EFFECT OF TEMPERATURE ON GROWTH OF THE PATHOGENIC OOMYCETE *PYTHIUM INSIDIOSUM*

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Abstract. *Pythium insidiosum* causes a potentially life-threatening infectious disease called pythiosis. An early, accurate diagnosis is important, since prompt treatment leads to a better prognosis. Unsuccessful attempts to isolate the organism have been associated with specimens subjected to lower temperatures. We analyzed growth of *P. insidiosum* at various temperatures. Culture at low (8ºC) and high (42ºC) temperatures resulted in death or inhibited growth of the organism. Culture under optimal temperatures (28 and 32ºC) was important for successful isolation of *P. insidiosum*.

Key words: *Pythium insidiosum*, pythiosis, oomycete, growth, temperature

INTRODUCTION

Pythium insidiosum is the causative agent of pythiosis, a potentially life-threatening infectious disease in humans and animals (*eg*, horses, dogs, cats, cattle) (Mendoza *et al*, 1996; Thianprasit *et al*, 1996; Kaufman, 1998; Krajaejun *et al*, 2006). It is normally found in swamp waters where it exists as either branching broad hyphae or biflagellate zoospores (Mendoza *et al*, 1993). These zoospores are the infective unit and follow chemoattractants exuded by host epidermal surfaces to which they attach, germinate and invade host tissues causing pathology (Mendoza *et al*, 1993). Phylogenetically, *P. insidiosum* belongs to oomycetes (organisms with a fungus-like morphology not classified as fungi in the supergroup Unikonts), which are classified as Stramenopiles of the supergroup Chromalveolates (Keeling *et al*, 2005).

Almost all cases of pythiosis are reported in tropical and subtropical countries. Human and animal patients usually present with an infection of arterial, ocular, cutaneous/subcutaneous or gastrointestinal tissue. A high rate of morbidity and mortality among patients with pythiosis is exacerbated by difficult early diagnosis and effective treatment (Mendoza *et al*, 1996; Thianprasit *et al*, 1996; Krajaejun *et al*, 2006). Conventional anti-fungal drugs are ineffective because oomycete pathogens do not possess the ergosterol biosynthesis pathway, the target of many fungicides (Krajaejun *et al*, 2006). Primary treatment for pythiosis is the surgical removal of infected tissues;

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Table 1
P. insidiosum isolated from Thai patients with pythiosis in this study.

Microorganism	Strain	Clinical source	Reference
Pythium insidiosum	Pins01	Artery	-
Pythium insidiosum	Pins02	Artery	CBS 119452
Pythium insidiosum	Pins03	Artery	CBS 119453
Pythium insidiosum	Pins04	Brain	CBS 119454
Pythium insidiosum	Pins05	Cornea	CBS 119455
Pythium insidiosum	Pins06	Cutaneous	-
Pythium insidiosum	Pins07	Artery	-
Pythium insidiosum	Pins08	Cutaneous	CBS 673.85
Pythium insidiosum	Pins09	Artery	-
Pythium insidiosum	Pins10	Artery	-
Pythium insidiosum	Pins11	Artery	-
Pythium insidiosum	Pins12	Stomach	-
Pythium insidiosum	Pins13	Artery	-

this must be pursued promptly to stem disease progression. Most human and animal patients with pythiosis require radical excision of infected body parts (*eg*, limb amputation, enucleation). Uncontrolled infection usually results in death.

Early and accurate diagnosis of pythiosis is important, since prompt treatment leads to a better prognosis. The most reliable method to diagnose pythiosis is identification on culture (Mendoza and Prendas, 1988), but efforts to isolate the causative organism have not been entirely successful. Unsuccessful attempts to isolate the organism have been anecdotally related to specimens subject to lower temperatures, such as an ice-box or refrigerator (Brown and Roberts, 1988; Patton *et al*, 1996). Since pythiosis is strictly endemic in tropical and subtropical locations (Mendoza *et al*, 1996; Thianprasit *et al*, 1996; Kaufman, 1998; Krajaejun *et al*, 2006), this implies a possible sensitivity to reduced temperatures. Freezing has reported to be deleterious to the organism (Bentinck-Smith *et al*, 1989).

