Lat. Am. J. Aquat. Res., 43(4): 755-765, 2015
DOI: 10.3856/vol43-issue4-fulltext-14


*Research Article*

# Isolation and risk assessment of *Geotrichum* spp. in the white shrimp (*Litopenaeus vannamei* Boone, 1931) from culture ponds

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**ABSTRACT.** The present study was done in order to identify the fungus invading some of the supralittoral ponds used for shrimp aquaculture in the CIBNOR facilities in La Paz, Baja California Sur (BCS), México during the summer season. From the walls and bottoms of the ponds, two strains of *Geotrichum* spp. were isolated and morphologically identified. Fungal adhesion towards hemocytes and primary cultures of various white shrimp (*Litopeneaus vannamei*) tissues (gill, tegument, and gut) was analyzed to determine infectivity. Extracellular protease, lipase, and amylase activity were evaluated as virulence factors. Survival of shrimp post-larvae (PL8) exposed to fungal culture supernatant or to their filaments was also investigated. The results showed that shrimp tegument cells and hemocytes were very susceptible to *Geotrichum* spp. invasion, and that this fungus provokes great mortality of post-larvae. Hence, *Geotrichum* spp. could be considered an opportunistic pathogen that might represent a serious health risk to shrimp in culture.

**Keywords:** *Geotrichum* spp., *Fusarium solani*, *Litopenaeus vannamei*, mycotoxins, extracellular enzymes, aquaculture.

# Aislamiento y evaluación de riesgos de *Geotrichum* spp. en el camarón blanco (*Litopenaeus vannamei* Boone, 1931) en estanques de cultivo

**RESUMEN.** El presente trabajo se realizó con el fin de identificar hongos que invaden algunos de los estanques supralitorales utilizados para el cultivo del camarón en la instalación del CIBNOR, en La Paz, BCS, México durante la temporada de verano. De las paredes y el fondo de los estanques se aislaron e identificaron morfológicamente dos cepas de *Geotrichum* spp. Se analizó la adhesividad de hongos hacia cultivos primarios de diversos tejidos (hemocitos, branquias, tegumento, e intestino) de camarón blanco (*Litopeneaus vannamei*) para determinar la infectividad. La actividad de lipasas, amilasas, y proteasa extracelular, fueron evaluadas como factores de virulencia. También se evaluó la supervivencia de post-larvas (PL8) de camarones expuestos a los sobrenadantes del cultivo o filamentos de hongos. Los resultados muestran que las células de tegumento y hemocitos de camarón son susceptibles a la invasión por *Geotrichum* spp. y que este hongo provoca gran mortalidad de post-larvas de camarón. Por lo tanto, *Geotrichum* spp. puede ser considerado un patógeno oportunista que podría representar un riesgo grave para la salud de los camarones en cultivo.

**Palabras clave:** *Geotrichum* spp., *Fusarium solani*, *Litopenaeus vannamei*, micotoxinas, enzimas extracelulares, acuicultura.

## INTRODUCTION

Shrimp aquaculture is at present an attractive economic activity of great impact and commercial importance in Mexico (Gillett, 2008). Unfortunately, disease incidence affects production and commercialization success. Thus, shrimp-farming success depends on the application of procedures aimed at preventing and controlling the presence of pathogenic microorganisms in the ponds. Fungi are considered opportunistic patho-

Corresponding editor: Cesar Lodeiros

gens in aquaculture because they usually affect stressed or immunocompromised animals (Pelczar *et al.*, 2001; Leslie & Summerell, 2006; Madigan *et al*., 2009). Lightner (1996) reported 100% mortalities of shrimp eggs and larvae exposed to *Lagenidium callinectes* while other researchers found that such fungus is also capable of infecting juvenile and adult shrimps in culture (Bertke & Aronson, 1992; Nakamura *et al*., 1994; Khoa *et al*., 2004, 2005; Cruz da Silva *et al*., 2011). Other fungi belonging to the genera *Haliphtrofos* and *Sirolpidium* provoke diseases in cultured shrimp larvae (Noga, 1990); *Fusarium*, on the other hand, is capable of affecting practically all developmental stages of shrimp (Bachere *et al.*, 2000; Bugni & Ireland, 2004). Some toxic strains of *Fusarium* have been found responsible for different epizootic episodes in cultures of *Penaeus chinensis* (Chen *et al.*, 1992), *P. californiensis* (Lightner & Hose, 1984), *P. stylirostris* (Lightner, 1996), *P. japonicus* (Lightner & Hose, 1984; Noga, 1990; Lightner, 1996), and *Litopenaeus vannamei* (Cruz da Silva *et al*., 2011; Lozano-Olvera *et al*., 2012).

