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Effectiveness of Sodium Hypochlorite against Spores of *Penicillium brevicompactum* in an Insect Rearing Facility

Peter M. Ebling 2007

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Ebling, Peter M
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Cover photo: Colonies of the fungus *Penicillium brevicompactum* growing on a plate of Sabouraud Dextrose Agar. (Photo by P. Ebling).

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ABSTRACT

The effectiveness of household bleach for reducing spore viability of the fungus *Penicillium brevicompactum* was evaluated for use in a multi-species insect rearing facility. Minimum exposure time to various bleach concentrations for the elimination of spore viability of this fungus is reported here. Viability was determined after exposure to 1.0, 5.7 and 10% bleach (i.e., 0.053, 0.299 and 0.525% sodium hypochlorite, respectively) over a range of contact times up to 30 min. Although we confirmed that the manufacturer's recommendation of a 10 min exposure time to 5.7% bleach (0.299% sodium hypochlorite) deactivated these fungal spores, successful results were also achieved with other concentrations and exposure times. Total elimination of spore viability required contact times of 30, 4 and 2 min at concentrations of 1.0, 5.7 and 10.0% bleach, respectively. Insect producers can use these baseline data to determine the level of disinfection that is achievable when the maximum contact time and bleach concentration, which is tolerated by the life stage of the insect species being treated, has been selected.

Keywords: bleach, insectary, *Penicillium brevicompactum*, sanitizer, sodium hypochlorite, spore viability.

RÉSUMÉ

La capacité d'un javellisant à usage ménager de réduire la viabilité des spores du champignon *Penicillium brevicompactum* a été évaluée dans une installation d'élevage d'insectes plurispécifique. La durée d'exposition minimale à diverses concentrations de javellisant requise pour inactiver les spores de ce champignon est indiquée. La viabilité des spores a été déterminée après exposition des spores à des concentrations de 1,0, 5,7 et 10 % de javellisant (ou 0,053, 0,299 et 0,525 % d'hypocrite de sodium, respectivement) pendant une période pouvant atteindre 30 minutes. Notre évaluation a confirmé l'allégation du fabricant selon laquelle une exposition de 10 min à une solution à 5,7 % de javellisant (0,299 % d'hypochlorite de sodium) inactive les spores de ce champignon, mais elle a aussi démontré l'efficacité d'autres concentrations et durées d'exposition. L'inactivation complète des spores a été observée après une exposition de 30, 4 et 2 minutes à des concentrations de 1,0, 5,7 et 10,0 % de javellisant, respectivement. Les producteurs d'insectes pourront utiliser ces valeurs de référence pour déterminer le niveau de désinfection optimal après avoir établi la durée d'exposition et la concentration de javellisant maximales tolérées par le stade de développement de l'insecte traité.

Mots-clés : javellisant, insectarium, *Penicillium brevicompactum*, désinfectant, hypochlorite de sodium, viabilité des spores.

INTRODUCTION

Some fungal species that were formerly thought to be innocuous are now recognized as producing pathogenic mycotoxins. *Penicillium brevicompactum*, commonly found as the primary colonizer in water- or moisture-damaged buildings, has been reported to produce carcinogenic, teratogenic and immunotoxic mycotoxins (Murphy et al. 2006). During periods of high humidity in our multi-species insect rearing facility, this fungus has become a serious impediment to various life stages of some of our colonies.

Our facility does not have the capability to sufficiently filter the incoming air to remove contaminants, nor do all areas have sufficient humidity control to maintain sub-optimum environmental conditions for this fungus. This becomes particularly problematic where insects are maintained in closed rearing containers, along with artificial diets or other substrates conducive to fungal growth. Holding insects on diet in closed containers results in condensation and often increases disease occurrence (Steinhaus 1953).

