RESEARCH

Cumulative mortalities in white leg shrimp, *Litopenaeus vannamei* Boone 1931, cultured in biofloc system in Egypt reflected new record of *Fusarium verticillioides* infection

Mortada M. A. Hussein[1] · Walid H. Hassan[2] · Nadia N.A. Abd El-Ghany[3] ·
Nourhan H. Ghanem[3]

Abstract

The Egyptian aquaculture sector is still struggling to establish *L. vannamei* culture. In an effort to stimulate shrimp production with minimal environmental impact and less water loss, biofloc technology (BFT) has been employed. Chronic mortality was recently observed in *L. vannamei* which was raised in the BFT system. Examining samples of moribund shrimp revealed sporadic melanization on the musculature and gills, suggesting the potential existence of black gill disease. The present work was carried out to isolate and identify potentially implicated fungus species based on mycological properties of interest. To isolate and serve as initial identification tools, wet mount preparations and colonial features, along with slide culture techniques, were used. PCR assays, gene sequencing, and phylogenetic analyses were used in the molecular identification process to clarify the confirmation of the fungal isolates that were acquired. The pathogenicity tests were designed to fulfil *Koch's* postulates in addition to clarifying the infectivity of isolated fungus. Moreover, histopathological examination was employed to notice tissues' alterations associated with experimentally infected shrimp. The results revealed the isolation of three fungal isolates, namely, BNS 1117, BNS 2117, and BNS 3117, that were confirmatively identified as *Fusarium verticillioides*, and their selected sequences were submitted to *Genbank*. Moreover, experimental infection using *F. verticillioides* selected isolate (BNS 31,117) at both high (5×10^5 CFU/mL, group A) and low (3×10^5 CFU/mL, group B) doses of its conidial suspensions experienced cumulative mortality of 72.2% and 21.1%, respectively. Besides, the selected isolate was re-isolated from the experimentally infected shrimp in a pure form, which satisfied *Koch's* postulates. Histopathological alterations demonstrated several degrees of either degenerative, oedema, or proliferative changes in the hepatopancreas, musculatures, and gills, respectively. This study sets a new record for *F. verticillioides* to be included in *Fusarium* species linked to shrimp mortality.

Keywords Biofloc · Chronic mortality · *L. vannamei* · *F. verticillioides*

Handling Editor: Brian Austin

Extended author information available on the last page of the article

Introduction

White leg shrimp, *Litopenaeus vannamei*, is the most popular cultured species worldwide as it has a better resistance to white spot syndrome virus (WSSV) and might be considered the most causative agent of catastrophic shrimp episodes (Benzie 2009). *L. vannamei* comprises more than 90% of the farmed shrimp production in the Western Hemisphere (Pazir et al. 2011). In China, white leg shrimp has been successfully cultured in freshwater since 2000 due to the continuous development of farming techniques (Ding et al. 2014) to reach an annual freshwater aquaculture production of over 591,000 tons in 2017 (Ministry of Agriculture and Rural Affairs of China, 2018). Ecuador and Viet Nam saw double-digit growth in harvest volumes in 2022, with Ecuador exceeding 1 million tons of *L. vannamei* production (FAO 2023). Egyptian aquaculture sector is still struggling to establish *L. vannamei* culture; however, trials were recorded in pond culture at coastal areas facing Mediterranean piscine. Besides, aquaponics and biofloc might share in the production on small scales through the private sector. The estimated Egyptian total yield shrimp production was 8614 and 2164 tons in 2020 for fisheries and cultured *L. vannamei*, respectively (GAFRD 2021). Globally, farmed shrimp production dropped in 2020 as a result of COVID-19 pandemic, however, setbacks to increase in 2022 and reached a new high record of 9.4 million tons over half of them accounted for *L. vannamei* shrimp (FAO 2023).

To stimulate shrimp production worldwide, biofloc technology (BFT) has recently been employed for several reasons, including the nutritional value of floc particles themselves, which practically reduces feeding costs. Additionally, BFT enhances shrimp health and physiological functions as a natural probiotic increasing biosafety on farms (Avnimelech 2009). Besides, biofloc becomes an additional feed source for shrimp, decreasing the feed conversion ratio of commercial feeds and thus reducing costs one more time (de Almeida et al. 2021). Moreover, BFT has a minor environmental impact with less water loss (El-Sayed et al. 2021; Manan et al. 2023). Several factors control the BFT system, including dissolved oxygen (DO), temperature, mixing of water, carbon/nitrogen (C/N) ratio, feeding ratio, and pH level, which are considered the most important ones affecting the success of the BFT system (Khanjani et al. 2022). Part of those factors is the pH level that keeps the beneficial bacteria in the system working in an equilibrium manner. The pH shifts to the acidic side that might cause softening of the shrimp shell, along with mitigation of mucus production on the gills (González-Vera and Brown 2017). Consequently, the possibility of black gill disease incidence caused by *Fusarium* species increased.

Genus *Fusarium* belongs to the Phylum Ascomycota, Class: Sordariomycetes, Order: Hypocreales, and Family: Nectriaceae. The genus contains more than 1500 species that present pathogenicity for both animals and plants (Arie 2019). Many *Fusarium* species are responsible for black gill disease in cultured shrimp, including *F. moniliforme* (Bower et al. 1994), *F. oxysporum* (Le and Hatai 2005), and *F. solani* (Ishikawa 1968; Khoa et al. 2005; Karthikeyan et al. 2014; Dewangan et al. 2015). From a clinical point of view, the disease signs are also associated with infections with some bacterial species, such as *Vibrio harveyi* and *Photobacterium damselae* subsp *damselae* (Dewangan et al. 2015; Wang et al. 2020). Recently, Frischer et al. (2022) reported that abiotic stressors and nutritional deficiencies might share the characteristic sign of melanized gills in shrimp. The first black gill disease was reported in Japan in cultured kuruma prawn, *Penaeus japonicas*, showing black gills, and *F. solani* was detected; however, subsequently, *F. oxysporum* was accused in the same shrimp species (Le and Hatai 2005).

Recently, chronic mortality occurred in *L. vannamei* which raised in the biofloc system. An investigation of sampled diseased shrimp showed scattered melanization on the musculature and gills, pointing to the possibility of black gill disease occurrence. From mycological aspects of interest, the presented study was aimed at isolating and identifying fungal species that might be incriminated. Additionally, pathogenicity experiments are designed not only to elucidate the infectivity of isolated fungal species but also to satisfy *Koch's* postulates. Moreover, histopathological examination was employed to notice tissues' alterations associated with experimentally infected shrimp.