Our work is the first report referring to the pathogenicity of *Geotrichum* strains towards American white shrimp *Litopenaeus vannamei.* This yeast like fungus, found in soil, water, and air worldwide is a colonizer of the intestinal tract. It may cause opportunistic infections (geotrichosis) in immunocompromised hosts, which usually acquire it via ingestion or inhalation (Buchta & Otcenasek, 1998). The isolation from walls and bottom of a shrimp pond in La Paz, Baja California Sur (BCS), México was conducted in order to determine the pathogenicity of isolated strains of filamentous fungi that implied a potential risk in shrimp farming success. Pathogenic fungi were assessed by determining their virulence and adhesive capacity on cells in primary culture of white shrimp (*Litopenaeus vannamei*).

## MATERIALS AND METHODS

### Fungus isolation and characterization

During the preparation of experimental ponds for cultivation of *L. vannamei* at Centro de Investigaciones Biológicas del Noroeste (CIBNOR) in La Paz, BCS, México, the presence of white spots of microbial colonies in the walls and bottoms of the ponds were frequently noticed. The isolation of the corresponding microorganisms by common microbiological procedures was done (Hyde *et al*., 2000). The samples, collected with a sterile scraper and poured in 250 µL of glycerol, were plated in PDA medium and incubated at 22ºC for 48 h (Newell, 2001). Purification was done streaking in various Petri dishes containing the same culture media and stored at -80°C and -20°C until use (Hernández-Saavedra, 1990). Microorganism identification was performed by morphological criteria using a Nikon Optihot-2 microscope (Nikon, Japan) according to Pitt & Hocking (1997). Distinctive morphological characteristics for the *Geotrichum* genus were observed (Pitt & Hocking, 1997; Kurtzman & Robnett, 1998; Smith *et al*., 2000). Identification keys were obtained from Tortora *et al.* (2012) and the identification was done only up to genus.

Growth kinetics determination was done using 125 mL Erlenmeyer flasks containing 25 mL of M-1 medium [glucose 2% (w/v), peptone 1% (w/v), yeast extract 0.5% (w/v)], and incubated at 25°C with constant orbital shaking (110 rpm) according to Hernández-Saavedra (1990). The mycelium was recovered from the culture by filtration using Whatman Nº1 paper, washed with distilled water, and afterwards placed in an oven at 80°C for 24 h to get a constant weight. The dry weight of the sample was determined with an analytical balance (Ohaus, AP210S) and plotted against the time of collection. The analysis of enzymatic activity, compared with collection strains obtained from infected shrimp; *Fusarium solani* (ATCC 46940), isolated from *Penaeus japonicus*; and *Fusarium javanicum* (CBS 420.76), isolated from *Penaeus californiensis.*

### Shrimp and primary shrimp tissue culture cells

The white shrimp juveniles (12-14 g) and post-larvae (PL8) were acquired from two local commercial shrimp farms (APSA, La Paz, BCS, México and Acuacultores Marh, La Paz, BCS, México, respectively). Primary cell cultures of different shrimp tissues (tegument, gill, intestine, and hemocytes) were prepared by an enzymatic disaggregation procedure modified from Fuerst *et al.* (1991), Jackson *et al.* (1993), and Alexopoulos *et al.* (1996). Hemocyte culture was prepared from haemolymph obtained by puncture at the pleopod base of the first abdominal segment near the genital pore from juvenile shrimp, with a 1-mL syringe (Hernández *et al.*, 1996). Essentially, aliquots of 100 µL of cell suspension (of $2.4x10^5$ cells $mL^{-1}$) were placed in 96-wells microplate, mixed with 90 µL Leibovitz's L-15 complete medium containing 10% (v/v) of fetal bovine serum (FBS), (Sigma, Chemical Co., St Louis, USA) and incubated at 22ºC in a $CO_2$ incubator (Shel-Lab, VWR 1810) for 16 h to obtain a primary culture. Cell counting in 100 µL aliquots of primary cultures was done with a hematocytometer using an Optiphot-2 microscope under the contrast phase mode. Trypan blue staining (Sigma, Chemical Co., USA), was carried out to estimate cell viability.

### *In vitro* cytotoxicity assay

The *in vitro* cytotoxic assays were done with 100 µL of shrimp tissue cell cultures that were first washed with 250 µL of PBS (137 mM NaCl, 0.2 mM KCl, 1.44 mM $Na_2HPO_4$, 0.24 mM $KH_2PO_4$; pH 7.2), and mixed with 100 µL of a cytotoxic preparation according to Varughese *et al.* (1999). Such preparations consisted of the supernatant and the fungal extract obtained by sonication. The culture fluid supernatants of *Vibrio alginolyticus*, *V. cholera*, and *V. parahaemolyticus*, which are known to be toxic for shrimps, were used as positive controls (Aguirre *et al.*, 2003). The plates were incubated in $CO_2$ atmosphere at 37ºC for 2 h. After, they were washed 3 times with PBS before adding 50 µL of cold methanol and allowed to evaporate under a hood for 2 min. Cell staining was done with 50 µL crystal violet in PBS by letting them standstill for 20 min; then the plates were washed three times with PBS, air dried before adding to each well 200 µL of sodium duodecyl sulfate (SDS) (1 g/50 mL ethanol), and incubated for 20 min. Finally, absorbance at 595 nm was determined in a plate reader (BioRad 3550-UV). Each toxin preparation was evaluated by triplicate with each culture.

