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Light, temperature, and aging dependent vegetative growth and sporulation of *Colletotrichum gloeosporioides* on different culture media

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The fungal organism *Colletotrichum gloeosporioides* is the causative agent of anthracnose disease of *Citrus* fruits. It is recently introduced as a potential producer of anticancer metabolite paclitaxel. Here, we introduce the optimal conditions for growth and sporulation of *C. gloeosporioides*. We have considered four fungal culture media, that is potato dextrose agar (PDA), carnation leaf agar (CLA), potato carrot agar (PCA) and water agar (WA), based on which sporulation inducers like Watman or Fabriano filter papers could be added, and evaluated both for vegetative growth and sporulation. Three light regimens, i.e. continuous light, 16/8 hrs light/darkness, and continuous darkness were applied in combination with the culture media. All experiments were tracked on 7th, 15th, 21st, and 30th day after incubation. At 28°C, PDA and PCA culture media, under continuous light, provided the best condition for *C. gloeosporioides* maximal growth. Decreasing light periods decreased the fungal growth. Furthermore, fungal sporulation showed a high dependence on light, temperature and culture medium in use. Under 16/8 h light/darkness interval at the same temperature *C. gloeosporioides* sporulation was at its maximum on Fabriano paper placed on PDA medium. At a lower temperature, that is 22°C, *C. gloeosporioides* sporulation on the same culture media was highly defected. Furthermore, aging generally increased the fungal sporulation.

Key words: *Colletotrichum gloeosporioides*, citrus, growth, conidiation, development.

INTRODUCTION

The ascomycetous fungus *Colletotrichum,* members of which are anamorphic *Glomerella* species (Sutton, 1992; Armstrong-Cho and Banniza, 2006; Pfenning et al., 2007), is one of the most economically important complexes of plant pathogens, causing post-harvest rots, anthracnose, and blights of aerial plant parts. The symptoms typically appear as small to large, dark-colored spots or slightly sunken lesions on the foliage, stems or fruits of a wide range of tropical, subtropical and temperate crops (Bailey and Jeger, 1992). Although *Colletotrichum* species are classified as virulent pathogens, several species including *Colletotrichum gloeosporioides* can express mutualistic lifestyles in non-disease hosts (Rodriguez and Redman, 2008). Individual isolates of *Colletotrichum* species can express either parasitic or mutualistic lifestyles depending on the host genotype colonized. Mutualistic benefits of *Colletotrichum* spp. to hosts include growth enhancement, disease

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resistance, and/or abiotic stress tolerance (Redman et al., 2001).

Virulent *Colletotrichum* species employ a hemibiotrophic strategy to invade host, in which biotrophic and necrotrophic stages of infection are sequentially established (Perfect et al., 1999). Upon germination of conidia of these fungi, the hyphae penetrate the host cell lumen through host cuticle and cell. These intracellular hyphae are biotrophic (O'Connell et al., 2004). Afterwards, necrotrophic hyphae forms, extensively spread and kills the host tissue.

Colletotrichum species, compared to obligate biotrophs, can be cultured *in vitro*. They are amenable to genetic transformation by a variety of methods, especially *Agrobacterium*-mediated transformation (de Groot et al., 1998; Tsuji et al., 2003; Takahara et al., 2004; Flowers and Vaillancourt, 2005; Talhinhas et al., 2008; Ushimaru et al., 2010; Nakamura et al., 2012; Yousefi-Pour Haghighi, 2013). Since *Colletotrichum* species are haploid, molecular genetics approaches are facile in these fungi. Hence, they have been served as excellent models for the study of fungal morphogenesis and pathogenicity.

The phytopathogenic fungus *C. gloeosporioides* (Penz) Penz & Sacc in Penz, (Teleomorph: *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk), causes anthracnose on many tropical, subtropical and temperate fruits (Waller, 1992), especially on *Citrus* species. Post-harvest problems caused by *C. gloeosporioides* especially in the tropics, often are a significant factor in limiting export (Fitzell and Peak, 1984). The economic cost of cryptic infections caused by *C. gloeosporioides* is about 25% greater than that for field losses (Jeger and Plumbley, 1988). These have grouped *C. gloeosporioides* among the most important post-harvest pathogens. In addition to its considerable detrimental economic importance, it has been shown that endophytic, apparently nonpathogenic, *Colletotrichum* species including *C. gloeosporioides* are a source of secondary metabolites with anticancer effects, that is paclitaxel (Taxol) (Gangadevi and Muthumary, 2008; Strobel et al., 1999).

