Saprolegnia parasitica Isolated from Rainbow Trout in Korea: Characterization, Anti-Saprolegnia Activity and Host Pathogen Interaction in Zebrafish Disease Model

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Abstract Saprolegniasis is one of the most devastating oomycete diseases in freshwater fish which is caused by species in the genus Saprolegnia including Saprolegnia parasitica. In this study, we isolated the strain of S. parasitica from diseased rainbow trout in Korea. Morphological and molecular based identification confirmed that isolated oomycete belongs to the member of S. parasitica, supported by its typical features including cotton-like mycelium, zoospores and phylogenetic analysis with internal transcribed spacer region. Pathogenicity of isolated S. parasitica was developed in embryo, juvenile, and adult zebrafish as a disease model. Host-pathogen interaction in adult zebrafish was investigated at transcriptional level. Upon infection with S. parasitica, pathogen/antigen recognition and signaling (TLR2, TLR4b, TLR5b, NOD1, and major histocompatibility complex class I), pro/anti-inflammatory cytokines (interleukin [IL]-1β, tumor necrosis factor α, IL-6, IL-8, interferon γ, IL-12, and IL-10), matrix metalloproteinase (MMP9 and MMP13), cell surface molecules (CD8+ and CD4+) and antioxidant enzymes (superoxide dismutase, catalase) related genes were differentially modulated at 3- and 12-hr post infection. As an anti-Saprolegnia agent, plant based lawsone was applied to investigate on the susceptibility of S. parasitica showing the minimum inhibitory concentration and percentage inhibition of radial growth as 200 μg/mL and 31.8%, respectively. Moreover, natural lawsone changed the membrane permeability of S. parasitica mycelium and caused irreversible damage and disintegration to the cellular membranes of S. parasitica. Transcriptional responses of the genes of S. parasitica mycelium exposed to lawsone were altered, indicating that lawsone could be a potential anti-S. parasitica agent for controlling S. parasitica infection.

Keywords Host-pathogen interactions, Lawsone, Saprolegnia parasitica, Saprolegniasis, Zebrafish

Saprolegniasis (also known as saprolegniosis) is considered as one of the most common and devastating oomycete diseases in hatcheries and farms of salmon and trout [1]. Saprolegnia species affect to decline of the wild fish [2] and cause diseases in amphibians and crustaceans [3]. The species belonging to the genus Saprolegnia (class Oomycetes) are considered to be the causative agents of the saprolegniasis, and often considered as opportunistic facultative parasites [4]. Saprolegniasis is closely associated with environmental stress, and it can be secondary invader to viral, bacterial and parasitic infectious agents to fish [5]. Among the Saprolegnia species, the fungal-like oomycete, S. parasitica is regarded as the most destructive pathogen, and causes significant economic losses to salmonid aquaculture. The virulent levels of some S. parasitica strains are very high and they cause primary infections in both salmon eggs and fish [6]. Jiang et al. [3] emphasized that it is urgently needed to have in-depth understanding of this pathogen related to epidemiology, biology and pathology which may support to find new control strategies of saprolegniasis. Outbreaks of S. parasitica infections have been significantly
increased during last decade in many parts of the world after banning of organic dye, malachite green, which was the most effective anti-Saprolegnia agent [7]. At present, effective control of S. parasitica is one of the main challenges in salmonid aquaculture. Wide array of chemicals [8], natural products [9], bacterial isolates, and UV irradiation [10] have been researched on to find the replacement or alternative strategies for banned malachite green and to develop environmentally safe treatment methods. Anti-Saprolegnia agents such as formalin [11], boric acid [1], clotrimazole [12], potassium permanganate [5], copper nitrate [13], and copper sulfate [14] have been tested, however, none of them have matched with the efficacy level for killing the pathogen. Few licensed anti-Saprolegnia agents such as pyceze [15] and hydrogen peroxide [16] are reported for treating salmon eggs to control S. parasitica infection although those agents are not very effective for high protection after hatching [17]. Thus, identifying effective anti-Saprolegnia compounds can lead new avenue for sustainable control of the disease.

Lawsone (2-hydroxy-1,4-naphtoquinone) is the principal active component in extract of henna (Lawsonia inermis) leaves [18], and exhibits wide range of bioactivities such as antibacterial, antifungal, antimalarial, antitumor, and antioxidant [19]. Therefore, we investigated the efficacy of extracted lawsone against isolated S. parasitica.

Zebrafish (Danio rerio) is considered as powerful animal model widely used in many research fields including various infection models upon bacteria [20], virus [21], yeast [22], and fungal infection [23]. Most of the studies related to pathogenicity and host pathogen interactions of S. parasitica have been investigated with naturally infected animals and aquaculture species such as salmon [24], trout [25], and tilapia [26]. Therefore, we initiated fundamental investigations for developing zebrafish disease model for S. parasitica infection in laboratory level for further understanding of saprolegniasis and its effects in host.

In this study, morphological and molecular identification were conducted for the isolated S. parasitica from farmed rainbow trout in Korea. We developed zebrafish-S. parasitica infection model for better understanding of pathogenicity and host (fish)-pathogen (oomycete) interactions. Furthermore, plant based natural lawsone was tested against isolated S. parasitica to investigate the efficacy of the compound, aiming to introduce new anti-Saprolegnia agent for disease control. The possible mode of action of lawsone on S. parasitica growth inhibition was also studied to search the potential of this compound for anti-Saprolegnia activity.