Materials and methods

Shrimp

Shrimp samples collection

A total of 45 shrimp samples, including freshly dead, moribund, and alive *L. vannamei*, were collected randomly from BFT culture facilitates (fiberglass tanks) and hygienically transported to the wet lab of the Fish Diseases Department, Animal Health Research Institute (AHRI), Cairo, Egypt. Samples were then subjected to parasitological examination, particularly alive ones, following the methodology of Noga (1996). Following laboratory scheme work, bacteriological examinations were carried out to exclude and/or isolate bacterial pathogens that might be incriminated. For such purposes, different cultural media including ordinary, enriched, and specific ones are used. Special considerations were taken for bacterial species that might be shared in the back gill disease, such as *Vibrio* and/or *Photobacterium* species. Accordingly, biochemical identification might be later confirmed molecularly. At virology investigation levels, PCR commercial kits (Bioingentech Ltd., Concepcion, Chile) were used to exclude and/or detect 5-shrimp viruses. These viruses included white spot syndrome virus (WSSV), yellow head virus (YHV), Taura syndrome virus (TSV), infectious hypodermal and haematobiotic necrosis virus (IHHNV), and penaeid shrimp infectious myonecrosis virus (IMNV). Five commercial products of Bioingentech Ltd., Concepcion, Chile, namely the VetPCR™ IHHNV Detection Kit, the VetPCR™ IMNV Detection Kit, the VetPCR™ TSV Detection Kit, the VetPCR™ SWSSV Detection Kit, and the VetPCR™ YHV Detection Kit with a reference number of PCR-V279-48D, PCR-V290-48R, PCR-V293-48R, PCR-V249-48D, and PCR-V294-48R, respectively, were used independently to exclude and/or detect the 5-shrimp viruses. The instructions and recommendations provided with the PCR commercial kits for each virus were followed while performing all of the PCR protocols needed for virus detection assays, and they are available at https://kitpcr.com/product-category/veterinary-pathogen-detection/shrimp/.

Experimental shrimp

A total of apparently healthy 450 white leg juvenile shrimp individuals with an average weight of 10 ± 2 g and an average length of 4 ± 1 cm were obtained from a private farm at Port-Said Governorate, Egypt. Shrimp juveniles were subjected to an acclimatization period of 3 weeks in 3 huge glass aquarium of 150 L × 65 W × 65 H cm, 150 individuals in each, filled with artificial sea water (23 ± 2‰, salinity), equipped with air stones,

and a power biomechanical recycle filter (Eheim® GmbH & Co.KG, Germany). During acclimatization, shrimp were fed on an artificial feed (Aller® Aller-Aqua Co., Giza, Egypt) containing 45% crude protein at a ratio of 2% of their body weight twice a day. Shrimp were monitored with special care for their appearance, swimming behaviour, feeding status, and growth development and randomly sampled to perform aforementioned laboratory scheme work. After the elapsing acclimation period, feeding was stopped and shrimp were grouped according to the experimental design. At all levels tested, acclimation and monitoring of the shrimp were performed as outlined by the Marine Products Export Development Authority and the Network of Aquaculture Centres in Asia-Pacific (2003).

Fungal isolation and identification

Fungal isolation

The fungi were isolated from suspected shrimp samples after being microscopically examined for the existence of hyphal elements. Then, shrimp-infected individuals were washed three times with sterile physiological saline (SPS), dried on sterile tissue towels, and mounted onto pre-prepared plate sets of Sabouraud Dextrose Agar (SDA, Difco®, Egypt) and Glucose Yeast Extract Agar (GYA, 1% glucose, 0.25% yeast extract, and 1.5% agar) (Khoa et al. 2004; Yu et al. 2020). Sequentially, traces of pulverized ampicillin (ADWIC®, El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt) were added onto and around the inoculums to inhibit the growth of bacteria (Hussein and Hatai 2001). Sets of inoculated plates were incubated at 28 ℃ and monitored for 5~7 days for fungal colonial growth. Obtained fungi were purified by subculturing on freshly prepared SDA and/or GYA plates.

Morphological identification

To identify the isolated fungus on the basis of morphological features, the basic slide culture technique was used as described and modified by Rosana et al. (2014). Briefly, agar blocks (1 × 1 cm) of GYA were obtained using a sterile scalpel blade, then transferred onto glass slides, one block onto each. Then, using a special sterile needle, inocula of fungal isolates that were actively growing and extracted from the fungus colonies' periphery were inserted into each of the agar blocks' four corners (angles). Sequentially, all inoculated blocks were covered with sterile cover slides with gentle pressure to ensure adherence. To ensure a humid environment during incubation, cultured slides were transferred to sterile Petri dishes supplemented with wet tissue towels and bent glass rods, where the cultured slides were placed. Then, all plates containing cultured slides were incubated at 28 ℃ and monitored for 3–5 days. Once the fugal growth was grossly detected, the cover slides were carefully withdrawn, mounted onto new sterile slide(s), and stained with Glycerol Lactophenol Cotton Blue (GLPCB). Before subjecting stained slides to microscopic examination (ZEISS, Olympus KHE, Japan), all the stained slides settled for a while, and then the four edges of the cover slides were closed through careful wiping with clear nail polish. Thus, such a promising process ensures complete avoidance of the stain escaping and/or dryness of the fungal structures. Microscopic investigations were performed on several slides at both low and high (10 and 40×) magnification powers to prove the typical morphological structures from various differential aspects. Highly qualified microscopic fields were simultaneously photographed (ZEISS Camera, Olympus KHE, Japan). At all levels tested, all agar blocks were collected and then condemned through autoclaving.

Molecular identification, PCR, sequencing, and *Genbank* submission

Culture conditions and cDNA extraction

Selected fungal isolates, BNS 1117, BNS 2117, and BNS 3117 (Table 1), were sub-cultured in Glucose Yeast Extract Broth (GYB) incubated at 25 °C for 5 days to obtain considerable hyphal mass. Prior to extracting DNA, the collected fungal growth was homogenized separately in Chinese mortars after the addition of liquid nitrogen to ensure complete destruction of hyphal walls. Immediately, broken hyphae were trans-ferred separately to Eppendorf tubes. Then, genomic DNAs of the selected fungi were extracted using genomic DNA extraction kits (GeneJet Plant DNA Purification Kit, Thermo Scientific®, Lithuania) following the manufacturer's protocol. Prior to perform-ing PCR assays, the obtained DNAs were estimated for their purity and concentration at optical density (OD) wavelengths of 260 and 280 nm using a spectrophotometer (SPEC-TROstar NANO® BMG Labtech), adjusted to 50 µg/mL, and kept at −20 °C until used as a template for PCR reactions.

Conventional PCR assay

The target gene and genus-specific oligonucleotide primer sequences are illustrated in Table 2, and they were manufactured by Macrogen®, South Korea. The PCR assay was performed according to the methodology described earlier by Konietzny and Greiner (2003). Briefly, the assay was performed in 50 µL PCR reaction tube mixtures by add-ing 25 µL PCR master mix (Thermo Scientific®, Lithuania), 20 pg (equal to 1 µL) of each primer, and 2 µL of template (extracted DNA), and 21 µL DNA/RNA purified water (Thermo Scientific®, Lithuania). The PCR reactions were carried out by subject-ing samples to a designated regime of amplification in a PCR gradient thermal cycler machine (Bio-RAD®, USA). The designated regime of amplification included an initial denaturation step of 95 °C for 5 min, 38 serial cycles of a denaturation step of 94 °C for 1 min, annealing at 59 for 1 min, extension at 72 °C for 1.5 min, and final extension at 72 °C for 5 min. Negative controls containing no template and a nonspecific fungal tem-plate (*Aspergillus niger*) were included in the PCR assay.