### Adhesion assay

The adhesion capacity of the isolated fungi (mycelium and spores) to primary cultures of shrimp tissues and hemocytes was estimated following the procedure described by Guzmán-Murillo & Ascencio (2001). In this case, the mycelium was obtained as recommended by Saha *et al.* (2008) from M-1 broth culture medium (pH 4.5) at 22ºC and constant orbital shaking (110 rpm). A sample was collected on the 5 th day of incubation and adjusted to 1.0 optical density (Beckman DU 640 Spectrophotometer).

The spores were collected from 15 mL assay tubes containing solid M-1 culture medium (pH 4.5) after incubation at 22ºC for 10-12 days and suspended in 10 mL of an aseptic 0.15 M NaCl solution containing 1% (w/v) Tween 60. This mixture was carefully transferred in portions and rotated slowly to a sterile tube. The recovered spores were counted with a hematocytometer, and their viability was evidenced by staining with malachite green.

Biotin labeling of fungal mycelium and spores was done according to Hernández *et al.* (1996). For this purpose, 15 mL of mycelium suspension with an optical density of 1.0 nm and a spore suspension at $1x10^7$ spores-$mL^{-1}$ in bicarbonate buffer (0.1 M de $NaHCO_3$, pH 8.0) were centrifuged at 6,000 rpm at 22°C for 5 min. The supernatant was discharged. Cell sediment was then suspended in 1 mL of bicarbonate buffer and 100 µL of biotin-DMSO (1.3 mg in 1.0 mL). Incubation was carried out at 22°C under darkness with manual stirring every 30 min for 2-3 h. After this period, 9 mL of PBS were added, and the mixtures were centrifuged at 6,000 rpm at 22°C for 20 min. Finally, spores and mycelium were suspended in 2.5 mL of PBS and stored under darkness at 4ºC until use. For the adhesion assay, 100 µL aliquots adjusted at $1x10^4$ spores $mL^{-1}$ were used. The adhesion assay was done in a 96-wells microplate containing the primary cell culture of the various shrimp tissues. The cells were fixed with 100 µL of 2.5% (v/v) glutaraldehyde and rinsed with PBS. To each well, 100 µL of labeled spore or mycelium suspension were added and incubated for 0, 30, 60, and 180 min. The plates were washed 3 times with PBS containing 0.1% (v/v) Tween 20 to eliminate non-adhered cells. Then, 100 µL of streptoavidine-POD (1 µL in 2 mL of PBS) were added to each well, and incubation was carried out at 37°C for 90 min. Finally, the wells were washed 3 times with PBS-0.1% (v/v) Tween 20 and re-suspended in 100 µL of OPD reagent (2 mg OPD, 12 mL sodium citrate plus 5 mL $H_2O_2$). The plates were incubated under darkness at 22ºC for 20 min, and the reaction was stopped by adding 100 µL at 1 M $H_2SO_4$. Absorbance was determined at 490 nm in a plate reader (BIORAD 3550-UV).

### Extracellular enzyme production as virulence factors

The extracellular production of amylase, lipase, protease, and chitinase was evaluated in plates containing M-1 modified medium (glucose 0.4%, peptone 0.2%, yeast extract 0.5% and agar 4%; all w/v). The medium in each case was supplemented with the specific substrate (starch 1% for amylase; 0.5% Tween 80 and 10 mM $CaCl_2$ for lipase; 1% partially hydrolyzed casein for protease; and 3% colloidal chitin for chitinase). The plates were inoculated by puncture with a needle and incubated at room temperature for 48-72 h. Amylase production was considered positive by the appearance of a translucent halo after overlaying 3 mL of fresh lugol (3.3 g Iodine crystals; 6.6. g KI; 1 L of distilled water) on the gel; lipase production was revealed by the formation of a precipitate surrounding the colony; protease and chitinase production was revealed by the appearance of a halo (Pierce & Leboffe, 2011).

### Acute toxicity test on white shrimp post-larvae (PL8)

Shrimp post-larvae (PL8) survival was evaluated under two conditions: (a) exposure to mycelium suspension; and (b) exposure to culture supernatant. In the first case, the isolated fungi were grown in 25 mL of liquid M-1 medium in 125 mL Erlenmeyer flasks, at 22ºC for 3

days and under constant orbital shaking (110 rpm) to reach the logarithmic phase. The cell suspension was adjusted to different optical densities (0.1, 0.25, 0.5 and 1.0) at 540 nm with a 0.85% NaCl solution. In the second bioassay, the culture was incubated at 22°C and constant orbital stirring (110 rpm) for 10 days (stationary phase).