Specimens of infected tissue collected by clinicians frequently require transport to distant diagnostic laboratories for culture analysis. This is particularly true in developing countries and rural areas where expedited delivery may be less available. Cool temperatures are the standard means of protecting samples from overgrowth with contaminating bacteria. However, such precautions may lead to difficulty recovering *P. insidiosum* on culture. In this study, we examined the impact of temperature on the growth of *P. insidiosum*.

MATERIALS AND METHODS

Thirteen culture-proven *P. insidiosum* isolates (strains Pins01-13, Table 1) from human patients with various forms of pythiosis (8 vascular, 2 cutaneous/subcutaneous and one each of ocular, brain and gastrointestinal tract infection) were used to determine the growth characteristics at 5 relevant temperatures: 8ºC (temperature in a cold room), 28ºC, 32ºC, 37ºC and 42ºC

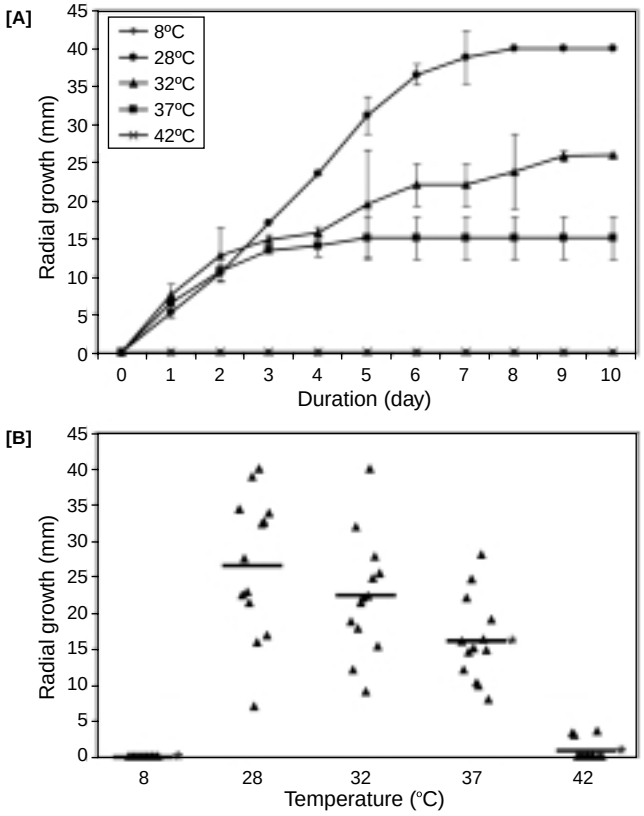

Fig 1–Growth of *P. insidiosum* at various temperatures and times. (A) Growth of a *P. insidiosum* isolate (strain Pins01) over a 10-day period. (B) Mean radial growth of all 13 *P. insidiosum* isolates (strains Pins01-13) (Table 1) at Day 7 (the bar indicates the average radial growth; an asterisk indicates statistical significance (p-value < 0.05) when compared to growth at 28ºC).

(temperature in an incubator). The growth assessment method of Brown *et al* (2008) was modified to analyze growth of *P. insidiosum* in this study. Briefly, *P. insidiosum* was sub-cultured on Sabouraud dextrose agar (SDA) comprised of 1% (wt/vol) bacto peptone (Becton Dickinson, MD, USA), 2% (wt/vol) dextrose (HiMedia, Mumbai, India) and 1.5% (wt/vol) agar (Agar bacteriological, Barcelona, Spain), then incubated at 37ºC for 3 days. A mycelial plug (5 mm in diameter) was cut from the margin of an actively growing colony and placed in the center of a fresh SDA plate with the mycelium in contact with the agar. The radial growth diameter of each *P. insidiosum* colony was measured daily for 10 days. The experiment was carried out in duplicate. The diameter of the mycelial plug (5 mm) was subtracted from the total diameter of each colony and divided by 2 to obtain the mean radial growth.