C. gloeosporioides sensu lato is a species complex with broad genetic and biological diversity. It is associated with at least 470 different host genera (Sutton, 1980). Several cultural and environmental factors affecting the growth of this species, *in vitro*, are studied in isolates from different host genera including papaya, green pepper and *Plumeria* (Nithya and Muthumary, 2009; Silveira et al., 2004). Because of the economically significance of *C. gloeosporioides* for *Citrus* species (Adaskaveg and Förster, 2000; Ramos et al., 2006) and its significance for fermentation-based paclitaxel production (Gangadevi and Muthumary, 2008; Strobel et al., 1999), here we aimed at quantifying and comparing vegetative mycelial growth and sporulation of a *Citrus* isolate of this fungus on twelve synthetic culture media, under different light and temperatures regimens.

MATERIALS AND METHODS

Fungal strain

Colletotrichum gloeosporioides wildtype strain JS-1389 (obtained from National Plant Protection Institute, Tehran, Iran), which was isolated as a plant pathogen from *Citrus* species in Iran, was used as the model. The fungal strain was grown on potato dextrose agar (PDA) medium (purchased from Merck, Darmstadt, Germany) at 28°C. For long term usage, the fungus was maintained under liquid paraffin at 4°C.

Culture media

Four standard fungal solid culture media that is, PDA, potato carrot agar (PCA), carnation leaf agar (CLA) and water agar (WA) were employed for developmental studies (purchased from Merck, Darmstadt, Germany). In combination with those culture media, two kinds of filter papers, that is Watman No. 41 and Fabriano No. 808, in total 12 synthetic culture media, were investigated. All experiments were performed in three replicates.

Light and temperature regimens

The effects of two light conditions, that is 24 h light versus 8 h dark/16 h light; as well as two temperatures, i.e. 22 and 28°C on fungal development were evaluated in combination with culture media which had shown supportive effect for fungal development.

Assessment of fungal development

Mycelial discs of 7 mm diameter cut from the growing margins of the fresh fungal culture were placed at the center of each 9 cm Petri plate. Two factors, that is diameter of mycelial growth (mm), and sporulation of the fungus (× 10) under different regimens were measured on a daily (4, 10, 15, 20, 30) interval.

Statistical analyses

Analysis of variance (ANOVA) and SAS procedures and programs were used for statistical analyses. In cases where the F-test showed significant differences among means, the differences among treatments were compared using least significant differences (LSD) test at 1% significance level (Steel et al., 1997). In cases where there was zero number, like absence of sporulation, the non-parametric statistics and Wilcokson's test were applied.

RESULTS

Fungal mycelia growth under 24 h light at 28°C on different culture media

At five daily intervals, that is 4th, 10th, 15th, 20th, and 30th days, fungal growth was measured under continuous light on solid culture media. Significant differences were observed for mycelial growth under 24 h light at 28°C, using different culture media, and filter papers ($P \leq 0.01$; not shown). Table 1.

The interaction of culture media and filter papers was also statistically significant ($P \leq 0.01$) for mycelia growth.