**MATERIALS AND METHODS**

**Isolation of S. parasitica.** Initially, diseased rainbow trout with necrotic skin near to fins and head was received from the fish farm in Wonju, Gangwon-do, South Korea. To isolate and identify pathogen(s) associated with the disease, skin tissue samples were collected and repeated sub-culturing was performed. In brief, tissue sample was transferred into a potato dextrose agar (PDA) plate containing ampicillin (100 μg/mL) and incubated at 20°C for 4 days. To obtain pure culture, sub-culturing was performed for 5 times by cutting and transferring a small section of growing margin of mycelium into new plate by using a sterile surgical blade.

**Morphological and molecular identification.** Morphological features and molecular based techniques were applied to identify the isolated pathogen. Gross and microscopic features of radial growth of pathogen were observed in PDA plate. To identify zoospore as asexual reproduction, the margin of advancing mycelium was collected using 0.5-cm cork-borer from 3-day old grown plate. Collected mycelium was transferred into petri dish filled with 30 mL of potato dextrose broth (PDB) and incubated at 20°C. After 24 hr, mycelium was collected and washed twice using sterilized tap water (STW) and incubated for another 24 hr in STW. Then mature sporangium and zoosporas were observed through light microscope. Additionally, secondary zoosporas were observed after stained with standard lactophenol cotton blue wet mount preparation as described by Sime et al. [27].

For molecular identification, internal transcribed spacer (ITS) region sequencing was carried out by using universal primers ITS1 (TCCGTAAGGTAAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) as described by White et al. [28]. Resulted sequence (including 18S ribosomal RNA gene partial sequence, ITS 1, 5.8S ribosomal RNA gene, ITS 2 and 28S ribosomal RNA gene partial sequence) was compared with other ITS region sequences in GenBank data base. Phylogenetic analysis was conducted using 22 counterparts of Saprolegnia species and other selected oomycete strains to understand the taxonomic relationship of isolated S. parasitica. Phylogenetic tree was constructed by neighbor-joining method based on ClustalW pairwise alignment using MEGA 6.0 program.

**Pathogenicity of S. parasitica against zebrafish and histological analysis.** Zebrafish AB line was obtained from Korea Zebrafish Organogenesis Mutant Bank (ZOMB) and maintained in zebrafish culturing system. To understand the pathogenicity and host pathogen interactions zebrafish embryo, juvenile and adult were exposed to secondary zoosporas of S. parasitica to induce artificial infection. Firstly, to observe the survival rate and hatching delay, zebrafish eggs were obtained from adult AB line zebrafish. In each group, 10 eggs were transferred into petri dish containing egg water with 6 replicates. Then the eggs were directly exposed to 5 × 10⁴ spores/mL at 12-hr post fertilization (hpf) and survival rate and hatching delay were observed simultaneously. Secondly, juvenile zebrafish (30–40 days old) were divided into 3 groups (10 fish per group) then directly exposed to secondary zoospore (5 × 10⁴ spores/mL) in a tank filled with 200 mL of zebrafish system water.
During these experiments, same conditions were maintained for the control group without zoospore exposure. Experimental temperature was maintained at 20 ± 2°C with 12 hr light/12 hr dark period. Survival rate and other morphological features of embryo, and juveniles were observed for 4 days at every 12-hr intervals. During observation of survival rate and hatching delay, embryos were counted as dead when they were coagulated without heart beat and viability. To investigate the level of infectivity of *S. parasitica*, the adult zebrafish were exposed to secondary zoospore (5 × 10⁵ spores/mL) with removal of 7–9 scales near to dorsal fin, and the infected fish samples were collected at 36-hr post challenge (hpc) for histopathological examinations. Then the samples were fixed with 10% buffered formalin followed by paraffin tissue processing. Then the histology slides were stained with periodic acid-Schiff according to the method described by Kligman and Mescon [29].

**mRNA expression analysis of zebrafish kidney immune and antioxidant enzyme genes by quantitative real-time PCR (qRT-PCR).** Separate experiment was conducted for the mRNA expression analysis of zebrafish immune and antioxidant genes after *S. parasitica* challenge. Ten fish were maintained in a tank with 1 L of water, and final concentration of *S. parasitica* secondary zoospore was adjusted at 5 × 10⁵ spores/mL. Then the tank was maintained at 20 ± 2°C with 12 hr light/12 hr dark period. Control group was set with same conditions without adding zoospore, and 3 replicate tanks were maintained for both control and challenge group. Kidney tissue was collected at 3- and 12-hr post infection (hpi) from 3 fish in each tank. Collected kidney tissues were immediately snap frozen in liquid nitrogen followed by storage at −80°C until further use for RNA isolation.

Total RNA was extracted from the 3 hr and 12 hr *S. parasitica* exposed adult zebrafish kidney using TRIzol regent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan) according to the manufacturer’s protocol [30]. RNA concentration was adjusted to 500 ng/μL of total RNA was used to synthesize the cDNA. cDNA was diluted 40-fold and stored at −20°C until further use. Transcript levels of selected immune and antioxidant functional genes were analyzed by qRT-PCR using a TaKaRa Thermal Cycler Dice TP 800 real time system. The qRT-PCR cycling protocol was performed with SYBR Premix Ex-Taq (Perfect Real Time) master mix (TaKaRa) in a total reaction volume of 10 μL, containing 4 μL of cDNA, 5 μL of 2× TaKaRa Ex-Taq SYBR premix, and 0.5 μL of each forward and reverse primer (10 μM). Standard 3-step thermal cycling profile of the machine with 55–60°C annealing was performed and single dissociation reading step at the end was followed to identify the specificity of the primers. The thermal reaction was included with single cycle of 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 55–60°C for 20 sec, and 72°C for 20 sec. The relative expression fold was determined by 2⁻ΔΔCT method described by Livak and Schmittgen [31]. The full name and annealing temperature of the gene specific primers of zebrafish used for qRT-PCR are listed in Table 1, and zebrafish β-actin was used as an internal reference gene to normalize the gene expression analysis. Expression fold units were calculated with dividing the normalized expression values of the *S. parasitica* challenged group by that of the respective control group at each time point.