The cultures were centrifuged at 10,000 rpm (Beckman J2-HS centrifuge) at 4°C for 10 min, to obtain both the supernatant and the pellet. To assess survival of post-larvae (PL8) exposed directly to the supernatant and mycelium. Before bioassay, the shrimp post-larvae (PL8) was collected in plastic bags with seawater for to acclimate at 22°C for 2 h. Specimens (20) were placed in a 6-well polystyrene plate with flat bottom (BD Falcon) containing 5 mL sterile seawater. To each well, 8 mL of the mycelium suspension of different optical densities and of the culture supernatant were added. The final volume in each well was adjusted to 15 mL with sterile seawater, and a coverlid was applied. The controls were prepared replacing the cell suspension and supernatant by sterile seawater. Observations were done with a stereoscope (SP Southern Precision 1839) at 5x magnification during a 24-h period every 2 h as described Sainz *et al.* (1998). All assays were performed in triplicate

### Statistical analysis

All data were normalized using their corresponding logarithms and ANOVA analysis was performed two-way. Type of cell culture and toxins were considered assuming absorbance as a dependent variable. Normalization was done by the Kolmogorov-Smirnov analysis and homoscedasticity by the Bartlett test. Whenever significant differences were found, the Tukey analysis was performed (Zar, 1996).

## RESULTS

### Fungus identification

The samples collected from CIBNOR´s shrimp ponds yielded two different yeast-like fungal strains. Both isolates showed similar morphological features with white, dry, and dusty colonies (http://www.doctor-fungus.org/thefungi/Geotrichum.htm). Hence, the Isolated strains were designed as *Geotrichum* sp. 1 (Gsp. 1) and *Geotrichum* sp. 2 (Gsp. 2). Table 1 summarizes the properties of Gsp. 1 and Gsp. 2. In Gsp. 1 and Gsp. 2 growth kinetics was similar (Fig. 1), which allowed us to employ similar incubation time for cell biomass preparation in both cases. Because the start of the exponential phase was observed very early between the first and the second day of incubation, and the stationary

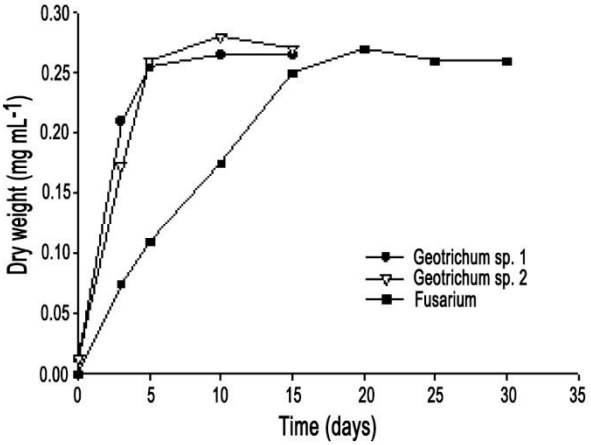


**Figure 1.** Growth kinetics of *Geotrichum* sp. 1, *Geotrichum* sp. 2 and *Fusarium solani*.

phase was reached after 7 days, it was decided to carry out mycelium collection at day 3, whereas the cell biomass was collected at day 20. It is important to mention that by adjusting growth to the equation: $y = K\cdot (1+Ae^{-bx})^{-1}$, an $R^2 = 0.9926$ was found for Gsp. 1 and an $R^2 = 0.9949$ for Gsp. 2; hence, a difference in growth rate between the two isolates became apparent, where Gsp. 1 was faster than Gsp. 2. In consequence, at least with regards to growth rate, Gsp. 1 and Gsp. 2 showed different properties, and thus they may correspond to different strains.

### *In vitro* cytotoxicity of *Geotrichum* strains on shrimp tissues

The various shrimp tissue cultures tested showed a distinct susceptibility to the extract or the supernatant of each fungal strain ($P < 0.001$). Hemocytes were more sensitive than tegument, intestine, and gill cells (Fig. 2). Interestingly, Gsp. 1 and Gsp. 2 toxicity was higher than the preparations obtained from *V. alginoliticus, V. parahemoliticus,* or *V. cholera* but lower than the *Fusarium* strains. Primary cell cultures of gill and intestine showed no differences ($P > 0.05$) with regard to their susceptibility towards the fungal extracts, and they were more affected than the tegument tissues. In general, no differences were observed between fungal extracts and their corresponding supernatant with primary shrimp tissue culture gill and intestine cells, which were also the least affected.

### Fungal adhesiveness

*Geotrichum* sp. 1 and *Geotrichum* sp. 2 showed a higher tendency to adhere to hemocytes than to other shrimp tissues (Fig. 3). However, some differences in adhesion between spores and filaments were observed. The spores were always less adhesive than the corres-

**Table 1.** Properties of *Geotrichum* sp. 1 and *Geotrichum* sp. 2 isolated from shrimp ponds in Baja California Sur, Mexico. *Strain used as reference.

| Characteristics | *Geotrichum* sp. 1 | *Geotrichum* sp. 2 | *Fusarium solani** |
|---|---|---|---|
| Colony diameter | > 45 mm | > 45 mm | 60-65 mm |
| Colony color (Stationary phase) | Black | Black | White-cream |
| Mycelium | Cottonish, hyaline | Cottonish, hyaline | Cottonish |
| Spore type | Arthrospore; cylindrical 3-6 x 6-12 µm | Arthrospore; cylindrical 3-6 x 6-12 µm | Macroconidia (half moon shape) 3-4 conidias |
| Type of hypha | Septed | Septed | Septed |

