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A method to obtain rapid zoosporogenesis of *Pythium insidiosum*

Leonel Mendoza & Jorge Prendas
Mycology Department, School of Veterinary Medicine, Universidad Nacional, Apt. 86, Heredia, Costa Rica

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Abstract

Nine strains of *Pythium insidiosum* the etiologic agent of pythiosis, were inoculated on 2% water agar plus grass blades and then incubated one day at 25 °C, 35 °C and 37 °C. Sporangium and secondary biflagellate-type zoosporas from the parasitized grass blades were noticed in induction medium after one hour of incubation at 35 °C and 37 °C. The number of sporangia and zoospores were lower at 25 °C, than 35 °C and 37 °C. Increasing the days of incubation of the parasitized grass blades resulted in the increase in the time of incubation in the induction medium. Corn meal agar, Schmitthenner medium and Sabouraud dextrose agar were also tested but the sporangium and zoosporas were always observed after five hours of incubation in induction medium.

Introduction

Pythiosis is a disease in horses [3, 15], dogs [9, 17], cattle [18] and humans [4], caused by a long-unnamed 'phycomycete', recently classified as *Pythium insidiosum* by de Cock *et al.* [4]. This microorganism belongs to the Kingdom Protoctista, Class Oomycetes and Family Pythiaceae [14]. The disease has been reported in tropical [5, 12, 15], subtropical [3, 7] and temperate [1] areas of the world.

The etiological agent was isolated for the first time in 1902 by de Haan [5]. He did not name the agent since no spores were found in the cultures. He called the disease in horses 'Hyphomycosis destruens' and later 'Hyphomycosis destruens-equi' [6]. Witkampt [22] isolated the agent for the second time in 1924, but no spores were found in the strains to help in its identification. Bridges and Emmons [3] isolated a similar microorganism in 1961 from eight horses in Texas. They called the microorganism *Hyphomyces destruens,* and although they used a wide variety of media, they did not induce sporulation.

In 1974, Austwick and Copland [2] were successful in obtaining sporulation by placing a portion of colonies grown on Sabouraud dextrose agar in a petri dish which contained pieces of sterilized aquous medium of rotten maize silage. They concluded that *H. destruens* 'could be included in the genus *Pythium* Pringsheim'.

In this paper we describe a simple method to obtain rapid zoosporogenesis of *Pythium insidiosum,* based on the characteristic of the genus *Pythium* to parasitize leafs on the acuatic environment [11].

Materiales and methods

Isolates of Pythium insidiosum *used*

ATCC 58643 = CBS 574.85 type strain, ATCC 58638 = CBS 579.85, ATCC 58640 = CBS 577.85, ATCC 28251 (*Pythium* sp. *sensu* Austwick and Coplan), USA 60932 (From R. I. Miller), ATCC 46947 (*Pythium gracile sensu* Ichitani & Amemiya), USA 25156 (from a dog with Pythiosis, R. I. Miller), H-19 (from a horse with pythiosis, Shipton, Australia), and CBS 673.85 (from a Thailand pythiosis human case).

In addition, *P. diclinun* CBS 664.79, *Lagenidium*

giganteus CBS 580.84 and *Saprolegnia* sp. isolated from a dead fish, were also tested.

Preparation of induction medium (IM)

Solution No. 1 K_2HPO_4, 87.09 g; M KH_2PO_4, 68.05 g; $(NH_4)_2HPO_4$; 66.04 g. Distilled water, 500 ml. Solution No. 2 $Cl_2 \cdot 6H_2O$, 25.42 g; $CaCl_2 \cdot 2H_2O$ 18.38 g; Distilled water 250 ml. Mixe 0.5 ml of solution No. 1 plus 0.1 ml of solution No. 2 in a 2 liters flask containing 1000 ml of distilled water [11].

The isolates were grown on Sabouraud dextrose agar pH 7.0 and corn meal agar (Difco) and transferred to a 2% water agar pH 6.9. The pH used in IM and 2% water agar was in accordance with those recommended by Shipton [19]. Grass blades of *Palpastum notatum* were cut in 25 mm pieces and then boiled 20 minutes.

Petri dishes containing 30 ml of 2% water agar were inoculated with the above cited isolates. The boiled grass blades were layed over each inoculum and incubated at 37 °C. After one day of incubation, the parasitized grass blades were transferred to a Petri dish containing 30 ml of IM and incubated 3 hours at 25 °C, 35 °C and 37 °C. The inoculum of the *P. insidiosum* strains plus the grass blades were also tested in IM, on days two through 30 at the same temperatures. The number of sporangium were counted on the border of parasitized grass blades under a light microscope.

We also used the medium suggested by Waterhouse [20], for comparison purposes, including Sabouraud dextrose agar media.

Results

All the tested isolates produced undiferenciate sporangia and released of secundary biflagellate-type zoospores at the end of incubation. The temperature was crucial for all *P. insidiosum* strains tested. While *P. declinum, L. giganteus* and *Saprolegnia* sp. developed more sporangium at 25 °C, *P. insidiosum* produced larger numbers of sporangium at 35 °C and 37 °C than at 25 °C.

Table 1. Comparison between the days of incubation in 2% water agar with strains of *P. insidiosum* plus grass blades and the number of sporangium obtained in 3 hours (IM)[1] at 25°, 35° and 37 °C.