**Determination of minimum inhibitory concentration (MIC) and percentage inhibition of radial growth (PIRG) of lawsone against *S. parasitica*.** To determine the MIC of lawsone against *S. parasitica*, grown mycelium plug was transferred into PDB containing 50, 100, 200, and 400 μg/mL of lawsone and mycelial growth was observed after 24 hr. In parallel, to determine the PIRG percentage, growth inhibition test was carried out using PDA plates which contained different concentrations of lawsone (100, 200, and 400 μg/mL). For both experiments, controls were prepared without adding lawsone. For each assay, 0.5-cm diameter of plug from the advancing margins of 5-day-old grown mycelium was seeded onto the center of each prepared plates and all the plates were incubated at 20°C. In the PIRG assay, radial diameters of the mycelium were recorded when the control plate of *S. parasitica* was fully grown up to margin of plate (at 60 hr). Then the growth inhibition was calculated based on the percentage inhibition of radial growth (PIRG %) as follows: PIRG % = [(R1 − R2)/R1] × 100%, where, R1 = radial growth of control plate, and R2 = radial growth of treatment plate.

**Analysis of membrane damage and viability of *S. parasitica* upon lawsone treatment.** To investigate the effects of lawsone on membrane damage and cell viability, field emission scanning electron microscopy (FE-SEM) (Sirion, FEI, Netherlands) analysis, and propidium iodide (PI) assay were performed after treatment with lawsone to *S. parasitica* mycelium. Mycelium from control, lawsone treated at MIC (200 μg/mL) and 2× MIC (400 μg/mL) were collected after 24-hr incubation, then fixed with 2.5% glutaraldehyde for 30 min in room temperature. Fixed samples were washed with phosphate buffered saline (PBS; pH 7.2), dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and 100%), passed through acetone and fully dried. The dried mycelium was mounted on copper stubs and sputter-coated with platinum (Quorum Technologies, Lewes, UK) and then observed using a FE-SEM. Subsequently, to test the effect of lawsone on cell viability, 24-hr lawsone treated mycelium was re-suspended in PBS and treated with PI (Sigma-Aldrich, St. Louis, MO, USA) at final concentration of 2.5 μg/mL for 15 min. Fluorescence images were observed using a Zeiss LSM 510 meta confocal laser scanning microscope (CLSM) integrated with the Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany).
| Gene for zebrafish | Forward primer sequence (5‘-3’) | Reverse primer sequence (5‘-3’) | Annealing temperature (°C) | Accession No. |
|-------------------|---------------------------------|---------------------------------|-----------------------------|---------------|
| **Housekeeping gene** |                                |                                 |                             |               |
| β-Actin           | AATCTTGCGGTATCCAGGAGACCA        | TCTCCTTCTGCATCCTGTCAGCAGA       | 60                          | AF025305      |
| **Pathogen/antigen recognition and signaling** |                                    |                                 |                             |               |
| TLR2              | TCTCCTGCTTGGGTTTCAC            | GGTCCCAAGTTAGTAGTATG            | 55                          | NM_212812     |
| TLR4b             | GGATAATGGACGACGGTAAGG          | AGCCAGACGAAAGACTATACTG         | 58                          | AY388400      |
| TLR5b             | GAAACATTCACCCCGCCGAGC          | CTCAACACGAGGAGCAAGAATG         | 58                          | BC163185      |
| NOD1              | CAGACACGAGTACGAGAAATCGG        | TGGAAATACGCCGAGGATCTCC         | 58                          | XM_0026650600 |
| **MHC class I**   |                                 |                                 |                             |               |
|                  | TGCAGAAATGATGTCGGCTATG         | GACACAGACTGGAGAAGAAG           | 60                          | NM_131471     |
| **Cytokines; pro/anti-inflammatory** |                                    |                                 |                             |               |
| IL-1β             | TCAAACCCCAATCCACAGAG           | TCACCTGACGCTCTGAGTG            | 55                          | AY340959      |
| TNFα              | AGAAGAGAGAATGGGCTTACACGCT      | AACACGCTCAGAGCCGACTTT          | 60                          | AY427649      |
| IL-6              | TCAACTCCCTCCAGGCTATG           | TCTTCTCCCTTTTCTCTCTG           | 60                          | JN698962      |
| IL-8              | CTTCCTGCTCAAGCAGGGAC           | GATGCGGCAATTGTCAGG             | 60                          | XM_00936855   |
| IFNγ              | AAATGTTGCTCTCTGTCGGG           | TGGACCGCTGAAATCTGATG           | 55                          | AB185361      |
| IL-12             | TCGACGGAACAGAGATTACCGG         | GATCTCTCCTAAAGGTCACCTG         | 60                          | AB183002      |
| IL-10             | CCAATGATGTCGGCTGATG            | CATATGCCGCTTTGGTGTCCTG         | 55                          | AY887900      |
| **Matrix metalloproteinase family** |                                    |                                 |                             |               |
| MMP9              | TTTTGCCCTGATGCTTGAGATC         | GGGAAACCTCCACGACTTT           | 55                          | AY151254      |
| MMP13             | GAGAAGTTTGGGGTCTCTATG          | TGAAGTCTGCTCTCTCTACTG          | 55                          | AF506756      |
| **Cell surface molecules** |                                    |                                 |                             |               |
| CD8+              | AAGGAGATGATCCGACCGGTA          | GACCTGTCGCTCCTGCTTGGG          | 60                          | NM_01040049   |
| CD4+              | GTGCTCTCATTGCGCTCTGTG          | AATCCTGTGCTGGTGTGTTT          | 60                          | NM_01135906   |
| **Antioxidant enzymes related** |                                    |                                 |                             |               |
| SOD               | AGGTGAATGGTAAATTTACTTTG        | GTCTCAACTATCGTGGTGCG           | 55                          | NM_131294     |
| Catalase          | CCAAGAATGTCAGTGCTACCAATAG     | GCTCAACTCCGGGAAATAA           | 55                          | NM_130912     |
| **Gene for Saprolegnia parasitica** |                                    |                                 |                             |               |
| Tub-β             | AGGAGATGGTTCACGCGGCT           | GATCCTATGTTGAGCTGGC           | 55                          | XM_012348507  |
| Htp1              | CGTATCATGACGGAATATCC           | CGCTTTGCTAAATGTTGCC           | 55                          | GU345745      |
| Ssp1              | CACGAAAGAATAGCAGGAA           | GGTTGAGGCTGAGTTGTA            | 55                          | XM_01253901   |
| Treh              | TGCCGCGCGATGCCGTCAGCA          | GATCAGATGGTACGGGTCCTCAC       | 55                          | XM_01255227   |
| Upase             | TACAGGCTCTCCGCAATTTTGGG        | CCTTGCTGACAGTTGCGGAGGAG       | 55                          | XM_012550441  |
| Igpd              | GCCGGCGCGCGCAGACAGTATG         | CTGTGCGCGTGGCGGCGGTA          | 55                          | XM_012338914  |
| Oatt              | GCCGGCGCGCGCAGACAGTATG         | CTGTGCGCGTGGCGGCGGTA          | 55                          | XM_012350140  |