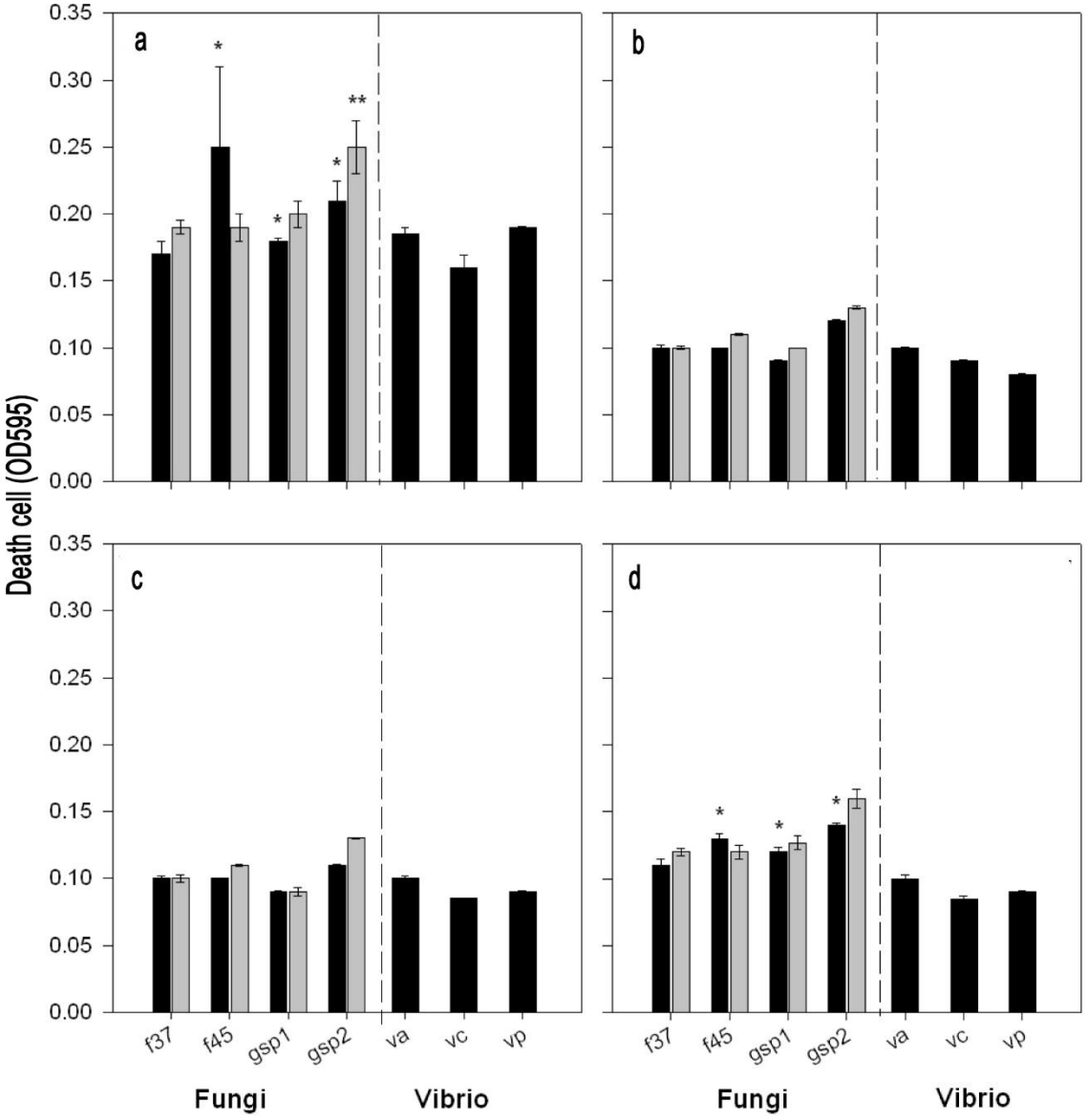


**Figure 2**. Cytotoxicity of the supernatant (black bars) and sonicated extracts (grey bars) of *Geotrichum* sp. 1, *Geotrichum* sp. 2, *Fusarium solani* (F45), *Fusarium javanicum* (F37), *Vibrio alginolyticus* (Va), *Vibrio cholera* (Vc), and *Vibrio parahemolyticus* (Vp) against primary cell cultures of a) hemocytes, b) gills, c) intestine, and d) tegument of *Litopenaeus vannamei*. Each point represents the mean of three experiments; bars indicate SD.