PCR-generated products (amplicons) were simultaneously analyzed by electrophore-sis of 5 µL of each amplification mixture in 1.5% agarose in Tris borate EDTA (TBE) buffer (100 V, 22 min; TBE running buffer, Mupid-exu®, Advance, Japan) gel contain-ing 0.001% ethidium bromide (Sambrook and Russell 2001). A 100–3000 bp DNA lad-der (Gene Ruler 100 bp Plus DNA Ladder, Thermo Scientific®, Lithuania) was included as a molecular weight standard on the left side of the gel. Consequently, after the com-plement of electrophoresis run, gel was photographed under UV-light transillumination (Winpact Scientific®, USA).

Table 1 Fungal isolates revealed from moribund and freshly dead *L. vannamei* raised in biofloc system

Isolate	Host	Source	Location
BNS 1117	*Litopenaeus vannamei*	Biofloc	Giza, Egypt
BNS 2117	*Litopenaeus vannamei*	Biofloc	Giza, Egypt
BNS 3117	*Litopenaeus vannamei*	Biofloc	Giza, Egypt

Table 2 Primer oligonucleotide sequences set for amplification of genus-specific for *Fusarium* species

Target species/ primer	Oligonucleotide sequences (5′–3′)	Target gene	Amplicon size (bp)	Reference
Fusarium spp. ITS *forward* ITS *reverse*	AACTCCCAAACCCCTGTG AACATA TTTAACGGCGTGGCCGC	5.8 S ribosomal RNA	431	Bluhm et al. (2002)

Gene sequencing, phylogenetic analyses, and *Genbank* submission

The DNAs of the selected fungal isolates (BNS 1117, BNS 2117, and BNS 3117) were extracted, and followed by amplification of their selected desired gene (5.8 S ribosomal RNA gene) by performing the PCR reactions as mentioned above. PCR products (amplicons) were purified using a PCR purification commercial kit (Thermo Scientific®, USA) following the manufacturer's instructions. Purified amplicons were subjected to gene sequencing in forward and reverse directions using the same amplification primers on an Applied Biosystems 310 automated DNA sequencer (Applied Biosystems®, USA) using cycle sequencing ABI prism Big Dye terminator chemistry (Perkin-Elmer, Applied Biosystems®, USA). High-quality fragments of the sequenced gene were obtained after trimming the initial and final sequences' portions. Then, sequences were exported in FASTA format and compared with the *Genbank* database (http://www.ncbi.nlm.nih.gov/genbank) using Mega software (MEGA version 6 programme Copyright ©, 1993–2013). Comparative analyses and phylogenic relationships of the obtained sequences with those of the *Genbank* database (Table 3) were determined through a bootstrap of 1000 trials using neighbour-joining alignment programme. The best substitution model was obtained by using the Clustral Walignment algorithm (Tamura et al. 2013). The sequences obtained were submitted to *Genbank* under accession numbers (acc. no) ON973077, ON973078, and ON973079.

Experimental infections to satisfy *Koch's* postulates

Regarding results obtained from both morphological and molecular identification, isolate BNS 3117 (acc. no. ON973079) was selected for induction of artificial infection in juvenile *L. vannamei*.

Preparation of conidial suspension filtrate

The selected fungal isolate was subcultured on GYA and incubated at 25 °C for full plate growth (8 days). The conidial suspension was obtained by dispensing 20 mL of sterile artificial sea water (SASW) onto the fungal surface with careful circular shaking movement, and then the conidial suspension was carefully aspirated using a sterile pipette (Eppendorf® AG, 100~1000 μL, Germany). Subsequently, aspirated conidial suspension was brought to purification through multi-layer sterile gauze filtration. Both designated low and high conidial doses (5×10^3 and 5×10^5 CFU/mL) were adjusted through hemocytometer (Tiefe Depth 0.100 mm, Medizinalbedarf, Germany) counting.

Experimental infection

Experimental infection was carried out through intramuscular (IM) injection of the designated conidial suspension doses following the methodology earlier outlined by Khoa et al. (2004). The experiments on artificial infection were performed in the same acclimation aquaria (no. 3) to avoid stress occurrences with special attributes. Briefly, the experimental individuals were randomly redistributed, grouped, and cohabitated in fenestrated plastic boxes (3 boxes A, B, and C; 30 individuals in each). Shrimp groups (A) and (B) are designated for injection with high (5×10^5 CFU/mL) and low (3×10^5 CFU/mL) doses of conidial suspensions, respectively. Group (C) is designated as the control group. Similarly, replicates of aquaria (no. 2 for each group) were treated as the experimental ones. All remaining shrimp were transferred and kept in an equipped fiberglass tank (ca. 800 L). Prior to performing the artificial injection, shrimp were stopped fed for 3 days and monitored for abnormal behaviours along with the removal of waste. On the injection day, experimental shrimp groups were IM injected with 0.3 mL conidial suspensions at designated doses, while control ones received SASW at the same doses instead. All experimental shrimp groups were observed daily for any clinical alterations or mortality for a month. Dead shrimp samples were collected with special concern to re-isolate the fungus to achieve *Koch's* postulates. Others were fixed in a 10% phosphate-buffered formalin solution (Khoa et al. 2004) for histopathological investigations. After the elapsing of the experimental period, all remaining shrimps in groups A and B were subjected to re-isolating the fungus, while controls were humanly sacrificed.

Histopathological investigation

Fixed specimens were trimmed, washed in water, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and embedded in paraffin. Thin Sect. (4–6 μ) were processed and either stained with Hematoxylin and Eosin stain (H&E) or counter-stained with

Table 3 *Fusarium* species used in molecular phylogenetic analyses

Isolate	Target gene	Origin	Accession number
BNS 1117	5.8 S ribosomal RNA	White leg shrimp, Egypt	ON973077
F. oxysporumis	5.8 S ribosomal RNA	Lentil crop, India	KU671030
F. acutatum	5.8 S ribosomal RNA	Tomato, Egypt	MF787612
BNS 2117	5.8 S ribosomal RNA	White leg shrimp, Egypt	ON973078
F. oxysporum	5.8 S ribosomal RNA	Citrus, Pakistan	KX834820
F. verticillioides	5.8 S ribosomal RNA	Panax ginseng, China	ON417444
BNS 3117	5.8 S ribosomal RNA	White leg shrimp, Egypt	ON973079
F. verticillioides	Fumonisins gene	Maize crop, Iraq	MN486508
F. virguliforme	5.8 S ribosomal RNA	Soybean, South Africa	KF648839
F. solani	28 S ribosomal RNA	Soybean, USA	L36629
Fusarium spp.	5.8 S ribosomal RNA	Soybean, USA	AF178396
F. solani	5.8 S ribosomal RNA	Soybean, USA	AF178418

Hematoxylin and Periodic Acid-Schiff (H&PAS) stain, then microscopically examined (Bancroft and Gamble 2008).

Statistical analysis

GraphPad Prism version 9.1.0.221 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com, was used for the statistical analysis, which consisted of a one-way ANOVA and the Brown-Forsythe test. Disparities with $p < 0.05$ are deemed significant. With the same programme, a comparison of survival curves and a log-rank test were conducted.

Ethical approval

All of the in vivo procedures are carried out strictly in compliance with the Egyptian Code of Practice's instructions and ethical guidelines for the care of animals used for experiments and other scientific reasons. Every experimental protocol has been permitted by the Beni-Suef University Institutional Animal Care and Use Committee (BSU-IACUC) and is assigned an approval number 022–480.