qRT-PCR, quantitative real-time PCR; TLR, toll-like receptor; NOD1, nucleotide-binding oligomerization domain containing 1; MHC class 1, major histocompatibility complex class 1 UBA; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; CD, cluster of differentiation; SOD, superoxide dismutase; Tub-β, tubulin beta; Htp1, host targeting protein 1; Ssp1, secreted serine protease 1; Treh, trehalase; Upase, uridine phosphorylase; Igpd, imidazoleglycerol-phosphate dehydratase; Oatt, ornithine-oxo-acid transaminase.
**Analysis of transcriptional responses of S. parasitica upon lawsone treatment.** To understand the transcriptional responses of *S. parasitica* mycelium with lawsone treatment, 2-day grown mycelium was separated into 3 groups for 3-time points and added into PDB (20 mL) tubes containing MIC level of lawsone then incubated at 20°C. Controls were kept without adding lawsone. *S. parasitica* mycelium (100 mg in each tubes) from treated and controls were collected at 1-, 3-, and 12-hr post treatment (hpt) and immediately snap frozen in liquid nitrogen followed by storage at −80°C. RNA extraction, cDNA synthesis and qRT-PCR were conducted as described above. The *S. parasitica* genes and gene specific primers used for the gene expression analysis were listed in the Table 1. *S. parasitica* β-tubulin was used as an internal reference gene to normalize the gene expression.

**Statistical analysis.** Statistical analysis of the data from immune and antioxidant enzyme gene expression against *S. parasitica* infection at 3 and 12 hpi was performed by one-way analysis of variance (ANOVA) followed by tukey test analysis for the mean comparison. The control and the treatment means at each time points were compared with unpaired, two-tailed *t*-test using GraphPad program ver. 6 (GraphPad Prism Software, Inc. La Jolla, CA, USA). Significant differences were defined at *p* < 0.05. All data are represented as mean ± SD for triplicate reactions.

**RESULTS AND DISCUSSION**

**Isolation, morphological, and molecular identification of *S. parasitica.** In this study, an oomycete strain was isolated from diseased rainbow trout in Korea and identified as a strain of *S. parasitica* based on the gross, microscopic morphology and genetic analysis. At the time of sampling, morbid fish had severe necrotic infection displaying heavily damaged caudal fin, hemorrhage around gills and dorsal part of the head and skin ulcers in the certain parts of the body. Fig. 1.

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**Fig. 1.** Disease signs in rainbow trout and the morphology of *Saprolegnia parasitica* culture. A, Farmed rainbow trout with necrotic lesions on fins, gills, mouth and head. Black dotted circles indicate severe *Saprolegnia* infection with hemorrhage; B, Radial growth of *S. parasitica* isolate with whitish cotton-like mycelium in potato dextrose agar; C, Microscopic observation of aseptate hyphae (×400); D–F, Releasing of moving primary zoospores from mature zoosporangia; G, Secondary encysted zoospore and hair-like structures on the surface of zoospore (arrows) (×1,000) stained with cotton blue; H, Germinating secondary cyst (×400).
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Therefore, skin tissue samples of the lesions were collected for isolating the causative agent. After repeating subculture with ampicillin containing PDA plate, the isolated pathogen showed the characteristic whitish cotton-like mycelium similar to *S. parasitica* (Fig. 1B) and long aseptate hyphae under light microscope (Fig. 1C).

