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# *In Vitro* Evaluation of Native Isolates of *Lecanicillium spp* (Berk & Broome) on *Hemileia vastatrix*

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**Abstract** **Original Research Article**

With the objective of developing biological management options for coffee rust, through the characterization and evaluation of native isolates of *Lecanicillium* spp. The inoculum was obtained from hyperparasitized rust pustules and purified in potato dextrose agar culture medium. *Lecanicillium* spp. isolates were studied by microscopic, macroscopic, and physiological characterization, as well as *In Vitro* tests to determine the parasitism of each isolate on uredospores and rust pustules, and the potential for mass production of conidia on organic substrates: soybean, sorghum and rice. Six native isolates of the genus *Lecanicillium* spp. were obtained from 20 samples, with conidia ranging in size from 2.5 to 7.5 µ in length and from 1.5 to 2 µ in width, in groups or solitary, hyaline in color and cylindrical or ellipsoidal in shape. The size of the phialides ranged from 20 to 28 µ with a base width of 2.5 µ. Phialides were found solitary or in whorls originating from straight conidiophores, the conidia generally in groups of two to six, sometimes solitary. The average radial growth of *Lecanicillium* spp. 20 days after inoculation was 18 mm in PDA, 15 mm in EMA and 16 mm in SDA. The Majada isolate presented the highest percentage of viability with 99.7% at 22 hours. The isolate La Gotera on the soybean-based organic substrate showed the highest conidial yield of 1.08E+09 per gram of substrate compared to the other substrates and the other isolates. The Jinotega isolate presented the highest parasitism of uredospores in Petri dishes 16.6%. The highest percentage of parasitism on pustules was presented by the isolate La Gotera with 88.3%.

**Keywords:** Biological control, hyperparasitized, pustules, parasitism, uredospores, uredospores.

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## INTRODUCTION

Coffee (Coffea arabica L.) is one of the most important crops in the world from an economic and social point of view. For the Nicaraguan economy, coffee has been over the years the item that has had the greatest contribution to the economy, representing about 30% of agricultural GDP and 50% of foreign exchange from exports (Moraga *et al.*, 2012). During the 2011-2012 cycle Nicaragua had a coffee export of 450,383,446.61 dollars and for the 2012-2013 cycle it had an export of 430, 721,041.16 (CETREX, 2020), this shows a decrease of 19,662,405.5 million dollars due to the high incidence of rust of 37.0%. The rust crisis has put the survival of hundreds of thousands of families in the region at risk (Avelino *et al.*, 2013).

Rust (Hemileia vastatrix Berk. & Br.) is the most important disease of coffee whose damage can cause defoliation between 30% and 50% (Avelino *et al.*, 2004). Pest control in coffee growing has been using chemicals, but due to undesirable effects on beneficial organisms, groundwater, food safety and development of insect resistance (Shahid *et al.*, 2012), these strategies have not been the ideal solution to the problem (Javed, 1987). Many importing countries are increasingly demanding specialty coffees such as organic coffee (Caixeta and Pedini, 2002). The price of organic coffee can reach prices between four and five times higher than conventionally cultivated coffee. The demand for organic coffee requires certain requirements, that it be free of certain pesticides that are normally applied to control pests and diseases. It is therefore necessary to develop organic production systems that benefit the environment and human health.

Entomopathogenic fungi are distributed approximately in more than 100 genera and 750 species, some of which present agronomic interest as microbial control agents (García *et al.*, 2019). These

fungi include *Lecanicillium* spp, Beauveria bassiana, Metarhizium spp, Isaria fumosorosea and among others, which are used for the control of dozens of pests in a wide variety of crops (Castillo-Arévalo, 2022, Castillo Arévalo, 2023).

The use of biopesticides is a tool to improve strategies for the rational use of insecticides, including microbial pesticides based on bacteria, fungi, viruses, and nematodes (Bautista *et al*., 2010). The fungus *Lecanicillium* spp. is one of the most common hyperparasites of coffee rust, occurs naturally in coffee plantations and could be a good candidate for biological control of coffee rust (Leguizamón *et al*., 1989). Given the importance of coffee rust disease, the purpose of the present study is to contribute to the development of new alternatives for biological management of the disease through characterization and evaluation under laboratory conditions of native isolates of *Lecanicillium* spp on coffee rust.

## MATERIAL AND METHODS

The research was conducted at the biopesticide laboratory of the National Agrarian University, Nicaragua in January 2018.

The study was carried out in two stages, the first consisted of collecting samples of *Lecanicillium* spp. from leaves with rust (*H. vastatrix*) parasitized by *Lecanicillium* spp. for subsequent isolation and characterization. In stage two, six native isolates of *Lecanicillium* spp. were evaluated in laboratory conditions to determine the level of parasitism on uredospores and on rust pustules on leaves from the field. The radial growth of all isolates was also evaluated in Petri dishes of 90 mm in diameter in potato dextrose agar, sabouraud dextrose agar and malt extract agar, and they were also evaluated on different organic substrates to determine promising strains for mass reproduction.

The methodology to isolate *Lecanicillium* spp. was dry isolation, which consisted of taking the inoculum directly from the leaves with parasitized rust pustules using a hypodermic needle and deposited in Petri dishes containing acidified potato dextrose agar potato culture medium. The samples were previously observed under the microscope, using staining with lactophenol blue to determine that the desired fungus was being isolated. Once confirmed by taxonomic keys (Zare and Gams, 2001) and purified on PDA culture medium.

**Macroscopic and physiological characterization of *Lecanicillium* spp. isolates**

This was done in three culture media, Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) and Malt Extract Agar (EMA). This study also included characteristics related to their mass production on natural substrates of rice, soybean, and sorghum. The germination reading of *Lecanicillium* spp. conidia was done at 24 hours and at least 200 conidia per Petri dish were quantified with a 40x objective. To proceed with the characterization, the fungus was transferred from a pure culture to the three-culture media in 90 mm diameter Petri dishes, in which the corresponding observations were made and six native isolates of *Lecanicillium* spp were evaluated, using 10 replicates for each isolate in the three culture media for a total of 30 dishes per isolate and one Petri dish was considered as a replicate.

**Microscopic characterization**

The size of the reproductive structures of the fungus was measured, such as: size of the phialides, size of the conidia and width of the base of the phialides, as well as the shape and color of the conidia. A previously calibrated LW-Scientific light microscope was used for this measurement, using a 40x lens. The data were taken 48 hours after inoculation of the culture medium and the number of structures taken was thirty for each variable for each culture medium (PDA, SDA, and EMA). To avoid contamination with bacteria, a 2% chloramphenicol-based antibiotic was used. Macroscopic characteristics included qualitative aspects such as colony color, growth type and growth rate.

**Radial growth**

This was done in 90 mm diameter Petri dishes based on the methodology described by French and Hebert (1982). The technique consisted of placing the inoculum in the center of the dish containing the culture medium and then drawing a cross on the back of the Petri dish; the center of the dish was the inoculation point; the cross delimits four radii identified with letters A, B, C and D on which the readings were taken in millimeters. The data were taken by measuring the growth of the fungus on each of the four marked radii. Measurements were taken every 48 hours for a period of 20 days. Thirty plates were used for each isolation, 10 for each culture medium for a total of 180 experimental units.