Results

Fungal isolation and identification

Isolation and morphological identification

Wet mounts examination of moribund and/or freshly dead shrimps resulted in the existence of septate delicate fungal hyphae together with illumination of banana-shaped conidia characteristic for the genus *Fusarium* (Fig. 1). Three fungal isolates were obtained on both selected media (GYA and SDA) with colonial characteristics of the genus (Fig. 2). The colonial characteristics appeared as white to purple/pink colonies in colour that gradually changed to pale towards the periphery, floccose, with dense aerial mycelium that turned to a white powdery appearance in old cultures (over 7 days). Mature colonies appeared with convex, elevated, dark purple centres together with irregular colonial peripherals. Colonies on the reverse side tend to have a pale pink colour that gradually darkens to the centre. As a result, the fungal isolate (BNS 3117) shared the same colonial characteristics, with an exception. The exception was that the colour tended to be whitish rather than pinkish. All colonial characteristics are illustrated in Fig. 2.

On the other hand, slide cultures confirmed that all of the three fungal isolates belonged to *Fusarium* spp. Characteristically, hyphae were delicate, funiculus with dense aggregation, branched, transversely septate, with a diameter ranging from 8.5 to 10 μ, together with the absence or lack of conidiophores (Fig. 3). Besides, eventually the distribution of much more microconidia will come at the expense of a small number of macroconidia. The macroconidia were curved falciform-shaped with blunt appressed apexes representing 3~5 septa; however, microconidia tend to be ovoid shape without septa.

Molecular identification, PCR, sequencing, and *Genbank* submission

Conventional PCR Selected oligonucleotide primer sets (Table 2) could detect the inter-transcribed spacer (ITS) gene related to genus *Fusarium* with the production of 3 amplicons of 431 bp characteristic for the 3 isolates selected (Fig. 4).

Sequencing, phylogenic analysis, and *Genbank* submission On the basis of sequence analyses, nucleotide blast homogeny search, and identity matrix revealed high homogeneities and intraspecies similarities (85~100%) between the three isolates and sequences published in the *Genbank* database (Table 4). Additionally, phylogenetic analysis confirmed the identity and close relationships between the three isolates and those of the *Genbank* database (Fig. 5). The three selected isolates, BNS 1117, BNS 2117, and BNS 3117, were confirmatively identified as *F. verticillioides* and their selected sequences were submitted to National Center for Biotechnology Information (NCBI) with accession numbers of ON973077, ON973078, and ON973079 (Table 3).
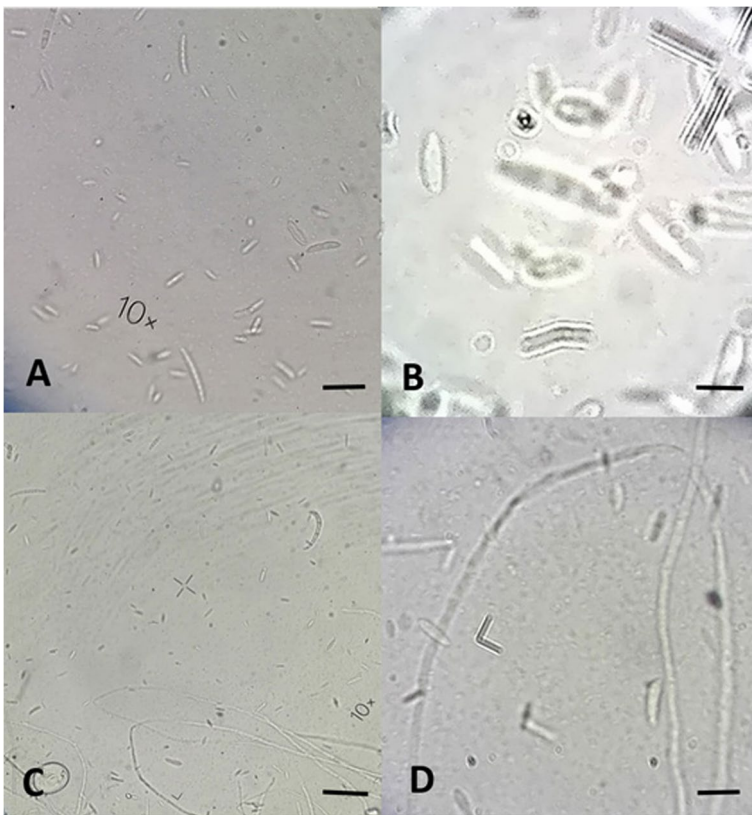
Fig. 1 Wet mount preparation showed microconidia and banana-shaped macroconidia (A and B). (C) and (D) illustrate septate delicate fungal hyphae with illumination of mico and macroconidia. Scale bar = 25 m