*S. parasitica* mycelium cultured in STW formed the mature zoosporangia filled with zoospores (Fig. 1D) and primary zoospores were discharged between 18 and 24 hr. The time series of zoospore releasing events are shown in Fig. 1D, 1E, and 1F. After 24 hr, secondary encysted zoospores were collected and lactophenol cotton blue stained image showed the formation of characteristic hair-like structures on the surface of spores (Fig. 1G). These hairs are considered as “attachment structures” and play an important role in adhesion to host. The secondary zoospore has started to germinate within 12 hr from the encysted zoospores (Fig. 1H). These gross and microscopic observations of isolated oomycete were perfectly matched with typical characteristic features of *Saprolegnia* spp. as described in previous reports [32, 35, 36].

ITS sequence of *S. parasitica* has 100% identity with that of *S. parasitica* (accession No. JN400038.1) available in Genbank. Phylogenetic position of isolated *S. parasitica* was clearly grouped with known clade of *S. parasitica* (Fig. 2). Constructed phylogenetic tree displayed representative species of *Saprolegnia* such as *S. ferax*, *S. australis*, and *S. torulosa* under different clusters. ITS sequencing and phylogenetic analysis further confirmed the isolated oomycete strain as a member of *S. parasitica*, hence, we named it as *S. parasitica* CNUaq1. And the sequence of rDNA gene cluster (including 18S ribosomal RNA gene partial sequence, ITS 1, 5.8S ribosomal RNA gene, ITS 2, and 28S ribosomal RNA gene partial sequence) from the isolated *S. parasitica* was deposited in Genbank under accession number of KX494868.1. Moreover, this *S. parasitica* strain was deposited in Korean Collection of Type Cultures (KCTC) under accession number of KCTC46454.

Pathogenicity of *S. parasitica* on embryo, juvenile, and adult zebrafish. Even though the host specificities of individual *Saprolegnia* strains have been reported, *Saprolegnia* species could be infected to multiple hosts [37]. Therefore, studying pathogenicity and host pathogen interactions of *saprolegniasis* using vertebrate aquatic animal
model will be beneficial for the sustainability of fisheries industry. Especially, different stages of zebrafish have been developed as various infection model with bacteria, virus, yeast, and fungi including fish pathogens like Edwardsiella tarda [20] and viral hemorrhagic septicemia virus [21], and host immune function was studied [38]. Also it is reported that Saprolegnia sp. infection can occur in zebrafish as a host [39, 40]. Developing of zebrafish disease model is beneficial for sophisticated study about specific diseases including saprolegniasis, therefore in this study, isolated S. parasitica CNUaq1 strain was used to challenge for all stages of zebrafish including egg, juvenile, and adult to establish a zebrafish disease model for understanding and comparing the level of pathogenicity and mortality. Firstly, zebrafish embryos (at 12 hpf) were challenged with secondary zoospores of S. parasitica (5 × 10^5 spores/mL). Zoospores

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**Fig. 3.** Morphology, survival rate, and hatching delay percentage of Saprolegnia parasitica challenged zebrafish embryos. A, B. *S. parasitica* zoospores were germinated and started growing on the surface of chorion at 3-hr post infection (hpi) and 6 hpi. White dotted line indicates the grown length of mycelium; C. *S. parasitica* mycelium penetrated into a whole embryo and grown inside the embryo at 30 hpi; D, E. Abundant mycelium growth in the dead embryos at 3 hpi and 12 hpi; F. Mycelium growth on the chorion which remained after hatching; G. Cumulative survival rate and hatching delay percentage of embryo exposed to *S. parasitica* secondary zoospores. Survival and hatching rate percentage of embryos were the means of 6 individual groups (10 embryos in each group).
started to grow on the surface of chorion (Fig. 3A) with 100% infection rate at 3 hpi and further grown at 12 hpi (Fig. 3B). The mycelium penetrated into the chorion and grown inside the chorion by 30 hpi (Fig. 3C). Importantly, there was no visual observations of the infections to the larvae, when it was inside the chorion before hatching. Contrastively, high mycelium growth was observed in the dead embryos at 3 hpi (Fig. 3D), 12 hpi (Fig. 3E), and chorion parts which remained after hatching (Fig. 3F). The embryos from the control suffered a mortality event of unresolved origin between 24 hr and 48 hr (93% cumulative percent survival by 60 hr) (Fig. 3G), and the mortality continuation stopped after 60 hr. Though the difference was not significant, low survival percentage was observed for the challenged embryos from 72–96 hpc. However, overall, mortality of S. parasitica challenged embryo was