**Viability of conidia**

It was determined by evaluating the average conidia germination time described by Monzon (2001). The methodology consisted of placing one gram of fungus on rice substrate in a test tube containing 9 ml of sterile distilled water, thus preparing serial dilutions 10-1, 10-2, 10-3. Once the first dilution was prepared, it was homogenized by vortexing at 3 thousand rpm and kept for three minutes to separate the conidia from the substrate. From the first dilution, the following dilutions were prepared, and the most appropriate dilution was selected to take the reading of the viability study. For the establishment of the test, a 5 µl aliquot of the *Lecanicillium* spp suspension was deposited with a micropipette in a 90 mm diameter Petri dish containing 1.5% Agar-water medium, autoclaved at 1.2 bar pressure and 121°C temperature. The inoculated Petri dishes were placed in a room at a temperature of 24°C with eight hours of light per day.

Germination readings were taken at 16, 18 and 22 hours, using a 40X LW-Scientific light microscope. For this study, three Petri dishes were used for each reading time, for a total of nine dishes for each isolate. At the time of viability reading in each dish 10 microscopic fields were observed, these were previously delimited with a marker on the back of the dish; a total of thirty fields were observed for each isolate in each reading, observing at least 200 conidia in each dish. The viability percentage was calculated using the following formula (French and Hebert, 1982); germinated conidia was one whose germinative tube reached twice its length.

$$\textbf{Germinated conidia}\ (\%) = \frac{\textbf{Number of germinated conidia}}{\textbf{Total number of conidia}}\boldsymbol{x}\mathbf{100} \quad \textbf{Equation 1}$$

**Mass Production**

Each isolate of *Lecanicillium* spp. was evaluated by the production of conidia on rice, soybean, and sorghum, based on a semi-industrial biphasic mass production method. The methodology consisted of reproducing the inoculum in a synthetic solid culture medium and in liquid matrices based on nitrogen, vitamins, phytohormones, sucrose and skim milk. The inoculum for inoculation of the natural solid substrates to be evaluated was taken from the matrix. Two hundred grams of each raw substrate were placed in thermos resistant bags and 50 ml of drinking water was added, then the bags were sealed with staples, then the bags were sterilized by moist heat at 1.5 bar pressure at a temperature of 121°C for 5 minutes. Once the bags with the substrates were sterilized and cooled, 10 bags were inoculated with each isolate, depositing 20 ml of fungal suspension (Monzon, 2001). The inoculation of the bags was carried out in a laminar flow chamber. After inoculation, the substrate inside the bags was manually homogenized to ensure uniform growth of the fungus; the bags were then placed in an incubation room at a temperature of 24°C in dark conditions.

Performance evaluation was performed 20 days after inoculation when the substrate had been fully colonized and sporulated by the fungus. Of the 10 bags of each isolate with a given organic substrate, five were randomly selected for counting. For the readings, one gram was weighed in rice and serial dilutions (10-2, 10-3, 10-4, 10-5) were prepared and the 10-3 dilution was selected for conidia counting. To homogenize and separate the conidia from the substrate, the tubes were shaken for three minutes in a vortex at 2400 rpm. Conidia counting was performed in a Neubauer chamber and an LW-Scientific microscope with a 40x lens was used. For the calculation of yield number of spores/gram of substrate, the following formula was used:

$$Yield = Number\ of\ conidia\ observed \times chamber\ factor \times dilution\ factor$$ **Equation 2**

**Parasitism *In Vitro* of *Lecanicillium* spp. on *H. vastatrix***

For the study of parasitism of *Lecanicillium* spp on uredospores of *H. vastatrix* consisted of scraping with a scalpel on the underside of leaves in rust lesions free of the hyperparasite, then deposited in a container with water, and a concentration of $1x10^{-4}$ uredospores/ml was prepared in sterile distilled water. The inoculum of *Lecanicillium* spp isolates was obtained from the growth of the fungus on rice substrate, from that substrate a suspension of 1x10-5 spores/ml was prepared. The parasitism test was performed in Petri dishes of 90 mm in diameter containing 1.5% Agar-water. For this study, three dishes were used for each isolate of *Lecanicillium* spp. 5µl of the uredospore suspension were deposited on the surface of the medium in five points previously delimited with a marker. Subsequently, 5 µl of the suspension of conidia of *Lecanicillium* spp. was deposited on the spore suspension of *H. vastatrix*. Once the test was established, the plates were placed in a room at a temperature of 24°C. To determine the percentage of parasitism of *Lecanicillium* spp. on the uredospores, five spots per Petri dish were observed and a minimum of 50 uredospores were quantified. For the reading a LW-Scientific light microscope was used to determine parasitism using the following formula:

$$\text{Parasitism} = \frac{\text{total uredospores}}{\text{uredospores par}} x100$$ ............... **Equation 3**

**Parasitism of *Lecanicillium* spp on rust pustules**

A bioassay was established on coffee leaves under laboratory conditions to determine the effectiveness of *Leacnicillium* spp on rust pustules. Leaves with fresh rust pustules, highly sporulated and free of *Lecanicillium* spp. were collected. Before setting up the study, the samples were analyzed with mounts and observed under the microscope to confirm the absence of the hyperparasite. To avoid the premature aging process of the leaves and to keep them turgid for 120 hours during the evaluation period, a cotton swab impregnated with 3 milliliters of a 2% solution of 6 BAP (Benzyl amino purine) at pH 5.8 was placed on the petiole of each leaf (Tórrez and Castillo, 2005).

Inoculation of Leacnicillium spp. on rust pustules was done by immersion at a concentration of 1x10-8 conidia/ml. After immersing the leaves with rust in the conidia suspension, it was left to stand for two minutes, then the leaves were placed in a humid chamber consisting of a plastic container 30 cm long x 18 cm wide x 10 cm deep, with a 2.5 cm high mesh, on which ten leaves were placed in each container and each leaf was considered as a repetition. To maintain relative humidity greater than 95%, 500 ml of water was placed at the bottom of each container. Finally, the containers were placed in a dark chamber, formed with plastic and moistened craft paper in such a way that all the leaves were covered with black plastic to ensure high humidity. The bioassay temperature ranged between 22°C and 24°C and a relative humidity above 95% inside the containers. For the evaluation of parasitism of *Lecanicillium* spp. on the pustules, observations were made at 36 hours, the time required for the hyperparasite to colonize the uredospores. For the calculation of the percentage of parasitism, mounts were made to observe mycoparasitism at the microscopic level and the following formula was used (Monzon, 1992).

$$\text{Parasitized pustules (\%)} = \frac{\text{Number of parasitized pustules}}{\text{Number of total pustules}} x100$$ **.........Equation 4**

## RESULTS

A total of 20 samples were collected, from which six isolates of *Lecanicillium* spp. were obtained, which were subjected to all the tests performed in the study. All isolates were obtained from coffee plants; these genetic materials are considered native isolates because no commercial applications of *Lecanicillium* spp. had been made.

**Microscopic characteristics**

All *Lecanicillium* spp. isolates were similar in the three media evaluated. Conidia size was similar and ranged from 2.5 to 7.5 µ in length and 1.5 to 2 µ in width, with conidia present at the end of the phialides in clusters or solitary, hyaline in color and cylindrical or ellipsoidal in shape (Figure 1). The size of the phialides ranged from 20 to 28 µ with a base width of 2.5 µ and thinning at the end in a kind of very small tip, solitary phialides were found or in whorls originating from straight conidiophores, the conidia generally in groups of two to six and sometimes solitary (Figure 1).