Experimental infection and satisfying *Koch's* postulates

The results obtained from the experimental infection with the selected fungal isolate were considerably varied. Shrimp in group A and its replicates showed cumulative mortality that started on day 6, followed by days 7, 17, 23, and 30. In the same manner, group B and its replicates exhibited the same cumulative mortality pattern, with exceptions. The exceptions were that mortality started late and in a lower number of the dead individuals. Ultimately, the total cumulative mortality in groups A and B experienced 72.2% and 21.1%, respectively, until the end of the experimental period (30 days). Comparing with control groups, group A showed a significant ($p<0.05$) value of cumulative mortality; however, group B exhibited no significant one (Fig. 6). At all levels tested, the obtained results showed no mortality occurred in the controls. The same fungal isolate was recovered from all shrimp samples collected from groups A and B during the experiment, thus satisfying *Koch's* postulates.
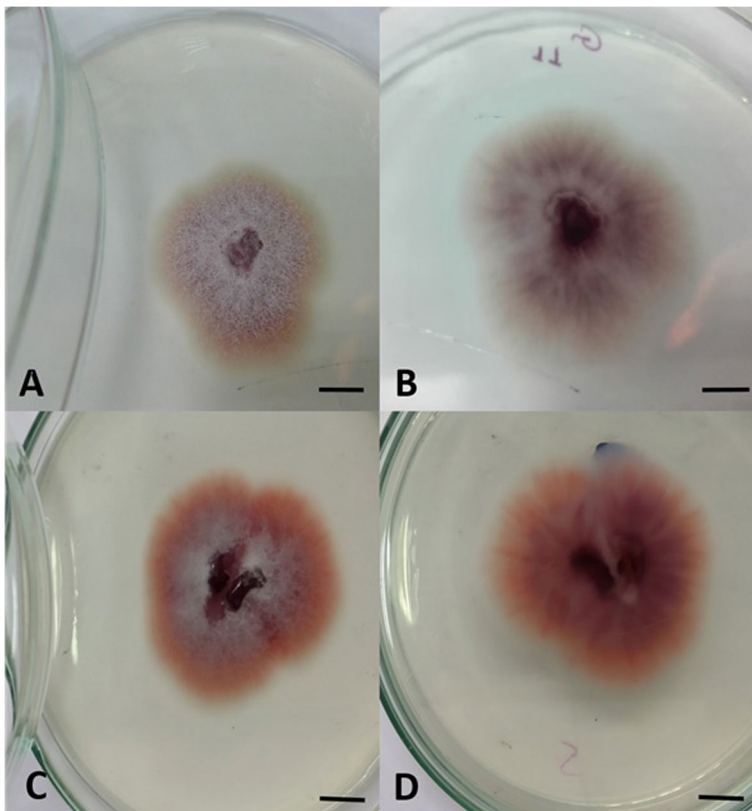
Fig. 2 *Fusarium verticillioides* on GYA. The colonial morphology on front side showed purple/pink coloured colony at the center and gradually changes to pale toward the periphery, floccose, and dense aerial mycelium (A and C). The reverse side showed white mixed with purple/pink coloured colony (B and D). *Scale bar* = 1 cm
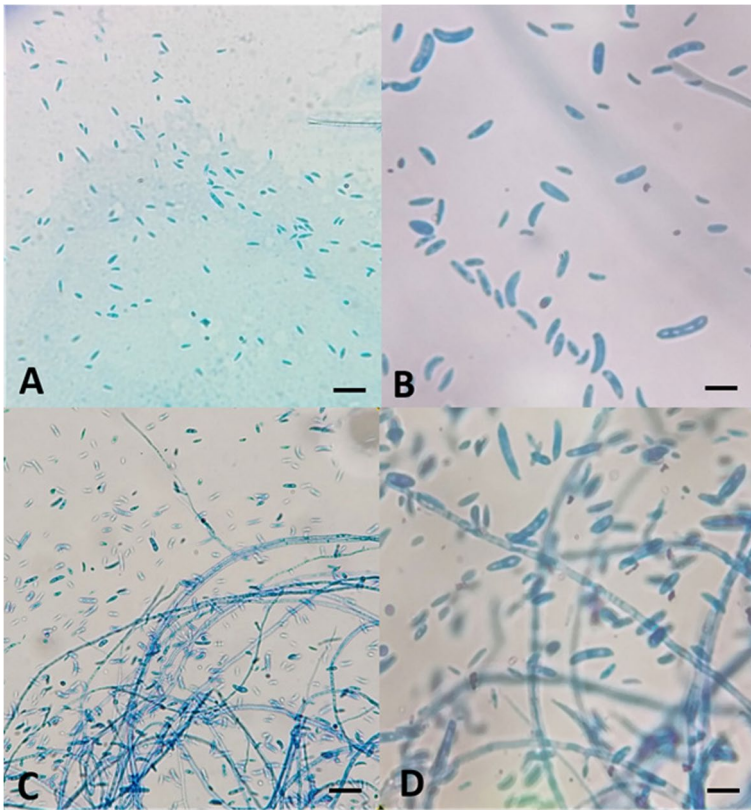
Fig. 3 Stained slide culture preparation showed distribution of transversely septate hyphae along with ovoid-shaped microconidia and banana-shaped macroconidia (falciform-shaped) characteristic for genus *Fusarium* (A and B). Dense aggregation of delicate, funiculous, branched, septate hyphae together with absence of conidophores (C and D). Scale bar = 25 m

Histopathological investigation

The most common tissue alterations revealed by histological examination were vacuolar degeneration in hepatopancreatic tubules. Some hepatopancreatic tubules showed mycelial vegetation of the injected fungus. No fungal conidia were demonstrated in any of the inspected hepatopancreatic tissues (Fig. 7). Gill tissues showed undifferentiated hyperplastic cell proliferation together with gill edema. However, musculature showed degrees of fibroblastic degeneration and oedema together with occupied fungal elements.

Discussion

BFT, principally an innovative system, tends to remove metabolic wastes from aquatic production systems by initiating beneficial bacteria, particularly heterotrophs and nitrotrophs (de Almeida et al. 2021). This action simulates that of biofilters in a classic clear water

Fig. 4 Electrophoresis gel transillumination of PCR amplified ITS gene from the three selected *Fusarium* isolates with amplicon sizes of 431 bp. Lane M. DNA marker 100–3000 bp; lanes 1 and 2 control negative; lane 3, 4, and 5 represented amplicons of the isolates, BNS 1117, BNS 2117, and BNS 3117
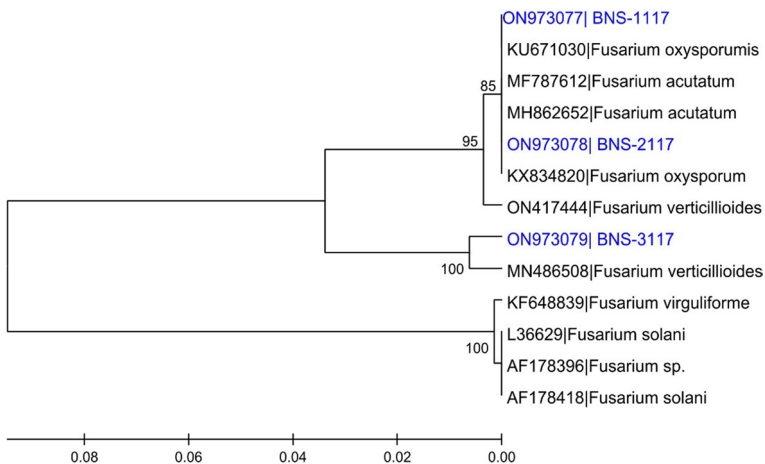

Fig. 5 The phylogenetic tree based on the intertranscribed spacer (ITS) gene related to genus *Fusarium* fragment sequences shows the clustering of the three *F. verticillioides* isolates revealed from the diseased *L. vannamei* in relation to the deposited *Fusarium* species sequences in the *Genbank* database. The tree was generated by using the neighbor-joining method. Bootstrap values (expressed as percentages of 1000 replicates) are shown at each branch nod (bar = 0.01 substitutions per nucleotide)

recirculating aquaculture system (RAS), converting harmful ammonia to nitrate within the rearing fish or shrimp holding facility. The biofloc particles mainly resulted from bacterial aggregates, algae, protozoa, uneaten feed particles, and other suspended elements within the water column. These biofloc particles are mainly gathered by the raised fish or shrimp, thus reducing feeding costs (Avnimelech 2009). Unfortunately, intensive BF shrimp cultures usually face erratic environmental considerations mainly associated with acidic pH shifting, resulting in shrimp shell softening and gill inflammation (González-Vera and Brown 2017). Sequentially, such a situation produces an entrance facility for pathogens, particularly *Fusarium*, to invade, proliferate, and attack the host's cuticle defense mechanism. This in turn explains the possibility of BGD incidence.

