Growth and protein content in *Colletotrichum circinans*, *Fusarium solani* and *Rhizoctonia solani* in liquid culture

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Abstract

The phytopathogenic fungi *Colletotrichum circinans, Fusarium solani*, and *Rhizoctonia solani* were incubated in aerated (0, 0.5, 1 dm^3 min^{-1}) potato dextrose broth (PDB) or Czapek-Dox broth (CDB), under 0-, 12- or 24-h photoperiods. Greater dry mass was produced in PDB. Higher air flows improved dry mass of *F. solani* and *R. solani*. The 24-h photoperiod improved *F. solani* dry mass. Except for *F. solani*, which was not affected, incubation in PDB increased protein content. The no air treatment increased protein content in *F. solani*, 0.5 dm^3 min^{-1} produced the highest protein content in *R. solani*, but air flow-rate did not affect *C. circinans*. Incubation in the dark produced the lowest protein content in *C. circinans*, the highest under the 24-h photoperiod for *R. solani*, and photoperiod did not affect protein content in *F. solani*. Protein content in *R. solani*, incubated in CDB, was lowest at the 0 dm^3 min^{-1} air flow at all photoperiods. Molecular masses of most proteins were under 30 kDa, and numbers of bands in SDS-PAGE gels varied with air flow. In CDB, especially under 12- or 24-h photoperiods, proteins in *F. solani* were between 1.6 and 310 kDa. For *R. solani* in PDB, at 0.5 dm^3 min^{-1} air flow and 12-h light, proteins were between 6 and 81 kDa.

Additional key words: air flow, fluorescent light, media.

Introduction

Length of exposure to, and quality of, light affect fungal growth (Cochrane 1958, Griffin 1994), which may be due to cell division or elongation initiating genes being turned on or off (Arpaia *et al.* 1995, Corrochano *et al.* 1995). Presence and amounts of various environmental gasses can affect fungal growth by affecting respiration (Griffin 1994).

Most physiological information for species of *Colletotrichum*, *Fusarium*, and *Rhizoctonia*, and other phytopathogenic fungi has been from incubation on semisolid media (Griffin 1994). Increased exposure to light increased colony diameter and conidia production in *C. dematium* var *circinans* (Berk.) v. Arx (Russo and Pappelis 1993), and induction of sporulation was more efficient in *C. graminicola* (Yang *et al.* 1991). For *F. solani* (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyder & Hansen, more conidia were produced under a 12-h photoperiod than in continuous dark (Mussa and Russell 1977), and only 4-h of light was needed to produce conidia (Das and Busse 1990). For *R. solani* Kühn, more sclerotia were formed in light than in the dark (Lokesha and Somashekar 1988, Moromizato *et al.* 1983). In light, on semisolid media, mycelial growth of *R. solani* isolates was either inhibited, unaffected, or stimulated (Kim and Chung 1992). However, when incubation was in shake culture, *R. solani* mycelial dry mass decreased as exposure to light increased (Durbin 1959).

Fungal proteins, comprising up to 44 % of thallus dry mass, are basic to fungal growth, and are important to the infection process (Albersheim and Valent 1974), and the degradation of host plant tissues (Bruton *et al.* 1998, Zhang *et al.* 1999). Exposure to air and light affect fungal

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Abbreviations: CDB - Czapex-Dox broth; PDB - potato dextrose broth.
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growth and metabolism. Aeration can affect dehydration and gaseous exchange. Light affects temperature, rate of dehydration, and formation and/or denaturation of proteins. This project was undertaken to determine how photoperiod affected growth, and protein content, of three fungal plant pathogens in aerated and non-aerated liquid culture.

Materials and methods

An apparatus to deliver filtered, compressed, air to fungi in liquid culture was modified from that of Gray and Abou-El-Seoud (1966). A flow meter [1 to 10 dm^3 min^{-1}; *Cole-Parmer*, Vernon Hill, USA] was attached through a primary filter filled with cotton to a non-sterile manifold in an incubator (Model *BOD50ABA*, *Revco*, Asheville, USA). The manifold was connected to restriction flowmeters that can deliver up to 1 dm^3 min^{-1}.