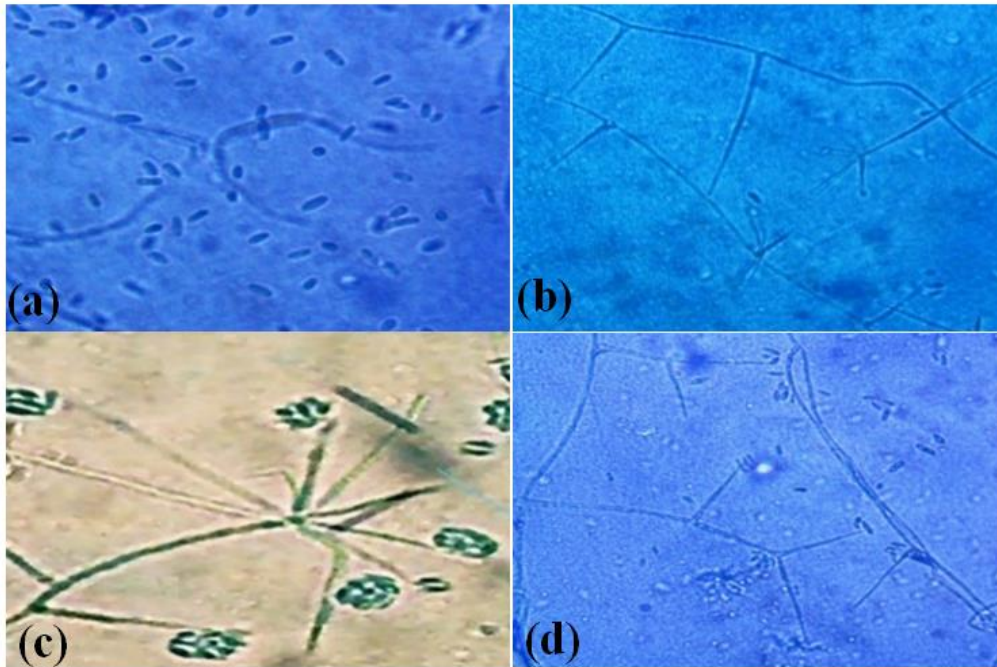


**Figure 1: Reproductive structures of *Lecanicillium* spp. cylindrical, ellipsoidal, and rounded conidia (a). Verticillate conidiophores with phialides and solitary conidia (b). Verticillate conidiophore with phialides and conidia in clusters (c). Solitary phialides with conidia in clusters (d)**

**Macroscopic characteristics**

The growth of the colonies was similar in color and shape, they presented an off-white coloration, with a cottony appearance, in addition the colonies presented greater vertical growth in the upper part due to the abundant mycelium, in the lower part of the plate a wrinkling or ridges were observed in the medium during their growth (Figure 2).

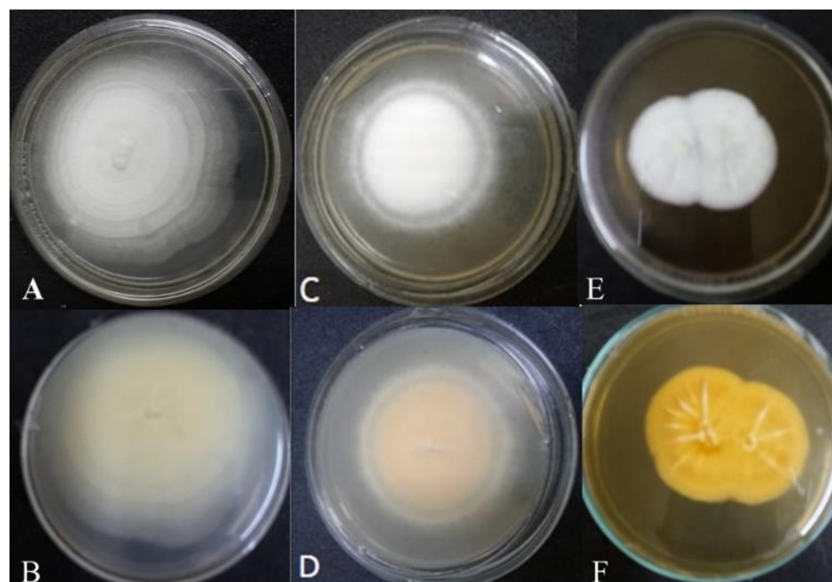


**Figure 2: *Lecanicillium* spp. colonies in different culture media. PDA culture medium. Front view (A), back view (B). SDA culture medium front view (C), rear view (D), EMA culture medium front view (D) rear view (F)**

The average radial growth of all native isolates of *Lecanicillium* spp achieved varied growth on the culture media evaluated. According to Hall (1984) the fungus *L. lecanii* grows well on almost any culture medium. In our study the PDA culture medium was where the highest growth values were recorded, followed by the SDA culture medium, the lowest growth values were recorded in the EMA culture medium (Figure 3).

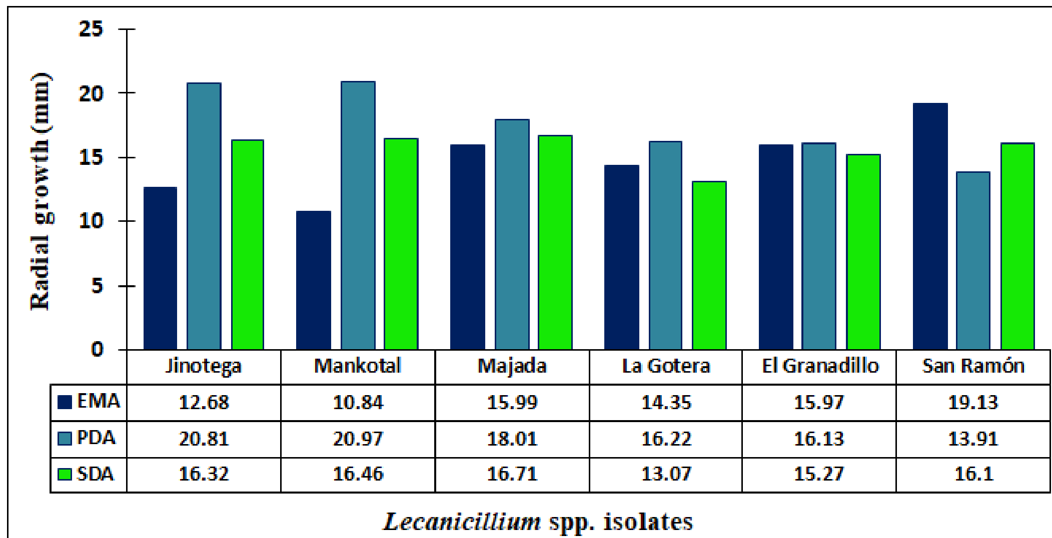


**Figure 3: Average radial growth of *Lecanicillium* spp. isolates 20 days after inoculation on PDA, EMA, and SDA medium**

The analysis of variance for the variable of radial growth in EMA culture medium, indicates significant statistical differences between the evaluation periods ($p < 0.0001$); it also indicates that there are significant differences ($p < 0.0001$) between isolates. Furthermore, it indicates that the Time*Isolate interaction is significant ($p = 0.002$). This same result was found in PDA culture medium. In the SDA culture medium, the radial growth of *Lecanicillium* spp isolates was significant for the evaluation period factor ($p<0.0001$) and the isolate factor ($p<0.0001$).