Generated data from the presented study revealed that almost all of the dead and moribund shrimp individuals exhibited either softening shells and/or complete shell loss. As a result, no evidence of viral, bacterial, or parasitical affections was recorded at the laboratory

Table 4 Nucleotides identities for selected *Fusarium* spp. isolated from diseased *L. vannamei* raised in biofloc system

Fusarium strains	1	2	3	4	5	6	7	8	9	10	11	12	13	Nucleotide identity %
1. ON973077|F. verticillioides BNS 1117		100	93	85	83	85	100	100	100	100	85	93	99	
2. ON973078|F. verticillioides BNS 2117			93	85	83	85	100	100	100	100	85	93	99	
3. ON973079|F. verticillioides BNS 3117				81	81	81	93	93	93	93	81	98	100	
4. AF178418|F. solani					100	100	83	83	83	83	99	82	84	
5. AF178396|Fusarium sp.						100	83	83	83	83	99	82	84	
6. L36629|F. solani							83	83	83	83	99	82	84	
7. KU671030|Fusarium oxysporumis								100	100	100	85	93	99	
8. KX834820|Fusarium oxysporum									100	100	85	93	99	
9. MF787612|Fusarium acutatum										100	85	93	99	
10. MH862652|Fusarium acutatum											85	93	99	
11. KF648839|Fusarium virguliforme												82	84	
12. MN486508|F. verticillioides													93	
13. ON417444|F. verticillioides														
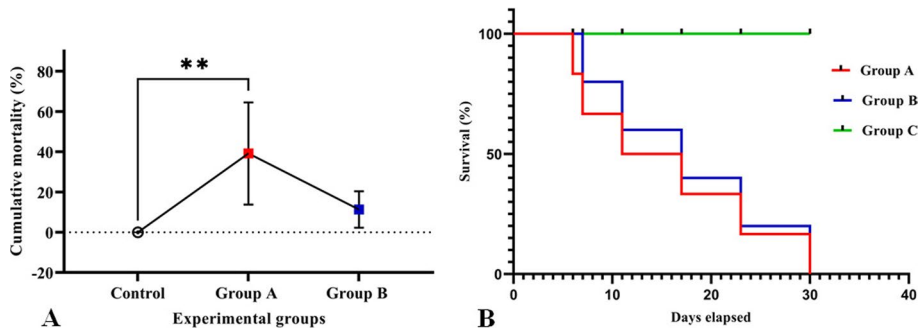
Fig. 6 Experimental infection for satisfying *Koch's* postulates. (A) shows cumulative mortality percentage differences between both high and low doses infected shrimp. (B) shows survival curve of both (A) and (B) infected shrimp groups over the experimental period (30 days)

scheme levels. However, wet mount preparations illustrated the existence of fungal elements, including hyphae and/or banana-shaped conidia (Fig. 1). These results are in accordance with those reported earlier by Khoa et al. (2004) and (2005), indicating a probability of *Fusarium* infection. Such a possibility was indicated by the isolation of three fungal isolates with colonial characteristics of *Fusarium* species, as described by Sempere and Santamarina (2009). They concluded that *F. verticillioides* colonies had different pinkish colour shades with scarce and whitish aerial mycelium. Accordingly, from a colonial characteristic point of view, the three isolates closely resembled those of Sempere and Santamarina. However, one of them was more pinkish with dense myceilium (Fig. 2). In parallel, Rhoobunjongde et al. (1991) described the colonial appearance of *F. moniliiform* as creamy white, changing to purple at the centre and lavender towards the periphery on potato dextrose agar. In this respect, *F. verticellioides* and *F. monilliform* were recently considered to be synonymous.

The colonial appearance colour difference might occur as a result of isolating media (GYA) components that probably affected the colour development. Additionally, the colonial development colour characteristics of the three obtained isolates act in the same manner as those earlier reported by Yao et al. (2022). Although they isolated *F. solani*, accused in BGD, they described its colonial colour development as pink colonies with black to brown centres, and some colonies were dark pinkish and changed to white at the periphery. Moreover, *F. oxysporum* on PDA exhibited white to beige in young colonies that darkened to violet and brown in aged ones (Le and Hatai 2005). It seemed that the graduation behaviour of colonial colour development in *Fusarium* species acted as a general aspect and could not be dependable for their exact identification. Supportively, Rhoobunjongde et al. (1991) noted that the rapid colonial colour changes and morphological characteristics of *Fusarium* spp. made them difficult to identify at the species level. Sequentially, morphological identification obtained from the screening of slides' cultures showed the existence of abundant production of microconidia and scattered numbers of macroconidia associated with the absence of chylamidospores (Fig. 3A, B). Additionally, hyphal elements were in a dense arrangement, with both conidia in between (Fig. 3C, D). Moreover, the modified slide culture technique employed could differentiate the intraconidial septa of both conidia types. These results are following the findings of Leslie and Summerell (2006). Besides, the absence of chylamidospores and/or pseudo-chylamidospores in investigated slides' culture categorized the 3 isolates as belonging to *F. moniliform*, which is a synonym of *F. verticillioides*.
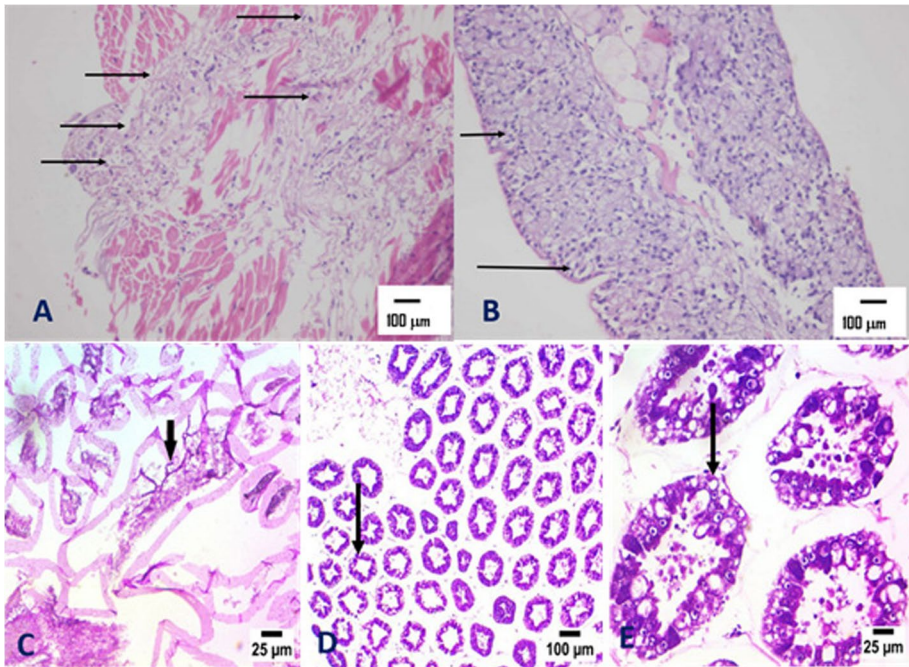
Fig. 7 Histopathological alterations associated with shrimp tissues experimentally infected by *F. verticillioides* (BNS 3117). (A) Photomicrograph showing oedema and degrees of fibroblastic degeneration with occupied fungal elements (*arrows*). (B) Photomicrograph showing undifferentiated hyperplastic cell proliferation (*arrows*) and gill oedema. (C) Photomicrograph showing fungal hyphae in the lumen of the hepatopancreas tubule (*arrow*) (PAS stain). (D, E) Photomicrograph showing vacuolar degeneration in some hepatopancrease tubules (*arrow*)

On the progression of molecular identification and phylogenetic analysis, data generated from the nucleotide sequence, identity matrix, and phylogenetic analysis of cDNA fragments showed high homogeneities of 85, 95, and 100% intraspecies similarities among the 3 isolates (BNS 1117, BNS 2117, and BNS 3117), respectively, with the deposited sequences in the *Genbank* database (Table 4; Fig. 5). As a result, the selected 3 isolates were presumptively identified as *F. verticillioides* and deposited in the *Genbank* database with accession numbers ON973077, ON973078, and ON973079, respectively. These results, together with those obtained from morphological characterization, showed high discriminatory power to identify and differentiate the divergence similarities between *Fusarium* species. Similarly, Yao et al. (2022) clarified the usefulness of sequence alignment and phylogenetic identification in the discrimination of intraspecies similarities between *F. solani* and related species.