A 250 cm^3 volume of potato dextrose broth (PDB; *Becton Dickinson*, Cockeysville, USA) or Czapek-Dox broth (CDB; *Sigma Chemicals*, St. Louis, USA) was decanted into individual 500 cm^3 flasks topped with neoprene stoppers having a 7 mm diameter glass inlet tube, and a 9 mm diameter glass outlet tube. Inlet and outlet tubes were filled with cotton. Flasks were autoclaved at 835 kPa for 15 min. Stock cultures of *C. circinans* (Berk.) Vogl. (ATCC #44202), *F. solani* (Mart.) Appel & Wollenweb. (OK 738), and *R. solani* (TX 970021) were maintained on potato dextrose agar (PDA; *Becton Dickinson*, Cockeysville, USA). Isolates of *Fusarium* and *Rhizoctonia*, supplied by Dr. B. Bruton, were from diseased plants in Oklahoma and Texas. Under sterile conditions, the stopper in an autoclaved flask was loosened, and an 8 mm diameter disc from a 6-d-old culture of one of the fungi was introduced into the flask. Other inoculum plugs were placed on plastic, preweighed, weigh boats, lyophilized in a freeze-dryer (*Model 18, Labconco*, Kansas City, USA), and weighed.

A restriction flowmeter was connected to the inlet of an inoculated flask, and either 0.5 or 1 dm^3 min^{-1} of air was allowed to flow into the flask. Controls were inoculated flasks connected to the manifold through a restriction flowmeter, but no compressed air entered the flasks. Inoculated flasks were incubated at 24.5 ± 0.5 °C under 0-, 12-, or 24-h photoperiods using cool white fluorescent tubes delivering 4.6 W m^{-2} at 25 cm (*Dura-Test*, North Bergen, USA).

After 72-h, contents of each flask were vacuum filtered through nylon parachute cloth (*Woven Ripstop, Type I, Coated Sales*, Lawrence Harbor, USA). The fungal biomass was recovered, dried, and the inoculum plug dry mass subtracted from the total mycelial dry mass. Freeze-dried tissue from each treatment combination, PDA powder, and the uninoculated PDB and CDB were analyzed with the methods of Bradford (1976) for total protein content using a UV-vis spectrophotometer (*Model UV-160A, Shimadzu*, Kyoto, Japan). Results were compared to calibration curves for known amounts of protein. Amounts of protein found in the media was subtracted from that found in fungi.

The experimental design was a randomized complete block for dry mass and total protein content with two media, three photoperiods, three air flows, and four flasks for each treatment combination. The entire experiment was repeated twice. Total protein analysis from fungi, liquid media, and dry PDA, was replicated three times for each sample. All data were subjected to *ANOVA* in the General Linear Models procedures in *SAS* (ver. 6.12, *SAS Inc.*, Cary, USA).

For SDS-PAGE protein analysis samples were prepared by grinding mycelium in a mortar and pestle with 0.1 M sodium phosphate buffer (pH 7), 50 mg of polyvinylpolypyrrolidone, and 100 mg of sterile quartz sand. The slurry was centrifuged for 5 min (12 000 g), and the supernatant passed twice through a 0.45 µm cellulosic filter. The extract, with loading buffer was boiled for 3 min, and centrifuged (12 000 g) for 3 min before loading onto the gel. Proteins were separated on 0.75 mm thick preformed 4 % stacking and 12 % separation slab gels (*Bio-Rad*, Richmond, USA) using the Laemmli (1970) buffer system, according to instructions from *Bio-Rad*. Gels were electrophoresed at 10 min at 100 V and then 30 min at 200 V until the bromophenol tracking dye front moved off the gel. Gels were stained with silver stain according to methods from Bio-Rad and compared to, 7.5 to 207 kDa, standards to estimate protein size.

Results

Media, air flow, or photoperiod affected fungal dry mass or protein content in the fungi, and the air flow by photoperiod interaction affected protein content only in *R. solani* (Table 1). Fungal dry mass was highest when fungi were incubated in PDB. Air flow did not affect *C. circinans* dry mass, while the greatest *F. solani* dry mass was at 1 dm^3 min^{-1}, and for *R. solani* at 0.5 and 1 dm^3 min^{-1}. *F. solani* had the highest dry mass under 24-h

light, and *C. circinans* and *R. solani* dry mass was not affected by photoperiod.