The results of the interaction between the two factors under study, isolates and evaluation periods for the radial growth of *Lecanicillium* spp, in EMA and PDA, indicate that the behavior of the radial growth of the isolates varies significantly through time in each isolate, that in some periods some isolates result with greater radial growth, but in others they are surpassed by other isolates. Since the results were significant for the interaction between the isolates and the evaluation periods for the EMA and PDA culture media, an analysis of variance was performed to compare the radial growth of the isolates in each evaluation period.

In the malt extract agar culture medium, the San Ramón isolate showed the highest growth rate in most of the evaluation periods, while the Mankotal isolate showed the lowest radial growth in all evaluation periods, except at 48 and 192 hours after inoculation. The analysis of variance performed for the different dates indicated differences in radial growth between isolates at 48 ($p<0.0001$), 96 ($p<0.0259$), 192 ($p<0.0001$), 240 ($p<0.0039$) and 336 hours ($p<0.0002$). Radial growth ranged from 0.31 mm to 3.16 mm. The San Ramón isolate recorded the highest growth with 3.6 mm, while the Mankotal isolate recorded the lowest radial growth with 0.31 mm.

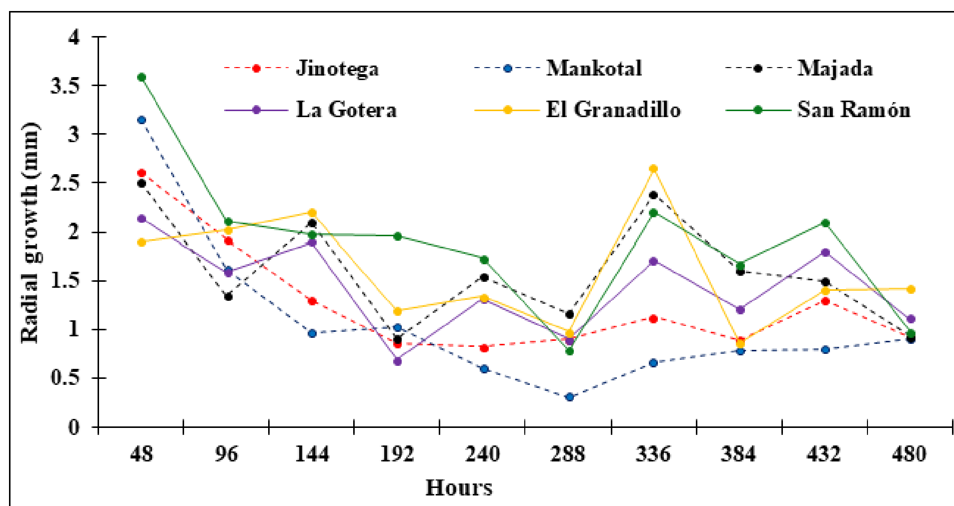


**Figure 4: Radial growth (mm) of *Lecanicillium* spp isolates at different times on malt extract agar culture medium**

The analysis of variance for the radial growth of *Lecanicillium* spp isolates in PDA culture medium indicates that there are significant differences at 48 ($p<0.0001$), 192 ($p<0.0001$) and 384 hours after inoculation ($p<0.0083$). In this culture medium, the radial growth values of the different isolates ranged from 0.71 mm to 2.98 mm every 48 hours. The isolate Majada presented the highest radial growth at 336 hours, while the isolate San Ramón presented the lowest radial growth at 288 hours and in most of the sampling periods. The isolate Mankotal presented the best growth in most of the sampling periods (Figure 5).

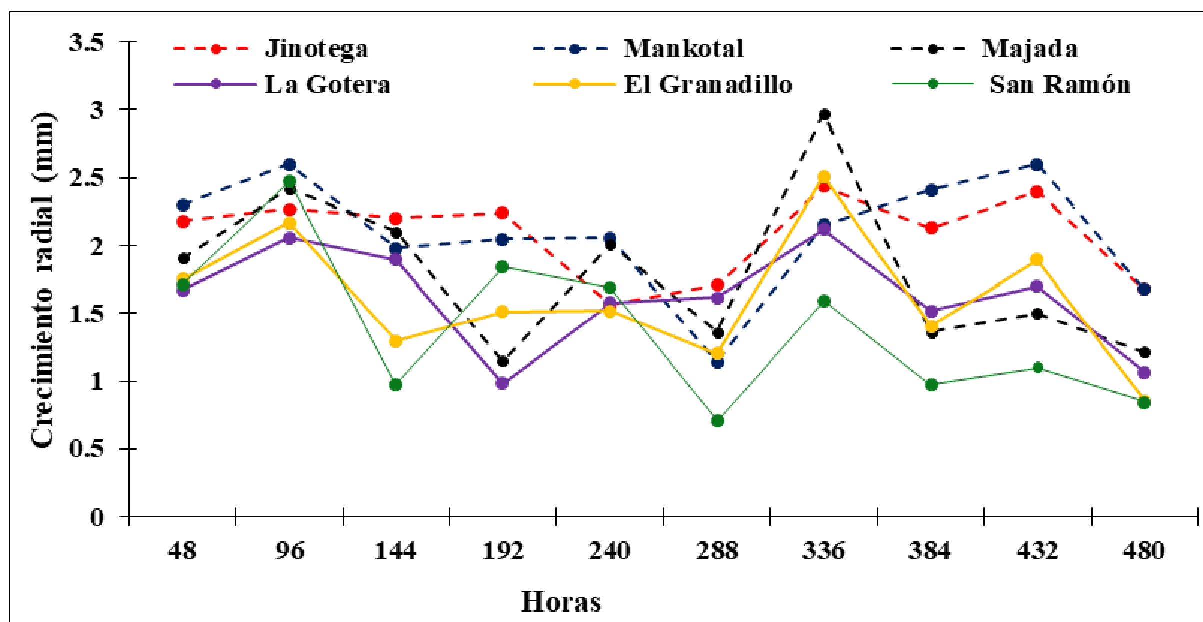


**Figure 5: Radial growth (mm) of *Lecanicillium* spp isolates at different times in potato dextrose agar medium**

The radial growth of *Lecanicillium* spp in SDA was significant for the evaluation period factor ($p<0.0001$) and the isolate factor ($p<0.0001$), however, the interaction between both factors was not significant. Radial growth ranged from 0.9 mm to 2.5 mm every 48 hours. The Mankotal isolate presented the highest average radial growth at 336 hours, while the San Ramón isolate presented the lowest radial growth of 0.9 mm at 48 hours (Figure 6).

