REVISTA DE CIÊNCIASAGRÁRIAS
Amazonian Journal
of Agricultural and Environmental Sciences
www.ajaes.ufra.edu.br

http://dx.doi.org/10.4322/rca.2013.063

ORIGINAL ARTICLE

Mycelia growth and sporulation of *Fusarium chlamydosporum* species complex under different culture conditions

Crescimento micelial e esporulação de Fusarium chlamydosporum species complex sob diferentes condições de cultivo

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KEYWORDS
Macroconidia
Morphology
Photoperiod

PALAVRAS-CHAVE
Macroconídios
Morfologia
Fotoperíodo

ABSTRACT: The *Fusarium* genus exhibits morphological, physiological and genetic variation among its different species, which may lead to differences in the environmental conditions required for their cultivation. This study aimed to verify the effects of different culture media and lighting regimes on the mycelial growth and sporulation of *Fusarium chlamydosporum* species complex isolates that are pathogenic to *Carya illinoinensis*. Four isolates from infected *Carya illinoinensis* roots and inflorescences were selected; these isolates were purified and subsequently transferred to four different culture media: potato sucrose agar (PSA), water agar (WA), potato dextrose agar (PDA) and carnation leaf agar (CLA). The isolates were then submitted to three different lighting regimes: a photoperiod of 12 h (TL), continuous light (CL) and continuous darkness (CD). After seven days of incubation under the three lighting regimes, the mycelial growth was evaluated on PSA, WA and PDA, and sporulation was evaluated on PSA, WA, PDA and CLA. In general, PSA and BDA media support mycelial growth and sporulation, particularly under CL. CLA medium also supports sporulation, particularly under CL. The CD lighting regime and the WA medium are not recommended for either the mycelial growth or sporulation of the *F. chlamydosporum* species complex.

RESUMO: *O gênero Fusarium apresenta diversas variações morfológicas, fisiológicas e genéticas entre suas espécies, portanto diferentes condições ambientais de cultivo podem ser requeridas. O objetivo deste estudo foi verificar os efeitos do uso de diferentes meios de cultura e regimes de luminosidade no crescimento micelial e na esporulação de isolados de Fusarium chlamydosporum species complex patogênicos a Carya illinoinensis. Foram selecionados quatro isolados patogênicos coletados de inflorescências e raízes infectadas de C. illinoinensis, os quais foram purificados e repicados para quatro meios de cultura: Batata-sacarose-ágar (BSA), Ágar-água (AA), Batata-dextrose- ágar (BDA) e Folha-de-cravo-ágar (CLA), e submetidos a três regimes de luminosidade: fotoperíodo de 12 h (FT), luz contínua (LC) e escuro contínuo (EC). Após sete dias de incubação, foi avaliado o crescimento micelial em BSA, AA e BDA, e a esporulação nos meios BSA, AA, BDA e CLA, nos três regimes de luminosidade. Os meios BSA e BDA favoreceram o crescimento em diâmetro e a esporulação, especialmente sob LC. O meio CLA também promoveu a esporulação, principalmente sob LC. O regime de luminosidade EC e o meio de cultura AA não são recomendados para crescimento e, especialmente, para esporulação de F. chlamydosporum species complex.*

Received: 05/20/2013
Accepted: 09/21/2013

1 Introduction

The genus *Fusarium* exhibits great morphological, physiological and ecological diversity. Unsurprisingly, the members of this genus occur in a vast array of ecological niches and geographical regions worldwide (BURGESS; SUMMERELL; BACKHOUSE, 1997). The species *F. chlamydosporum* (*stricto sensu*) belongs to the Sporotrichiella section (GERLACH; NIRENBERG, 1982) and has been previously reported as a pathogen by Nahar and Mushtaq (2006). These authors tested the pathogenicity of six *Fusarium* species isolated from sunflower seeds (*Helianthus annuus*) naturally infested by these pathogens, including *F. chlamydosporum*, and observed that all were pathogenic for this crops' seedlings; *F. chlamydosporum* in particular was responsible for stem wilting and rot. Chehri et al. (2011) identified several pathogenic *Fusarium* species that caused head blight of wheat in Iran, including *F. chlamydosporum*.

