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The radiation resistance of ascospores and sclerotia of *Pyronema domesticum*

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The Food and Drug Administration has become aware of several instances where supposedly sterile medical surgical products made of Chinese cotton have been found to contain viable *Pyronema domesticum*. The aim of this research was to determine the gamma and electron beam radiation resistance values for the two dormant phases (ascospores and sclerotia) of *P. domesticum*. The resistance values were obtained by developing a standardized system to cultivate, purify, and harvest biological indicators containing sclerotia or ascospores. Ascospores were more resistant to radiation than sclerotia. The D_{10} values for sclerotia were 0.79 and 1.09 kGy for strains 32030 and 14881, respectively. The resistance value for wild type ascospores was 2.83 kGy. The current standard for assuring radiation sterilization of medical devices is ISO 11137. This standard was developed to address the propensity for highly radiation-resistant organisms such as *P. domesticum*. Prior to the standard, biological indicators such as *Bacillus pumilus*, having a nominal D_{10} value or 1.7 kGy, were used to determine the sterility of many medical devices.
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Introduction

In 1993, a manufacturer of sterile laparotomy sponges announced a voluntary recall of the sponges because of fungal contamination [4]. In April, 1994, the US Food and Drug Administration (FDA) found that the prevalent organism, isolated from various lots of manufacturers' laparotomy sponges, was a mold identified as *Pyronema domesticum* [8].

P. domesticum is a member of the Ascomycetes class of Dikaryomycotan fungi. It reproduces through the formation of sexual spores known as ascospores. The ascospores are found within a multihyphal structure known as an apothecial ascoma or apothecium. Under stressful conditions, *P. domesticum* produces a second long-term survival mechanism known as a sclerotium. This consists of a hard mass of hyphae, which germinates upon the return of favorable conditions, giving rise to a fruiting body or mycelium [9].

The FDA found that several companies using cotton had been sterilizing it with ethylene oxide gas and were experiencing problems killing *P. domesticum* [4,8]. All affected companies were found to have purchased the cotton used in the manufacturing of the sponges from a supplier in China [8]. The FDA reported that radiation sterilization was not killing *P. domesticum*, and issued a memorandum to all medical device manufacturers serving as an advisory notice for recommended cotton product testing [8]. The memorandum indicated that several companies had experienced problems with mold, resulting in changes to the companies' sterilization methods. The memorandum referenced an unpublished paper by Kendall Healthcare Products [10] stating that there was a problem with the sterilization resistance of *P. domesticum*. Kendall Healthcare Products performed a study on *P. domesticum* and reported that the organism was resistant to gamma radiation doses between 18 and 28 kGy. They recommended steam sterilization of cotton products derived from Chinese cotton [10].

The present study was carried out to obtain additional results on the radiation resistance of *P. domesticum* and to compare the resistance of the two distinct resting phases in the mold's life cycle — ascospores and sclerotia.

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Materials and methods

P. domesticum cultures

Two American Type Culture Collection (ATCC) *P. domesticum* strains (ATCC strain numbers 14881 and 32030, deposited by Elizabeth Moore-Landecker) were obtained. Upon receipt, all cultures were transferred to rabbit food agar (RFA) slants (25 g of pet store rabbit food, boiled in 1 l of H_2O, steeped for 30 min, filtered through four layers of cheesecloth, mixed with 15 g of agar, and autoclaved for 15 min [1]) and incubated at 20–25°C until hyphal growth was observed.

The wild type strain was obtained from cultures isolated from laparotomy sponges involved in the FDA recall. To verify viability and morphology, all cultures were transferred at 3-month intervals. All strains were subcultured to potato dextrose agar (PDA; Difco, Detroit, MI) [3] plates. All strains revealed abundant growth at 20–25°C and 30–32°C on PDA.

Sclerotia propagation medium: PDA was prepared by combining 39 g of PDA mix (Difco) with 1 l of distilled water. The mixture was then sterilized at 121–124°C for 15 min and a final pH of 7.0 was obtained.

Ascospore propagation medium: Claussen's agar without casein was prepared by mixing 0.5 g of KH_2PO_4, 0.2 g of

$MgSO_4 7H_2O$, 0.5 g of NH_4NO_3, 0.01 g of $FeCl_3$, 20 g of agar, and 1 l of deionized water (Millipore Ultrapure RO/DI System; Millipore, Bedford, MA). The pH was adjusted to 7.0. The medium was autoclaved and dispensed into 15×100-mm Petri plates (Fisher Scientific, Pittsburgh, PA) [11].

