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Culture Medium, Light Regime and Temperature Affect the Development of *Sirosporium diffusum*

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Abstract

Sirosporium diffusum is the causal agent of the brown leaf spot disease on pecan trees that seriously damages the foliage of adult plants and seedlings. This fungal species is difficult to grow satisfactorily in a culture medium. Therefore, the aim of this study was to evaluate the effects of different physical conditions on the development of *S. diffusum*. In the first assay, eight culture media and five light regimes were combined, while in the second, the three treatments that promoted highest sporulation were combined with three temperatures. The trials were conducted in a two-factorial arrangement in a fully randomized design with six replicates. V8, V8$CaCO_3$, and CA media under a 24-h photoperiod produced the highest respective sporulations: 29×10^4, 35×10^4, and 41×10^4 conidia ml^{-1}. The best temperature for sporulation was 20±1 °C for all culture media, especially V8$CaCO_3$ and CA. The best artificial conditions for obtaining good mycelial growth and sporulation consisted of a photoperiod of 24 h, temperature of 20±1 °C and V8$CaCO_3$ or CA culture medium.

Keywords: *Carya illinoinensis*, brown leaf spot, sporulation, *in vitro* cultivation

1. Introduction

Pecan [*Carya illinoinensis* (Wangenh) K. Koch], from the Juglandaceae family, is a deciduous tree species native to the temperate zones of North America (Poletto et al., 2015). The species is one of the oldest and most important exotic forest systems in the southern region of Brazil, especially in Rio Grande do Sul State, but only in recent years has been valued commercially (Poletto et al., 2015). Moreover, in 2017, the State created the Pecanculture Development Program (Pró-Pecan) to increase the cultivated area and the production, generating employment and income in the rural areas and encouraging industries and suppliers of equipments for this production chain (SEAPI, 2017). In Brazil, the plantations with the greatest extensions are located in the Taquari Vale, Rio Pardo and central Rio Grande do Sul regions (Poletto et al., 2015).

Pecan cultivation is also present in numerous small farms in the state where it is used in agroforestry systems, shading of poultry houses, for wood and ornamental purposes. The cultivation of this species is growing rapidly, with new orchards stablished, not only in Rio Grande do Sul, but also in the states of Santa Catarina and Paraná. In 2015, the Brazilian production was 4,138 tons of nut (dry fruit), totaling 2,319 hectares planted (IBGE, 2016).

Among the main limiting factors in the production, the incidence of diseases such as stem canker caused by *Lasiodiplodia subglobosa* Machado & Pereira, leaf spot of cladosporium, caused by fungi belonging to the *Cladosporium cladosporioides* complex and leaf spot of pestalotiopsis caused by *Pestalotiopsis clavispora* (G.F. Atk.) Steyaert are widely reported, both causing damages in nursery and field plantations (Lazarotto et al., 2012; Poletto et al., 2016; Walker et al., 2016).

Sirosporium diffusum (Heald & Wolf) Deighton is afungal pathogen of pecan being reported to cause leaf spot in several countries, including the United States, Mexico and South Africa (Crous & Braun, 2003). In Brazil, the disease was first observed in 2016, causing leaf spot and subsequent defoliation, impairing the yield and quality of the fruits. The disease also affects seedlings in the nursery, delaying their development by defoliation (Poletto et al., 2017).

Despite the importance of this pathogen, no studies are found in the literature on suitable physical conditions for *in vitro* growth, information essential for conducting research. The cultivation of phytopathogenic fungi under artificial conditions is of importance in studies that demand a supply of pure inoculant material for testing genetic resistance and verifying taxonomic classification. It is therefore essential to determine optimal conditions for *in vitro* growth and sporulation. Studies involving fungal species with slow growth and low sporulation, like *S. diffusum,* can even be rendered impracticable by this lack of information. Cruz et al. (2009) pointed out that due to the difficulty to standardize ideal conditions for phytopathogenic fungi sporulation research with some might be a challenge.

