; Johnston, P. R.; Kirk, P. M.; Malosso, E.; May, T.; Meyer, W. T. and Schoch, C. L. 2020. Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding?. IMA Fungus. 11(1):1-32. 10.1186/s43008-020-00033-Zty-jour.

14 Meena, R. S.; Kumar, S.; Datta, R.; Lal, R.; Vijayakumar, V.; Brtnicky, M.; Sharma, M. P.; Yadav, G. S.; Jhariya, M. K.; Jangir, C. K.; Pathan, S. I.; Dokulilova, T.; Pecina, V. and Marfo, T. D. 2020. Impact of agrochemicals on soil microbiota and management: A Review. Land. 9(2):34-54. https://doi.org/10.3390/land9020034.

15 Moricca, S. and Ragazzi, A. 2008. Biological and integrated means to control rust diseases. *In*: Ciancio, A. and Mukerji, K. G. Ed. Integrated management of diseases caused by fungi, Phytoplasma and Bacteria. Springer 3. Dordrecht, Netherlands. 303-329 pp. https://doi.org/10.1007/978-1-4020-8571-0-15.

16 Munsell Color Company. 1988. Munsell Soil Color Charts. Baltimore, Maryland, USA.

17 Promecafé. 2013. Programa cooperativo regional para el desarrollo tecnológico y la modernización de la caficultura y Instituto Interamericano de Cooperación para la Agricultura (IICA). La crisis del café en Mesoamérica, causas y respuestas apropiadas. Guatemala. Boletín Promecafé. 135:10-15.

18 Safavi, S. A. 2010. Isolation, identification and pathogenicity assessment of a new isolate of entomopathogenic fungus *Beauveria bassiana* in Iran. Journal of Plant Protection. 50(2):158-162. 10.2478/v10045-010-0027-z.

19 Santiago-Elena, E.; Zamora-Macorra, E. J.; Zamora-Macorra, M. and Elizalde-Gaytan, K. G. 2020. Interaction between *Mycodiplosis* and *Hemileia vastatrix* in three scenarios of coffee crop management (*Coffea arabica*). Revista Mexicana de Fitopatología. 38(3):320-336. https://doi.org/10.18781/r.mex.fit.2005-2.

20 VanderMolen, M. K.; Raja, A. H.; El-Elimat, T. and Oberlies, H. N. 2013. Evaluation of culture media to produce secondary metabolites in a natural products screening program. Amb. Express. 3(1):71-78. 10.1186/2191-0855-3-71.

21 Zare, R. and Gams, W. 2004. A monograph of *Verticillium* section Prostrata. Rostaniha. 5(3):51-109.

Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i1.3392

# Identification of five strains of *Simplicillium* associated with pustules of coffee rust in Costa Rica


| Journal Information |
| --- |
| **Journal ID (publisher-id):** remexca |
| **Title:** Revista mexicana de ciencias agrícolas |
| **Abbreviated Title:** Rev. Mex. Cienc. Agríc |
| **ISSN (print):** 2007-0934 |
| **Publisher:** Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias |

| Article/Issue Information |
| --- |
| **Date received:** 01 November 2024 |
| **Date accepted:** 01 February 2025 |
| **Publication date:** 20 March 2025 |
| **Publication date:** Jan-Feb 2025 |
| **Volume:** 16 |
| **Issue:** 1 |
| **Electronic Location Identifier:** e3392 |
| **DOI:** 10.29312/remexca.v16i1.3392 |

**Categories**

**Subject:** Articles

**Keywords:**

**Keywords:**

cultural characterization
natural biological control
physiological characterization

**Counts**

**Figures:** 6
**Tables:** 3
**Equations:** 0
**References:** 21
**Pages:** 0