Additional media: Fluid D was prepared with 1 g of peptone per liter of deionized water and 1 ml of Tween 80, and the pH was adjusted to 7.0. Phosphate-buffered water (PBH_2O) included the addition of 1.25 ml of KH_2PO_4 to 1 l of deionized water to reach a neutral pH of 7.0. Sabouraud dextrose broth (SDB) was prepared by combining 30 g of SDB mix (Difco) with 1 l of deionized water. The solution was sterilized at 121–124°C for 15 min and maintained at pH 5.6.

Radiation: All radiation treatments of *P. domesticum* samples were conducted in the Whiteshell Irradiator (ACSION Industries, Pinawa, MB, Canada).

Electron beam accelerator: Electron beam sample irradiations were done with an I-10/1 Accelerator (Atomic Energy of Canada, Mississauga, ON, Canada). The I-10/1 is an S-band standing wave RF LINAC. As installed in the Whiteshell Irradiator, it delivers a scanned beam of high-energy electrons, free in air, across a stainless steel belt conveyor. The scan can be adjusted from 15 cm wide to the full 60-cm width of the conveyor. The delivered beam energy is typically 9.2 MeV and the peak current — delivered in 4-μs pulses, 300× s^{-1} — is 70 mA. The average beam current is, therefore, about 85 μA and the average beam power is about 0.77 kW.

At the conveyor level, the beam is approximately Gaussian in cross-section with a 10-cm mean diameter (FWHM). The average dose rate is scan width-dependent but is approximately 0.012 kGy/s at a typical scan width of 50 cm. The instantaneous dose rate is about 8 Gy/pulse or about 2×10^3 kGy/s. The applied dose variation is less than ±6% over the entire radiation field.

Gamma cell irradiator: Samples were exposed to gamma radiation in a ^{60}Co Gammacell 2000 irradiator (MDS Nordion, Kanata, ON, Canada) with a dose rate of about 1.33 Gy/s (chamber center). The dose rate throughout the 15.5-cm diameter×20-cm-high irradiation chamber varies by ±20%.

Dosimetry: As required by the American National Standard, ISO 11137 [6], doses were measured for each set of samples by placing radiochromic dye film dosimeters (Far West Technology, Ventura, CA) calibrated traceably to National Physical Laboratories (Teddington, UK) with each pair of samples during irradiation. GAFchromic dosimeters were used for doses below 2 kGy. FWT-type dosimeters were used for doses of 2 kGy and above.

Sclerotia D_{10} value radiation experiments: Each ATCC strain of *P. domesticum* was transferred to PDA plates and incubated at 20–25°C for 14 days. Plates were placed near fans in the incubator to create conditions to optimize spore production and allow agar to dry to potato chip-like form. A sterile hand paper punch was used to punch out 8-mm circles from the agar, the discs were counted, and it was found that one plate yielded approximately 100 discs. The discs from plates with evenly distributed sclerotia were transferred to sterile Petri dishes and sclerotia were counted using an Olympus stereo microscope. Duplicate counts were made and the average count was recorded. Each spore disc was placed into a sterile glassine bag (Westvaco, Envelope Division, Springfield, MA) and labeled with the sclerotia amount. For each strain, a total of 220 enumerated sclerotia discs were shipped to the Whiteshell Irradiator. Thus, a set of 20 sclerotia discs was submitted to each type and dose of radiation for each strain. Because excess moisture rendered the sclerotia nonviable, the discs were shipped inside a ziplok™ poly bag with a desiccant ($CaCO_3$-Drierite™) (Fisher Scientific). A ziplok™ poly bag with desiccant

Table 1 Electron beam radiation results for sclerotia from strains 32030 and 14881 and for wild type apothecia