**Fig. 4.** Survival rate, gross and histopathological observation of zebrafish challenged with Saprolegnia parasitica. A, Cumulative survival percentage of juvenile zebrafish challenged with S. parasitica; B, C, Typical whitish mycelium of S. parasitica near to gill and trunk (B) and methylene blue stained S. parasitica mycelium (C) in all over the trunk of infected juvenile zebrafish; D, E, S. parasitica infection near to the gills (D) and trunk (E) of adult zebrafish; F, G, Histopathological observations of Saprolegnia infected skin tissue. Damage on the epithelium (F and G; black arrow), tissues near to scales (F and G; white arrows) and muscle (F and G; m). S. parasitica hyphae (F and G; h) grown on the epithelial tissue. Over-activation of epithelial goblet cells (F and G; black dotted arrows). Large number of immune cells (F and G; i) infiltration near to infection area (×200); G, Magnified image (×400) of Fig. 4F (scale bars: F = 100 μm, G = 50 μm). Stained with Periodic acid Schiff.
less than 20%. Importantly, the hatched larvae in the challenge group had no signs of *S. parasitica* infection or mortality. Van West [41] reported that abundant mycelial growth on fish eggs could result in high mortality. Interestingly, in this study, wide range of pathogenicity and mortality patterns were observed in different growth stages of zebrafish. At early embryonic stages, (before hatching) growing embryos were protected from infection, even high mycelia growth around the surface and inside the chorion. This was further supported by similar survival rate of *S. parasitica* infected embryo until 60 hpf (before hatching) with control. This gives the evidence that growing embryo may develop resistant, and chorion may provide the protection to inside larva (act as barrier) to reduce mortality, even the hyphae have penetrated into the chorion. However, very high mycelia growth was observed in dead embryos and also on detached chorion and it could be due to no immune resistance and/or high organic matter in dead embryo [42]. Furthermore, there were no any clinical signs or infection in larvae until hatching even though the unhatched egg had mycelium inside. And, after hatching, survival rate of larvae was 100% (data not shown) in *S. parasitica* challenged group, suggesting that it could be due to the host innate immune responses. Apart from that, the overall low pathogenicity in the embryo as well as hatched larvae could be due to the low virulence effect of the *S. parasitica* with the time.

Another important phenomenon we observed in this study was the delayed hatching rate of the *S. parasitica* challenged embryos (Fig. 3G). Hatching was delayed continuously (72–96 hpf), and challenged embryos had 50% of delayed hatching rate compared to respective control groups at each time points. Generally, the zebrafish started to hatch around 48 hpf under the normal fish rearing conditions at 28.5°C. In this study, initiation of the hatching in un-challenged zebrafish embryos was also delayed by 12 hr, which may be due to the low level of temperature (20°C) maintained during the experiment. Though the *S. parasitica* infection had less effect on mortality, observed significantly extended hatching duration (2–4 days hatching delay) suggests that mycelium growth in the chorion may change the enzymatic and other metabolic functions associated with hatching process [43].

Secondly, juvenile challenge was conducted and it showed significantly lower survival rate from 24 hpf onwards compared to control group (Fig. 4A) and it was gradually decreased with characteristic clinical signs (typical whitish mycelium of *S. parasitica* near to the gills and trunk) (Fig. 4B and 4C). Consequently, the survival rate decreased to 35% at 72 hpc. None of the juveniles in the control were died throughout the experimental period. Moreover, when adult fish were challenged with *S. parasitica* zoospores, fish showed extensive range of symptoms not only around the scale removal area but also head, gills, trunk and at the base of pectoral fins (Fig. 4D and 4E). Also, mortality of *S. parasitica* infected adult fish was similar to that of juvenile, resulting 25% of mortality (data not shown). This progressed infection indicates that zebrafish can be developed as disease model to figure out the pathogenicity and host-pathogen interactions against *S. parasitica*.

Microscopic histopathology analysis results showed the invasive infection of *S. parasitica* in zebrafish diseased model (Fig. 4F, ×200 and 4G, ×400). The data showed critical damage to the epithelium (Fig. 4F, black arrow), tissues near to scales (Fig. 4F, white arrows) and muscle (m in Fig. 4F) of the *Saprolegnia* infected areas. Hyphae were shown to be grown onto the epithelial tissues and affected to the epithelial loss (h in Fig. 4F). *Saprolegnia* infection also induced over-activation of epithelial goblet cells (Fig. 4F, black dotted arrows) and infiltration of large number of immune cells near to infected area (i in Fig. 4F). The Fig. 4G shows the magnified photograph (×400) of Fig. 4F, including all the histopathological features described. In fish, *Saprolegnia* hyphae not only invades epidermal tissues and causes cellular necrosis leading dermal and epidermal damage [34, 35], but also penetrates into the muscle and blood vessels of infected fish [24]. In contrast, Álvarez et al. [44] described that some of the *S. parasitica* infections take place rapidly, therefore, inflammatory responses in the fish appear to be completely absent, and it can affect to the goblet cells in the skin resulting to intensify the pathogenicity of *S. parasitica*. Nevertheless, overall our histopathology observations demonstrated the critical damage to the epithelium tissues near to the skin and muscle of infected adult fish.

**Analysis of transcriptional responses of immune and antioxidant enzyme genes in adult zebrafish kidney.** Though the C-type lectin-like receptors are the major receptor group for the recognition of fungi, it has been reported that Toll-like (TLR) and NOD-like receptors (NLR) also play an important role in fungal recognition and clearance [45]. In antifungal immunity, NLRs are important in forming inflammasome complex, which helps to cleave pro-interleukin (IL)-1β/-18 into their biological active forms. Recently it has been shown that proinflammatory cytokines and chemokines (IL-1, IL-8, and tumor necrosis factor [TNF]) are strongly induced upon *S. parasitica* infection, together with several components of the innate cellular responses in rainbow trout cell lines (RTG-2, RT-Gill, RTL, and RTS11) [46]. Similar results on proinflammatory cytokine expression and some degree of suppression of host major histocompatibility complex (MHC) class II molecules and associated molecules have been also reported for RTS-11 cell line [46]. Belmonte et al. [47] reported that *S. parasitica* is able to trigger strong inflammatory responses (e.g., induction of IL-1β, IL-6, and TNFα) in Atlantic salmon gills and head kidney, whereas drastic suppression of adaptive immunity associated genes, via down regulating T-helper cells cytokines, antigen presenting machinery and immunoglobulin.

