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Research Article
Circadian Rhythms, Such as Light Regimes Influencing *in vitro* Growth of *Pestalotiopsis mangiferae* from Mango Tree

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Abstract

Background and Objective: The fungus *Pestalotiopsis mangiferae* causes a gray leaf spot, which can reduce fruit production. This work aimed to carry out the pathogenic and physiological characterization of *Pestalotiopsis mangiferae*. **Materials and Methods:** For the physiological evaluation, isolates were streaked on the Petri dishes containing PDA medium and incubation in BOD chamber at 25°C under three light regimes (0, 12 and 24 h) for 6 days. Evaluations were performed daily with a digital caliper to obtain growth measures. After 6 days of incubation, a total of 10 mL of sterile distilled water was added to each Petri dish for sporulation evaluation. For the pathogenic characterization, the isolates were inoculated in leaves and kept in transparent acrylic boxes. The design was completely randomized, with 6 leaves of *Mangifera indica* per isolate of *P. mangiferae*. The lesions were measured at 2, 4, 6, 8 and 10 days of inoculation with the aid of a digital caliper. **Results:** The N-01-15 isolate was statistically superior to the other isolates in terms of the spotted leaf area and the area under the progress disease curve, all isolates were able to cause injuries to the mango leaves. Regarding physiological evaluation, the daily light hour regime directly influences mycelial growth and spore production of *P. mangiferae.* **Conclusion:** The amount of daily light hours directly influences mycelial growth and spore production of *P. mangiferae*. The isolates were capable of lesions ranging from 0.44-1.46 cm^2 in mango leaves.

Key words: *Mangifera indica*, mycelial growth, pathogenicity, spores production, lesions

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mango (*Mangifera indica* L.) is a tropical fruit, with special flavor and high alimentary worth, it is well accepted globally and due to its characteristics, it is considered as "king of fruits"[1]. Brazil is the world's third largest fruit producer and the seventh largest mango producer[2]. Mango can be grown in all Brazilian regions and has become one of the most exported fruit motivate greater investments in the country and representing the largest source of revenue for fresh fruit exported from Brazil[3,4]. The Sao Francisco Valley, located in the Brazilian semiarid region, is the largest mango-producing and exporting site in Brazil. In 2018 approximately 148,000 t of mangos produced in the valley were exported and considered one of the best fruits for trade[5]. The appearance of the fruit plays a significant role in the success of its commercialization[6]. However, many hurdles are found in the production chain, the main requirement of the consumer market is greater phytosanitary treatment and post-harvest treatment of the fruits[7]. The post-harvest life of mangoes is limited due to development of pathogens, especially fungi that cause rot and by the natural ripening and senescence of the fruit, which can cause losses of up to 30% of the fruits[8].

Pestalotiopsis mangiferae fungi is responsible for causing leaf ash spot while after post-harvest it cause stem rot in fruits[9] fungi reduce productivity and have a vast range of hosts and are gaining the recognition as a pathogen and for its secondary metabolites world widely[10,11]. Its dissemination occurs easily through spores, penetrating plant tissues through injures or natural openings[12].

The study based on relationship between a disease and a causal agent can only be confirmed after completing a series of steps, known as Koch's Postulates[13]. In carrying out the pathogenicity test, the ability of a pathogen to cause diseases in the host is proven through its pathogenic potential in which the degree of pathogenicity can vary between fungal isolates as well as the type of host, since some fungal species they are able to infect a substantial range of hosts, while others are specific to few host[14,15]. Environmental factors are vital for the production and growth of microorganisms, small changes generate crucial influence on its metabolism[16]. Studies related to the influence of light in circadian rhythms reduce the difficulty in obtaining sporulated isolates and help to standardize the conditions for sporulation of phytopathogenic fungi, which is one of the main problem faced by research groups[17]. The pathogenic power of *P. mangiferae* in causing damage to mango leaves in Brazil are scanty, even at the global level there is no clarification of the fungus as to its pathogenic and sporulation capacity. This study aimed to evaluate the effect of light regimes on the growth and sporulation of the isolates and to estimate the pathogenic potential of *P. mangiferae* isolates in mango leaves cv. "Amrapali".

MATERIALS AND METHODS

Study area: This work was carried out at the Phytopathology Laboratory of the State University of Goiás-Ipameri University Unit, which started in March, 2018 and ended in February, 2020.