Although the *F. chlamydosporium* species complex is not often referred to as a pathogen of species with agricultural importance, isolates of the complex have previously been identified in the inflorescences and roots of *Carya illinoinensis* (Wangenh.) K. Koch, and these isolates were pathogenic for the seedlings of the crop following the inoculation of the substrate. Several species that belong to the Sporotrichiella section in which *F. chlamydosporum* is classified, such as *F. sporotrichioides* and *F. tricintum*, are part of the *F. chlamydosporum* species complex (GERLACH; NIRENBERG, 1982).

A number of studies have been performed to establish better conditions for the growth and sporulation of *Fusarium* species, including *F. solani* (SILVA; TEIXEIRA, 2012), *Gibberella zeae*, the teleomorph of *F. graminearum* (BIZZETTO; HOMECHN; SILVA, 2000) and *F. oxysporum* (SHARMA; PANDEY, 2010). Bragulat et al. (2004) tested the growth of several *Fusarium* species (*F. anthophilum*, *F. culmorum, F. dlamini, F. graminearum, F. napiforme, F. nygamai, F. oxysporum, F. proliferatum, F. semitectum, F. solani, F. subglutinans* and *F. verticillioides*) in several different media. Because species from the *F. chlamydosporum* species complex are not usually associated with diseases of important crop cultures in Brazil, studies on their characterization, identification and *in vitro* growth conditions are scarce, making it difficult to perform pathogenicity and resistance studies with *F. chlamydosporum* isolates.

Favorable conditions for several nutritional and environmental factors are required for the *in vitro* reproduction of fungi. Namely, temperature and light have been reported as important for mycelial growth and spore production (NOZAKI; CAMARGO; BARRETO, 2004). However, the conditions which favor growth are not always the same as those favoring sporulation because light has a direct effect on the fungus, inducing or inhibiting the formation of reproductive structures.

Culture media including complex carbohydrates are more favorable for the sporulation of fungi because these carbohydrates are better suited for the production of spores than of vegetative hyphae. Culture medium composition, temperature and light have been found to determine the quantity and quality of the mycelial growth and sporulation of phytopathogens (DHINGRA; SINCLAIR, 1995), and environmental factors such as pH, light and temperature have been reported as limiting factors in the optimization of the production of a fungus (MONTEIRO et al., 2004).

The difficulty of obtaining sporulating isolates, or even of establishing optimal conditions for the sporulation of phytopathogenic fungi, is one of the main problems faced by research groups trying to identify resistant cultivars (CRUZ; PRESTES; MACIEL, 2009).

The present study thus tested the effects of different culture media and light regimes on the mycelial growth and sporulation of the macroconidia of *Fusarium chlamydosporum* species complex isolates that were pathogenic to *Carya illinoinensis*.

2 Materials and Methods

The experiment was performed at the Phytopathology Laboratory of the Federal University of Santa Maria (Universidade Federal de Santa Maria – UFSM), state of Rio Grande do Sul (RS). Four *Fusarium chlamydosporum* species complex isolates, shown to be pathogenic to *Carya illinoinensis*, were used. The isolates had been previously identified by sequencing of the Internal Transcribed Spacer (ITS) and Transcription Elongation Factor-1 alpha (TEF-1α) regions. The isolates could not be identified at the species level but were identified at the species complex level. The identification and origin of the isolates are shown in Table 1.

Pathogen structures were transferred from the infected plant parts into Petri dishes containing potato dextrose agar culture medium (PDA) using a histology needle, according

Table 1. Plant organ collected, origin, date and accession number of the *F. chlamydosporum* species complex isolates tested.

Code	Plant organ collected[1]	Origin	Collection month/year	ITS[2] Accession	TEF-1α Accession
F1	Inflorescence	Santa Maria 29°43'13.0"- 53°43'1.9"	Oct/10	KC662466	KF022232
F5	Root	Cachoeira do Sul 29°59'45"-52°55'22"	Nov/10	KC758963	KF022233
F6	Inflorescence	Santa Maria 29°43'13.0"- 53°43'1.9"	Oct/10	KC778405	KF022234
F7	Inflorescence	Santa Maria 29°43'13.0"- 53°43'1.9"	Oct/10	KC778406	KF022235

[1]Plant organ from the pecan tree (*Carya illinoinensis*) from which the isolate was collected; [2]GenBank accession code.