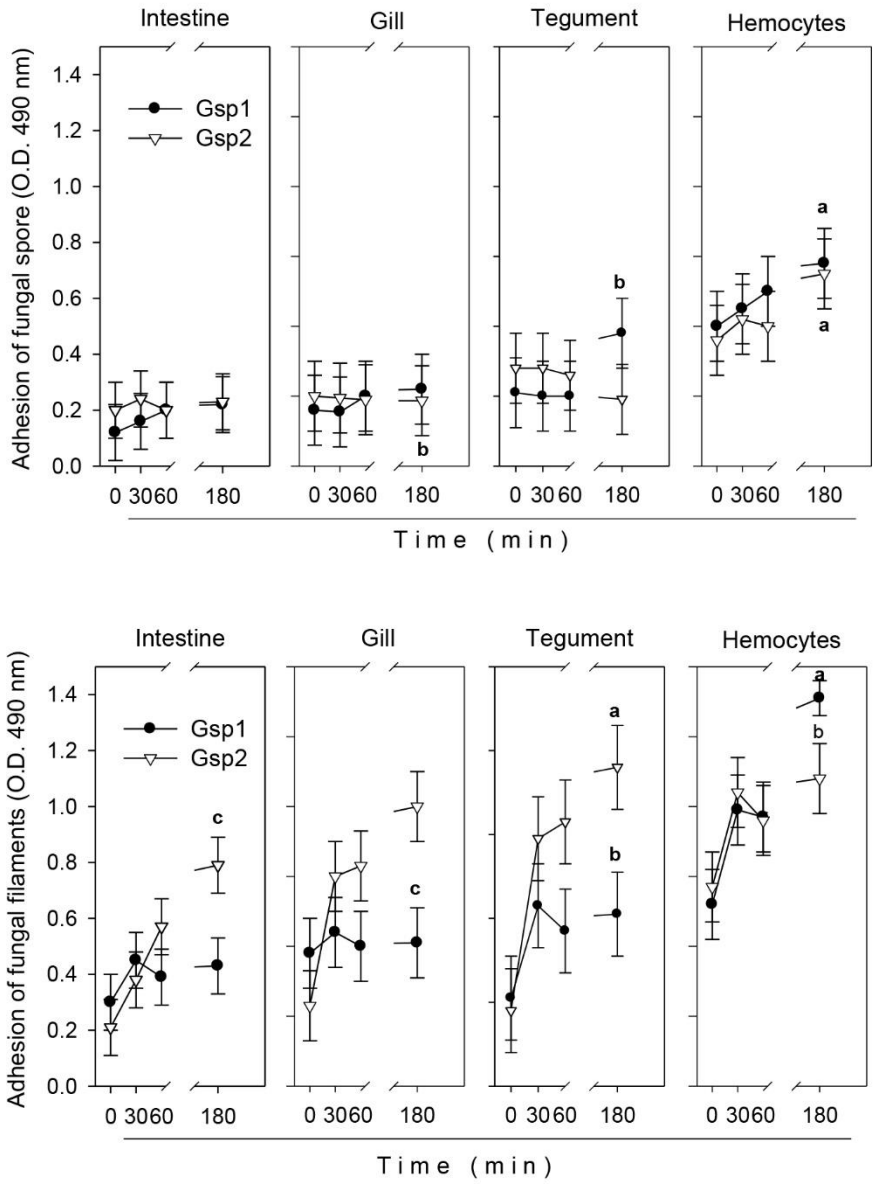


**Figure 3.** Adhesion time-kinetics of *Geotrichum* sp. 1, *Geotrichum* sp. 2 spores (top panel), and filaments (bottom panel) to primary cell cultures of intestine, gills, tegument, and hemocytes of *Litopenaeus vannamei*. Each point represents the mean of three experiments; bars indicate SD.

ponding filament stage, indicating some advantage for the multiple-point attachment that a filament can exert. Also, a significant increase in adhesion tendency was observed with elapsed time. No difference in spore adhesion between the strains was observed, but their corresponding filament stages showed different attachment abilities, where Gsp. 2 was more adhesive than Gsp.1 ($P < 0.001$; Fig. 3).

### Toxicity study on shrimp post-larvae (PL8)

*Geotrichum* sp. 1 supernatant at a concentration of 0.1 optical density (O.D.) caused 25% post-larvae (PL8) mortality after 7 h and total loss at 20 h. Lower doses were innocuous. On the other hand, the culture supernatant of *Geotrichum* sp. 2 showed a similar effect but at earlier times, 25% mortality after 5 h and total loss at 16 h. It was noted that the toxicity is dose-dependent as it is observed that post-larvae mortality tends to be higher with increasing the optical density at 540 nm of the extract. In the case of *Fusarium* strains, the supernatant caused 100% mortalities after only 2 h (Table 2).

### Fungi extracellular enzymatic activity

The strains *Geotrichum* sp. 1 and *Geotrichum* sp. 2 produced less amylase than *Fusarium* reference strains.