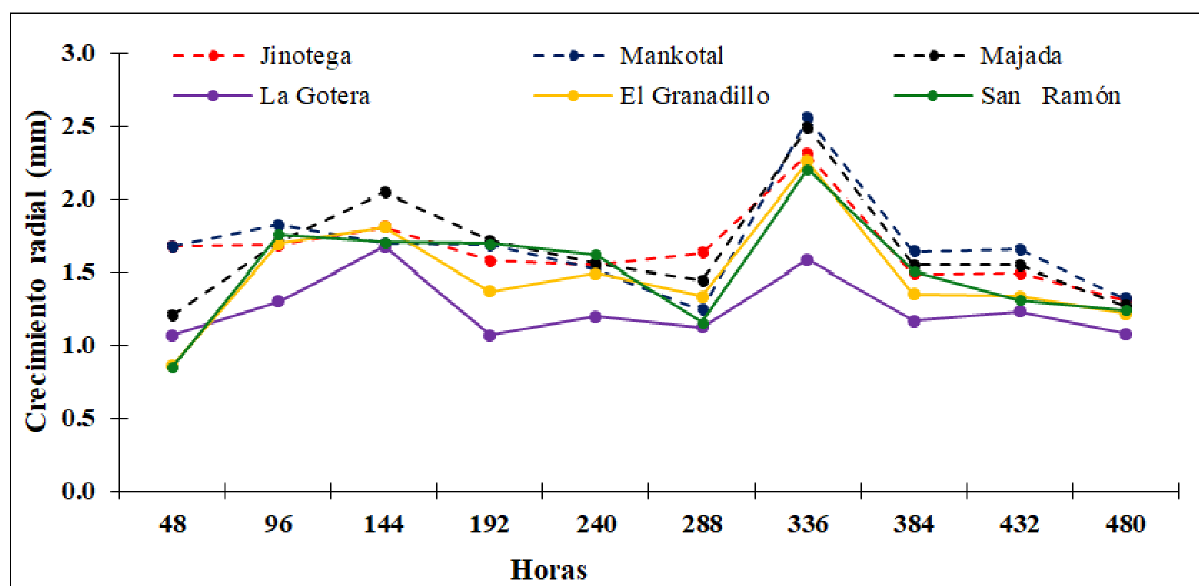


**Figure 6: Radial growth (mm) of *Lecanicillium* spp. isolates at different times on sabouraud dextrose agar medium**

**Viability of conidia**

For the variable viability of conidia of *Lecanicillium* spp isolates ranged from 71.4% to 99.7%. The highest viability was recorded in the Majada isolate at 22 hours after the test was established. The analysis of variance carried out for conidia viability determined significant differences between the different hours of evaluation (p<0.0001), between isolates (p<0.001), likewise the interaction hour*isolate was significant (p<0. 0001), which indicates that the viability of the fungus isolates depends on the hour in which the readings were taken, that the isolate that presented the highest viability of conidia at a certain hour was not the same at another hour, therefore we proceeded to perform an analysis of variance for each evaluation time to determine the behavior of the isolates.

The analysis of variance performed for the data at different times indicated that there were significant differences between the different isolates at 16 (p<0.0001), 18 (p<0.0001) and 22 hours (p<0.0001). The average percentage of viability at 16 hours was 83%, with the Majada isolate presenting the highest percentage of viability with 89%, while the Jinotega isolate presented the lowest viability with 71%. At 18 hours, the average viability percentage was 93%. The San Ramón isolate presented the highest viability of 98%, while the Mankotal isolate presented the lowest viability percentage of 87%; the average viability percentage at 22 hours was 96%. The Majada isolate was the one that presented the highest percentage of viability with 100%. Most of the isolates presented a high germination percentage of more than 95% at 22 hours (Table 1).

**Sampling periods**

**Table 1: Percent viability of conidia of *Lecanicillium* spp. isolates on three sampling dates using Tukey's test**

| **Isolates of *Lecancillium* spp** | **Sampling periods** | | |
|---|---|---|---|
| | 16 hours | 18 hours | 22 hours |
| | | | |
| Majada | 89 a | 95 a | 100 a |
| San Ramón | 89 a | 98 a | 97 a |
| La gotera | 89 a | 94 a | 98 a |
| El Granadillo | 87 a | 93 a | 98 a |
| Mankotal | 76 b | 87 b | 92 b |
| Jinotega | 71 b | 90 a | 91 c |
| **Average** | **83** | **93** | **96** |

Means with equal letters, in the same column, do not differ significantly according to Tukey (p≤0.05).

**Mass Production**

In general, the results of this study showed that the organic substrates based on soybean and rice were the ones that presented the best results compared to the sorghum-based substrate. All native isolates of *Lecanicillium* spp produced conidia, but with different results, some substrates favored some isolates in the multiplication of the fungus, while those same isolates were not favored in other organic substrates. The analysis of the yield in number of conidia per gram of substrate indicated that there were significant differences between substrates (p<0.0001), between isolates (p<0.0039) and the interaction isolate*substrate was significant (p<0.0001), which indicates that the type of substrate influences in different ways on the multiplication of the fungus of each isolate. Due to these results we proceeded to perform an analysis of variance for each substrate with each of the isolates to determine the performance in terms of yields of each isolate.

**Performance of *Lecanicillium* spp. isolates on soybean-based substrate**

All isolates of *Lecanicillium* spp. achieved highly variable growth and sporulation on soybean-based substrate, some isolates were favored in multiplication. The analysis of variance determined significant differences in the yield in the number of conidia per gram, which indicates that each isolate has certain very particular characteristics in its multiplication (p<0.0002). The isolate La Gotera presented the highest yield with 1.08E+09 conidia per gram on substrate, while the isolate Mankotal presented the lowest yield with 1.90E+08 conidia per gram. The isolates Majada, Jinotega, Granadillo and San Ramón presented similar intermediate yields between 7.42E+08 and 4.21E+08 conidia per gram of substrate (Figure 7).

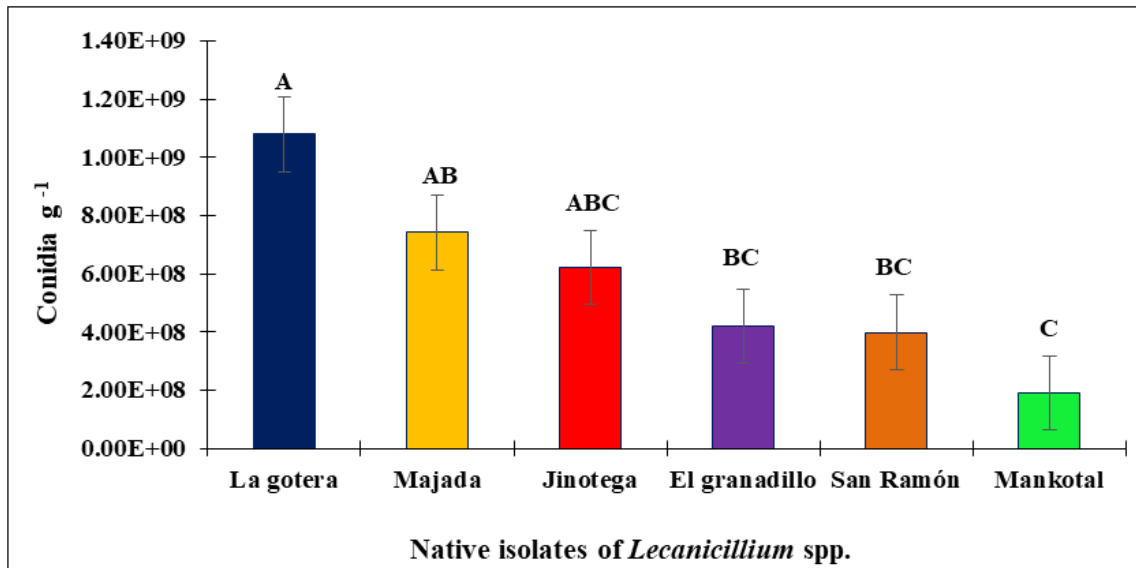


**Figure 7: Average yield of native isolates of Lecanicillium spp. after 20 days of incubation on the soybean-based organic substrate. soybean-based organic substrate error**

**Performance of *Lecanicillium* spp. isolates on rice substrate**

All native isolates of *Lecanicillium* spp were able to grow, but with different yields, some substrates favored some isolates in the multiplication of the fungus, while those same isolates were not favored in another organic substrate. The analysis of variance determined significant differences in the yield in the number of conidia per gram on rice-based substrate (p:0.0337), which indicates that each isolate has certain very particular characteristics in multiplication. All the isolates presented very variable yields. The San Ramón isolate presented the highest yield of 5.95E+08 conidia per gram on substrate, the Jinotega, Majada and Mankotal isolates were statistically like the San Ramón isolate, while the El Granadillo and La Gotera isolates presented lower yields of 2.35E+08 and 2.14E+08 conidia per gram (Figure 8).