to Alfenas et al. (2007). Following ten days of growth, the isolates grown in PDA were used to establish monosporic cultures and stored in PDA medium for subsequent use. The following measurements were performed on the purified isolates (Figure 1):

Mycelial growth. Mycelial growth was determined using 6 mm diameter PDA medium disks obtained from seven-day-old colonies, which were then transferred to 90 mm Petri dishes containing different growth media and kept under different light regimes, as described below. Four replicates were performed, with each replicate consisting of one individual Petri dish. The mycelial growth for each isolate was evaluated daily over seven days of incubation by measuring the mean colony diameter in two diametrically opposed directions.

Sporulation. Sporulation was measured, following seven days of incubation, by adding 20 mL sterile distilled water to each Petri dish used for the measurement of mycelial growth. Colonies were scraped using a Drigalski spatula, the suspension was filtered using double gauze, and the conidia concentration was estimated (conidia mL^{-1}). Counting was performed using a Neubauer chamber. All Petri dishes were kept in an incubator at 25±2 °C under the different light regimes.

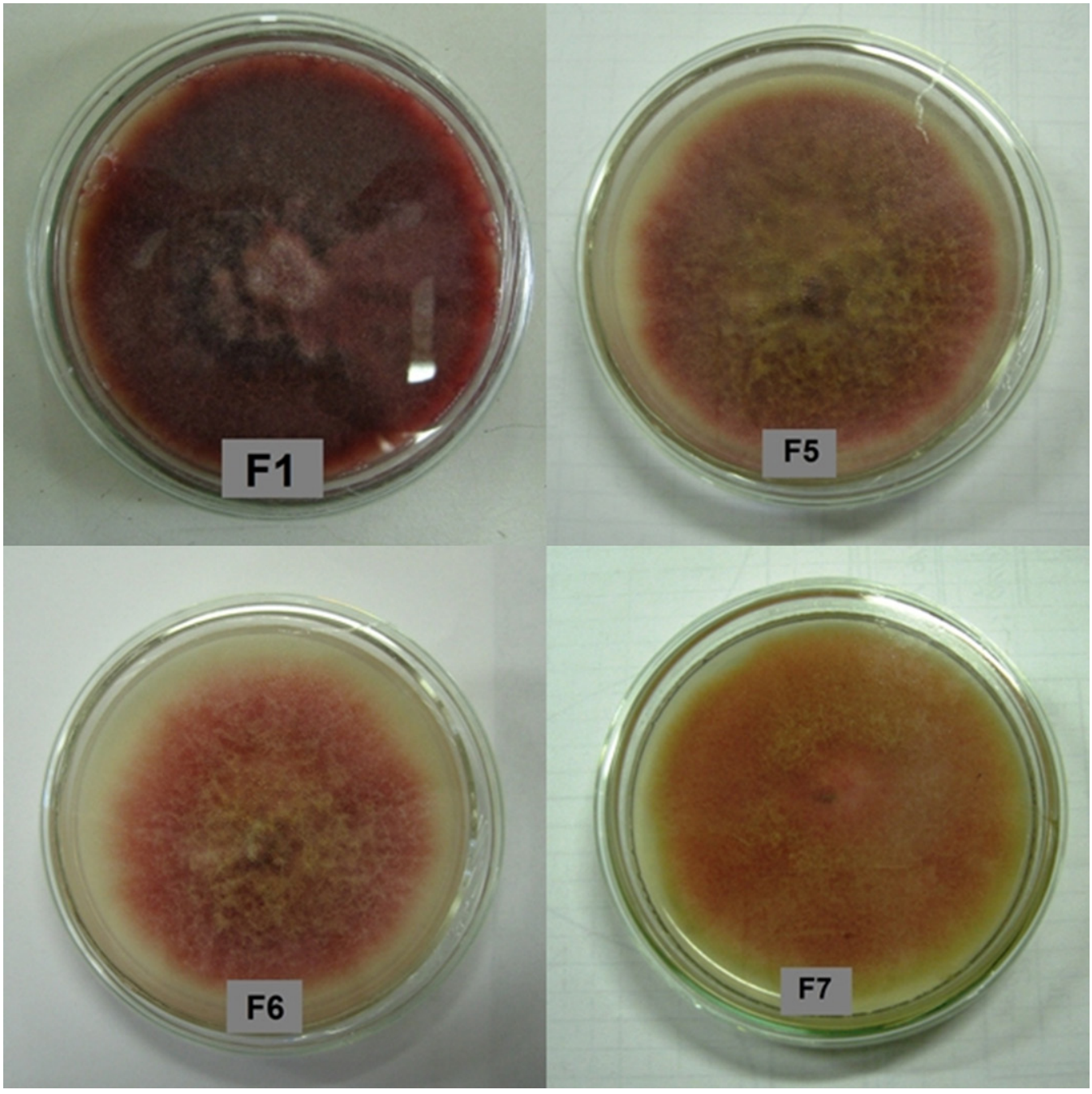

Figure 1. *Fusarium chlamydosporum* species complex isolates purified in PDA medium following seven days of incubation.

The following culture media were tested for mycelial growth and sporulation: potato sucrose agar (PSA) (200 g potato extract, 20 g sucrose, 20 g agar and 1,000 mL distilled water), water agar (WA) (20 g agar and 1,000 mL distilled water) and potato dextrose agar (PDA) (200 g potato extract, 20 g dextrose, 20 g agar and 1,000 mL distilled water). Carnation leaf agar (CLA) (≈ 3-5 mm^2 *Dianthus caryophyllus* leaf fragments, 20 g agar and 1,000 mL distilled water; LESLIE; SUMMERELL, 2006) was also tested for sporulation. The three tested light regimes were 12 h white light (TL), continuous white light (24 hours light – CL) and continuous darkness (CD).

A completely randomized experimental design with four replicates was used, with a 4 × 3 × 3 factorial scheme (isolates x light regimes x culture media) for mycelial growth and a 4 × 3 × 4 factorial scheme (isolates × light regimes × culture media) for sporulation. Means were compared using Tukey's test at the $p < 0.05$ significance level. Statistical data analysis was performed using the SISVAR v. 4.0 software (FERREIRA, 2008).