**Table 2.** Survival percentage of PL8 white shrimp post-larvae exposed to *Geotrichum* sp. 1, *Geotrichum* sp. 2, and *Fusarium javanicus* CBS, and *Fusarium solani* ATCC culture media supernatants for a 24-h period. Data correspond to averages (Standard deviation).

| Dilution factor | *Geotrichum* sp. 1 | *Geotrichum* sp. 2 | *F. javanicus* | *F. solani* |
|---|---|---|---|---|
| 1 | 0.00 (0.00) | 1.66 (2.88) | 0.00 (0.00) | 0.00 (0.00) |
| 0.5 | 3.33 (2.88) | 6.66 (2.88) | 0.00 (0.00) | 3.33 (2.88) |
| 0.25 | 11.66 (7.63) | 61.66 (16.07) | 16.66 (20.81) | 26.66 (7.63) |
| 0.10 | 15.00 (5.00) | 96.66 (2.88) | 65.00 (8.66) | 71.66 (7.63) |
| 0.01 | 90.00 (13.22) | 96.66 (2.88) | 66.66 (14.43) | 50.00 (45.00) |

**Table 3.** Extracellular enzymes of *Geotrichum* sp. 1, *Geotrichum* sp. 2, *Fusarium javanicum*, and *Fusarium solani* strains. Data correspond to averages. Standard deviation is indicated in parenthesis. *Hydrolysis halo (mm), **Appearance of precipitate.

| Strain | Amylase* | Lipase** | Protease* | Chitinase** |
|---|---|---|---|---|
| *Geotrichum* sp. 1 | 1.36 (0.170) | + | 0.63 (0.050) | - |
| *Geotrichum* sp. 2 | 0.27 (0.075) | + | - | - |
| *Fusarium javanicum* CBS | 2.83 (0.170) | + | 1.83 (0.038) | - |
| *Fusarium solani* ATCC | 1.53 (0.050) | + | 3.16 (0.340) | - |

All strains tested produced lipase. Strains *Geotrichum* sp. 2 not produce protease, and none of the tested strains, produced chitinase (Table 3).

## DISCUSSION

As pointed out, from the walls and bottoms of ponds utilized for shrimp culture at CIBNOR, we isolated 2 *Geotrichum* strains using M-1 marine culture medium that favors the growth of marine yeasts and fungi (Deacon, 2005). The isolated strain, were grown on PDA medium prepared with distilled water, which suggests that it may be regarded as facultative marine fungi strains, and were identified as members of *Geotrichum* genus. Their growth characteristics were at temperatures in the ranges 25-30°C, and both strains were capable of growing with very low oxygen tension but not under anaerobic conditions (Pitt & Hocking, 1997).

All pathogenic microorganisms possess some attributes known as virulence factors by which they can invade and cause damage to host organisms (Atlas, 1995). Virulence depends to a large extent on two properties: Invasion capacity and toxin production. Invasion refers to the capacity of the microorganism to adhere to host tissues, attack the cells, and proliferate inside the tissues causing an infection. Toxicity refers to the ability of the microorganism to produce toxins capable of altering the normal function of cells or tissues and/or destroy them. Some toxins are secreted outside the host and cause severe damage when they penetrate the body (Atlas, 1995). Based on these facts, the virulence of Gsp. m1 and Gsp. 2 towards various shrimp primary cultures and hemocytes was demonstrated, showing that both have a significant cytotoxic effect, especially against hemocyte and tegument cells (Fig. 2). This finding is important because hemocytes are known to play an important role in shrimp defense mechanism (Bachere *et al.*, 2000; Vargas-Albores & Yepiz-Plasencia, 2000), being the first line of cells that detect invading microorganisms, and their response or reaction may determine the susceptibility of the organism towards infection (Bachere *et al.*, 2000). Adhesion as a virulence factor has been studied in other pathogens because it is known that through this mechanism colonization and infection development is facilitated (Rhem *et al*., 2000; Krachler *et al*., 2011). Other reports have focused on the nature of the host-pathogen interaction and have identified the corresponding host receptors to which the pathogens show affinity (Guzmán-Murillo & Ascencio, 2001; Wiles *et al.*, 2008). In this case, we observed that the filaments of both Gsp. 1 and Gsp. 2 strains showed a significantly higher adhesion capacity ($P < 0.001$) than that the spores (Fig. 3).

Such difference may be attributed to the fact that filaments are developing structures that help the fungus to attach to different substrates through multiple contacts, while spores are reproductive structures aimed to warrant species conservation rather than to facilitate attachment to a host surface (Tortora *et al.*, 2012). Alexopoulos *et al.* (1996), for instance, suggests