Results obtained from artificial infection using selected isolate BNS 3117 (ON9373079) revealed different pathogenic levels of the isolate when injected intra-abdominal in experimental shrimp depending on the dose used. Instantly, both high and low doses caused different cumulative mortality percentages; however, no clinical signs were observed (Fig. 6). Despite this result, which confirmed the pathogenicity of the selected *Fusarium* isolate, no melanization lesions occurred within experimentally infected shrimp, even at injected sites. The major aspect of cumulative mortality is probably related to moulting failure and

the mycotoxins produced by the injected *Fusarium*. Most *F. verticillioides* strains produce fumonisins, which have high toxicity values for animals and plants and are mainly produced within 24 h to reach their toxic level after a period of time, according to the infected host (Sánchez-Rangel et al. 2012). That might explain why the cumulative mortality pattern occurred 7 days post-injection for both groups (A and B), and that was evident in their replicates as well. Additionally, the mycotoxin produced by injected *Fusarium* elements might contribute to the moulting failure and result in increasing the damage to the host (Moret and Moreau 2012; Yao et al. 2022). Supportively obtained results showed that control groups and their replicates exhibited successful moulting with no evidence of mortality. Interestingly, almost all *F. verticillioides* were mainly concerned with plant mycotic affections (de Oliveira Rocha et al. 2011; Aiyaz et al. 2016; Ortiz et al. 2015; Mwangi et al. 2021; Zitnick-Anderson et al. 2021); however, there was a lack of research addressing the affections of this species in aquatic animals. The obtained results within the presented study highlighted the role of the fungus as a mycotic etiological agent connected to long-term mortality in biofloc-cultured *L. vannamei*. Such distinct findings might be included as a new indication in the *F. verticillioides* literature platform.

Histopathological alterations associated with infected tissues demonstrated pictures of several degrees of either degenerative, oedema, or proliferative changes in the hepatopancreas, musculatures, and gills, respectively (Fig. 7). Degenerative changes are related to the effects of mycotoxin liberation inside infected tissue and were indefinite in hepatopancreas cells, elucidating such vacuolar degeneration within hepatopancreatic tubules (Deepa and Sreenivasa 2019). On the other side, although the proliferative changes that occurred, particularly in gill tissues, might be related to irritation of gill lamellae, it seemed that they were not specific to a fungal infection because no fungal elements were demonstrated at the site. However, these histopathological findings revealed within the presented study were under those described earlier by Khoa et al. (2004) and Yao et al. (2022).

In conclusion, the presented study demonstrated a new record for *F. verticillioides* to be included in *Fusarium* species accused of shrimp mortality. The fungal elements associated with mount preparation of dead and moribund shrimp gills and tissues in the study indicated involvement of the fungus in mortality patterns. In addition, isolation, morphological, and molecular identification confirmed *F. verticellioides* as the causative agent of such deaths. Moreover, although the typical lesions of BGD (melanization) were not demonstrated in this study, *Koch's* postulates experiment and histopathological findings showed the pathogenicity of the fungus for *L. vannamei* shrimp.

Declarations

Competing interests The authors declare no competing interests.

References

Aiyaz M, Thimmappa Divakara S, Mudili V, George Moore G, Kumar Gupta V, Chandra Nayaka S, Ramachandrappa Niranjana S (2016) Molecular diversity of seed-borne Fusarium species associated with maize in India. Curr Genom 17(2):132–144

Arie T (2019) Fusarium diseases of cultivated plants, control, diagnosis, and molecular and genetic studies. J Pesticide Sci 44(4):275–281. https://doi.org/10.1584/jpestics.J19-03

Avnimelech Y (2009) Biofloc technology: a practical guide book. World Aquaculture Society

Bancroft JD, Gamble M (eds) (2008) Theory and practice of histological techniques. Elsevier health sciences

Benzie JA (2009) Use and exchange of genetic resources of penaeid shrimps for food and aquaculture. Reviews Aquaculture 1(3–4):232–250. https://doi.org/10.1111/j.1753-5131.2009.01018.x

Bluhm BH, Flaherty JE, Cousin MA, Woloshuk CP (2002) Multiplex polymerase chain reaction assay for the differential detection of trichothecene- and fumonisin producing species of Fusarium in cornmeal. J Food Prot 65(12):1955–1961. https://doi.org/10.4315/0362-028x-65.12.1955

Bower SM, McGladdery SE, Price IM (1994) Synopsis of infectious diseases and parasites of commercially exploited shellfish. Annu Rev Fish Dis 4:1–199. https://doi.org/10.1016/0959-8030(94)90028-0. (https://www.sciencedirect.com/science/article/pii/0959803094900280)

de Almeida MS, Carrijo-Mauad JR, Gimenes RMT, Gaona CAP, Furtado PS, Poersch LH, Fóes, G K (2021) Bioeconomic analysis of the production of marine shrimp in greenhouses using the biofloc technology system. Aquacult Int 29(2):723–741. https://doi.org/10.1007/s10499-021-00653-1

de Oliveira Rocha L, Reis GM, Da Silva VN, Braghini R, Teixeira MMG, Corrêa B (2011) Molecular characterization and fumonisin production by *Fusarium verticillioides* isolated from corn grains of different geographic origins in Brazil. Int J Food Microbiol 145(1):9–21. https://doi.org/10.1016/j.ijfoodmicro.2010.11.001

Deepa N, Sreenivasa MY (2019) Molecular methods and key genes targeted for the detection of fumonisin producing *Fusarium verticillioides* – an updated review. Food Bioscince 32:100473. https://doi.org/10.1016/j.fbio.2019.100473

Dewangan NK, Gopalakrishnan A, Kannan D, Shettu N, Singh R (2015) Black gill disease of Pacific white leg shrimp (LitoLitopenaeus Vannamei) by aspergillus flavus. J Coastal Life Med 3(10):761–765. https://doi.org/10.12980/jclm.3.2015j5-70

Ding S, Wang F, Dong S, Li Y (2014) Comparison of the respiratory metabolism of juvenile Litopenaeus vannamei cultured in seawater and freshwater. J Ocean Univ China 13:331–337. https://doi.org/10.1007/s11802-014-2134-3

El-Sayed A, Aleya L, Kamel M (2021) Microbiota's role in health and diseases. Environ Sci Pollut Res 28(28):36967–36983. https://doi.org/10.1007/s11356-021-14593-z

FAO (2023) Guidelines for Sustainable Aquaculture (GSA). FAO Fisheries and Aquaculture Report No. 1359 NFIAN/R1359 (En). https://www.proquest.com/openview/ca455bca0be17cd9f536ede6d3370b9e/1?pq-origsite=gscholar&cbl=237321