3 Results and Discussion

A significant interaction was found between the different tested light regimes and culture media regarding the colony diameter only, as measured following seven days of growth (Table 2). Under the 12 h photoperiod (TL), the largest colony diameter for the F_1 isolate was observed in the PSA medium, followed by PDA, and the smallest diameter was seen in WA. Under continuous light (CL) and continuous darkness (CD), growth was highest in both the PSA and PDA media, while the smallest colony diameter for this isolate was observed in WA. When PSA or WA media were used, the light regime did not influence colony diameter, as no statistically significant differences in this variable were observed with the different regimes tested. For the PDA medium, the largest diameter was observed under CL, followed by CD and TL.

For the F_5 isolate, colony diameter was higher in the PSA and PDA media for the TL and CL light regimes. Under CD, the largest diameter was observed in the PSA medium, followed by PDA, and the smallest diameter was seen in WA (Table 2). The light regime influenced growth when PDA was used, with the highest growth under TL and CL, and also when using WA, with the highest growth under TL.

The F_6 isolate, when kept under the TL regime, exhibited larger colony diameters in the PSA and PDA media. Under CL, the largest diameter was observed in the PDA medium, while under CD, the largest diameter was seen in PSA. The best light regimes for diameter growth were TL and CD for the PSA medium and CL for the PDA and WA media. For the F_7 isolate, the best culture media for diameter growth were PSA and PDA under all light regimes. Growth in the PSA and WA culture media was influenced by the light regime and was highest under CL for both media.

In general, the best culture media for the growth of the four *F. chlamydosporum* species complex isolates tested, as indicated by colony diameter, were PDA and PSA. The latter was particularly suitable, as its use always resulted in higher mean growth for all isolates and light regimes. Modified PSA medium has been previously reported as the most appropriate for recovery of *Fusarium solani* soil propagules, followed by Nasch-Snyder medium (NS) (COSTAMILAN, 2003).

Table 2. Colony diameter (mm) of *F. chlamydosporium* species complex isolates, measured following seven days of incubation, in different culture media and under different light regimes.