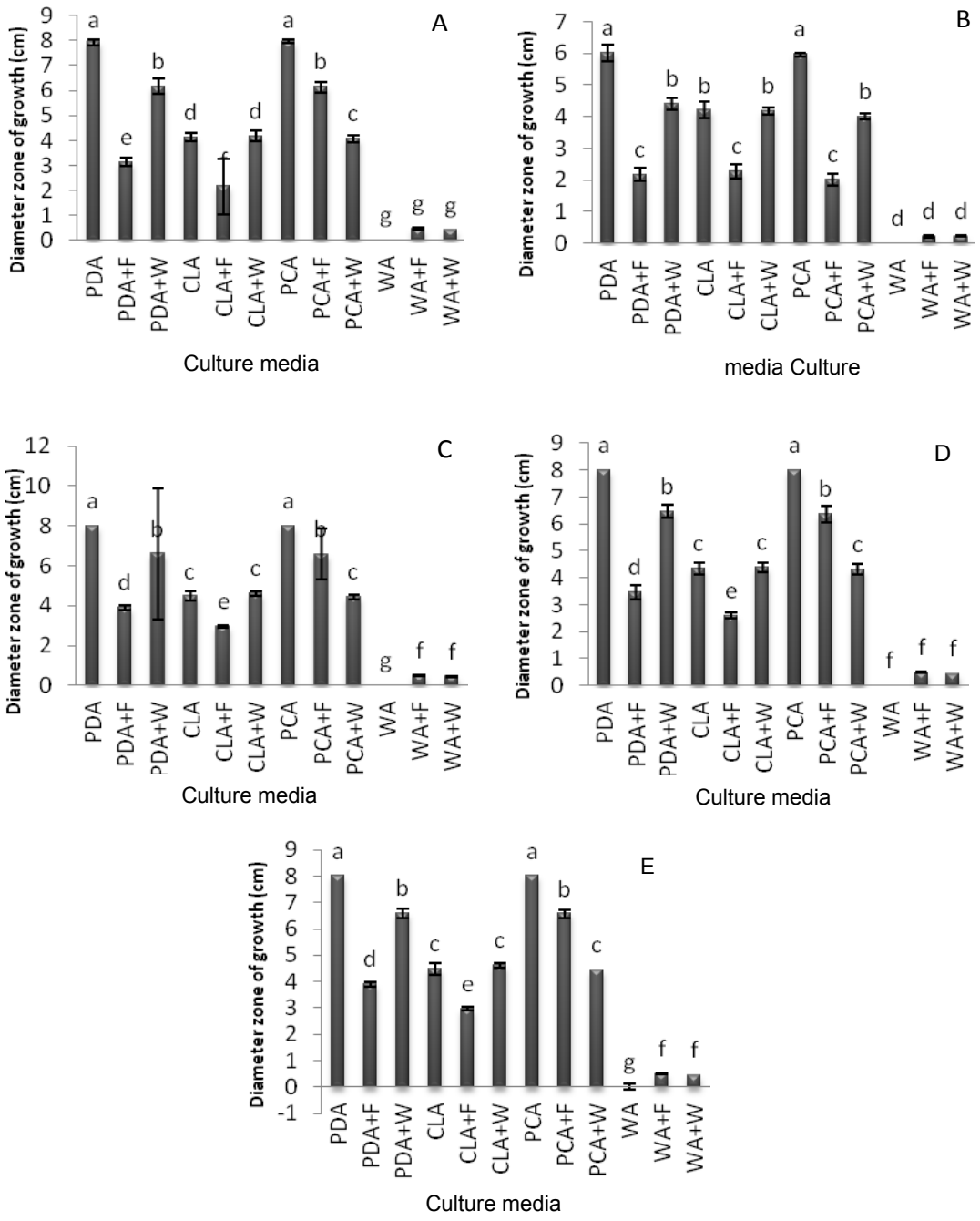

Figure 1. Mycelial growth of *C. gloeosporioides* on 12 culture media under 24 h light at 28 to 30°C on daily intervals. (A) 4th, (B) 10th, (C) 15th, (D) 20th, (E) 30th day. Data (significant at $P \leq 0.01$) are averages of three replicates. Error bars indicate standard errors. Similar letters indicate no significant difference.

As seen in Figure 1, under 24 h light, the best culture media for fungal mycelia growth were PDA and PCA, on which *C. gloeosporioides* JS-1389 could establish itself over 4 days incubation, and after 10 days fungus had covered the whole Petri plate. Among other culture media tested, PDA + Watman filter and PCA + Fabriano filter better favored fungus growth, but it took almost 20 days for fungus to establish its colony. The fungus could grow on CLA-based culture media over 4 days, but hardly could grow further. It seems that on PDA- and CLA-based media the growth induction effect of Watman filter was more than Fabriano filter. However, on PCA-based media the opposite effect was seen. WA-based culture media provided the worst condition for fungal mycelia growth