RESULTS

The growth kinetics of *P. insidiosum* over 10 days (strain Pins01, Table 1) at various temperatures are depicted in Fig 1A. *P. insidiosum* did not grow at 8ºC or 42ºC. It grew optimally at 28ºC, and more slowly at 32ºC and 37ºC. Radial growth at 28ºC plateaued at Day 7 while growth at 37ºC plateaued by Day 4. Similar growth kinetics were observed in other isolates (Table 2). The mean radial diameters of the isolates (strain Pins01-13, Table 1) obtained on Day 7 are shown in Fig 1B. The growth differences among the 5 groups of isolates growing at different temperatures were statistically calculated using a non-parametric test with 2-independent samples. The majority of *P. insidiosum* isolates grew optimally at 28ºC. The organism grew more slowly at 32ºC, and its growth was significantly reduced at 37ºC. Isolate growth was completely inhibited at 8ºC. At 42ºC, 10 isolates (77% of isolates) failed to grow, whereas the remainder (3 isolates) grew minimally.

Table 2
Mean radial growth and hyphal elongation rates of all 13 *P. insidiosum* isolates (strain Pins01-13 in Table 1) at various temperatures (8ºC, 28ºC, 32ºC, 37ºC, and 42ºC) and times (1-10 days). SD, standard deviation.

Temp	Mean growth (mm)/ elongation rate (mm/day)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
8ºC	Radial growth (SD)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Elongation rate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28ºC	Radial growth (SD)	4.4 (1.9)	8.8 (3.4)	14.0 (5.6)	17.5 (7.0)	22.0 (9.0)	24.3 (9.5)	26.6 (9.8)	28.0 (10.2)	29.0 (10.6)	29.5 (10.6)
	Elongation rate	4.4	4.5	5.2	3.5	4.5	2.3	2.3	1.5	0.9	0.5
32ºC	Radial growth (SD)	5.8 (2.2)	10.6 (3.9)	13.5 (5.4)	16.3 (6.7)	19.1 (7.8)	21.5 (7.9)	22.2 (8.3)	23.5 (8.8)	24.1 (9.0)	24.5 (9.4)
	Elongation rate	5.8	4.8	2.9	2.8	2.8	2.4	0.7	1.3	0.6	0.4
37ºC	Radial growth (SD)	5.0 (2.2)	8.3 (3.3)	11.0 (4.1)	12.9 (4.2)	14.3 (5.0)	15.5 (5.3)	16.2 (5.9)	16.7 (6.4)	17.2 (7.2)	17.6 (7.9)
	Elongation rate	5.0	3.3	2.8	1.9	1.4	1.2	0.7	0.5	0.6	0.4
42ºC	Radial growth (SD)	0.4 (0.8)	0.8 (1.4)	0.8 (1.4)	0.8 (1.4)	0.8 (1.4)	0.8 (1.4)	0.8 (1.4)	0.8 (1.4)	0.8 (1.4)	0.8 (1.4)
	Elongation rate	0.4	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

A second growth assessment study was carried out to evaluate whether *P. insidiosum* colonies maintained at 8ºC can recover and subsequently grow at optimal temperatures. Isolates were incubated at 8ºC for 5 days then incubated at 28ºC. Using this regimen only 4 isolates (31%) exhibited hyphal elongation (data not shown). This demonstrates exposure to reduced temperatures can prevent culture of *P. insidiosum*, depending the individual strain.

DISCUSSION

Variations in temperature have a substantial impact on the growth and recovery of *P. insidiosum*. Temperatures ranging from 28 to 32ºC were found to be optimal for the culture of this microorganism, while its growth may be profoundly inhibited at 8ºC and 42ºC. Temperature sensitivity is a critical characteristic of *P. insidiosum* in regard to its successful isolation from clinical specimens, especially since transport of specimens on ice has not been previously contraindicated. In order to maximize recovery rates for *P. insidiosum*, and the likelihood of its identification on culture, it may be necessary to explore alternatives to the transport of clinical specimens on ice. Cultures of clinical specimens should be maintained at an optimal temperature (28ºC and 32ºC).

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