Medium	F_1 Isolate – CV (%): 2.9		
	TL	CL	CD
PSA	90.00 Aa*	89.06 Aa	87.41 Aa
PDA	76.89 Bb	90.00 Aa	82.78 Aab
WA	68.05 Ca	69.58 Ba	65.20 Ba
Medium	F_5 Isolate – CV (%): 3.7		
	TL	CL	CD
PSA	90.00 Aa	83.22 Aa	90.00 Aa
PDA	90.00 Aa	90.00 Aa	81.58 Bb
WA	63.80 Ba	55.19 Bb	62.85 Cab
Medium	F_6 Isolate – CV (%): 2.5		
	TL	CL	CD
PSA	90.00 Aa	80.60 Bb	90.00 Aa
PDA	82.57 Aab	90.00 Aa	77.03 Bb
WA	60.28 Bb	81.54 Ba	61.34 Cb
Medium	F_7 Isolate – CV (%): 2.9		
	TL	CL	CD
PSA	80.71 Ab	88.88 Aa	82.75 Aab
PDA	80.68 Aa	86.33 Aa	83.16 Aa
WA	54.96 Bb	66.50 Ba	52.92 Bb

*Means followed by the same upper case letter within the same column and the same lower case letter within the same row are not significantly different according to Tukey's test ($p < 0.05$). TL: 12 h photoperiod, CL: 24 h continuous light, CD: continuous darkness; PSA: potato sucrose agar, WA: water agar, PDA: potato dextrose agar.

Silva and Teixeira (2012) observed the highest *F. solani* mycelial growth when the fungus was grown under continuous light, followed by the 12 h photoperiod and continuous darkness. The same authors also found that the absence of light inhibited *F. solani* mycelial development in almost all of the tested culture media, whereas continuous light favored mycelial growth. These authors further reported that PSA and PDA media were adequate for the physiological requirements of the fungus.

These findings are in accordance with the present results: continuous light was the most favorable regime for *F. chlamydosporum* mycelial growth, while continuous darkness was the least favorable. In addition, PSA and PDA culture media were also found to be adequate for the growth of the isolates.

However, the behavior of the isolates in terms of sporulation must also be considered. This variable is often the most important, both for isolate characterization (size, shape and number of macroconidial cells) and for studies of pathogenicity and resistance, which often require sporulation to be performed.

For the F_1 isolate, under the 12 h photoperiod (TL) and continuous darkness (CD) regimes, the WA medium displayed lower sporulation than the other media. Under continuous light

(CL), the highest sporulation was observed in PSA and the lowest in WA. The light regime only influenced sporulation when PSA was used, with CL being the most favorable regime for the sporulation of this *F. chlamydosporum* species complex isolate (Table 3).

The CLA culture medium was the most favorable for the sporulation of the F_5 isolate under TL. Under CL, this culture medium, together with WA, were the least favorable for the sporulation of the isolate. Under CD, sporulation was not significantly different between PSA and CLA, both of which had higher sporulation than WA (Table 3). The light regime influenced sporulation when PSA or CLA culture media were used. Sporulation was highest under CL with PSA and under TL with CLA.

For the F_6 and F_5 isolates, the highest sporulation under TL was observed in the CLA medium, while no sporulation was observed in WA (Table 3). Under CL, the highest sporulation was observed in the PDA and CLA media, and under CD, the highest sporulation was seen in CLA. For the F_6 isolate, no significant differences were observed between the different culture media that indicated an influence of the light regime.

Table 3. Sporulation (x 10^6 spores/mL) of *F. chlamydosporium* species complex isolates, measured following seven days of incubation, in different culture media and under different light regimes.

Medium	F_1 Isolate – CV (%): 3.0		
	TL	CL	CD
PSA	7.67 Ab*	32.08 Aa	6.27 Ab
PDA	6.94 Aa	9.89 Ba	7.68 Aa
CLA	4.44 Aa	3.77 BCa	8.80 Aa
WA	0.14 Ba	0.19 Ca	0.06 Ba
Medium	F_5 Isolate – CV (%): 3.8		
	TL	CL	CD
PSA	4.58 Bb	14.63 Aa	3.90 Ab
PDA	6.28 Ba	12.33 Aa	5.87 Aa
CLA	12.68 Aa	1.51 Bb	6.43 Aab
WA	0.00 Ca	0.07 Ba	0.04 Ba
Medium	F_6 Isolate – CV (%): 2.5		
	TL	CL	CD
PSA	5.70 Ba	1.50 Ba	1.65 Ba
PDA	2.11 Ba	5.65 Aa	2.07 Ba
CLA	11.09 Aa	4.52 Aa	9.37 Aa
WA	0.00 Ca	0.25 Ba	0.01 Ca
Medium	F_7 Isolate_CV (%): 3.1		
	TL	CL	CD
PSA	5.13 ABb	27.00 Aa	3.52 Ab
PDA	10.31 Aa	5.48 Ba	4.85 Aa
CLA	7.19 Aa	1.97 Ba	7.21 Aa
WA	0.05 Ba	0.11 Ba	0.07 Ba