Although all life stages of our colonies are always handled aseptically within biological safety cabinets to minimize the introduction of microbials, our synthetic diet-making operation is potentially exposed to air-borne fungal spores that may be unintentionally propagated within our rearing systems. Our diets not only provide nutrients essential to the growth of insects but to the growth of *P. brevicompactum*. Chemical preservatives and inhibitors are routinely added to our diets to reduce the incidence of this fungus, but total elimination is difficult to achieve while maintaining nutritional quality for the insects.

To further minimize or eliminate microbial contaminants, we employ strict sanitation measures including routine disinfection of facilities, equipment and various life stages of our insects using dilutions of household bleach (Javex® brand, Colgate-Palmolive Canada Inc.; 5.25% sodium hypochlorite). Sodium hypochlorite is one of the most commonly used anti-microbial chemicals because of its broad anti-microbial spectrum, good solubility in water, stability in aqueous solutions, availability and low price (Ignoffo and Dutky 1963). It is an ideal disinfectant that offers maximum anti-microbial efficacy without harm to insects when used at appropriate concentrations and duration of exposure. Bleach is used in our facility at dilutions of 1.0, 5.7 and 10% v/v (i.e., final sodium hypochlorite concentrations of 0.053, 0.299 and 0.525%, respectively) for 10 min durations for multiple decontamination procedures, but there is insufficient data on the efficacy of this product against *P. brevicompactum*. Due to the recent rise in the incidence of *P. brevicompactum* in the egg hatching pans of our spruce budworm (*Choristonerua fumiferana*) colony, evaluation of the effectiveness this anti-microbial chemical against this fungus was initiated. Because our rearing facility produces and maintains a variety of insect cultures, all of which may be affected by the presence of *P. brevicompactum*, it was necessary to establish the minimum contact time required to achieve complete surface sterilization using concentrations of sodium hypochlorite that are tolerated by specific insect life stages. A plethora of publications identify bleach concentrations and exposure times that are antagonistic to *P. brevicompactum* in an array of applications, however, spore viability over contact time has not previously been reported.

MATERIALS AND METHODS

Isolation of *P. brevicompactum* spores

A culture of *P. brevicompactum* was initiated by scraping a small quantity of mycelium and spores from the surface of contaminated spruce budworm eggs using an inoculation loop. The mycelium and spores were streak plated onto solid isolation media [Difco™ Sabouraud Dextrose Agar (SAB)] that supported the growth of fungi. These fungi initially grow in isolated colonies that do not overgrow one another, making them easy to isolate and quantify. To ensure that a pure culture was developed, an individual colony was selected and passaged five times using this technique before identification by an independent contract laboratory (EMC Scientific Incorporated, Mississauga, Ontario). Plates were maintained at 28°C and 30%RH.

Fungal spores used in three replicated tests were collected from the seventh, eighth and ninth passage of the pure culture, respectively, nine days after inoculation of the isolation media. Fungi were scraped from the agar, re-suspended in distilled water and spores disassociated from mycelium by vortexing for 1 min before quantification using a stained film method (Wigley 1980). The stock suspensions were standardized by dilution with water to a concentration of 4 x 10^7 spores ml^{-1}, since preliminary tests indicated that 500µl at this concentration was sufficient to yield a pellet that was visible to the eye after centrifugation. Twenty-four aliquots of 500µl (i.e., 2 x 10^7 spores in each) were prepared from each replicated stock suspension, seven for treatment with each of three sodium hypochlorite concentrations and three for positive controls. Spores were separated from the mixtures by pulse centrifugation (16,000 x g for 5 seconds), followed by siphoning and discard of the supernatants. Mean recovery efficiency was determined for each replicate by post-centrifugation quantification (Wigley 1980) of spores from three positive controls. These were examined microscopically to ensure that potentially viable mycelium and fragments had been removed. The viability of the spores recovered was determined using a pour plating method (see below). Spores were used for testing within 4h of removal from the stock culture plate and suspension in water.