Abiotic factors, such as temperature, luminosity and composition of the culture medium, are capable of inducing or inhibiting the vegetative and reproductive development of the majority of fungi (Dhingra & Sinclair, 1995; Brunelli et al., 2006). On *Magnaporthe grisea* (Hebert) Barr, for example, the composition of the culture medium influences the cultural characteristics of fungal colonies and sporulation (Dias Neto et al., 2010). The proteins and enzymes production is influenced by temperature and are responsible for the maintenance of the fungal cell. While luminosity has direct action on the fungal cell, meaning that it may induce or inhibit the formation of reproduction structures, some species are indifferent to the quantity and/or quality of light (Hawker, 1957; Griffin, 1994).

Studies on the effect of culture media, light regimes and temperatures for culturing fungi have already been conducted on some fungi, such as *Mycosphaerella fijiensis* (Morelet) Deighton (Hanada et al., 2002), *Cercospora zeae-maydis* Tehon & Daniels (Brunelli et al., 2006), *Magnaporthe grisea* (Dias Neto et al., 2010), *Cercospora coffeicola* Berk. & Cooke (Silva et al., 2016), *Pseudocercospora vitis* (Lév.) Speg. (Maia et al., 2015), and *Passalora sojina* (Hara) Poonam Srivast. (Camera et al., 2014). The aim of this study was to evaluate the effects of different culture media, light regimes and temperatures on the growth and sporulation of *S. diffusum*.

2. Material and Methods

A representative and well characterized isolate from *S. diffusum* (AG-RM-1) obtained from symptomatic pecan leaves collected in Anta Gorda in the Brazilian State of Rio Grande do Sul, was used to perform the tests. The isolate was deposited at the Phytopathology Laboratory of the University and in the SMDB Herbarium of the Department of Biology at the Federal University of Santa Maria under accession code 16.434. The inoculum was produced by placing the fungus culture into Petri dishes containing V8 medium and kept in incubator at 24±1 °C under a 12-h photoperiod for 56 days.

2.1 Effects of Culture Medium and Light Regime

Different culture media and light regimes were combined. The design was fully randomized in a two-factorial arrangement, with six replicates. The following culture media were used: V8 (100 ml of V8 (Spicy Hot), 20 g of agar and 900 ml of distilled water); V8 $CaCO_3$ (100 ml of V8 (Spicy Hot), 2 g of $CaCO_3$, 20 g of agar and 900 ml; PCA (20 g of potato juice and 20 g of carrot juice extract, 20 g of agar and distilled water, enough for 1000 ml); CA (200 g of carrot extract, 20 g of agar and distilled water, enough for 1000 ml); CWA (1000 ml of coconut water (Ducoco®) and 20 g of agar); STJ (200 ml of tomato juice (Superbom®), 20 g of agar and distilled water, sufficient for 1000 ml); PDA (200 g of potato extract, 20 g of dextrose, 20 g of agar and distilled water, sufficient for 1000 ml); and OA (50 g of oatmeal, 15 g of agar and 1000 ml of distilled water). All culture media were autoclaved at 120 °C for 30 min.

Light regimes were as follows: CD (continuous darkness), SEQ D-UV (seven days darkness/seven days under black light), SEQ D-L (seven days darkness/seven days under fluorescent light), FT-12h (photoperiod of 12 h under fluorescent light), FT-24h (photoperiod of 24 h under fluorescent light).

For light regimes consisting of periods of darkness, the plates were wrapped in aluminum foil and placed in a cardboard box. For the SEQ D-UV regime, a compact fluorescent black light (Golden, 46 W) emitting near-ultraviolet (NUV) radiation was used, located at a distance of 45 cm from the plates. To provide fluorescent light, four daylight fluorescent lamps were used (General Electric F40T, 40 W).

To set up the assay, a 5 mm diameter V8 culture medium disk containing 56 days old *S. diffusum* mycelium was transferred to the center of the Petri dishes (90 mm diameter) containing the different culture media and all replicates were kept in incubator at a temperature of 24±1 °C. For each treatment the total mycelial growth was evaluated at 56 days (the colony was measured in two diametrically opposed directions). To evaluate sporulation at 56 days, 4 ml of distilled, autoclaved water were added to each plate (sufficient for a small colony). Next, the surface of the colony was scraped to release the conidia. The suspension was then pipetted and a two-layered

gauze used to retain the mycelial fragments and culture medium. Spore concentration was counted using a Neubauer chamber.