In this study, we conducted the qRT-PCR analysis of 18 immune gene expressions with focusing on the pattern/
antigen recognition receptors, pro/anti-inflammatory cytokines, matrix metalloproteinases (MMPs) family members, components of adaptive immune responses and antioxidant enzymes related genes to understand the immune responses in zebrafish upon *S. parasitica* artificial infection. In agreement with previously reported in vitro [46] and in vivo data [47], our study also showed different gene expression modulation patterns at 3 and 12 hpi. The relative mRNA gene expression fold values in challenge groups compared to same time point controls were presented in the Figs. 5 and 6. In brief, fish that were infected with *S. parasitica* showed up-regulated expression pattern of TLR2, TLR5b, IL-1β, TNFa, IL-6, interferon γ (IFNg), IL-10, MMP9, and CD8+ at both 3 and 12 hpi compared to uninfected control group at their respective time points. Among these, increased mRNA expression patterns were observed for TLR5b, IL-1β, IFNg, and IL-10 at 12 hpi compared to 3 hpi. Though the IL-8 mRNA expression was basal at 3 hpi, it was highly up-regulated at 12 hpi. Moreover, TLR4, NOD1, IL-12, and MMP13 genes were up-regulated at 3 hpi, and became basal or slightly down-regulated at 12 hpi. Additionally, MHC class I, CD4+, superoxide dismutase (SOD), and catalase genes were exhibited down-regulated expression pattern at both 3 and 12 hpi compared to control group.

In this study, the induction of three important pro-

**Fig. 5.** Transcriptional responses analysis of zebrafish immune and antioxidant enzyme genes in kidney upon *Saprolegnia parasitica* infection. mRNA expression patterns of selected genes were analyzed at 3- and 12-hr post infection (hpi) of *S. parasitica*. mRNA expression fold-changes were analyzed according to the Livak method ($2^{-\Delta\Delta CT}$) using zebrafish β-actin as a house keeping gene. TLR, toll-like receptor; NOD1, nucleotide-binding oligomerization domain containing 1; MHC class 1, major histocompatibility complex class 1 UBA; IL, interleukin; TNFa, tumor necrosis factor α; IFN, interferon; MMP, matrix metalloproteinase; CD, cluster of differentiation; SOD, superoxide dismutase. Data are presented relative to that of un-challenged fish (control) at 3 and 12 hpi each. Significant differences were defined at $p < 0.05$ and all data are represented as mean ± SD.

**Fig. 6.** Comparison of relative gene expression in kidney of zebrafish against *Saprolegnia parasitica* challenge at 3 hr and 12 hr. TLR, toll-like receptor; NOD1, nucleotide-binding oligomerization domain containing 1; MHC class 1, major histocompatibility complex class 1 UBA; IL, interleukin; TNFa, tumor necrosis factor α; IFN, interferon; MMP, matrix metalloproteinase; CD, cluster of differentiation; SOD, superoxide dismutase; V, relative expression fold value.
inflammatory (IL-1β, TNFα, and IL-6) and anti-inflammatory (IL-10) cytokines suggests the possibility of activation and regulation of the pro-inflammatory cytokine cascade, the macrophages functions and also the adaptive immune system. However, the high mortality in infected fish indicates that these responded gene expression levels/patterns may not sufficient to clear the infection, once the fish severely infected. Nevertheless, when considering most of the genes, individuals have high difference in gene expression levels. This also might suggest that, in some individuals, important immune responses might have occurred to determine the fish survival or death. The failure of the adequate responses in immune system also can be explained by the suppression of components of adaptive immune system seen in this study. Another interesting result was that the antioxidant enzymes (SOD and catalase) genes (Figs. 5 and 6) were down-regulated at both 3 and 12 hpi. However, increasing trend was observed with the time and this increment was statistically significant between 3 and 12 hpi. This down regulation pattern might occur because of initiating of the reactive oxygen species production for cellular innate immune responses (e.g., respiratory burst activity) or stress caused by Saprolegnia infection, which needs to be studied in detail. Even the severity of the infection, physiological conditions of the fish and thermal conditions could influence the gene transcription, ultimately it causes fish to survive or die. Hence, S. parasitica challenge under different zoospore doses and thermal conditions may support for better understanding of host pathogen interactions and understanding for the role of these genes during S. parasitica infection and elucidating detail mechanism of anti-saprolegniasis.

**Anti-Saprolegnia activity of lawsone.** With banning of Malachite green in 2002, searching for alternative agents for controlling S. parasitica becomes vital for sustainability of aquaculture industry. Recently, it was shown that copper sulfate is a potential agent against S. parasitica infection in grass carp [48]. Thus, in this study, plant based lawson was chosen to treat S. parasitica due to its known antimicrobial properties [49, 50]. To investigate the anti-Saprolegnia effects of lawson, growth profile of S. parasitica was determined under different concentrations of lawson (50, 100, 200, and 400 μg/mL) in PDB. There was no pH change after adding lawson into PDB. In other studies, anti-fungal activity of lawson has been compared at MIC level against different pathogens including E. oxysporum (12 μg/mL), Aspergillus flavus (50 μg/mL), A. niger (150 μg/mL) [49], and Candida albicans (512 μg/mL) [50], suggesting that it varies with the type of pathogens and the strain. In this study, results revealed that the MIC of lawson against S. parasitica was 200 μg/mL. Base on the MIC result, different lawson doses were tested against S. parasitica in lawson containing PDA to determine growth inhibition and PIRG in time series experiment. The data shows that the anti-Saprolegnia effect of lawson was concentration dependent (Fig. 7A). The highest growth inhibition was observed at 400 μg/mL, and lower lawson doses (100 and 200 μg/mL) exhibited the moderate level of inhibition compared to control. To determine the growth inhibition quantitatively, we compared the growth fold of control and lawson treated S. parasitica at 60 hr. As expected, lawson treated with 400 μg/mL showed the highest PIRG (88.03%) compared to untreated control (13.8%) (Fig. 7B).