There was no detectable protein in uninoculated media. Except for *F. solani*, which was not affected, protein content was highest for fungi incubated in PDB (Table 1). Air flow did not effect *C. circinans* protein content, while for *F. solani* the highest protein content was in the no air control, and for *R. solani* in flasks receiving 0.5 dm^3 min^{-1}. The 12- and 24-h photoperiods produced the highest protein content in *C. circinans*. Photoperiod had no affect on protein content of *F. solani*, and the highest protein content for *R. solani* was under 24-h light. The air flow by photoperiod interaction on protein content in *R. solani* indicated that in the dark there was no difference due to air flow, but under 12- and 24-h photoperiods the highest protein content was at 0.5 dm^3 min^{-1} (Table 2).

Table 1. Fungi dry masses [g] and protein contents [μg g^{-1}] as effected by medium, air flow [dm^3 min^{-1}] and photoperiod [h] (values in columns followed by the same letter are not significantly different, Ryan-Einot-Gabriel-Welsch test, $P \leq 0.05$; ns, *, ** non-significant, or significant at $P < 0.05$ or $P < 0.01$, respectively, *ANOVA*).

		Dry mass			Protein content		
		C. circinans	*F. solani*	*R. solani*	*C. circinans*	*F. solani*	*R. solani*
Medium	CDB	0.020	0.192	0.031	0.002	0.028	0.001
	PDB	0.191**	0.383**	0.091**	0.007*	0.026ns	0.043**
Air flow	0	0.066a	0.088c	0.009b	0.005a	0.048a	0.012c
	0.5	0.101a	0.320b	0.094a	0.006a	0.016b	0.044a
	1.0	0.090a	0.421a	0.085a	0.004a	0.011b	0.023b
Photoperiod	0	0.105a	0.253b	0.031a	0.001b	0.024a	0.004c
	12	0.094a	0.250b	0.062a	0.006a	0.031a	0.032b
	24	0.107a	0.327a	0.067a	0.007a	0.024a	0.044a
Medium		**	**	**	**	ns	**
Air-flow (A)		ns	**	**	ns	**	**
Photoperiod (P)		**	*	ns	**	ns	**
A x P		ns	ns	ns	ns	ns	**

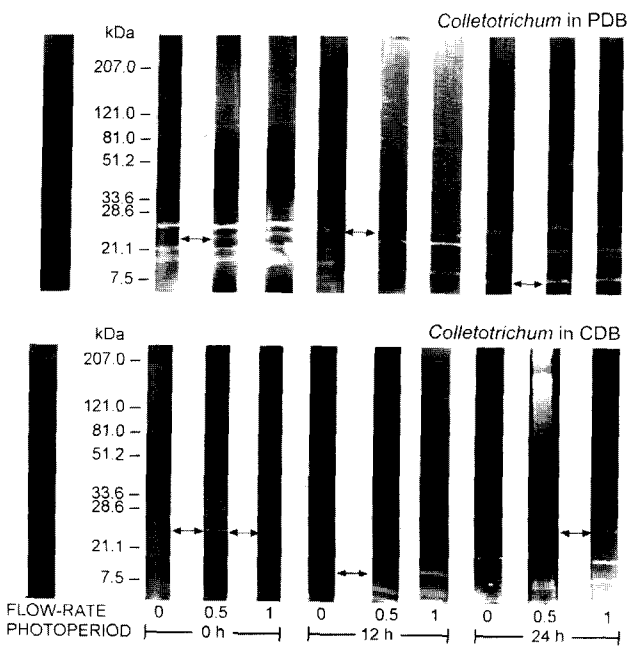

Fig. 1. Protein bands on SDS-PAGE gels for *C. circinans* incubated in potato dextrose broth (PDB) or Czapex-Dox broth (CDB) under 0-, 12-, or 24-h photoperiods and air flows of 0, 0.5, or 1 dm^3 min^{-1} for 72 h. The gel on the far left represents the extracted dry medium. *Double ended arrows* indicate protein bands that are present or absent in at least one gel within a light exposure and flow rate treatment.