2.2 Effect of Culture Medium and Temperature

A second experiment was carried out to evaluate the behavior of *S. diffusum* when subjected to different temperatures. The statistical design was fully randomized in a two-factorial arrangement with six replicates.

The culture media found to promote higher mean sporulation in assay 1 (V8, V8$CaCO_3$, and CA), together with the PDA medium, were selected as standard media for fungal growth. They were combined with the light regime found to stimulate higher sporulation in the previous assay (continuous photoperiod: FT-24h). The isolate used to inoculate the different culture media was subjected to three different temperatures (20±1 °C, 24±1 °C, and 28±1 °C). These temperatures were chosen considering the optimum growth emperature for most fungi and therefore verify if small variations are sufficient to induce or inhibit the development of the fungus and not to test the thermal limits of growth of the species. The culture media, assay setup and variables evaluated were identical to those described for assay 1.

2.3 Statistical Procedure

In this study, BioEstat 5.0 software (Ayres et al., 2007) was used to verify the normal distribution of the data from both assays, based on the Shapiro-Wilk test. Sporulation data showing abnormal distribution were transformed into $\sqrt{x + 0.5}$ before analysis of variance. The Scott-Knott test at 5% error probability was run on SISVAR 5.3 software (Ferreira, 2008) to compare the means of different treatments.

3. Results

3.1 Effects of Culture Media and Light Regimes

There was a significant interaction between the culture medium and light regime demonstrating that fungal development, mycelial growth, mycelial growth rate index and sporulation are influenced by the two integrated factors (culture medium and luminosity) to which the fungus was submitted, diminishing the statistical contribution of the isolated factors. The sporulation results showed a behavioral variation of the fungus as a function of the luminosity and nutrient conditions to which it was subjected (Table 1). Colonies subjected to the dark-light regime (SEQ D-UV) did not sporulate in any of the culture media, indicating that this light regime is very detrimental to fungal development, as was observed also for the variable mycelial growth.

Continuous darkness (CD) did not stimulate sporulation of *S. diffisum*. However, under a photoperiod of 12 h (FT-12h), the onset of sporulation was detected in CA, PCA, V8, and V8$CaCO_3$ media, indicating that light is essential to induce sporulation in this species. The 24-hour photoperiod (FT-24h) and sequential darkness and light (SEQ D-L) induced sporulation in all culture media tested, especially CA, V8 and V8$CaCO_3$. In this assay, the culture media that most induced sporulation were CA, V8, and V8$CaCO_3$. However, the conidia produced by CA bore a closer resemblance to those of the initial isolate obtained from infected leaves, and this medium may be the most suitable for studies involving morphological characterization (Table 1).

Table 1. *Sirosporium diffusum* sporulation as a function of interaction between culture media and light regime

Sporulation ($\times 10^4$ conidia ml^{-1})[3]					
Culture Medium[1]	**Light Regimes**[2]				
	SEQ D-UV	FT-12h	CD	SEQ D-L	FT-24h
V8	0.0 aC*	6.0 aB	0.0 aC	15.0 aA	29.0 aA
$V8CaCO_3$	0.0 aC	4.3 aB	0.0 aC	25.0 aA	35.0 aA
PDA	0.0 aB	0.0 aB	0.0 aB	2.4 bA	3.4 bA
CWA	0.0 aB	0.0 aB	0.0 aB	1.9 bA	4.9 bA
PCA	0.0 aA	4.1 aA	3.9 aA	2.0 bA	5.0 bA
CA	0.0 aB	7.1 aB	3.6 aB	21.0 aA	41.0 aA
STJ	0.0 aA	0.0 aA	0.0 aA	1.4 bA	0.5 bA
OA	0.0 aB	0.0 aB	0.0 aB	1.0 bB	4.5 bA
CV[4]: 58.65%					

Note. * Means followed by the same lowercase letter in the column and means followed by the same capital letter in the row did not differ in the Scott-Knott test at 5%.