Number of days of incubation[2]	1:00*			2:00*			3:00*		
	25°	35°	37°	25°	35°	37°	25°	35°	37°
1	0	80	88	3	13	15	0	3	2
2	0	75	76	3	18	17	0	2	1
3	0	95	106	2	17	20	0	1	0
4	0	27	25	1	52	50	0	16	14
5	0	0	0	4	64	66	1	18	15
6	0	0	0	3	69	68	0	17	19
7	0	0	0	6	77	81	0	17	20
11	0	0	0	9	91	90	2	33	35
13	0	0	0	5	32	37	3	40	43
15	0	0	0	2	28	27	4	55	50
20	0	0	0	0	5	10	7	53	60
22	0	0	0	0	0	0	5	63	65
25	0	0	0	0	0	0	10	33	30
27	0	0	0	0	0	0	4	23	26
30	0	0	0	0	0	0	0	8	6

[1] Sporagium obtained in induction medium (IM)

[2] Number of days in which the strains of *P. insidiosum* were incubated along with grass blades on 2% water agar.

*Time in hours incubate in IM

The parasitized grass blades obtained from the above mentioned strains in 2% water agar, took one hour to develop sporangium and zoosporas in IM, after one day of incubation at 35 °C and 37 °C. At 25 °C the sporangium and zoosporas were noticed after two hours of incubation in IM. Increased in the time of incubation of the parasitized grass blades, resulted in the increase in the time of incubation in the IM, to obtain sporangium and zoospores (Table 1).

Strains of *P. insidiosum* inoculated on Sabouraud dextrose agar, cornmeal agar and Schmitthenner's medium along with the grass blades were incubated four days at 37 °C, then were transferred to a petri dish containing 30 ml of IM and were incubated 5 hours at 25 °C and 37 °C. Under these conditions, the sporangium and resease of the secondary-type zoosporas were achieved after five hours of incubation. No sporangium and zoospores were observed before five hours. The time in which the sporangium and zoosporas were developed in IM varied according to the medium used (Table 2).

The maximum number of sporangium and zoospores was obtained using 2% water agar after one and half hours in IM, whereas using other mediums the zoosporogenesis took place after 5½ h of incubation. In the parasitized grass blades incubated at 25 °C in IM, the number of sporangia and zoospores was lower that those incubated at 37 °C, independent of the media used (Table 2).

The production of sporangium and zoospores were observed even 2 hours after the first sporangia was noticed. No sporangium and zoospores were observed after eight and a half hours of incubation in IM. Each sporangium produced 5 to 40 biflagellate zoospores. The zoospores swam from 15 to 25 minutes and then became encist. Thirty minutes later, a germinated tube was noticed.

Discussion

Some authors [8, 16, 20, 21] have suggested that conditions favoring the sporulation of several aquatic fungi are based on the reduction of their nutrient supply. In these conditions, most of the aquatic fungi developed sporangia and zoospores. However, presence of certain ions as Ca^{2+}, Mg^{2+} and K^{+} in IM medium, the pH, age of the strains, agitation to provide O_2, temperature, have also been noted as important factors for sporulation of water molds [10, 13, 16, 20, 21].

In our case, the largest number of sporangium was achieved in medium lacking in nutrient (2% water

Table 2. Coparison between the diferent medium inoculate with *P. insidiosum* plus grass blades, and the number of sporangium obtained during 7 h in IM at 25 ° and 37 °C.

Medium used days of incubation[1]		1*		5*		7*	
		25 °	37 °	25 °	37 °	25 °	37 °
Sabouraud	1	0	0	0	0	0	0
	4	0	0	5	50	0	2
	7	0	0	2	10	0	0
Cornmeal agar	1	0	0	0	0	0	0
	4	0	0	10	60	0	1
	7	0	0	1	9	0	0
Scmitthenner	1	0	0	0	0	0	0
	4	0	0	7	25	0	3
	7	0	0	2	3	0	0
2% Water agar	1	0	88	9	21	0	2
	4	0	25	3	9	0	0
	7	0	0	2	5	0	0

[1] Days in which the inoculum of *P. insidiosum* plus grass blades were incubated.
* Time in hours in which the parasitized grass blades were incubated in IM.

agar). In such a culture medium, the time of sporulation was reduced with respect to other mediums also used. Explanation for rapid sporulation under these conditions could be the absence of a nutrient-supply, not only in the IM medium, but in the culture medium used before induction. Shipton [20], working with strains isolated from horses afflicted with pythiosis in Australia, reported that 'A reduction in nutrient levels rather than removal of staling products best explains zoospore induction'. Our findings supported his observations. The temperature was crucial in obtaining sporulation of *P. insidiosum*. The optimum temperatures to develop sporangium were between 35 °C and 37 °C.

Temperatures under 35 °C did not favor the development of high numbers of sporangium. Similar observations were previously reported by Shipton [19] working with two Australian strains.

Sporangium and secondary-type zoospora formation were favored using 24 hour-old parasitized grass blades from 2% water agar. The use of five day old or older colonies resulted in an increase in the time of incubation in IM. The same results were found by Shipton [20].

Our findings indicated that the use of a medium lacking in nutrients before induction increased the number of sporangium and bipflagellate zoospores and reduced the time of incubation and sporulation in the nine *P. insidiosum* strains tested herein.

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