Table 2. Interaction of photoperiod [h] and air flow rate [dm^3 min^{-1}] as it affects protein content [μg g^{-1}] in *R. solani* (ns - non-significant, ** - significant at $P < 0.01$, least squares means).

Photoperiod	Air flow	Protein content
0	0	0.000
	0.5	0.005ns
	1.0	0.010ns
12	0	0.015
	0.5	0.061**
	1.0	0.020**
24	0	0.022
	0.5	0.069**
	1.0	0.040**

In *C. circinans* only a few bands were formed and none were above 30 kDa. In PDB, density of bands appeared to be greatest when incubation was in the dark (Fig. 1). Distribution of protein bands formed by *C. circinans* in CDB was variable within, and between, treatments. For *F. solani*, in PDB, bands below 30 kDa, were found (Fig. 2). In the dark there were more bands at 0.5 dm^3 min^{-1} than at 0 or 1 dm^3 min^{-1} air flow. At 12- and 24-h photoperiods the fewest bands were formed at 0 dm^3 min^{-1} air flow. However, when incubated in CDB, in the dark, few bands were formed regardless of air flow, and these were above 81 kDa. Under 12- or 24-h photoperiods protein bands were from 1.6 to 310 kDa for *R. solani* in both media, protein masses generally were below 30 kDa, with exceptions at 0.5 dm^3 min^{-1} air flow and 12-h photoperiod where several bands above 30 kDa were present (Fig. 3). In CDB bands generally were at, or below, 30 kDa regardless of photoperiod and air flow. Exceptions were for 24-h photoperiod and 0 dm^3 min^{-1} air flow, where a band at about 40 kDa was found, and a band at about 16 kDa, which was present at other light exposures, was not present. At 1 dm^3 min^{-1} air flow a band at about 32 kDa was present that was not present at other air flows.

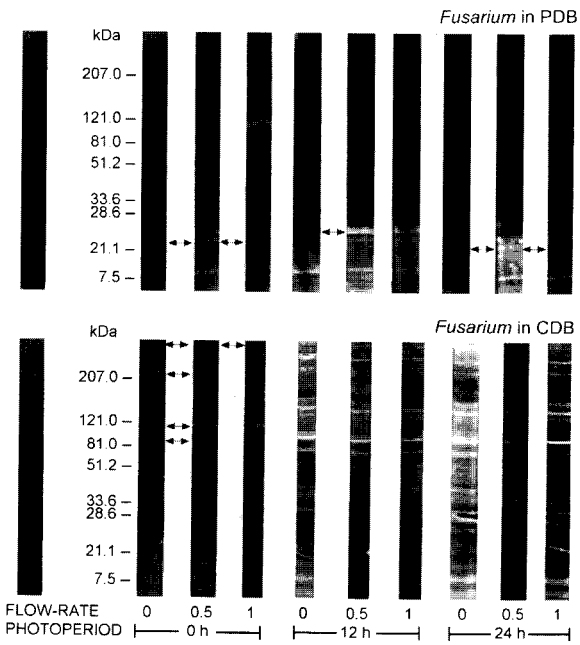

Fig. 2. Protein bands on SDS-PAGE gels for *F. solani* incubated in potato dextrose broth (PDB) or Czapex-Dox broth (CDB) under 0-, 12-, or 24-h of light and air flows of 0, 0.5, or 1 dm^3 min^{-1} for 72 h. The gel on the far left represents the extracted dry medium. *Double ended arrows* indicate protein bands that are present or absent in at least one gel within a light exposure and air flow rate treatment.

Discussion

Exposure to light can affect physiological processes mediating fungal mycelial growth (Chern and Ko 1993, Johnson and Coolbaugh 1990). Responses to light were different for these fungi suggesting that presence, and length of exposure to light were important to growth and protein physiology.

