

Fenton Reaction Facilitates The Fungal Infection On The Unicellular Alga *Haematococcus Pluvialis* and Discovery of a Biosafe and Environment-Friendly Chemical That Blocks The Infection

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Research

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Abstract

Background

The green microalga *Haematococcus pluvialis* is used as cell factories for producing astaxanthin, the high-value natural compound with multiple biological functions. However, *H. pluvialis* is prone to the infection by a parasitic fungus *Paraphysoderma sedebokerense*, which is the most devastating threat to the mass culture of *H. pluvialis* all over the world. Little is known for the mechanisms underlying the infection process, though it is of great essential for developing effective measures to mitigate the pathogen threatening for the natural astaxanthin industry.

Results

We observed that there were heat-stable substances with small molecular weight produced during the infection process, which significantly stimulated the parasitism process and enhanced the susceptibility of *H. pluvialis* cells to the pathogen. Systematic approaches including multi-omics, biochemical and imaging analysis were deployed to uncover the identity of the metabolites and the underlying mechanisms. Two metabolites, 3-hydroxyanthranilic acid and hordenine were identified and proved to stimulate the infection via driving fenton reaction mediated oxidative stress to *H. pluvialis*. The reaction generated hydroxyl radicals to disrupt the subcellular components of the algal cells and to make the algal cells more susceptible to the infection. Based on these findings, a biosafe and environment-friendly antioxidant butylated hydroxyanisole was selected to inhibit the fungal infection, which completely abolished the infection at 12 ppm.

Conclusions

This study provide for the first time, a framework to dissect the functions of secondary metabolites in the interaction between the unicellular algal *H. pluvialis* and its fungi parasite, indicating that oxidative degradation is a strategy used for the fungal infest. Eliminating the oxidative burst through adding antioxidant butylated hydroxyanisole could be an effective measure to reduce parasitic infection in *H. pluvialis* mass culture.

Background

Microalgae are increasingly used for producing high-value compounds for food, nutraceuticals and cosmetics applications and are promising resources for biofuels production (Borowitzka & Vonshak, 2017; Torres-Tiji et al., 2020). Microalgae also possess great potentials in greenhouse gas emission mitigation as well as for wastewater treatment (Choi et al., 2019; Maity et al., 2014; Specht et al., 2010). However, contaminations of parasites in mass culturing system are keeping threatening the sustainable production of biomass and bio-products by utilization of microalgae (Carney & Lane, 2014).

Consequently, it is difficult to produce bulk volume of microalgal biomass at low cost due to the occurrence of various predators and pathogens in mass cultivation.

The green unicellular microalga *Haematococcus pluvialis* is a freshwater biflagellate and belongs to the class Chlorophyceae, order Volvocales, which is well known for its ability in accumulating up to 5% of the dry weight biomass of natural bio-active compound astaxanthin under stresses conditions such as nitrogen deficiency and high light irradiation (Damiani et al., 2010; Han et al., 2013; Harker et al., 1996; Ren et al., 2021). Therefore, *H. pluvialis* is considered as the most sustainable feedstock for the commercial production of astaxanthin (Khoo et al., 2019), which has an estimated market value of USD 240 million in 2021 (Ren et al., 2021). However, the development of *H. pluvialis* mass culture industry has been retarded by contaminations of fungal parasite, which is often accompanied by reduced biomass yield and astaxanthin productivity (Ding et al., 2020; Shah et al., 2016). A parasitic fungus *Paraphysoderma sedebokerense* (Blastocladiomycota) infect the *H. pluvialis* cells in a highly species-specific manner, which is the most devastating threat to the mass culture of *H. pluvialis* all over the world (Ding et al., 2020; Hoffman et al., 2008; Hwang et al., 2019). Once the pathogen appears in the mass culturing system, the infected algal cells are dying during very short period of time, causing severe economic loss for the natural astaxanthin manufacturing industry (Gutman et al., 2009; Strittmatter et al., 2016). Several strategies have been developed to control the pathogenic fungi, such as maintaining the pH of culture system at the acidic condition and application of sodium dodecylbenzene sulfonate (SDBS) (Ding et al., 2020; Hwang et al., 2019). More environment-friendly and biosafe measures are desired and necessary to improve the sustainability of *H. pluvialis* mass culture.