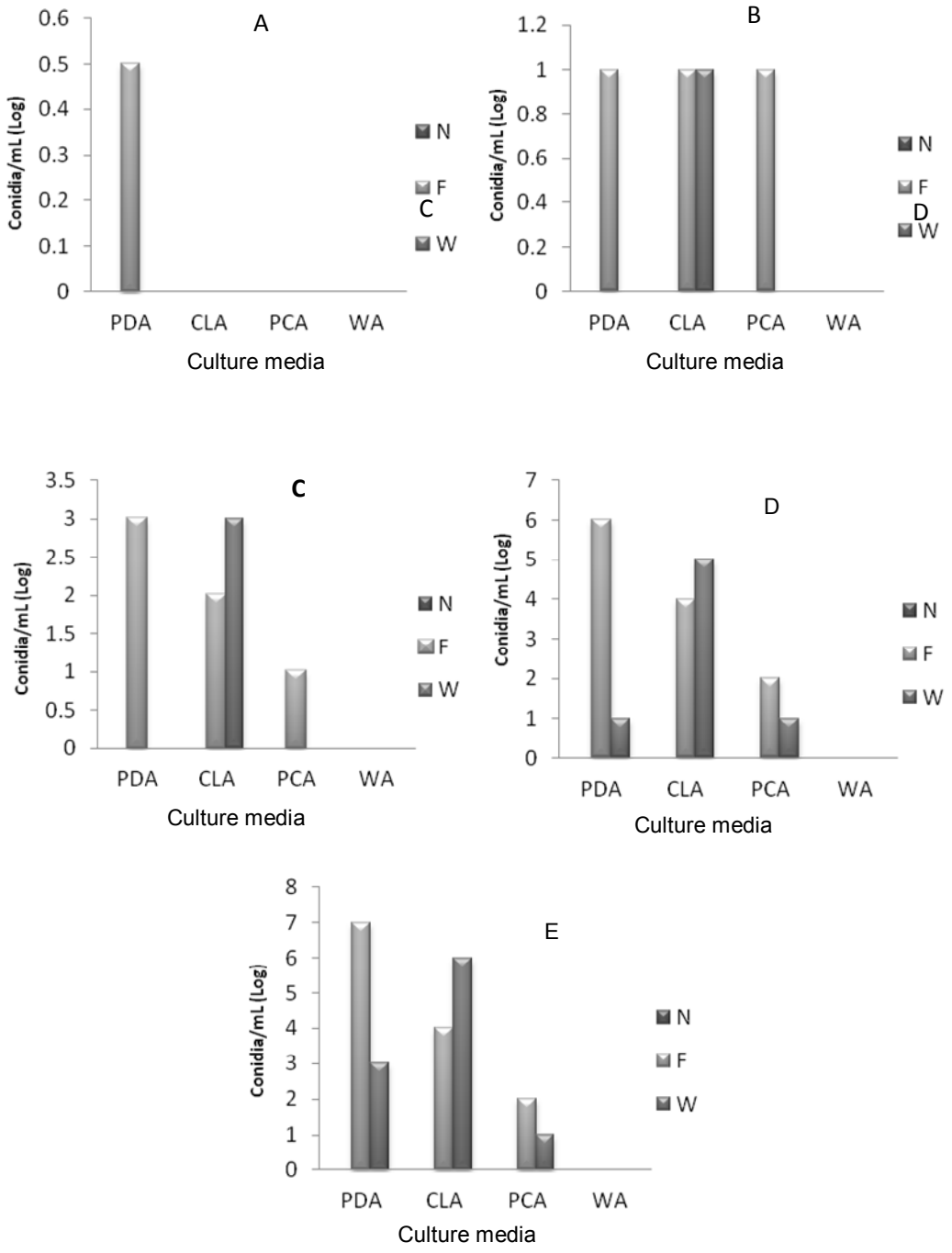

Figure 2. Sporulation concentration (spore ml^{-1}) of *C. gloeosporioides* on 12 culture media under 24 h light at 28 to 30°C on daily intervals. (A) 4th, (B) 10th, (C) 15th, (D) 20th, (E) 30th day.
Data (significant at P ≤ 0.01) are averages of three replicates.

growth under continuous light. Including filter papers on such media improved fungal growth.

Fungal sporulation under 24 h light at 28°C on different culture media

At five daily intervals, that is 4th, 10th, 15th, 20th, and 30th days, fungal sporulation was measured under continuously light on solid culture media. Significant differences were observed for sporulation under 24 h light at 28°C, using different culture media, and filter papers (P ≤ 0.01; not shown) Table 2. The interaction of culture media and filter papers was also statistically significant (P ≤ 0.01). As seen in Figure 2, under 24 h light the best culture