Obtaining *Pestalotiopsis mangiferae* strains: In this study, 6 strains of *P. mangiferae* obtained from leaves (showing lesions and with fungal growth on infected tissue) of *M. mangiferae* adult plants cv. 'Amrapali ́6-year-old located at the State University of Goiás (UEG), Ipameri campus (17°43'20" S, 48°09'44" W, 800 m) by removing fragments of injured plant tissue that were submitted to disinfestation[18]. Then, the fragments of the leaves tissue were sown in Petri dishes (5 mm^2 $plate^{-1}$) containing Potato Dextrose Agar (PDA) medium with antibiotics tetracycline. After 7 days at 25°C and 12 h photoperiod, the fungal colonies obtained were subjected to new subcultures in PDA medium until the colonies were purified. The purified strains were preserved in BDA medium at 5°C using Castellani method. These strain Ccs were part of the collection of phytopathogenic Fungi of the UEG phytopathology Laboratory.

Mycelial growth and sporulation characterization of *Pestalotiopsis mangiferae* under different light regimes at 25°C: The *P. mangiferae* strains were sown through an agar plug (5 mm^2 Ø) containing mycelium in Petri dishes containing PDA medium and incubated in a DBO chamber at 25°C under three light regimes (0, 12-24 h), using fluorescent lamps of 20W, 75RS (Philips®), for 10 days. Then, colonies were measured daily by using a digital caliper to obtain growth measures (based on the average of two diametrically opposed measurements) until 4 days after inoculation (DAI). After 6 DAI at 25°C, a total of 10 mL of Sterile Distilled Water (SDW) were added to each Petri dish, followed by the release of the spores with a Drigalski loop. Subsequently, the spores were collected in a beck and filtered through sterile gauze. The concentrations of the suspensions obtained were measured in a Neubauer chamber performing spore counting in 5 quadrant of the chamber for each plate.

Inoculation of *Pestalotiopsis mangiferae* on leaves of *Mangifera indica* cv. 'Amrapali': Young and healthy leaves

of adult *Mangifera indica* cv. 'Amrapali' were washed under running water and left to dry in a laminar flow chamber for 10 min. For inoculations, five wounds were made on the left side and five on the right side of the leaf blade with the help of a sterile needle and an agar disc (5 mm^2) containing mycelium from each isolate was deposited. The inoculated leaves were subjected to controlled conditions of a humid chamber in transparent acrylic boxes gearbox type (11×11×3.5 cm), containing a sheet of blotting paper with constant humidity maintenance only on the paper. The design was completely randomized, with 6 leaves of *M. indica* per isolate of *P. mangiferae* and one leaf of *M. indica* per gearbox. The control consisted of the application of 25 µL of SDW in the region of the holes in the leaf blade. For the evaluations, measurements of the lesions on the abaxial (below) surface of the leaves were performed at 2, 4, 6, 8 and 10 DAI, with the help of a digital caliper, obtaining the size of the lesions in cm^2.

Statistical analysis: The results related to the pathogenicity test (10 DAI), mycelial growth (4 DAI) and sporulation (6 DAI) with *P. mangiferae* strains were submitted to variance analysis, to the Scott-Knott test ($p \leq 0.05$) and regression analysis to obtain significant models for the development of injuries, with the help of the SISVAR 5.3 software[19].

RESULTS

Evaluation of mycelial growth and sporulation of *P. mangiferae* under different light regimes at 25°C: The 0, 12 and 24 h light regimes showed the following growth averages, 25.4, 30.2 and 47.7 cm^2, respectively, demonstrating that continuous light cultivation provides larger colonies than the other regimes tested given in Table 1. As for the mycelial growth of the isolates, the N-01-10 isolate was superior to the others when its average of 39.5 cm^2 was observed under the three tested regimes, followed by the N-01-02, N-01-03 and N- 01-06, with an average of 37.4, 37.3 and 36.5 cm^2, respectively. The isolate N-01-15 was statistically inferior to the other isolates, presenting an average of 26.3 cm^2.

As for sporulation, *P. mangiferae* isolates when grown under 0 h light (dark) did not produce conidia. The averages were 2.5×10^5 conidia mL^{-1} in the 12 h regime and 62.4×10^5 conidia mL^{-1} in the 24 h light regime given in Table 2. The results demonstrate that the continuous light regime (24 h) in addition to providing colonies of larger diameters also stimulates greater sporulation. The isolate N-01-06 was shown to be superior to the others in terms of conidia production in continuous light, although it did not show production in the partial light regime (12 h), a fact that was also observed for N-01-15.