Test concentrations of Sodium Hypochlorite

The survival of *P. brevicompactum* spores was determined after exposure to 1.0, 5.7 and 10% v/v bleach (i.e., 0.053, 0.299 and 0.525% sodium hypochlorite, respectively) over a period of 30 min. The pH of each bleach solution was 10.41, 11.21 and 11.41 (same order). These dilutions reflect the concentrations currently used for a range of activities within our multi-species rearing facility. One or 10% dilutions are used for the surface sterilization of several life stages of various insect species, depending on the tolerance of that insect/stage to sodium hypochlorite. Routine sanitation of the facility and pieces of equipment that cannot withstand autoclaving is performed using 5.7% bleach, as recommended by the manufacturer for general disinfection. Preliminary tests indicated reduced insect survival upon exposure to higher concentrations of sodium hypochlorite, therefore, only the dilutions currently used in the rearing facility are examined here.

Exposure times

To determine the minimum amount of time required for complete inactivation of *P. brevicompactum* spores, a range of exposure times was selected for each test concentration of bleach. Preliminary tests indicated that almost all inactivation occurred within minutes of exposure, therefore, samples were taken extensively during the first 10 minutes. Only two of the spore pellets were exposed for longer durations to ensure that the time for complete inactivation could be determined, particularly when low concentrations of bleach were used. Contact times chosen were 1, 2, 4, 6, 8, 10, 20 and 30 min.

Treatment of spores with bleach

Pellets consisting of 2 x 10^7 fungal spores were re-suspended in 1ml of each bleach test solution (i.e., 1.0, 5.7 or 10% dilutions), mixed by inversion to ensure full contact, then incubated at room temperature (22±1°C) for the required duration of exposure (i.e., 1-30 min). These samples were mixed by inversion at least twice during incubation period, then pulse centrifuged (16,000 x g for 5 seconds), followed by siphoning and discard of the supernatants. Pellets were washed immediately with distilled water to remove residual quantities of bleach, then pulse centrifuged. The resulting pellets were re-suspended in 1ml distilled water. Positive controls were treated similarly, with the exceptions that distilled water replaced the bleach and there was no incubation period. The quantity of viable spores in each of the samples treated with bleach was assumed to be equivalent to the mean of the three positive controls for the respective replicate.

Plating and quantification

To facilitate the determination of the number of viable spores remaining after treatment, four 10-fold serial dilutions were prepared from each sample and plated on SAB using a pour plating method (i.e., incorporation within the agar). Ten microlitres from each 10-fold dilution and un-diluted sample (i.e., maximum of 2 x 10^5 viable and non-viable spores) were added to a sterile disposable Petri plate; 10ml SAB (46±2°C) were then added and the mixture was gently swirled to evenly distribute the spores. Preliminary testing (not reported here) indicated that this agar temperature did not affect spore viability, nor were significant differences found between counts when using pour, spread or drop plate quantification methods. Plates were sealed and maintained at 28±1°C and 35±5%RH. Two negative controls were prepared for each replicate to evaluate the sterility of procedures and materials, one consisting of SAB only and the other consisting of SAB inoculated with 10µl of the same batch of water that was used for preparing spore suspensions and dilutions of bleach.

Cumulative counts of the number of fungal colonies observed on dilution plates derived from each treatment and exposure time were determined 3, 4, 5 and 6 days post inoculation (p.i.). Only those plates with no fungal growth 6d p.i. were examined again on day 10 to ensure that slow developing spores were not missed. Plates were viewed under 10x magnification and only those with fewer than 100 colonies three days p.i. were quantified, thereby optimizing the accuracy of the counts. Mean counts were determined from a minimum of two of the serial dilutions from each treatment and exposure time. To eliminate the inclusion of colonies derived from secondary infection, cumulative counts were suspended when aerial hyphal development began.