Understanding about the mechanisms underlying the infection process is essential for developing effective measures to mitigate the pathogen threatening for the natural astaxanthin industry. Interactions between *H. pluvialis* and *P. sedebokerense* have been investigated in previous studies. Sugar moiety on the *H. pluvialis* cell wall was proposed to play a role as binding molecule for recognition by *P. sedebokerense* (Gutman et al., 2011). Heat-stable recognition sites on the algal cell were necessary for the fungal attachment and encystment, while signal transduction in *H. pluvialis* cells was obligated for the fungal sporangium development and epidemic (Asatryan et al., 2019). Interaction between the cell walls of *H. pluvialis* and carbohydrate activated enzymes in *P. sedebokerense* was believed to be essential for the parasitism process (Lin et al., 2021). These studies are focused on the specific recognition of *H. pluvialis* cells by *P. sedebokerense* and the warfare between them during the early-stage of infection, but little is known for the mechanisms underlying the post-infection process, especially for the fast material degradation in algal cells.

In this study, we observed that the accumulated secondary metabolites in the infection system significantly stimulated the parasitism process and enhanced the susceptibility of the algal cells to the pathogen. With the aim to uncover the identity of them and to understand the underlying mechanisms, systematic approaches including multi-omics, biochemical and imaging analysis were deployed herein. Two metabolites, i.e. 3-hydroxyanthranilic acid (3-HAA) and hordenine, were identified and proven stimulating the infection process via non-enzymatic fenton reaction in a congruent manner. The hydroxyl

radicals produced from the reaction promoted the infection through causing strong oxidative stresses to the host cells and impairing the algal cell structures along with degradation of the intracellular components. Intentionally, application of the antioxidant butylated hydroxyanisole (BHA) to the algal-fungal system reduced the infection ratio effectively, indicating that oxidative degradation is a strategy used for the fungal to successfully infect algal cells while eliminating the oxidative stress was practicable for mitigating fungal infection in *H. pluvialis* mass culture. This study provided a framework to dissect the functions of secondary metabolites in the interaction between the unicellular algal and its fungal pathogen, and developed a new crop protection measure to improve the sustainability of algal mass cultivation.

Results And Discussion

Fungal infection caused cell death of *H. pluvialis* and the supernatant post infection (SPI) enhanced the infection process

When the *H. pluvialis* cells cultivated in 360 L panel photobioreactors were infected by *P. sedebokerense*, algal cells died rapidly and seriously (Fig. 1A-B). We collected the supernatant post infection (SPI) by removing both the host and fungal cells via centrifugation, and investigated the effects of SPI on the newly infection process. When the healthy algal cells were pre-treated with SPI and then challenged with the fungal swarmers, the infection process was significantly promoted (Fig. 1C). On the second day post inoculation, the color of the control cell culture was dark-green and a few algal cells were attached with the fungal swarmers. By contrast, a large number of dead algal cells were observed in the culture of algal cells pre-treated with SPI, which settled down to the bottom of flask. On the third day post inoculation, the color of the culture of algal cells pre-treated with SPI turned to brownish, whereas the control remained green. Pre-treating the algal cells with SPI prior to the pathogenic challenge caused significantly higher infection ratio after the second day post inoculation than the control (Fig. 1D). Additionally, the SPI remained the infection enhancing activity after pre-treated in 98°C water bath for 15 min, indicating that the activity of SPI was not eliminated by heating. It was further observed that in the 48h-SPI treated algal cells, the pigments were partially degraded and the cellular starch granules disappeared (Fig. 1E). Quantitative analysis revealed that when compared to the control, the contents of the total cellular carbohydrates and pigments (i.e. carotenoids and chlorophyll) were reduced in the algal cells treated with SPI by 50% and 20%, respectively (Fig. 1F). Additionally, after filtered with 3000 Da cut-off membrane, the activity of the filtrate did not significantly altered in degrading carbohydrates and pigments in the algal cells, suggesting that the activity in the SPI was most likely attributable to small molecules. These results together suggested that some heat-stable substances with small molecular weight produced during the infection process and were capable of enhancing the susceptibility of *H. pluvialis* cells to the pathogen by affecting the algal cell integrity.