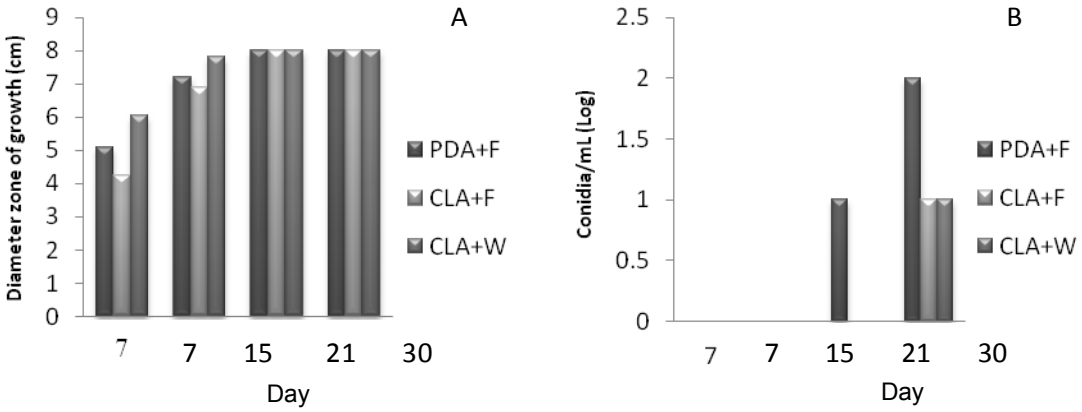

Figure 3. *C. gloeosporioides* mycelial growth (A) and sporulation (B) under 24 h darkness at 28 to 30°C on three different culture media.
Data are averages of two replicates.

medium for fungal sporulation was PDA + Fabriano filter, on which sporulation started as early as 4 days, and continuously raised up to × 10^7 conidia per ml on 30th day. Moreover, after 10 days, *C. gloeosporioides* JS-1389 could sporulate also on CLA + Whatman filter, CLA + Fabriano filter and PCA + Fabriano filter, but in the range of 10 conidia per ml. Afterwards, filter paper containing CLA media favored fungal sporulation, up to × 10^6 conidia per ml on 30th day. However, filter paper containing PCA media hardly favored further fungal sporulation. Notably, without providing filter papers, the fungus could sporulate neither on WA-based media, nor on PDA, CLA, and PCA.

Fungal mycelia growth and sporulation under 24 h darkness at 28°C on different culture media

At four daily intervals, that is 7th, 15th, 21th, and 30th days, fungal development was measured under darkness on three solid culture media which had shown the best effects on fungal mycelia growth and sporulation, that is PDA + Fabriano paper, CLA + Fabriano paper, and CLA + Whatman paper (Figures 1 and 2). As indicated in Figure 3A and B, significant differences were observed for sporulation under 24 h darkness at 28°C, using different culture media and filter papers. As seen in Figure 3A, until day 15, the *C. gloeosporioides* JS-1389 mycelia growth was better on CLA + Whatman paper, than two other media, under continuous light at 28°C. On this culture medium, the fungus had covered the whole Petri plate after 15 days. However, the difference was not so significant, as on day 21 the fungus had covered the whole Petri plate on all three culture media. As seen in Figure 3B, under continuous light, on neither culture media *C. gloeosporioides* JS-1389 could sporulate over 15 days. However, as shown, on PDA + Fabriano paper up to 10 per ml conidia was produced on 21th day, and up to × 10^2 on 30th day. However, after 30 days, the fungus could also sporulate on CLA + Fabriano paper, and CLA + Whatman paper under continuous light at 28°C, albeit about 10-fold less than on PDA + Fabriano paper. This indicates that aging also improved the fungal sporulation under this condition.

Fungal mycelia growth and sporulation under 16/8 h light/darkness at 28 and 22°C on different culture media

At four daily intervals, that is 7, 15, 21, 30th days, *C. gloeosporioides* JS-1389 development was measured under ligh/darkness on three solid culture media which had shown the best effects on fungal development, that is PDA + Fabriano paper, CLA + Fabriano paper, and CLA + watman paper. Here, two temperatures for incubation period were applied, that is 28 and 22°C. As indicated in Figure 4A and B, and in Figure 5A and B, significant differences were observed for sporulation under 16/8 light/darkness h at 28 and 22°C, using different culture media, and filter papers. As it is shown in Figure 4A, at 28°C under 16/8 h light/darkness, mycelia growth was almost identical on all three culture media. On day 21, *C. gloeosporioides* JS-1389 could cover the whole Petri plate on all media. However, as shown in Figure 4B, under this condition the maximum sporulation was appeared on PDA + Fabriano paper medium. It is seen that this medium favored fungal sporulation, ~ × 10^4 conidia per ml on 15th day, which rose to ~ × 10^6 on 21th day, and ~ × 10^8 conidia per ml on 30th day. This suggests that aging also improved the fungal sporulation under this condition. On both CLA + Fabriano paper and CLA + Whatman paper media, the fungus could only sporulate ~ × 10^5 conidia per ml on 30th day. As it is shown in Figure 5A, at 22°C, mycelia growth under 16/8 h light/darkness did not depend on culture media. On day