Table 1: Mycelial growth of *P. mangiferae* (cm^2) using different light regimes

	Mycelial Growth (cm^2) on the 4th day[1]			
Isolated	0 h	12 h	24 h	Mean
N-01-01	19.7^{cC}	30.1^{bB}	38.9^{cA}	29.6^{c}
N-01-02	32.2^{aB}	30.5^{bB}	49.5^{bA}	37.4^{b}
N-01-03	30.9^{aB}	29.4^{bB}	51.7^{bA}	37.3^{b}
N-01-06	22.1^{cC}	30.7^{bB}	56.7^{aA}	36.5^{b}
N-01-10	25.4^{bC}	36.4^{aB}	56.7^{aA}	39.5^{a}
N-01-15	22.1^{cB}	24.0^{cB}	32.8^{dA}	26.3^{d}
Mean	25.4^{C}	30.2^{B}	47.7^{A}	-
Coefficient of variation (%)	10.48	8.81	6.91	8.40

[1]Means followed by the same lower case letters in the columns and upper case letters in the rows do not differ statistically from each other, according to the Scott-Knott test ($p \leq 0.05$)

Table 2: Average number of *P. mangiferae* conidia mL^{-1} using different light regimes

	Conidia mL^{-1} at 6th DAI[1]		
Isolated	0 h	12 h	24 h
N-01-01	-	3.2×10^{5aB}	28.6×10^{5dA}
N-01-02	-	$1.9\times10^{5\,bB}$	72.6×10^{5bA}
N-01-03	-	2.2×10^{5bB}	55.3×10^{5cA}
N-01-06	-	-	93.7×10^{5a}
N-01-10	-	3.6×10^{5aB}	75.2×10^{5bA}
N-01-15	-	-	$49.6\times10^{5\,c}$
Mean	-	2.5×10^{5B}	62.4×10^{5A}
Coefficient of variation (%)	-	25.00	20.34

[1]Means followed by the same lower case letters in the columns and upper case letters in the rows do not differ statistically from each other, according to the Scott-Knott test ($p \leq 0.05$), (-) There was no sporulation

Table 3: Leaf spot area (LSA) of *Mangifera indica* by *Pestalotiopsis mangiferae*, regression models for increasing leaf lesions and area under the disease progress curve (AUDPC)

Isolated	LSA at 10th DAI (cm^2)[1]	Regression model	R^2	($P \leq X$)	AUDPC[1]
N-01-01	1.15[b]	Y = 0.0883x+0.2140	0.95	0.01	5.81[b]
N-01-02	0.97[b]	Y = 0.0617x+0.3620	0.99	0.01	5.85[b]
N-01-03	1.10[b]	Y = 0.0737x+0.3796	0.96	0.01	6.56[b]
N-01-06	0.44[d]	Y = 0.0251x+0.1548	0.87	0.01	2.38[d]
N-01-10	0.79[c]	Y = 0.0491x+0.2803	0.97	0.01	4.56[c]
N-01-15	1.46[a]	Y = 0.0714x+0.7615	0.97	0.01	9.55[a]
Coefficient of variation (%)	23.87	-	-	-	23.89

[1]Means followed by the same lower case letters in the columns do not differ statistically from each other, according to the Scott-Knott test ($p \leq 0.05$)

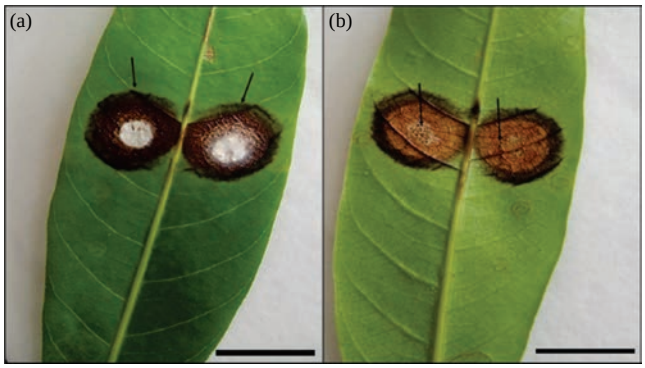

Fig. 1(a-b): Symptoms and lesions typical of the Pestalotiopsis spot in leaves of *Mangifera indica*, inoculated with PDA plugs containing mycelial structure of *P. mangiferae* (N-01-10), 10 DAI
The arrows showed, (a) Necrotic lesions and mycelial growth in plant tissue and (b) The formation of reproductive structures, Bars (A and B = 2.5 cm)

Evaluation of the pathogenic potential of *P. mangiferae* isolates: After inoculations with mycelium plug, spotted typical of the symptoms of the *P. mangiferae* spot developed in the leaf of *M. indica*, occurring the development of brown necrotic spots with a lighter center and darker edges forming a ring. The injuries also presents mycelium growth of the fungus developed in the necrotic leaf tissue at 10 DAI in the adaxial face in Fig 1a. In the abaxial face of the leaves, the formation of reproductive structures of the fungus was observed in Fig. 1b.