[1] Culture medium: V8: V8 (Spicy Hot®)-agar; $V8CaCO_3$: V8 (Spicy Hot®)-$CaCO_3$-agar; PDA: potato-dextrose-agar; CWA: coconut water-agar; PCA: potato-carrot-agar; CA: carrot-agar; STJ: Superbom® tomato juice-agar; and OA: oatmeal-agar. [2] Light regimes: SEQ D-UV (seven days darkness/seven days black light); FT-12h (12 h fluorescent light); CD (continuous darkness); SEQ D-L (seven days darkness/seven days fluorescent light); and FT-24h (24 h fluorescent light). [3] Spore concentration obtained by adding 4 ml of water per plate. [4] Coefficiente of variation.

For the mycelial growth variable, the highest mean was obtained under a 24-hour photoperiod (FT-24h), although other light regimes such as CD and SEQ D-L also showed good results when combined with V8, PCA, and OA (Table 2). The black light sequential regime (SEQ D-UV) impaired the development of the colonies and produced the lowest growth. The fungus was probably exposed to excessive doses of black light or is intolerant to this type of light.

Table 2. Mycelial growth (mm) of *Sirosporium diffusum* as a function of the interaction between culture media and light regimes

Mycelial Growth					
Culture Medium[1]	**Light Regimes**[2]				
	SEQ D-UV	FT-12h	CD	SEQ D-L	FT-24h
V8	8.5 aD*	16.2 aC	22.0 cB	27.0 aA	28.8 aA
$V8CaCO_3$	9.6 aB	18.1 aA	19.3 cA	21.1 bA	22.9 bA
PDA	9.4 aC	17.4 aB	24.8 bA	25.0 aA	23.3 bA
CWA	9.1 aB	17.6 aA	21.6 cA	20.3 bA	22.2 bA
PCA	9.6 aD	19.8 aC	24.3 bB	29.0 aA	31.2 aA
CA	8.2 aB	18.9 aA	19.4 cA	20.8 bA	20.9 bA
STJ	7.2 aB	15.1 aA	18.6 cA	17.9 bA	14.9 cA
OA	8.1 aC	20.4 aB	29.9 aA	21.7 bB	21.8 bB
CV[3]: 20.1%					

Note. * Means followed by the same lowercase letter in the column and means followed by the same capital letter in the row did not differ in the Scott-Knott test at 5%.

[1] Culture medium: V8: V8 (Spicy Hot®)-agar; $V8CaCO_3$: V8 (Spicy Hot®)-$CaCO_3$-agar; PDA: potato-dextrose-agar; CWA: coconut water-agar; PCA: potato-carrot-agar; CA: carrot-agar; STJ: Superbom® tomato juice-agar; and OA: oatmeal-agar. [2] Light regimes: SEQ D-UV (seven days darkness/seven days black light); FT-12h (12 h fluorescent light); CD (continuous darkness); SEQ D-L (seven days darkness/seven days fluorescent light); and FT-24h (24 h fluorescent light). [3] Coefficiente of variation.

The variety of culture media tested (eight) promoted significant differences in the visual characteristics of the colonies formed (Figure 1). In the OA medium, for example, the colonies developed light-colored cottony aerial mycelium. In other interactions, including the PDA and V8 media, the mycelium was predominantly dark in color. In the CA medium, the colonies were formed by reddish-brown mycelium with a strong purple halo.