Frischer ME, Landers SC, Walker AN, Powell SA, Lee RF (2022) Black gill in marine decapod crustaceans: a review. Reviews Fisheries Sci Aquaculture 30(4):498–519. https://doi.org/10.1080/23308249.2022.2047153

GAFRD (2021) General authority for fish resourses development in fish statistics year book. Agriculture Ministry, Cairo, Egypt

González-Vera C, Brown JH (2017) Effects of alkalinity and total hardness on growth and survival of postlarvae freshwater prawns, Macrobrachium rosenbergii (De Man 1879). Aquaculture 473:521–527. https://doi.org/10.1016/j.aquaculture.2017.03.016. (https://www.sciencedirect.com/science/article/pii/S0044848617304659)

Hussein MM, Hatai K (2001) In vitro inhibition of Saprolegnia by bacteria isolated from lesions of salmonids with saprolegniasis. Fish Pathol 36(2):73–78. https://doi.org/10.3147/jsfp.36.73

Ishikawa K (1968) A guide to quality control. Asia productivity organization. Tokyo

Karthikeyan V, Gnanamoorthy P, Gopalakrishnan A (2014) Incidence of Brown-Gill (fungal) disease in three Penaeus species grow out ponds of Vellapallam. Nagapattinam district of Tamil Nadu, India

Khanjani MH, Mozanzadeh MT, Fóes GK (2022) Aquamimicry system: a sutiable strategy for shrimp aquaculture–a review. Annals Anim Sci 22(4):1201–1210. https://doi.org/10.2478/aoas-2022-0044

Khoa LV, Hatai K, Aoki T (2004) Fusarium incarnatum isolated from black tiger shrimp, Penaeus monodon Fabricius, with black gill disease cultured in Vietnam. J Fish Dis 27(9):507–515. https://doi.org/10.1111/j.1365-2761.2004.00562.x

Khoa LV, Hatai K, Yuasa A, Sawada K (2005) Morphology and molecular phylogeny of Fusarium solani isolated from kuruma prawn Penaeus japonicus with black gills. Fish Pathology 40(3):103–109. https://doi.org/10.3147/jsfp.40.103

Konietzny U, Greiner R (2003) The application of PCR in the detection of mycotoxigenic fungi in foods. Brazilian J Microbiol 34:283–300. https://doi.org/10.1590/S1517-83822003000400001

Le VK, Hatai K (2005) First case of Fusarium oxysporum infection in cultured kuruma prawn Penaeus japonicus in Japan. Fish Pathol 40(4):195–196. https://doi.org/10.3147/jsfp.40.195

Leslie JF, Summerell BA (2006) The Fusarium Laboratory Manu. The Fusarium Laboratory Manual

Manan H, Kasan NA, Ikhwanuddin M, Kamaruzzan AS, Jalilah M, Fauzan F, Amin-Safwan (2023) Biofloc technology in improving shellfish aquaculture production–a review. *Annals of Animal Science*. https://doi.org/10.2478/aoas-2023-0093

Moret Y, Moreau J (2012) The immune role of the arthropod exoskeleton. Invertebrate Survival J 9(2):200–206

Mwangi MW, Muiru WM, Kimenju JW (2021) Characterisation of Fusarium species infecting tomato in Mwea West sub-county, Kirinyaga County, Kenya. Can J Plant Pathol 43(1):56–61. https://doi.org/10.1080/07060661.2020.1788645

Noga EJ (1996) Fish diseases diagnosis and treatment. Mosby Electronic Publishing, USA, p 163

Ortiz CS, Richards C, Terry A, Parra J, Shim WB (2015) Genetic variability and geographical distribution of mycotoxigenic Fusarium verticillioides strains isolated from maize fields in Texas. Plant Pathol J 31(3):203. https://doi.org/10.5423/PPJ.OA.02.2015.0020

Pazir MK, Afsharnasab M, Jalali Jafari B, Sharifpour I, Motalebi AA, Dashtiannasab A (2011) Detection and identification of white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) of Litopenaus vannamei from Bushehr and Sistan and Baloochestan provinces (Iran), during 2009–2010. http://hdl.handle.net/1834/11424

Rhoobunjongde W, Hatai K, Wada S, Kubota SS (1991) Fusarium moniliforme (Sheldon) isolated from gills of kuruma prawn Penaeus japonicus (Bate) with black gill disease. Nippon Suisan Gakkaishi 57(4):629–635

Rosana Y, Matsuzawa T, Gonoi T, Karuniawati A (2014) Modified slide culture method for faster and easier identification of dermatophytes. Microbiol Indonesia 8(3):7. https://doi.org/10.5454/mi.8.3.7

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sánchez-Rangel D, Sánchez-Nieto S, Plasencia J (2012) Fumonisin B1, a toxin produced by *Fusarium verticillioides*, modulates maize β-1,3-glucanase activities involved in defense response. Planta 235:965–978. https://doi.org/10.1007/s00425-011-1555-0

Sempere F, Santamarina MP (2009) The conidia formation of several Fusarium species. Ann Microbiol 59:663–674. https://doi.org/10.1007/BF03179206

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30:2725–2729

Wang Z, Shi C, Wang H, Wan X, Zhang Q, Song X, Huang J (2020) A novel research on isolation and characterization of Photobacterium damselae subsp. damselae from Pacific white shrimp, Litopenaeus vannamei, displaying black gill disease cultured in China. J Fish Dis 43(5):551–559. https://doi.org/10.1111/jfd.13153

Yao L, Wang C, Li G, Xie G, Jia Y, Wang W, Kong J (2022) Identification of Fusarium solani as a causal agent of black spot disease (BSD) of Pacific white shrimp, Litopenaeus vannamei. Aquaculture 548:737602. https://doi.org/10.1016/j.aquaculture.2021.737602. (https://www.sciencedirect.com/science/article/pii/S0044848621012655)

Yu P, Wang T, Ye H, Shan H, Ma S (2020) Isolation and identification of pathogenic Vibrio spp. retrieved from diseased Litopenaeus vannamei and beneficial role of some functional probiotic bacteria for control. Aquaculture International 28(4):1403–1420. https://doi.org/10.1007/s10499-020-00530-3

Zitnick-Anderson K, Gargouri Jbir T, Carlson A, Postovit S, Pasche J, Kalil A (2021) Fusarium species associated with root rot of lentil (Lens culinaris) in North Dakota. Plant Health Progress 22(4):524–528. https://doi.org/10.1094/PHP-03-21-0063-RS

Authors and Affiliations

Mortada M. A. Hussein[1] · **Walid H. Hassan[2]** · **Nadia N.A. Abd El-Ghany[3]** · **Nourhan H. Ghanem[3]**

✉ Mortada M. A. Hussein
   mortadahussein@hotmail.com

   Walid H. Hassan
   walidhamdyhassan@yahoo.com

   Nadia N.A. Abd El-Ghany
   noniaelghany@yahoo.com

   Nourhan H. Ghanem
   Nour_707@yahoo.com

[1] Fish Diseases and Management Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62513, Egypt

[2] Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62513, Egypt

[3] Department of Fish Diseases, Animal Health Research Institute (AHRI), Agricultural Research Centre (ARC), Dokki 12618, Cairo, Egypt