**Effects of lawson on cell membrane damage and viability of S. parasitica.** Morphological changes and viability analysis of S. parasitica mycelium were carried out using FE-SEM and PI analysis, respectively, upon lawson treatment. Damage on cell wall structure was clearly distinct at MIC (200 μg/mL) and 2× MIC (400 μg/mL) doses of lawson treated S. parasitica samples compared to untreated control. Smooth exterior surface on the cylindrical shaped mycelium was observed in control (Fig. 8A), whereas, in the treatment groups, the mycelium surface was damaged and those damages were aggravated when the treatment doses increased up to 400 μg/mL (Fig. 8B and 8C). In detail, irregular shrinkages were exhibited in the mycelium surfaces at MIC and 2× MIC level, and the mycelium walls were severely shrunken and ruptured.
The morphological data obtained by scanning electron microscopy (SEM) analysis was correlated with the PI uptake results, demonstrating that lawsone affected S. parasitica cell viability. After PI uptake, non-viable (dead) hyphae can be identified in red color by CLSM image analysis. In this study, undetectable levels of red colored hyphae were observed in untreated mycelium indicating 100% viability of S. parasitica (Fig. 8D). In the lawsone treated samples, distinctive red colored mycelia were observed at both MIC (Fig. 8E) and 2× MIC levels (Fig. 8F). Our FE-SEM and PI staining results revealed that lawsone can disrupt the mycelium surface, thereby causing mycelia death of S. parasitica. Overall anti-Saprolegnia activity results support our hypothesis that lawsone is able to alter the membrane permeability and it may cause irreversible cellular membrane damage and disintegration.

**Analysis of transcriptional response of S. parasitica genes upon lawsone treatment.** To investigate the possible mechanism which might involve in the inhibition of S. parasitica growth upon lawsone treatment, mRNA expression of selected S. parasitica genes related to protein synthesis, metabolism and virulence factors was studied. The varied expression patterns were observed in lawsone treated S. parasitica, following the time. For the qRT-PCR, three technical PCR replicates were used for each time point without biological replicates. Up-regulated mRNA expression patterns of Htp1 (15-fold) and Ssp1 (16-fold) were observed in lawsone treated S. parasitica compared to the control, at each time points with the highest expression levels at 12 hr (Fig. 9A and 9B). Similarly, up-regulated Treh mRNA expression (Fig. 9C) was observed at 1, 3, and 12 hr showing expression peak at 12 hr (40-fold). The function of the Ssp1 is predicted as a subtilisin-like serine protease due to significant similarity with other organism’s serine proteases [51]. Jiang et al. [3] reported that metalloproteinases and serine proteases secreted by S. parasitica are capable of degrading trout immunoglobulin IgM, suggesting this protein as a potential virulence factor with a role in suppression of host immune responses. Contrast to this phenomenon, Ssp1 has been shown as producing antibodies against saprolegniosis resistant fish [51]. Htp1 is translocated into host cells [52], and it has been found that copper sulfate treatment did not alter Htp1 mRNA expression [53]. Thus, in this study, the question arises whether lawsone has any influence on S. parasitica for inducing or suppressing the virulence effect, which needs to be studied more detail in future. In contrast, expression levels were down-regulated in some of the genes such as Upase (Fig. 9D), Igpd (Fig. 9E), and Oatt (Fig. 9F) at each tested time points. Similarly, Upase and Oatt mRNA expression have been down-regulated in copper sulphate treated S. parasitica, however, contrastively, Treh mRNA expression has been up-regulated [53]. As a whole, our data suggest that lawsone has ability to directly altering the expression of genes encoding key cellular processes such
Saprolegnia parasitica isolated from Rainbow Trout in Korea

The present study described the successful isolation, morphological and molecular based identification of S. parasitica from diseased rainbow trout in Korea. Pathogenicity of isolated S. parasitica was developed in embryo, juvenile and adult zebrafish as a disease model, and we demonstrated that adult zebrafish immune genes were modulated in kidney against S. parasitica. This provides an insight into immune responses of S. parasitica infected zebrafish, and will be aided in the identification of markers associated disease status in zebrafish. This paper also describes the in vitro experiments that plant based natural lawsone is able to inhibit the growth and even causes the death of S. parasitica. Moreover, we attempted to investigate a possible mechanism of S. parasitica growth suppression by studying S. parasitica transcriptional responses. Further studies will be required to find suitable S. parasitica infectivity levels and survival for understanding host pathogen interactions in zebrafish, and also to get clear understanding of the mechanism of anti-Saprolegnia activity of lawsone.

ACKNOWLEDGEMENTS

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2014R1A2A1A11054585), research fund of Chungnam National University and the project titled ‘Fish Vaccine Research Center’, funded by the Ministry of Oceans and Fisheries, Korea.

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