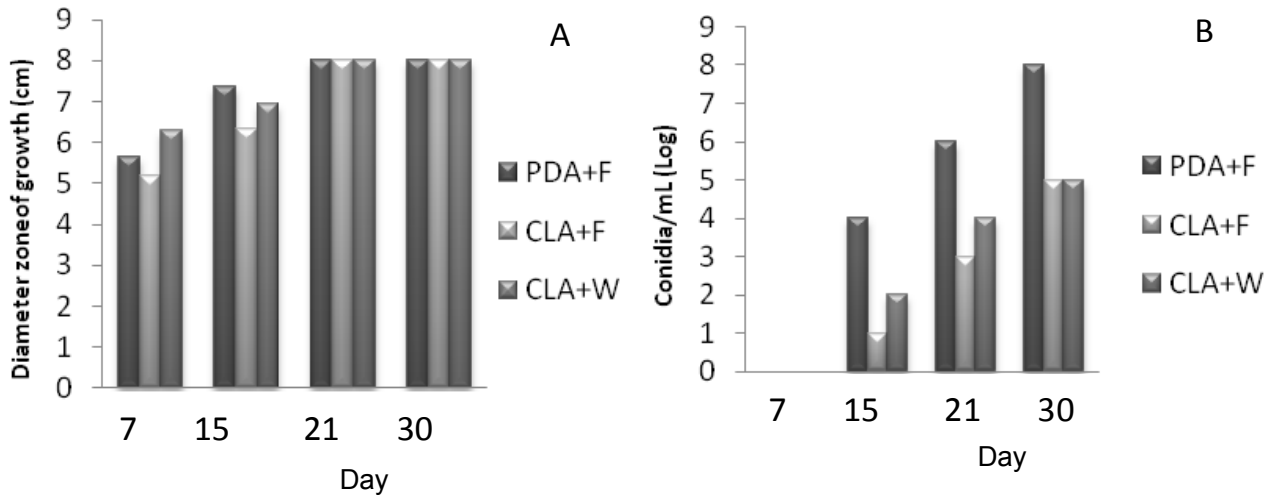

Figure 4. *C. gloeosporioides* mycelial growth (A) and sporulation (B) under 16/8 h light/darkness at 28 to 30°C on different culture media on three different culture media. Data are averages of two replicates.

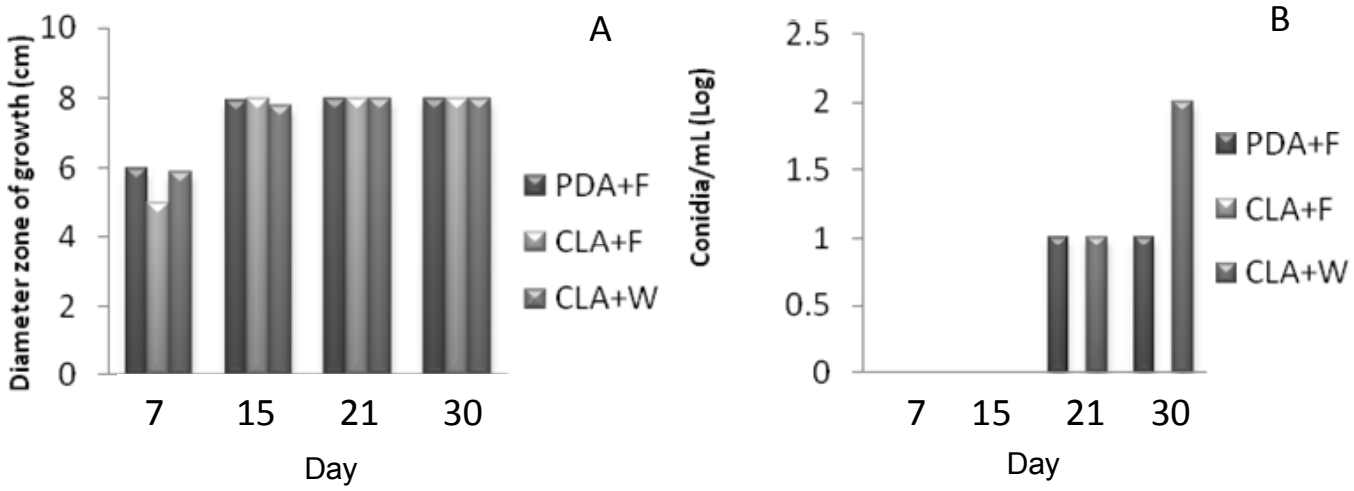

Figure 5. *C. gloeosporioides* mycelial growth (A) and sporulation (B) under 16/8 h light/darkness at 22°C on different culture media on three different culture media.
Data are averages of two replicates.