*Means followed by the same upper case letter within the same column and the same lower case letter within the same row are not significantly different according to Tukey's test ($p < 0.05$). TL: 12 h photoperiod, CL: 24 h continuous light, CD: continuous darkness; PSA: potato sucrose agar, WA: water agar, PDA: potato dextrose agar; CLA: carnation leaf agar.

Sporulation of the F_7 isolate was highest in the PDA medium, followed by CLA, and was not different from PSA under TL. Under CL, the best medium was PSA. Under CD, sporulation in WA was very low (0.07×10^6 spores/mL), statistically significantly lower than sporulation in the other culture media.

Overall, the PSA, PDA and CLA media were found to be adequate for the sporulation of *F. chlamydosporum* under the different light regimes tested, while WA is not recommended for the mass production macroconidia in these species. The use of continuous light (CL) favored the sporulation of most of the isolates in the different culture media tested.

Silva and Teixeira (2012) reported the greatest conidia production of *Fusarium solani* using PDA and PSA media under continuous light, a result due to the greater nutritional richness and complex carbohydrate contents of these media. Sharma and Pandey (2010) observed more intense *F. oxysporum* sporulation in lignocellulose agar medium than in PDA.

The CLA medium is composed of only water agar and plant material (carnation leaves) and is therefore poor in carbohydrates. However, Nozaki, Camargo and Barreto (2004) reported that the inclusion of plant material in the culture medium stimulates the production of spores in some fungi. Moreover, this medium is commonly used in the characterization of the macroconidia of several *Fusarium* species, and carnation leaves (*Dianthus caryophyllus*) have been recommended for the promotion of growth and sporulation, as well as the storage, of *Fusarium* spp. cultures (FISHER et al., 1982).

Silveira and Menezes (2006) studied the production of chlamydospores of seven *Fusarium* species grown in CLA medium containing carnation leaves of two different species (*Dianthus caryophyllus* and *Targetis* sp.) and observed that both plant species were equally efficient for the production of chlamydospores. The authors highlighted the positive effects of the carnation medium associated with continuous darkness (CD) for inducing chlamydospore formation in species able to form these structures, which have high taxonomic value.

Regarding the light regime, the present study indicates that continuous light induces the greatest sporulation of the *F. chlamydosporum* species complex. This result is in accordance with those of Devi and Singh (1994), who observed increased sporulation of *F. moniliforme* under continuous light. Burgess et al. (1994) reported that a 12 h photoperiod regime, associated with temperatures of 25 and 30 °C during incubation, was very favorable for sporulation. However, the light requirements for sporulation may vary between different species of fungi, and the ideal conditions for mycelial growth are not always the same as those for sporulation.

Silveira and Menezes (2006) studied the effects of different light regimes on the colony development of seven *Fusarium* species (*F. dlamini*, *F. beomiforme*, *F. napiforme, F. nygamai, F. moniliforme, F. anthophilum* and *F. subglutinans*) and found that the production of microconidia was not influenced by the light regime. However, the production of macroconidia was absent for some species in several of the treatments.

4 Conclusions

The best culture media and light regime for both the mycelial growth and sporulation of the *Fusarium chlamydosporum* species complex were potato sucrose agar and potato dextrose agar under continuous light; carnation leaf agar medium was also suitable for sporulation under the same light regime.

Continuous darkness was not adequate for the mycelial growth or sporulation of the *Fusarium chlamydosporum* species complex, and water agar culture medium is not recommended when the mass production of macroconidia is required.

Acknowledgements

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior- CAPES) for granting the Ph.D. scholarship to the first author.

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