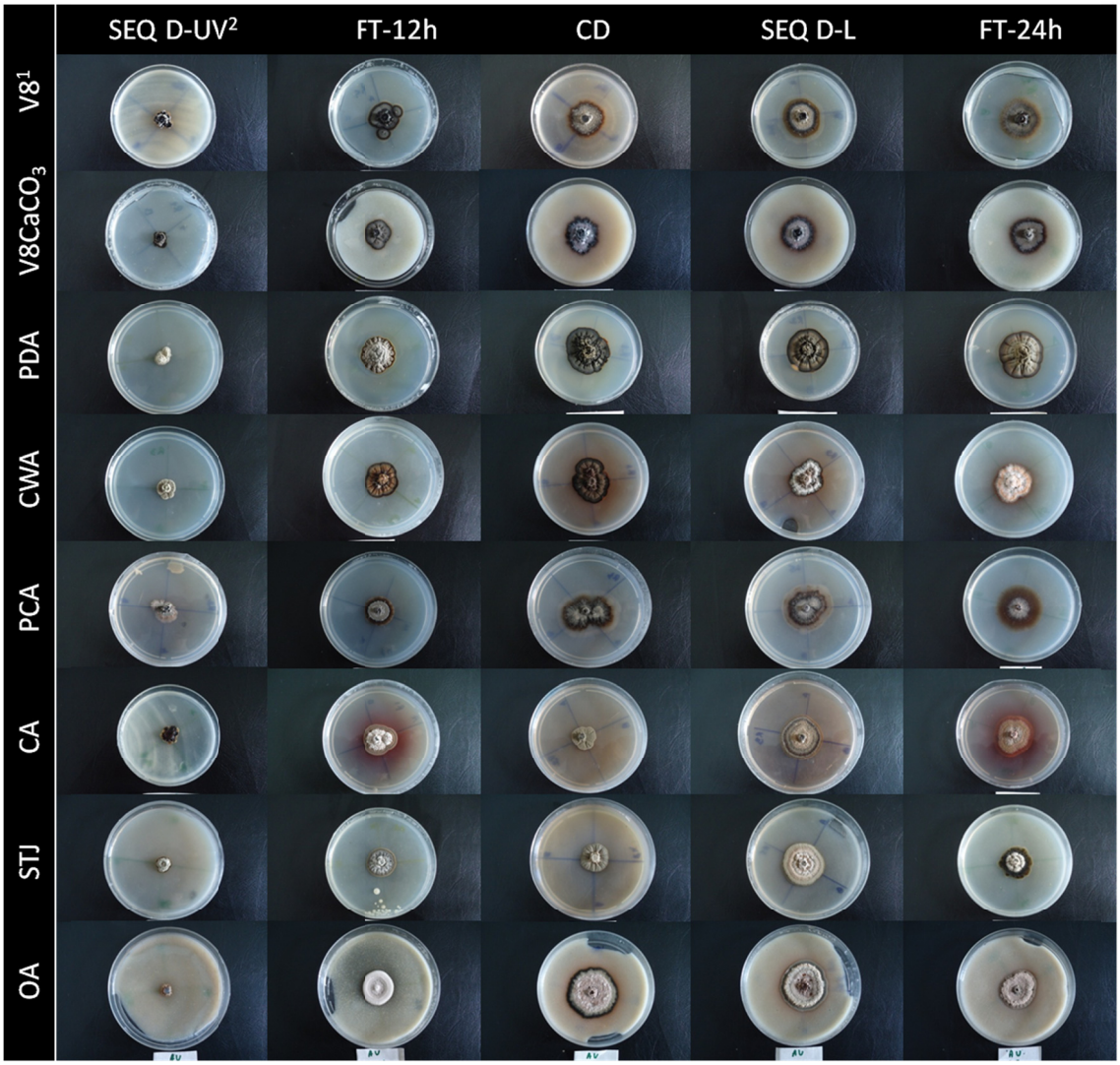

Figure 1. *Sirosporium diffusum* colonies after 56 days of growth in different culture media and under different light regimes

Note. [1] Culture medium: V8: V8 (Spicy Hot®)-agar; $V8CaCO_3$: V8 (Spicy Hot®)-$CaCO_3$-agar; PDA: potato-dextrose-agar; CWA: coconut water-agar; PCA: potato-carrot-agar; CA: carrot-agar; STJ: Superbom® tomato juice-agar; and OA: oatmeal-agar. [2] Light regimes: SEQ D-UV (seven days darkness/seven days black light); FT-12h (12 h fluorescent light); CD (continuous darkness); SEQ D-L (seven days darkness/seven days fluorescent light); and FT-24h (24 h fluorescent light).