For *F. solani*, incubation in 24-h photoperiod increased dry mass. Under lengthening exposure to light,

R. solani dry mass decreased when incubation was in standing liquid culture (Durbin 1959). In this study aeration induced agitation negated the limiting effect on growth of *R. solani* imposed by stationary culture. These observations may be due to differential responses reported for *R. solani* isolates where exposure to light either increased, decreased, or had no effect on growth (Kim and Chung 1992).

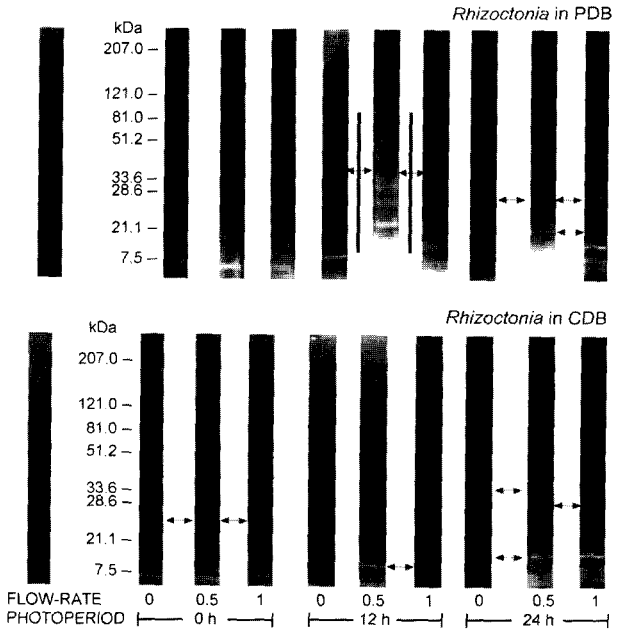

Fig. 3. Protein bands on SDS-PAGE gels for *R. solani* incubated in potato dextrose broth (PDB) or Czapex-Dox broth (CDB) under 0-, 12-, or 24-h of light and air flows of 0, 0.5, or 1 dm^3 min^{-1} for 72 h. The gel on the far left represents the extracted dry medium. *Double ended arrows* indicate protein bands that are present or absent in at least one gel within a light exposure and air flow rate treatment.

Impeller or turbine produced agitation affects biomass and metabolite production in fungi (Cui *et al.* 1997, Gibbs and Seviour 1996). Liquid media can be agitated by aeration. Gray and Abou-El-Seoud (1966) determined that species of *Linderina* and *Cladosporium* cultured in aerated liquid medium did not produce as much dry mass when compared to incubation in non-aerated shake culture. However, in that study, air flow was not adjusted, nor was the effect of exposure to light examined. Flasks had 2 to 40 % less medium at harvest than prior to incubation for no air controls to 1 dm^3 min^{-1} air flows. Some of the medium was converted to fungal mass, but the majority was likely lost to evaporation. Loss of medium did not greatly affect fungal growth since dry masses from flasks exposed to 1 dm^3 min^{-1} air flow were generally the same as, or better than, those from control flasks. This suggests that there was sufficient nutrition present in the flasks, and that the length of the experiment was not a factor.

Medium type affected fungal growth. Lower dry mass for fungi incubated in the defined CDB indicates that it is less suitable than PDB to support growth. Aerated PDB in flasks containing *R. solani* changed color. Melanin, phenols or tannin were not present in the colored medium. Oxidation of sugars in the medium was not the likely cause of the pigmentation since aerated flasks with *C. circinans* and *F. solani*, containing the same medium, did not change color. It is likely that a metabolite associated with *R. solani* was involved.

Proteins concentrations and band patterns were affected by treatment. Similar proteins were found in the various fungi, but it is not known whether they perform the same physiological functions. Correll *et al.* (1993) developed DNA profiles for isolates of *C. orbiculare* (Berk. & Mont.) v. Arx [= *C. lagenarium* (Pass.) Ellis and Halst]. Differences found in those isolates may explain raciation of that fungus as regards pathogenicity (Wasilwa *et al.* 1993).

Fungi are in competition with each other, and other organisms. Responses of the fungi to the identical environment were different, and probably reflect specialized adaptation. Organisms that can adjust when some factor in the environment becomes limiting tend to gain an advantage over their competitors. Culture conditions need to be considered when interpreting affects on growth and protein production.

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