RESULTS

Although the recovery efficiency of spores from the solid isolation media averaged 82.5±13.5, 53.1±10.1 and 38.5±8.2% for the three replicates, the primary goal of separating spores from mycelium and fragments by pulse centrifugation was achieved. Microscopic observation of stained preparations revealed that the spore suspensions used for testing had only trace amounts of mycelium fragments, which were too few to enumerate.

The number of fungal colonies growing on each plate was maximized either 4 or 5d p.i., with aerial hyphal development evident on some plates by 6d. No colonies were observed 10d p.i. on plates that had zero counts 6d p.i., thereby confirming that slow developing spores were not overlooked in the earlier counts. Negative control plates for each replicate did not show signs of fungal growth, thereby validating the sterility of procedures and materials used.

Spore viability prior to treatment with bleach, determined by averaging the number of fungal colonies on the positive control plates within a replicate, was found to be 50.4±4.5, 50.2±5.1 and 72.7±8.2% for the three replicates. Mean reduction of spore viability due to treatment with bleach is shown in Table 1. Total elimination of viability was achieved within 30, 4 and 2 min exposure when spores were treated with 1.0, 5.7 and 10.0% v/v bleach, respectively.

Table 1. Mean reduction (percent) of *P. brevicompactum* spore viability due to treatment with bleach.

Concentration of Bleach [a]	Contact Time (min)							
	1	2	4	6	8	10	20	30
1.0%	19.95 (±16.29)	30.33 (±17.49)	54.80 (±17.95)	79.16 (±15.00)	86.03 (±11.56)	95.80 (±4.79)	99.99 (±0.00)	100.0 (±0.00)
5.7%	98.21 (±1.85)	99.99 (±0.01)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)
10.0%	99.98 (±0.03)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)	100.0 (±0.00)

[a]1.0, 5.7 and 10.0% dilutions (v/v) of Javex® bleach contain 0.053, 0.299 and 0.525% sodium hypochlorite, respectively.

DISCUSSION

Any insect life stage may be disinfected to minimize or eliminate micro-organisms (Shapiro 1984). In our multi-species rearing facility, household bleach is used to treat different stages of our various colonies and sometimes multiple stages of the same species, particularly to eliminate the viability of spores from the fungus *P. brevicompactum*. The minimum exposure time to various concentrations of bleach for elimination of spore viability of this fungus has been reported here. Although the manufacturer's recommendation of a 10 min exposure time to 5.7% bleach (0.299% sodium hypochlorite) for general disinfection has been confirmed as successfully deactivating these fungal spores, successful results were also achieved over a range of concentrations and exposure times.

Results indicate that concentrations below that recommended by the manufacturer may yield satisfactory disinfection, however, longer exposure times are required. Insect life stages that can only tolerate low concentrations may drown due to the required submersion time. In some insect rearing applications, life stages that can only withstand short periods of submersion could possibly be treated with higher concentrations of bleach to achieve successful disinfection. However, this may still be detrimental to the insects. For example, treatment of eggs with sodium hypochlorite causes partial dechorionation, resulting in susceptibility to desiccation and mechanical injury (van Frankenhuyzen et al. 2004). Therefore, the effect of bleach treatment on insect survival at any stage of development should be evaluated before establishing the practice operationally. Also, some degree of spore survival must be anticipated when treating sensitive life stages with a suboptimal, albeit tolerable, bleach concentration or duration of exposure.

Life stages that can tolerate neither long periods of submergence, nor high bleach concentrations, may possibly withstand a brief treatment with a low concentration bleach solution where the pH has been lowered. The addition of vinegar to reduce the pH to an acidic value of about 6.8 or lower will raise its antimicrobial efficacy (Feirtag 2007), possibly eliminating spore viability with minimal effect on insect viability. Testing of these intolerant life stages is required.

Now that baseline data have been established to determine minimum exposure times to various concentrations of bleach that are antagonistic to *P. brevicompactum* spores, subsequent testing of its effect can proceed on various life stages of all of the species that we rear.

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