3.2 Effects of Culture Media and Temperatures

There was a significant effect of the interaction between the culture medium and temperature. The interaction that promoted better mycelial growth occurred with the combination of V8 culture medium and temperature of 24±1 °C. The interactions between the culture media $V8CaCO_3$, BDA and CA and the temperatures of 20±1 °C and 24±1 °C did not differ from each other. Increasing the temperature to 28±1 °C influenced negatively the development of colonies (Table 3).

Table 3. Mycelial growth (mm) and sporulation of *Sirosporium diffusum* in four culture media and at three temperatures under continuous light

Mycelial Growth (mm)			
Culture Medium[1]	Temperature±1 °C		
	20 °C	24 °C	28 °C
V8	21.6 aB	28.8 aA	13.3 aC
V8$CaCO_3$	23.6 aA	22.9 bA	13.9 aB
PDA	19.2 aA	23.3 bA	10.0 aB
CA	18.8 aA	20.9 bA	14.0 aB
CV[3]:18.6%			
Sporulation ($\times 10^4$ conidia.ml^{-1})[2]			
Culture Medium	Temperatures±1 °C		
	20 °C	24 °C	28 °C
V8	99.7 cA	29.0 aB	3.5 aB
V8$CaCO_3$	769.3 aA	35.0 aB	8.1 aB
PDA	91.8 cA	3.4 aB	0.8 aB
CA	198.3 bA	41.0 aB	26.2 aB
CV:32.3%			

Note. * Means followed by the same lowercase letter in the column and the same uppercase letter in the row did not differ in the Scott-Knott test at 5%.

[1] Culture medium: V8: V8 (Spicy Hot®)-agar; V8$CaCO_3$: V8 (Spicy Hot®)-$CaCO_3$-agar; PDA: potato-dextrose-agar; CA: carrot-agar; [2] Spore concentration obtained by adding 4 ml of water per plate. [3] Coefficiente of variation.

Sporulation was found to increase when *S. diffusum* colonies were grown at 20±1 °C in all the culture media tested. However, the highest mean was obtained in V8$CaCO_3$, followed by CA which also resulted in satisfactory sporulation. Means were lower for V8 and PDA. This experiment shows how temperature strongly affects fungal sporulation.

4. Discussion

Data of this study showed that *S. diffusum* did not develop well when submitted to continuous dark and dark-NUV light regimes. Moreover, a considerable dependence of *S. diffusum* on brightness was observed for promoting sporulation on artificial environment in all culture media used. Colony injuring factors such as source of light are commonly used to induce sporulation of fungi in an artificial environment. The NUV light used in this study, negatively influenced the development of *S. diffusum*. Pulz et al. (2009) emphasize that there is a very tenuous relationship between the effects of NUV light on sporulation and the damaging effect of this type of radiation on nucleic acids, its main target. Leach (1962) pointed out that ultraviolet (UV) or near-ultraviolet (NUV) near-ultraviolet light usually induces sporulation, but excessive dosages may inhibit sporulation. Luminosity may have an inducing or inhibitory effect on the formation of reproductive structures. Trione and Leach (1976) reported that, for fungi which sporulation is induced by light, this physical agent acts directly on the activation of key enzymes involved in sporogenesis. However, they show that the quantity and quality of light required to induce the formation of reproductive structures vary according to the fungal species.

Luminosity was a fundamental factor in the sporulation of *Mycosphaerella fijiensis* Morelet, where as there was no sporulation under continuous darkness (Hanada et al., 2002). The luminosity was also a determinant factor for sporulation of *Corynespora cassiicola* (Berk. & Curtis) Wei, independent of the culture medium used (Melo & Reis, 2010). This trend was observed for *Fusarium solani* (Mart.) Sacc., since in the continuous light regime the highest averages for sporulation were statistically different from 12 h photoperiod and continuous dark (Silva & Teixeira, 2012). The continuous light also stimulated sporulation in *Corynespora cassiicola*, while in total darkness the fungus presented low spore production (Celoto et al., 2015). Kuhnem Júnior et al. (2012) concluded that the luminosity was fundamental for spore production of *Stenocarpella maydis* (Berk.) Sutton. In addition, Brunelli et al. (2006) reported good sporulation results for *Cercospora zeae-maydis* cultured in V8 under sequential light. These authors also emphasized that V8 medium contains more complex carbohydrates that can induce sporulation in mitosporic fungi. This may also apply to the CA medium. Luminosity was undoubtedly a