Pyronema strain	Dose (kGy)	Number irradiated/number sterilized (n/r)[a]	D_{10} value[b] (kGy)	SD[c]
32030 — sclerotia	2	20/20	–	
	4	20/20	–	
	6	20/20	–	
	8	20/20	–	
14881 — sclerotia	2	20/0	–	
	4	20/18	1.34	0.13
	6	20/20	–	
	8	20/20	–	
WT — apothecia	4	20/0	–	
	6	20/0	–	
	8	20/1	2.67	0.13
	9.3	20/1	3.10	0.16
	10.9	20/19	2.29	0.11
	12.3	20/19	2.58	0.13
	16.8	20/20	–	
	17.8	20/20	–	

[a] n = number of sclerotia or spore discs irradiated; r = the number of discs sterilized by irradiation.
[b] D = dose/($\log N_0 - \log N_u$), where $N_u = \ln(n/r)$ and N_0 is the number of sclerotia or ascospores per disc. N_0 was 100±10 sclerotia, or 3000±150 ascospores.
[c] SD = standard deviation.

Table 2 Gamma radiation results for sclerotia from strains 32030 and 14881 and for wild type apothecia

Pyronema strain	Dose (kGy)	Number irradiated/number sterilized (n/r)[a]	D_{10} value[b] (kGy)	SD[c]
32030 — sclerotia	2	20/18	0.67	0.07
	3	20/19	0.91	0.09
	5	20/20	–	–
	8	20/20	–	–
	10	20/20	–	–
	16	20/20	–	–
	2	20/19	0.61	0.06
14881 — sclerotia	2	20/16	0.75	0.08
	3	20/19	0.91	0.09
	5	20/20	–	–
	8	20/20	–	–
	10	20/20	–	–
	16	20/20	–	–
	2	20/20	–	–
WT — apothecia	2	20/0	–	–
	9.3	20/2	2.99	0.15

[a]n=number of sclerotia or spore discs irradiated; r=the number of discs sterilized by irradiation.
[b]D=dose/($\log N_0 - \log N_u$), where $N_u = \ln(n/r)$ and N_0 is the number of sclerotia or ascospores per disc. N_0 was 100±10 sclerotia, or 3000±150 ascospores.
[c]SD=standard deviation.

was also submitted as a control. Before the bags were sealed and shipped, the relative humidity (RH) in both bags was tested using a calibrated RH meter (Cole Palmer Instrument, Vernon Hills, IL).

The sclerotia discs were exposed to electron beam or gamma radiation doses ranging from 2 to 16 kGy. The two control discs included in the study were not exposed to any radiation. After processing, the sclerotia discs were returned to the research laboratory and RH readings (25–30% RH) were taken in both the sample bag and the unopened control bag.

To visually validate counting methods, representative sclerotia discs that had not been exposed to radiation were placed into a sterile tube containing 10 ml of PBH_2O and vortexed until all the sclerotia were extracted from the disc. Each tube was then filtered through a sterile glass membrane filtration apparatus (Millipore) containing a 47-mm, 0.45-μm cellulose acetate filter (Gelman Sciences, Ann Arbor, MI). Additional aliquots of PBH_2O were used to wash the tube and filter apparatus free of remaining sclerotia.

After filtration, the filter was removed from the apparatus and placed into plates containing Claussen's agar without casein. All plates were incubated upright in a 25±1°C incubator for 5–7 days. Plates were macroscopically and microscopically (40×) examined and all sclerotia exhibiting hyphal growth were counted. Recovery experiments were carried out to determine the percent loss comparing microscopic counts to viable counts.

Apothecia D_{10} value radiation experiments: The *P. domesticum* wild type strain was inoculated to Claussen's agar without casein and incubated at 20–25°C for 24–48 h. Fungal growth was subjected to 12 h of light and 12 h of darkness at ambient temperature. Plates were examined daily for salmon-colored apothecia at the perimeter of the plates. Apothecia were aseptically transferred to 2.5×0.6 cm sterile paper strips (Whatman, Clifton, NJ). Population studies were determined by transferring five wild type apothecium strips to 15-ml sterile centrifuge tubes containing 0.1 ml of USP Fluid D. Using sterile tissue grinders, the apothecia were ground until there were no longer large sections of apothecia visible in the liquid. Additional Fluid D was added to the tubes and serial dilutions were made to determine the approximate ascospore population per apothecium using the most probable number (MPN) three tube test [5].