SPI induced oxidative stresses within algal cells

Alterations in the subcellular structures of the algal cells challenged with both *P. sedebokerense* and SPI were observed with transmission electron microscopy (TEM) (Fig. 2A). Degradation of the subcellular membrane systems were observed in the algal cells treated with SPI without the involvement of fungus. Additionally, the algal cell walls were loosened after being treated with SPI. These results indicated that the substances in SPI degraded the algal cellular components and destructed membranes.

To uncover the identity of the secondary metabolites in SPI, transcriptomic analysis was conducted to facilitate understanding the effects of SPI on the algal cells. A total of 998 and 490 genes were up- and down-regulated in *H. pluvialis*, respectively, after the SPI treatment for 24 h. Expression of many genes involved in biotic stresses responses were significantly altered in the algal cells treated with SPI (Fig. 2B). Several genes coding for the anti-oxidative enzymes were found to be significantly up-regulated while genes coding for synthesis and transportation were down-regulated. Up-regulation of the genes involved in oxidative stress responses indicated that SPI may contain substances that can cause the generation of ROS (Hasanuzzaman et al., 2020; Torres et al., 2006). To test this hypothesis, the oxidative activities of SPI were measured by using the thiobarbituric acid (TBA) assay with fenton reagent as the positive control because it is a known reaction that generates oxidative stress through small molecules (Arantes et al., 2012; Eastwood et al., 2011). The results showed that the SPI possessed strong oxidative activity *in vitro* (Fig. 2C). In addition, the SPI showed lipid peroxidation activity when acting on the algal cellular membranes, leading to formation of malondialdehyde (Fig. 2D). To further identify the ROS produced by SPI, the dimethyl sulfoxide trapping method was used and the results suggested that SPI could produce hydroxyl radical *in vitro* (Fig. 2E).

Based on the transcriptomic results and a suite of observations and biochemical assays, it can be concluded that SPI contained substances that exerted oxidative stresses via generation of ROS in the algal cells. Oxidative degradation of the algal subcellular structures might be the cause of decreased resistance to fungal infection.

Secondary metabolites mediated fenton reaction facilitates the fungal infection

Metabolomic analysis was performed for identification of the small molecules causing the oxidative stresses. The SPIs were collected at different infection stages, i.e., 1, 3 and 5 day post inoculation of the fungus into the algal cell cultures. It was found that the degradation activity of SPI collected on Day (D) 5 was significantly higher than that on D1, suggesting that the concentration of the metabolites of target increased over 5 days. Based on this, 62 metabolites which showed over 2-fold increases on D5 than that on D1 were selected, most of which were organic acid, dipeptide, amino acid and derivatives. Ten metabolites, including tyramine, trimethoprim, indole-3-carboxylic acid, hordenine, deoxycytidine, 4-pyridoxic acid, lumichrome, 3-hydroxyanthranilic acid (3-HAA), baclofen and cyclohexylamine, with the phenol/quinone/aromatic structure, were retrieved manually (Fig. 3), since such types of compounds are known to be able to mediate the fenton reaction producing hydroxyl peroxide (Arantes et al., 2012; Eastwood et al., 2011).

When these substances were added into the algal cell culture, it was firstly observed that hordenine and 3-HAA significantly reduced the contents of carbohydrates while tyramine, hordenine and cyclohexylamine caused degradation of the pigments in the treated algal cells (Fig. 4A). Secondly, the potential infection-promoting effect of the candidate metabolites was checked. On the 3rd dpi (day post infection), the algal cells pre-treated with either hordenine or 3-HAA showed significantly enhanced infection ratio than that of the control and other compounds (Fig. 4B).