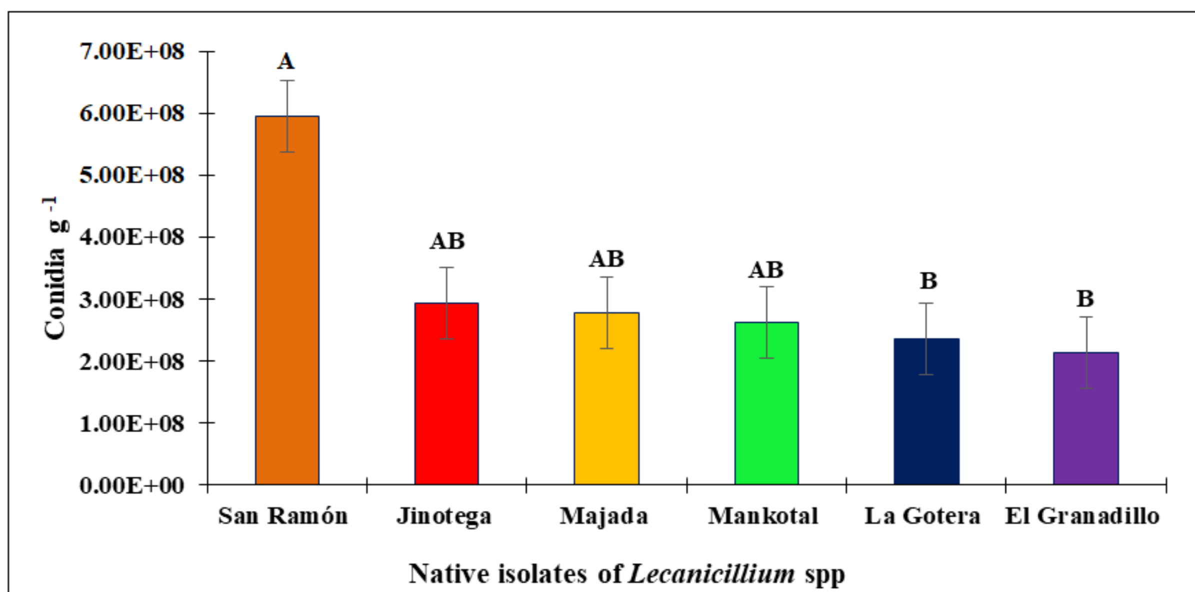


**Figure 8: Average yield of native isolates of *Lecanicillium* spp. after 20 days incubation on the rice-based organic substrate. Lines above the bars indicate the standard error**

**Performance of *Lecanicillium* spp. isolates on sorghum-based substrate**

In general, all isolates achieved highly variable growth and sporulation on sorghum-based organic substrate, some isolates were favored in the multiplication of some isolates, while those same isolates were not favored on other organic substrate evaluated. The analysis of variance indicates that there

are significant differences among the isolates evaluated (p:0.001). The isolates with the highest yields were Mankotal 3.29E+08 and San Ramón 2.83E+08, while the Majada isolate presented an intermediate yield of 1.74E+08 conidia per gram of substrate, while the isolates La Gotera, Jinotega and El Granadillo presented yields below the average production of the isolates evaluated with values of 6.90E+07, 6.60E+07 and 5.70E+07, respectively (Figure 9).

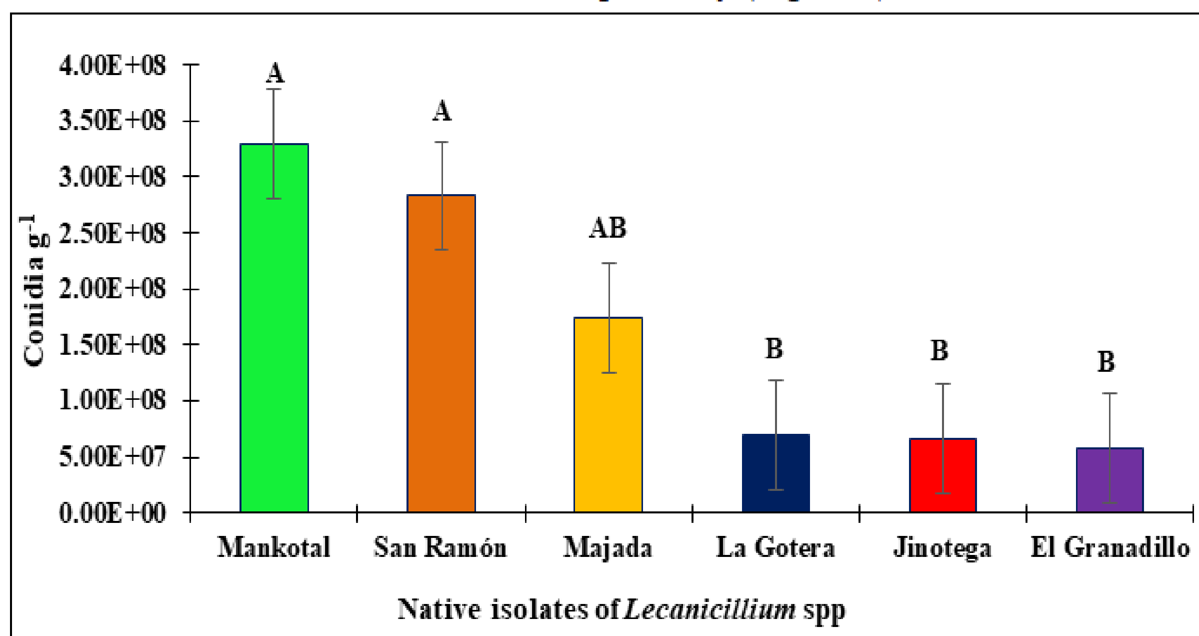


**Figure 9: Average yield of native isolates of *Lecanicillium* spp. after 20 days incubation on sorghum-based substrate. Lines above the bars indicate the standard error**

**Parasitism of *Lecanicillium* spp under *In Vitro* conditions**

All isolates caused parasitism to *H. vastatrix* uredospores except for the Majada isolate and the control for the duration of the trial. The analysis of variance performed for *In Vitro* parasitism of different isolates of *Lecanicillium* spp on the uredospores of *H. vastatrix*, determined significant statistical differences among the isolates (p:0.0014), indicating that each isolate presents different degrees of parasitism on the uredospores. The percentage of parasitism ranged from 16.6% to 2.36%, with the Jinotega isolate showing the highest percentage of parasitism. The isolates La Gotera, El Granadillo and San Ramón were statistically like the Jinotega isolate, while the Mankotal isolate presented the lowest percentage of parasitism of 2.36%, and the control isolate did not present parasitism as expected (Table 2).