that fungal spores may require from several hours to various days to germinate and produce infection under proper conditions, which is in agreement with our results. Lozano-Olvera *et al.* (2012) have reported that the filaments of *Fusarium solani* cause death by gill blockage and the resulting melanization, making the gill to appear black, causing the death by asphyxia (Lightner, 1996; Lignot *et al.*, 2000; Nosanchuk *et al.*, 2002; Pantoja & Lightner, 2008). In the case of the crab *Astacus leptodactyles*, Lignot *et al.* (2000) observed that *F. oxysporum* hyphae produced black spots in the gills after a 36-h exposure, which was attributed to melanization; on the other hand, Arala-Chavez & Sequeira (2000) observed an increased hemocyte proliferation in *Penaeus monodon* and *Drosophila* using various fungal antigens. In our case, exposure of *L. vannamei* post-larvae (PL8) to Gsp. 1 and Gsp. 2 filaments produced mortality within the 24 h (Table 2), and perhaps in this short interval melanization could not occur (Lignot *et al.*, 2000). Johansson *et al.* (2000) reported in shrimp hemolymph, the presence of a protein which specifically binds β-1,3 glucan in response to a fungus infection. Apparently, this protein triggers the shrimp immune system designed to combat the infection that also agrees with the results of our experiments in which the filaments reacting with hemocytes may contribute to accelerate shrimp response against fungal infection.

Because fungi are unable to produce their own food, they are prepared to ingest nutrients from the surrounding medium making use of several extracellular enzymes that degrade large molecules into smaller and more assimilated compounds. Hence, extracellular enzyme production may favor the infection process. Among the enzymes produced by fungi for this purpose, lipases and amylases are the most common (Bugni & Ireland, 2004). In our study, we observed that both Gsp. 1 and Gsp. 2 strains produce extracellular lipases, amylases, and proteases (Table 3), which could promote colonization as it occurs in the case of *G. candidum* (Mukkerjee & Kiewitt, 1996). Nakamura *et al.* (1994) also confirmed the production of polygalacturonase as a mechanism of citrus infection and the development of sour rot in fruits by *Geotrichum*.

Finally, we observed that when post-larvae (PL8) are in contact with the culture supernatant of Gsp. 1 and Gsp. 2 strains, shrimp started to die after 2 h and up reaching a total mortality within 20 h (Table 2). This observation is similar to those made with other crustacean eggs and post-larvae (PL8) exposed to filamentous fungi (Noga, 1990; Nakamura *et al.*, 1994). In particular, *Lagenidium*, *Haliphthoros* and *Sirolpidium* produce severe mycosis in shrimp protozoa and mysis with 100% mortality after only 1-2 days. Also *Fusarium* strains tested produced 100% mortality after only a 2-h exposure, which could be attributed to the excretion of potent toxic secondary metabolites (Nelson *et al*., 1995); in the case of *Geotrichum*, ammonia excretion could be toxic to shrimp (O'Donnell, 1996; Aldarf *et al*., 2002; Bugni & Ireland, 2004). Le Moullac & Haffner (2000) consider that ammonia is very toxic to aquatic organisms and found that the number of hemocytes is severely reduced in shrimp exposed to 3.0 mg $L^{-1}$ of ammonia. The dose at which a pathogen may cause damage to a given host is of extreme importance (Atlas, 1995). In our case, mortality increased with higher concentrations of the fungus cells or supernatant (Table 2). In the present study, it was possible to show that *Geotrichum* species may indeed constitute a serious threat in shrimp culture, and that monitoring and good management practices are the only strategies that could reduce the risk of collapse and total culture loss. Isolated fungal strains, and partially identified as *Geotrichum* sp. 1 and sp. 2, produce proteolytic enzymes (Table 3), and these filamentous fungal strains were cytotoxic for primary shrimp cell culture, where hemocytes showed greater susceptibility followed by tegument cells. In comparison, the adhesion of spores and filaments of *Geotrichum* fungi to primary cultured cells was higher in hemocytes than in any other cell types tested. Shrimp postlarval PL8 exposed to *Geotrichum* filaments showed mortalities in a dose-dependent manner. Based on our results, we can suggest that isolated strains of *Geotrichum* may represent a health risk for white shrimp culture. It should be noted that *Getrichum* is a saprophytic fungus that develops in decaying organic matter and that its presence can be avoided if proper measures are taken to minimize the unfavorable conditions at the bottom and the water column at pound; thus, having optimal conditions in the cultures, the chances of that organisms be susceptible to infection decreases.

It is necessary to mention that micro-flora at ponds are closely associated with trophic conditions, ecological factors and physic chemical parameters; so it has been considered, that the presence of yeast structures in the digestive tract of organisms in culture or in aquatic environment can be used as a tool for monitoring environmental quality to be a valid instrument for the assessment of eutrophication of the environment. Thus, the presence of these microorganisms can be used as a bio-marker that allows us to assess environmental changes; correlating either genus recovered from various species in different environmental conditions, taking into account the presence of

pollution sources; or, by evaluating the phenotypic changes in organisms recovered disturbed habitats (Coelho *et al.*, 2010; Brilhante *et al.*, 2012).

## ACKNOWLEDGEMENTS

This work was part of NOA's M.Sc. thesis. We thank Drs. Francisco Magallón & Guillermo Portillo, CIBNOR for introducing us to this problem. We also acknowledge the assistance and material provided by Dr. Hector Nolasco to carry out the enzyme test and to Diana Dorantes for editorial services.

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*Received: 22 October 2013; Accepted: 10 July 2015*