The fenton reaction is initiated from the reduction of Fe^{3+} to Fe^{2+} , which is the key factor for driving fenton reaction (Kameshwar & Qin, 2018). Thus the reducing activities of the 10 candidate metabolites were tested. Among them, 3-HAA showed the strongest activity in reducing Fe^{3+} to Fe^{2+} at 4 h (Fig. 4C). Additionally, 3-HAA and hordenine generated hydroxyl radical in the assay with DMSO as substrate (Fig. 4D). The intracellular concentration of hydrogen peroxide (H_2O_2), an important intermediate of the fenton reaction, was measured after staining with the fluorescence DCFH-DA. The result showed that in the hordenine-treating algal cells the concentration of H_2O_2 continued increasing during 48 h, while the level of H_2O_2 in the 3-HAA-treating algal cells transiently increased during 24 h and the gradually decreased (Fig. 4E).

These results taken together indicated 3-HAA and hordenine are the components in SPI which caused oxidative stresses in the algal cells through mediating fenton reaction. The metabolite 3-HAA is an intermediate of kynurenine pathway, and has been found in bacteria, yeast, fungi, plants and mammals and was reported to be a fungal producing mediator that was widely applied in fenton processes in dye decolorization due to its ability in reducing Fe^{3+} to Fe^{2+} (Santana & Aguiar, 2015; Santana et al., 2019). It is also a generator of free radicals through its auto-oxidation (Breton et al., 2000; Li et al., 2001). Thus, the generation of H_2O_2 from the auto-oxidation of 3-HAA could not be excluded here. Hordenine, originally detected in barley and also isolated from marine algal *Phyllophora nervosa* (Guven et al., 2010; Mann et al., 1963), is a phenethylamine alkaloid with various bioactivities, including antibiotic activity against microorganisms, inhibition of quorum sensing and biofilm formation (Rao, 1970; Zhou et al., 2018). It was reported that hordenine was responsible for the protective responses of plants to various stresses through jasmonate dependent defense pathway (Ishiai et al., 2016), and also acted as an plant allelochemical that can inhibit the growth of weed or defend against pathogens attack (Kotzamani et al., 2021; Lebecque et al., 2018).

Application of antioxidant to inhibit the fungal infection

As the oxidative stresses caused by the SPI impaired the algal cell structures and promoted the fungal infection process, an exogenous antioxidant was introduced to relieving such oxidative stress in the culture to inhibit the infection. BHA is one of the most commonly used synthetic antioxidants in food and biodiesel fuels to prevent oxidation for its low cost, high stability and effectiveness (Rodil et al., 2012; Ryu, 2010; Sahraee et al., 2019; Xu et al., 2021). Additionally, BHA is biosafe and environmental-friendly, rendering its application in the aquaculture industry (Additives et al., 2018; Williams et al., 1999). BHA was added into the infection system at different final concentrations (i.e. 2 ppm, 7 ppm and 12 ppm) (Fig.

5). The infection ratio of the newly infected algal cells was calculated to reflect the infection inhibitory effect. Compared to the untreated *H. pluvialis* culture, addition of BHA at 2 ppm delayed the complete fungal infection for 1 day. Elevating the concentration of BHA to 7 ppm decreased the infection greatly, and the infection was only about 30% on day 5, while the untreated group was 100% infected. Application of 12 ppm BHA to the system completely suppressed the fungal infection.

According to all the results described above, a model was proposed herein to illustrate the major findings of this study. Production of secondary metabolites such as 3-HAA and hordenine in the infection system mediated the generation of hydroxyl radical via the fenton reaction, the most reactive free radicals among various ROS, which disrupt the subcellular components of the *H. pluvialis* cells and make the algal cells more susceptible to the infection. However, by adding 12 ppm of the antioxidant BHA to the culture, the fungal infection was completely abolished, indicating the oxidative burst is essential for the pathogens to infest.