15th, *C. gloeosporioides* JS-1389 could cover the whole Petri plate on all three media. However, as shown in Figure 5B, under this condition the maximum sporulation was appeared on CLA + Whatman paper medium, only on 30th day, and maximally ~ × 10^2 conidia per ml. This was 10-fold less on PDA + Fabriano paper and CLA + Fabriano paper media.

DISCUSSION

C. gloeosporioides is currently a fungal model for plant-microbe interaction studies, as well as, a microbial source for anticancer drug fermentation. At fungal natural niches, light and nutrients are among the most environmental factors affecting the success and fitness of the fungus. However, for a better handling of the fungus under laboratory conditions there is a need for understanding its development that is vegetative growth and sporulation. Several researches have introduced a number of mixed solid media for gaining the maximal colonization of the fungus isolated from different hosts, and to obtain enough conidia for further experiments. We initially aimed at using a Citrus isolate of *C. gloeosporioides* for functional genetic studies in our laboratory, but by using the formerly introduced culture media we could not obtain sufficient

Table 1. Analysis of variance and means comparison for *C. gloeosporioides* mycelial growth under 24 h light at 28 to 30°C.

Parameter	No.	Mean square (Mycelial growth cm)				
S.O.V	F	4th day	10th day	15th day	20th day	30th day
Filter paper	2	17.45**	12.75**	10.59**	8.32**	8.32**
Media	3	32.53**	63.77**	67.68**	70.71**	70.71**
F×M	6	3.06**	6.82**	6.33**	5.69**	5.69**
Error	24	0.027**	0.029**	0.029**	0.012**	0.012**
CV%	-	5.54	4.39	4.22	2.6	2.6

**Significant at 1% level.

Table 2. Analysis of variance and means comparison for *C. gloeosporioides* sporulation under 24 hrs light at 28 to 30ºC by the non-parametric statistics and Wilcokson's test.

Parameter	Mean square (Sporulation spores/ml)				
S.O.V	4th day	10th day	15th day	21th day	30th day
Filter paper × Media	10^{1}*	10^{5}*	10^{4}*	10^{7}*	10^{7}*

**Significant at 5% level

sufficient conidia. Hence, we tried first to find the optimized condition in which *C. gloeosporioides* grows and sporulates best. We first considered four basic fungal culture media, i.e. PDA, CLA, PCA and WA, based on which sporulation inducers like filter papers could be added (in total twelve culture media), and evaluated both for vegetative growth and sporulation. Three light regimens that is, continuous light, 16/8 h light/ darkness, and continuous darkness were applied in combination with three top culture media. *C. gloeosporioides* mycelia growth and sporulation were measured at five daily intervals, that is 4th, 10th, 15th, 20th, and 30th days. However, because of the improving effects of 16/8 h light/ darkness on fungal development at 28°C, this condition was also applied at 22°C.

As the initiative experiments showed, under continuous light at 28°C, PDA and PCA culture media provided the best media for *C. gloeosporioides* maximal mycelia growth. However, under this condition, the maximum sporulation was observed on PDA + Fabriano paper medium followed by CLA + Whatman paper and CLA + Fabriano paper media. We then evaluated *C. gloeosporioides* mycelial growth and sporulation on those three culture media under continuous darkness at 28°C. Under this condition the growth rate was decreased. Notably, the sporulation of the fungus was defected. This indicates the significance of light for *C. gloeosporioides* vegetative growth and sporulation, independent of the culture media in use. Furthermore, under alteration of 16/8 h light/ darkness at 28°C, *C. gloeosporioides* vegetative growth was decreased compared to continuous light, and the growth was almost identical to continuous darkness. However, the maximum sporulation of the fungus was observed at this situation. This indicates that 16/8 h light/darkness intervals at the same temperature improve *C. gloeosporioides* sporulation compared to both continuous light and continuous darkness. We then evaluated fungal development under 16/8 h light/darkness at 22°C on the same culture media. *C. gloeosporioides* vegetative growth was almost identical, but the sporulation of the fungus was highly decreased at 22°C compared to that of 28°C. This indicates that sporulation of *C. gloeosporioides* is significantly temperature-dependent (Figure 6).