key factor in the sporulation of *S. diffusum.* Furthermore, the V8, V8$CaCO_3$, and CA culture media proved more suitable for sporulation under conditions of continuous light and/or sequential darkness and light.

No influence of the luminosity regimes on mycelial growth of *S. diffusum* was observed, except for the dark light-black light regime, which reduced it. Leach (1967), cited by Teixeira et al. (2001), demonstrated that fungal behavior can vary according to how much the fungus is dependent on light for growth and sporulation. This applies to *Curvularia* sp., which can grow in the presence or absence of light. The same characteristic was reported for *Fusarium moniliforme* Sheld, whose mycelial growth remains the same irrespective of the presence of a light source (Teixeira et al., 2001). For *Corynespora cassiicola* (Berk. & Curtis) Wei, continuous light regimes, 12 h photoperiod and absence of light did not influence mycelial growth (Celoto et al., 2015). Santos et al. (2005) also found no difference in the mycelial growth of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara races when grown in continuous darkness, under a photoperiod of 12 h, and black light, but sporulation did vary according to race. Specific studies of the behavior of the genus *Sirosporium* under artificial conditions are scarce. Berbegal et al. (2012) reported that growth and sporulation occurred when a single isolate of *Sirosporium celtidis* (Biv.) Ellis was cultured in PDA medium and kept in continuous darkness.

The colonies of *S. diffusum* presented great variation on morphological characteristics due to the different physical conditions. Dias Neto et al. (2010) also observed a wide variation in the characteristics of cultured *Magnaporthe grisea* colonies grown in different culture media (colony staining, sporulation, and mycelial growth). This variation was also observed in isolates of *Cryptosporiopsis perennans* (Zeller & Childs) Wollenw. when grown in different culture media (Bogo et al., 2008). *S. diffusum* could be considered a slow growth since it takes around two months to form a colony of about 30 mm (Table 3). *Sirosporium celtidis*, cultured in PDA, took 2 months to form structures for morphological examination (Berbegal et al., 2012).

Mycelial growth was impaired at 28±1 °C, whereas the temperature of 20±1 °C and 24±1 °C favored its growth, not differing between them. Similar results were obtained by Maia et al. (2011) showed that the best mycelial growth of isolates of *Colletotrichum* spp., pathogen to *Mangifera indica* L., presented better mycelial growth in the temperature range of 20 °C to 25 °C. Sporulation was strongly influenced by temperature, being higher at 20±1 °C and decreasing as temperature increased. Vivas et al. (2015) showed that isolates of both *Hansfordia pulvinata* (Berk. & Curtis) Hughes and *Acremonium* spp. exhibited stronger growth and sporulation at temperatures between 20 °C and 22 °C, whereas temperatures above 25 °C were not conducive to development. On the contrary, optimal temperature for the production of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. spores occurred at 28 °C (Poltronieri et al., 2013). The same occurred for *Thielaviopsis paradoxa* (De Seynes) Höhn. which ideal temperature for mycelial growth and conidia production is around 28 °C (Costa e Carvalho et al., 2011).

Our results showed the most favorable culture medium, light regime and temperature for culturing *S. diffusum*. The best conditions for culturing *S. diffusum* to stimulate good mycelial growth and sporulation consist of a 24 h photoperiod, temperature of 20±1 °C and V8$CaCO_3$ or CA culture medium. These findings could serve as a basis for future studies on the large-scale production of inoculant material, such as studies involving plant pathogenicity testing in order to identify sources of pathogen resistance, studies for taxonomic characterization, and studies on sensitivity to agents.

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