Conclusion

In this study, two secondary metabolites (i.e. 3-HAA and hordenine) were identified from the fungal infection system, which can drive fenton reaction thereby causing severe oxidative stresses on the host cells. Based on these findings, the antioxidant BHA was used to successfully block the fungal infection. This proof-of-concept study indicated utilization of antioxidants represents a novel strategy to reduce the fungal infection in microalgal mass culture.

Methods

Strains and culturing conditions

H. pluvialis cells were maintained in our laboratory (Lin et al., 2021) and cultured in the BG11 growth medium at 21-23°C under continuous illumination ($20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The fungal parasite *P. sedebokerense* used in this study was isolated in the previous study (Lin et al., 2021). *P. sedebokerense* cells were grown in the fungal growth medium supplemented with yeast extract and peptone (Hoffman et al., 2008) on an orbital shaker at a speed of 150 rpm maintained at 30°C.

Preparation of the supernatant post infection (SPI) and SPI incubation assay

When the OD₆₀₀ of the fungal cell cultures reached 0.03 within 5 days, 1% (v/v) of the fungal cells was inoculated in the algal cell cultures (about 3.0×10^5 algal cells mL⁻¹). The mixture was then incubated on an orbital shaker at a speed of 150 rpm at 30°C. Samplings were performed on a daily basis and the infection ratios (Gutman et al., 2011) were determined by cell counting under microscope (Olympus, BX53 with a DP70 CCD camera). To prepare for the SPI, when the algal cells were completely infected by the parasitic fungus, the culture mixture was centrifuged at 2,100 g for 5 min to collect the supernatants and was filtrated by using double-layered 1.2 µm glass microfiber filters (Whatman, UK), heated at 95°C in water bath for 15 min and were stored at 4°C, such liquid was termed as supernatant post infection (SPI).

For detecting the infection enhancing activity of the SPI, 100 mL of algal cells with density of 5.0×10^5 cells mL^{-1} from exponential growth phase was harvested at 700 g for 3 min, the pellet was pre-treated with 100 mL SPI or BG11 medium for 48 h at 30 °C, under light intensity of 20 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 150 rpm. The liquid was removed via centrifugation at 700 g for 3 min, and the cell pellet was re-suspended in 100 mL of BG11 medium. Fungal cells were inoculated in the algal cell cultures, samples of each experiment were checked with microscope to count the prevalence of infection every day as described above. Three independent biological replicates were conducted for each sample and the quantitative data were presented as mean \pm S.D. ($n=3$). **, $p < 0.01$ (Student's t-test).

Transmission electron microscopy and starch staining

Samples of healthy *H. pluvialis* cells that grown at logarithmic phase, or incubated with SPI for 3 days were centrifuged at 700 g for 3 min, respectively. The cell pellet of each sample was washed twice with fresh BG11 medium, and was fixed overnight in 2.5% (w/v) glutaraldehyde at 4°C. The flowing sample processing procedures included osmium fixation, dehydration, infiltration, polymerization, section and staining (Wayama et al., 2013). The fixed and stained samples were examined with transmission electron microscope (FEI, Tecnai G2 20 TWIN, 0.24 nm/200 kV). For starch staining, 2% (w/v) of the Lugol's solution was added to the algal cell samples and incubated for 5 min. The starch granules were observed under light microscope.