Taking all together, our data indicate that at 28 to 30°C, PDA and PCA culture media provide the best condition for *C. gloeosporioides* maximal growth, especially under continuous light. Shortage

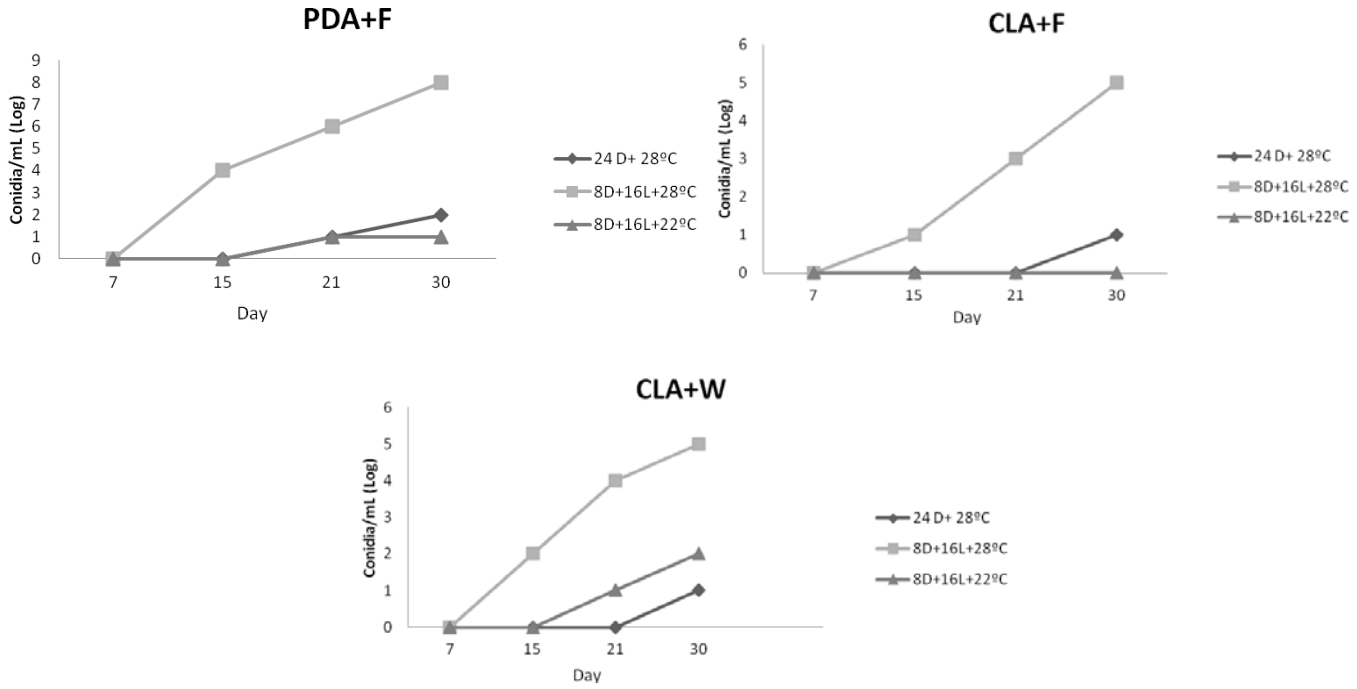

Figure 6. Comparison of sporulation of *C. gloeosporioides* on PDA+F, CLA+F, and CLA-W culture media, under three different light and temperature regimens, at four daily intervals.

of light could decrease the fungal growth. So, *C. gloeosporioides* vegetative growth is light dependent. Furthermore, our data suggest that fungal sporulation is highly light- , temperature- and culture medium-dependent. Indeed, under 16/8 h light/darkness intervals at the same temperature *C. gloeosporioides* sporulation was at its maximum on PDA + Fabriano paper medium, compared to its sporulation under both continuous light and continuous darkness. This suggests that both light alteration and culture media influence fungal sporulation. Moreover, decreasing incubation temperature to 22°C, highly decreases *C. gloeosporioides* sporulation on the same culture media. It should also be noted that in all experiments it was obvious that aging increased the fungal sporulation.

Fungal vegetative growth on nine different culture media under three different light regimens was almost identical. Indeed, the fungus could grow on all the culture media tested, regardless of light conditions. This means that mycelia growth was independent of light; although an alternation of 16/8 h light/darkness improved the fungal vegetative growth compared to either continuous light or continuous darkness. In total, the PDA culture medium could provide the best condition for *C. gloeosporioides* vegetative growth, regardless of light.

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