**Tabla 2: Parasitismo *In Vitro* de aislados nativos de *Lecanicillium* sp sobre uredosporas de *H. vastatrix***

| **Isolates** | **Percentage of parasitism** | **Categories Tukey (0.05)** |
|---|---|---|
| Jinotega | 16.61 | A |
| La Gotera | 15.37 | AB |
| El Granadillo | 6.39 | ABC |
| San Ramón | 2.74 | ABC |
| Mankotal | 2.36 | BC |
| Majada | 0.00 | C |
| Testigo | 0.00 | C |

Means with letters in common within the same column did not differ significantly according to Tukey ($p \leq 0.05$).

The isolate Majada and the control did not present parasitism during the bioassay evaluation time. In the case of the Majada isolate that failed to parasitize any uredospores, it was perhaps because it failed to find any uredospores in its growth trajectory as observed in the follow-up at the microscopic level.

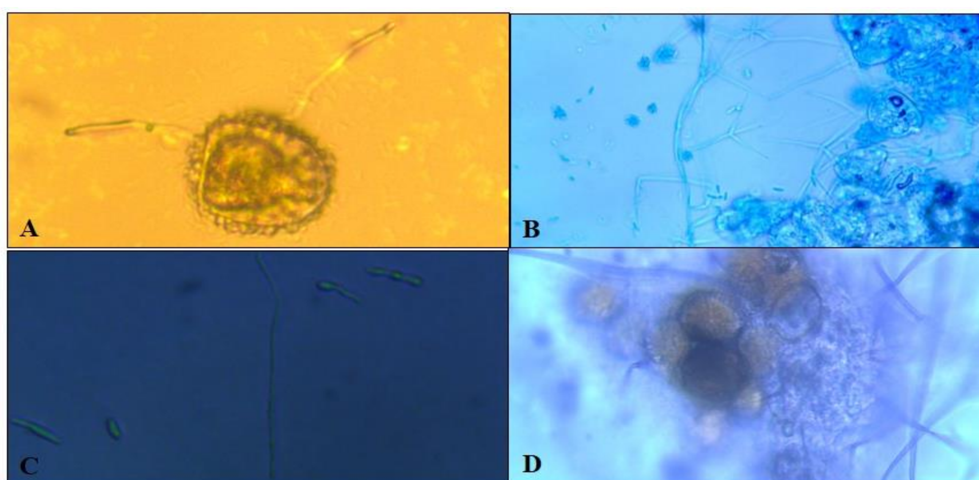


**Figure 10: *In Vitro* parasitism test. Ungerminated spore of H. vastatrix (A). Structures of *Lecanicillium* spp verticillate conidiophores emerging from uredospores of H. vastatrix (B). Germinated conidia of *Lecanicillium* spp (C). Interaction between *Lecanicillium* spp and *H. vastatrix* disintegrating uredospores (D)**

***In Vitro* parasitism of *Lecanicillium* spp on *H. vastatrix* pustules**

The percentage of parasitism caused by native isolates of *Lecanicillium* spp on leaf rust pustules under laboratory conditions ranged from 88.33 to 66.43 at 72 hours after establishing the test. No parasitism was observed in the control during this same evaluation period (Table 3). The Kruskal Wallis non-parametric analysis of variance for *In Vitro* parasitism of *Lecanicillium* sp on *H. vastatrix* uredospores on leaves found significant differences among the treatments evaluated (p:0.0001), all isolates caused parasitism on H. vastatrix pustules. However, no statistical differences were found within the isolates evaluated during the evaluation period, all isolates behaved in a similar way on leaf parasitism (Table 3).

**Table 3: Comparative means of *In Vitro* parasitism of native isolates of *Lecanicillium* sp on pustules of *H. vastatrix***

| Isolated | Parasitism % | Categories |
|---|---|---|
| Testigo | 0.00 | A |
| Mankotal | 66.43 | B |
| Majada | 70.08 | B |
| San Ramón | 71.95 | B |
| Jinotega | 78.75 | B |
| El Granadillo | 86.48 | B |
| La Gotera | 88.33 | B |

Means with letters in common, within the same column do not differ significantly according to Tukey($p \leq 0.05$).

## DISCUSSION

The results of our study with respect to microscopic morphometric characterization were similar to those of Icochea (2004), describing erect phialides, wide at the base and ending in a thin tip from which the conidia emerge, generally in groups of two to six, these conidiogenous cells measure 11 to 30 µ in length x 1.5 to 2 µ in diameter, the conidia are ellipsoidal from 2 to 4 µ x 1 to 1.5 µ. The phialides are solitary or in whorls originating from straight conidiophores. Likewise, Zare and Gams (2001) describe relatively short conidiogenous conidiogenous cell phialides 11 to 30 µ x 1.4 to 1.8µ, agglomerated and strongly tapered, produced singly or in groups of up to 6 directly on prostrate hyphae, or on short, erect conidiophores sometimes also produced secondarily on earlier phialides. Conidia formed on heads at the apex of the phialides, typically short ellipsoidal in shape ranging from 2.5 to 4.2 µ x 1 to 1.5 µ.

The physiological characteristics were like the studies carried out by González (2001), who describes that *Lecanicillium* spp. presents creamy white colonies, with striations on the back and cottony colonies, this is a characteristic of this fungus which produces a compacting in the whole colony that makes it difficult to detach its mycelium. In our study the wrinkling of the fungus was observed mostly in the SDA and EMA culture media. In the PDA culture medium, the growth of the colonies of the isolates presented the same off-white coloration, but showed little vertical growth and little cottony, in addition, a ringed growth of concentric spaced circles was observed, since the hyphae seek a better use of nutrients, adhesion and dissemination on the nutrient medium (Argueta, 2011). The radial growth of native isolates of *Lecanicillium* spp. is like studies reported by Zare and Gams (2001), who describe that this fungus presents a colony growth that reaches 15 to

25 mm in diameter in 10 days at 24°C in PDA culture medium. It is important to mention that all isolates of *Lecanicillium* spp presented cottony colonies, but not powdery colonies, which were observed in the three media evaluated (Figure 2).

The radial growth of native *Lecanicillium* spp isolates at different times for the SDA culture medium was less variable (Figure 6) compared to PDA and EMA culture media. It is important to emphasize that in SDA culture medium the growths were quite uniform compared to those obtained in EMA and PDA. (2007) cited by Retamal (2008) indicate that the culture medium in which L. lecanii grows and sporulates best is Sabouraud Dextrose Agar, which is one of the most widely used culture media by insect pathologists.

The radial growth of *Lecanicillium* spp. isolates in the EMA culture medium was lower compared to the other culture media; this medium has a pH of 4.7 ± 0.2, which could be unfavorable for mycelial growth (Figure 4). Pereira *et al*., (2007) affirm that the variation of pH in the culture medium is a determining factor in the behavior of fungal species. Studies by López and Carbonell (1999) confirm these findings where V. lecanii grows better at pH close to 7 (Figure 4). Espinosa and Vallejos (2016) comparing different culture media, found that the highest average radial growth rate of Beauveria bassiana was in PDA culture medium; in our study the same particularity was found when comparing different culture media with different native isolates of *Lecanicillium* spp finding that the highest growth was recorded in PDA culture medium (Figure 5). Some culture media could stimulate fungal growth and influence some physiological characteristics such as viability and sporulation. Pereira *et al*., (2007) indicate that the variation of pH in the culture medium is determinant in the behavior of fungal species. The demand for a particular nutrient often varies not only within a single species, but also for individual strains of a species (Iskandarov *et al*., 2004).