Biochemical analysis

SPI or BG11 medium pretreated algal cells were pelleted via centrifuged at 700 g for 3 min and were washed with distilled H₂O. To extract the pigments, the cell pellets that grinded in mortar on ice under dim light were extracted with dimethyl sulfoxide (DMSO, for several times until the cell pellets turned colorless). The cellular contents of pigments chlorophyll and carotenoid were quantified by spectrophotometers. To analyze the content of remaining carbohydrates, the cells were hydrolyzed by 3 M trifluoroacetic acid (TFA) for 4h at 98°C, dried by nitrogen gas and re-established in distilled H₂O. After filtrated with 0.22 μm micro-aperture filter membrane (Merck Millipore, USA), the sugar content in the sample was analyzed by an ICS 5000+ Ion Chromatography (IC) system (Thermo-Fisher Scientific, USA). Thiobarburic acid (TBA) assay was used for detecting oxidative activity of SPI. Fenton reagents (0.83 mM ferrous ions and 30 mM hydrogen peroxide) were used as the positive control. The Malondialdehyde (MDA) kit (Jiancheng Bioengineering Institute, Nanjing, China) was introduced to measure the in vivo lipid peroxidation in the SPI treated *H. pluvialis* cells. Ferrozine assay was conducted to measure the ferric ions reducing activity (Gibbs, 1976).

Analysis of the hydroxyl radical and hydrogen peroxide production

The hydroxyl radicals oxidize dimethyl sulfoxide (DMSO) and generate formaldehyde, which can react with 2, 4-dinitrophenylhydrazine (DNPH) and form hydrazone (HCHO-DNPH). HCHO-DNPH can be analyzed by HPLC, eluted peaks of HCHO-DNPH with maximum absorbance at 355 nm was

targeted (Tai et al., 2004). Fenton reagent containing 0.2 mM Fe²⁺ and 8 mM H₂O₂ was used as the positive control to generate hydroxyl radicals. To measure the intracellular hydrogen peroxide (H₂O₂), the fluorescence dye 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma, USA) was introduced to stain the treated algal cells (Gwak et al., 2014). The stained cells were analyzed by using the flow cytometer and the fluorescence intensity (excitation wavelength at 488 nm) of the cells reflected the intracellular H₂O₂ level.

Metabolomics

The SPI on day 1, 3 and 5 were collected respectively, and the supernatants were used for the ultra-high performance liquid chromatography coupled with hybrid quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) analysis. Two independent biological repeats were conducted for each sample. Fold change (peak intensity of D5/D1) > 3 was screened and compounds with phenol/quinone/aromatic moiety (Korripally et al., 2013) were particularly targeted. Target reagents were purchased from Sigma-Aldrich, cyclohexylamine was dissolved as 2% (v/v) solution in BG11 medium and the other 9 reagents were dissolved as 0.2% (w/v) solution in BG11 medium. These solutions were put in a 95°C water bath for 15 min and cooled down and applied appropriate ultrasonic treatment to enhance the dissolution, the supernatants of each solutions were collected to further test their activity.

RNA extraction and RNA-seq analysis

For analysis of algal response to SPI treatment, 100 mL of the *H. pluvialis* cell culture (5.0×10^5 cells mL⁻¹) were incubated in SPI or BG11 medium (as control), respectively for 24 h. One mL of the algal cell cultures was then centrifuged to collect the pellet, and was frozen with liquid nitrogen immediately. The RNA extraction and RNA-seq analysis were performed according to the methods described by Ma et al. (Ma et al., 2020). Differentially expressed (DE) genes with Log₂FC > 1.5 was considered, and the up- and down-regulated gens was checked and analyzed manually. Three independent biological repeats were conducted for each sample.

Declarations

Credit authorship contribution statement

Hailong Yan: Data curation and Writing - original draft. Haiyan Ma: Data curation and Writing - original draft. Yanhua Li: Methodology. Liang Zhao: Data curation and Software. Juan Lin: Resources. Qikun Jia: Data curation. Danxiang Han and Qiang Hu: Funding acquisition and Writing - review.