Apothecia placed onto sterile paper strips were used for both gamma and electron beam radiation experiments. A total of 200 strips were sealed in glassine bags (Westvaco, Envelope Division) and placed in poly bags containing Drierite™ desiccant (Fisher Scientific) for shipment to the irradiator. Dessicant was used to prevent excess moisture, which may lead to spore germination. Thus, a set of 20 strips was exposed to each type and dose of radiation. The administered doses ranged from 2 to 17.8 kGy. After irradiation, sample strips were returned and transferred to sterile 15-ml tubes of SDB. All tubes were incubated at 20–25°C and

Table 3 Summary of D_{10} values for sclerotia from strains 32030 and 14881 and for wild type apothecia

Pyronema strain	Electron beam radiation average D_{10} value (kGy) [SD[a]]	Gamma radiation average D_{10} value (kGy) [SD]	Average D_{10} value (kGy) [SD]
32030 — sclerotia	–	0.73 [0.16]	0.73 [n/a[b]]
14881 — sclerotia	1.34 [n/a]	0.83 [0.11]	1.09 [0.36]
WT — apothecia	2.66 [0.34]	2.99 [n/a]	2.83 [0.23]

[a]SD=standard deviation.
[b]Not applicable.

observed periodically for growth up to 28 days. All questionable growth-positive tubes were streaked to Claussen's agar without casein and PDA plates and incubated at 20–25°C for 7 days for strain confirmation.

D_{10} value calculations: The data were tabulated and D_{10} values were determined using the equation, $D = \text{dose}/(\log N_0 - \log N_u)$, which assumes exponential inactivation. This equation includes the Halvorson and Ziegler equation (the MPN equation), $N_u = \ln(n/r)$, which is the probability of the "no survivors term" of the Poisson equation [2]. The calculation of D_{10} values from MPN data is based on the assumption that the survivor curve is a straight line between N_0 and N_u.

Results and discussion

The nonirradiated control discs and test strips, which were sent along with those to be irradiated, showed normal growth upon enrichment. The electron beam data (Tables 1 and 3) indicated that *P. domesticum* strain 32030 revealed no growth in any of the enrichment tubes after processing. The results for strain 14881 led to a D_{10} value of 1.34 kGy. Wild type apothecia were found to be more resistant than sclerotia, with a D_{10} value of 2.66 kGy.

The gamma radiation data (Tables 2 and 3) indicated that the D_{10} value for strain 32030 was 0.73 kGy and the D_{10} value for strain 14881 was 0.83 kGy. Wild type *P. domesticum* apothecia subjected to gamma radiation had a D_{10} value of 2.99 kGy.

Prior literature on *P. domesticum* indicated that sclerotia were more resistant to radiation than the ascospores [10]. The results shown in Table 3 demonstrate that ascospores were more resistant than the sclerotia. The averages of the D_{10} values from the electron beam and gamma irradiation experiments were 0.73 and 1.09 kGy for ATCC strains 32030 and 14881, respectively. Wild type ascospores were more resistant than sclerotia, with D_{10} values of 2.66 and 2.99 kGy for electron beam and gamma radiation, respectively.

These results characterize *P. domesticum* as a highly radiation-resistant organism. The problem of microorganism resistance to ionizing radiation is of great importance to the medical device industry. Since *P. domesticum* was found on cotton products, analysis of these products for this organism is essential for declaration of sterility by the manufacturer. The average of the D_{10} values for *P. domesticum* ascospores (2.83 kGy) is substantially higher than the D_{10} value of 1.7 kGy for *Bacillus pumilus* spores, which were formerly used as one method for validating the radiation sterilization process. The current standard for assuring radiation sterilization of medical devices, ISO 11137, does not support the use of biological indicators for this purpose [6]. ISO 11737 [7]-directed bioburden testing and sterility testing may not address the use of optimum culture conditions for *P. domesticum*. Standards for sterilization of medical products, especially those involving foreign cotton, should be revised so that *P. domesticum* is routinely included in bioburden testing. This would require the use of alternate media, longer incubation times, and a range of incubation temperatures. In addition, further work should be done to determine the ethylene oxide resistance values of this organism.

The US FDA now recommends that companies perform sufficient and thorough validation studies for every sterilization cycle used, and that bioburden focus should include bacteria, yeasts, and molds [8] such as *P. domesticum*. These recommendations on *P. domesticum* product testing are important in terms of determining the product bioburden and using appropriate resistance models to provide sterility assurance.

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