The growth dynamics of *Lecanicillium* spp. isolates on PDA medium in the last three evaluation periods showed no statistical differences in the radial growth of the isolates, and it is possible that they are entering a period of low metabolism, which could allow the fungus to develop survival strategies. Milner *et al*., (1991) affirms that it is possible that some isolates enter a type of physiological dormancy, which would allow them to survive for prolonged periods in the field outside the host and give them better adaptability characteristics and better performance as biological controllers. All isolates showed the same growth pattern over time. At 480 hours, most of the isolates on SDA medium showed growth rates of less than 1.3 mm (Figure 6). Radial growth in this culture medium was lower on most of the dates evaluated compared to those obtained in PDA; radial growth is determined by several factors such as: pH, N/C ratio, as well as other nutritional values of the culture media. The growth dynamics of *Lecanicillium* spp. isolates in PDA medium showed that most of the isolates presented a similar growth pattern over time with less variability compared to that found in the EMA culture medium; however, radial growth in PDA culture medium was higher in most of the sampling periods. The PDA culture medium has a pH of 5.6 ± 0.2 which could allow the isolates to have a radial growth to be favored and present a uniform growth. The growth dynamics of the isolates on SDA culture medium was more regular with the highest growth of all isolates at 336 hours (Figure 6).

Viability Most of the isolates presented a germination close to 100% (Table 1), with adequate characteristics for mass production in terms of viability since this parameter is determinant for mass reproduction according to Monzon (2001). The results obtained in the test of viability of conidia of different isolates of *Lecanicillium* spp, show that in the majority the viability of conidia has a tendency to increase as the hours pass, on each date the isolates presented a variability in viability from one date to another; that is, those that were better at 18 hours were not necessarily better at 22 hours in their germination, a rapid germination helps to decrease the time of exposure to environmental conditions and achieve a rapid effect on the pest (Malpartida *et al*., 2013). The effectiveness of the fungus in the field depends on its ability to colonize a substrate and infect its host, which in turn is determined by its viability (Monzon, 2001). For this reason, the determination of this characteristic is important in the selection of strains for biological control; the higher the viability in a shorter time under laboratory conditions, it is presumed that it will present better adaptability to environmental conditions and better effectiveness on the host; also, Steinkraus (2006) suggests that viability is related to the ability to spread within a host population and determines its potential as a microbial control agent.

Spore yield in organic substrates the general analysis shows that the isolate La Gotera stands out above the others in the soybean-based substrate, possibly there is some specificity between the vegetative material and genetic characteristics of the isolate, this substrate provides better nutritional conditions in relation to carbon / nitrogen and other essential elements for its multiplication (Figure 7). For the rice-based substrate, the isolates that presented the best yields were San Ramón and Jinotega (Figure 8). In this study, it was observed that this rice substrate absorbs moisture efficiently in the process, and presents adequate aeration; however, it was observed that the growth in the substrate grows in a compact manner that hinders the separation of the conidia and substantially affects its yield. In some fungi such as *Lecanicillium lecanii,* the amount of conidia can decrease due to the high compaction exerted by the fungus on the rice,

which prevents internal aeration and reduces conidial production (Cortez, 2007). There are very important characteristics to use a certain substrate for mass reproduction, one of them is that it should have easy separation of conidia at the time of harvest, and another is that the substrate should have good consistency throughout the process of mass reproduction. In the sorghum-based substrate, the isolates that were favored in spore production were Mankotal and San Ramón (Figure 10).

To consider a substrate as suitable for mass production, it must stimulate a high concentration of conidia (Cortez, 2007). Similarly, Roberts and Yendol (1971) argue that a substrate with good attributes to produce conidia of a strain of an entomopathogenic fungus should be considered low cost and easy to acquire. In our study, this soybean substrate, although good conidia production and low cost were obtained, does not have a good capacity to absorb moisture in the preparation of the substrate or in the incubation period, which makes it difficult to handle, and for this reason this substrate is very susceptible to bacterial contamination.

Cortez (2007) affirms that conidial production varies according to the substrate used for its reproduction, and that the fungus *L. lecani*i produces a greater quantity of conidia when cultivated in substrates that allow greater aeration; however, in the results obtained in this study it was observed that to obtain greater production of conidia it is also necessary for the substrate to have good nutritional values. For this reason, we can say that the nutritional values of a substrate are a determining factor in obtaining a greater number of conidia. Volcy and Pardo (1994), who mention that a good substrate to produce conidia must have a high content of carbohydrates, nitrogen, microelements, B complex vitamins and a high concentration of ions necessary for growth and sporulation.

Aceves (2008) by means of a proximal chemical analysis of different organic substrates for mass reproduction showed that there is a correlation between nutritional values and spore production. Figueroa *et al*., (2007) argue that sporulation performance on a substrate can also be influenced by factors such as culture technique, initial inoculum concentration, temperature, humidity, aeration, and incubation time. In our study it was also observed that this substrate presented a deficiency in terms of moisture absorption during the production process, which generates a longer drying time, being somewhat tedious for its use in the mass reproduction of fungi, for such reasons the use of this substrate is not recommended for such purposes, and because there is a potential risk of bacterial contamination as observed.

The parasitism of *Lecanicillium* spp. on uredospores showed different degrees of parasitism (Table 2). Gonzalez (2001) obtained similar results where he found different degrees of parasitism on uredospores of H. vastatrix. Parasitism *In Vitro* was evidenced 72 hours after inoculation of *Lecanicillium* spp. conidia on rust uredospores, destruction of the cell wall of the uredospores could be observed through the light microscope (Figure 10). This same phenomenon of inhibition of germination of uredospores of *H. vastatrix* occurred in a study by Eskes (1987) who determined that in addition to being a mycoparasite of *H. vastatrix*, it was also an inhibitor of uredospore germination.

The percentages of parasitism on rust pustules of native isolates of *Lecanicillium* spp are high in this study, the values fluctuated between 88.33 and 66.43% (Table 3). Gonzalez (2001) found similar parasitism percentages on *H. vastatrix* sori and reduced uredospore germination to 0% and reached 81.03% parasitism. In this study, parasitism on pustules was quantified by visual observation and a pustule with whitish mycelium was parasitized. Mahfud *et al*., (2006) point out that the effects of some species of this genus produce discoloration of uredospores, formation of white mycelium on them or necrosis, depending on the time of evaluation. The findings found in this study under laboratory conditions could be very useful and of great importance. The collected isolates cause parasitism to the pathogen, and it was also demonstrated that the isolates present different degrees of parasitism on the uredospores of *Hemlieia vastatrix*. These results could motivate others to develop effective and inexpensive methods for mass reproduction, and their potential use for biological control of coffee rust should be considered by incorporating disease management strategies under field conditions.

## CONCLUSION

Six native isolates of the genus *Lecanicillium* spp. were obtained, the microscopic and macroscopic characteristics were similar in the three-culture media. The growth rate is influenced by the artificial culture medium and by the type of isolate. The viability of conidia of native isolates of *Lecanicillium* spp. depends on the isolate and increases with time; the best viability reading time was at 22 hours, since most of the spores are already germinated, and mycelium production makes the reading more accurate. The performance of *Lecanicillium* spp. isolates on different organic substrates is determined by a good initial inoculum, good aeration of the substrate, adequate multiplication technique, as well as the ratio of nitrogen and carbon content. In the *In Vitro* parasitism tests, the Jinotega and La Gotera isolates showed the highest percentages of parasitism on uredospores and rust pustules, respectively, indicating that the level of parasitism is also determined by the type of isolate.

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