The Isolate N-01-15 was superior to the others in terms of the leaf spot area (LSA) at 10 DAI given in Table 3. Consequently, this isolate had an area under the disease progress curve (AUDPC) greater than the others. After regression analyzes for the increase of leaf lesions over time, a simple linear model adjusted the six isolates. All models were significant and with a high coefficient of determination (R^2). The isolates caused lesions from 0.44-1.46 cm^2 in the leaves and presented AUDPC between 2.38-9.55.

DISCUSSION

Abiotic factors such as temperature and light are able to inhibit or induce the vegetative and reproductive development of most fungi by influencing the pathogen's circadian cycle[20]. Belonging to the same phylum of *Pestalotiopsis mangiferae*, the fungus *Fusarium solani* demonstrated a similar behavior, in which the light regime directly influenced the growth and sporulation of the colonies, according to Silva and Teixeira[21] the exposure to continuous light provided colonies with significantly larger diameters and grew at greater speed, just as there was greater production of conidia when compared to other light regimes. In Poletto *et al.*[22], there was a similar behavior, in which isolates of *Sirosporium diffusum* were subjected to three light regimes, continuous dark, 12 h photoperiod and continuous light. Thus, in dark cultivation, the isolates reached growth between 8.1 and 9.6 mm and did not produce spores, growth below the 24 h light regime that reached values between 14.9 and 31.2 mm in diameter and induced greater amount of sporulation for all isolates.

The evaluations on the influence of the light regime on the sporulation of the isolates corroborated with the study of Ding *et al.*[23], in which he used the same light regime methodology just to verify whether or not there would be spore production, isolates *of P. mangiferae* did not produce spores in cultivation in the dark. The conjuncture of the results confirms that exposure to light be decisive in reproduction, since in their absence, *P. mangiferae* isolates proved to be incapable of producing spores. As for the issue of mycelial growth, light proved to be a limiting factor as the isolates were able to grow, however the growth is slow and limited. The greater sporulation in continuous light may be related to the stress caused by the constant light intensity, since this would cause a greater dryness of the culture medium, inducing the fungus to generate a greater number of descendants[17].

The pathogenicity evaluations with the same methodology and host were presented by Ismail *et al.*[24], in which pathogenicity tests with six isolates of *Pestalotiopsis*

obtained from six mango orchards in the southern region of Italy was conducted. In this study, three isolates of *Pestalotiopsis clavispora* and three isolates of *Pestalotiopsis uvicola* were obtained, which were inoculated into mango leaves by means of PDA plug containing mycelium and subsequently, induced lesion growth between 15-17.5 mm and 10 and 12.5 mm in diameter, respectively. In another study by Steinrucken *et al.*[25], an isolate of *P. mangiferae* was subjected to a pathogenic study in seedlings of Parkinsonia, a perennial tree species, the isolate was inoculated by depositing mycelium under 8-10 mm incisions made with a scalpel on the stem of the seedlings and then induced lesions of 4.62-5.44 mm. There are no reports of characterization of *P. mangiferae* in mango leaves and it is expected that the variation in the size of the lesions has been influenced by the level of virulence of the isolates, guest and environmental conditions.

The isolates showed the same behavior model but differed statistically in terms of LSA and AUDPC, demonstrating that pathogenic variation occurs within the same species, which also explains the variation in the determination coefficient (R^2)[26]. Lima *et al.*[27] states that within the same species naturally occurs pathogenic variability, which corroborates the behavior of *P. mangiferae* isolates. The appearance of lesions in inoculation conditions were visualized from the 2° DAI. The symptoms consisted of necrotic lesions with a light brown color in the center and dark brown margins, afterwards with agglomeration of black colored spores, the size of the lesions ranged from 0.44-1.46 cm^2. This last parameter is important so that future research with *Pestalotiopsis*, even in other cultures can be used for the purposes of diagnosis and symptoms.

CONCLUSION

The amount of daily light hours directly influences mycelial growth and spore production of *P. mangiferae*. Isolate N-01-15 was superior to the others in terms of SLA and AUPDC. In addition, *P. mangiferae* isolates were capable of causing spotted from 0.44-1.46 cm^2 in size at 10 DAI on mango leaves.

SIGNIFICANCE STATEMENT

This study verified the influence of light regimes on the physiological development of the fungus *Pestalotiopsis mangiferae* and on the characterization of the pathogenicity of Brazilian isolates that can be beneficial for the elucidation of the intrinsic characteristics of the pathosystem of the species. This study will help researchers to discover the best light regimes for the *in vitro* production of fungal structures for future characterization studies and to highlight the pathogenic potential of populations located in Brazil, which few researchers have explored.

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