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Data availability

Transcriptome data is available at NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) with accession number PRJNA720251.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

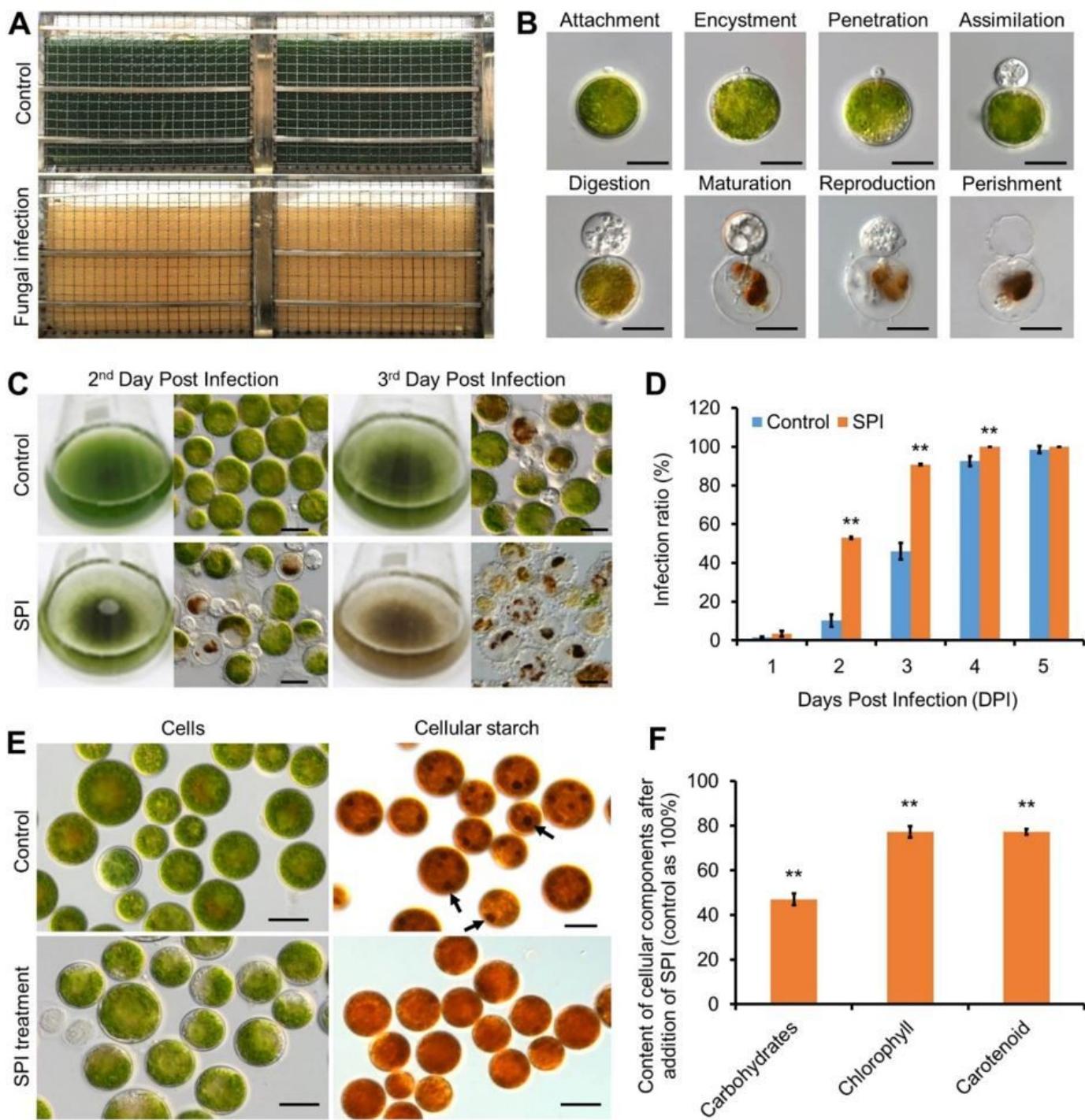


Figure 1

Fungal contamination crushed the growth of *Haematococcus pluvialis* culture and the supernatant post infection (SPI) enhanced the fungal infection process. (A-B) Crush of *H. pluvialis* algal culture after contaminated by fungal parasite *P. sedebokerense*. (C-D) SPI enhanced the fungal infection process. (E-F) Decomposing effects of SPI on the algal cell. Bars=20 μ m.

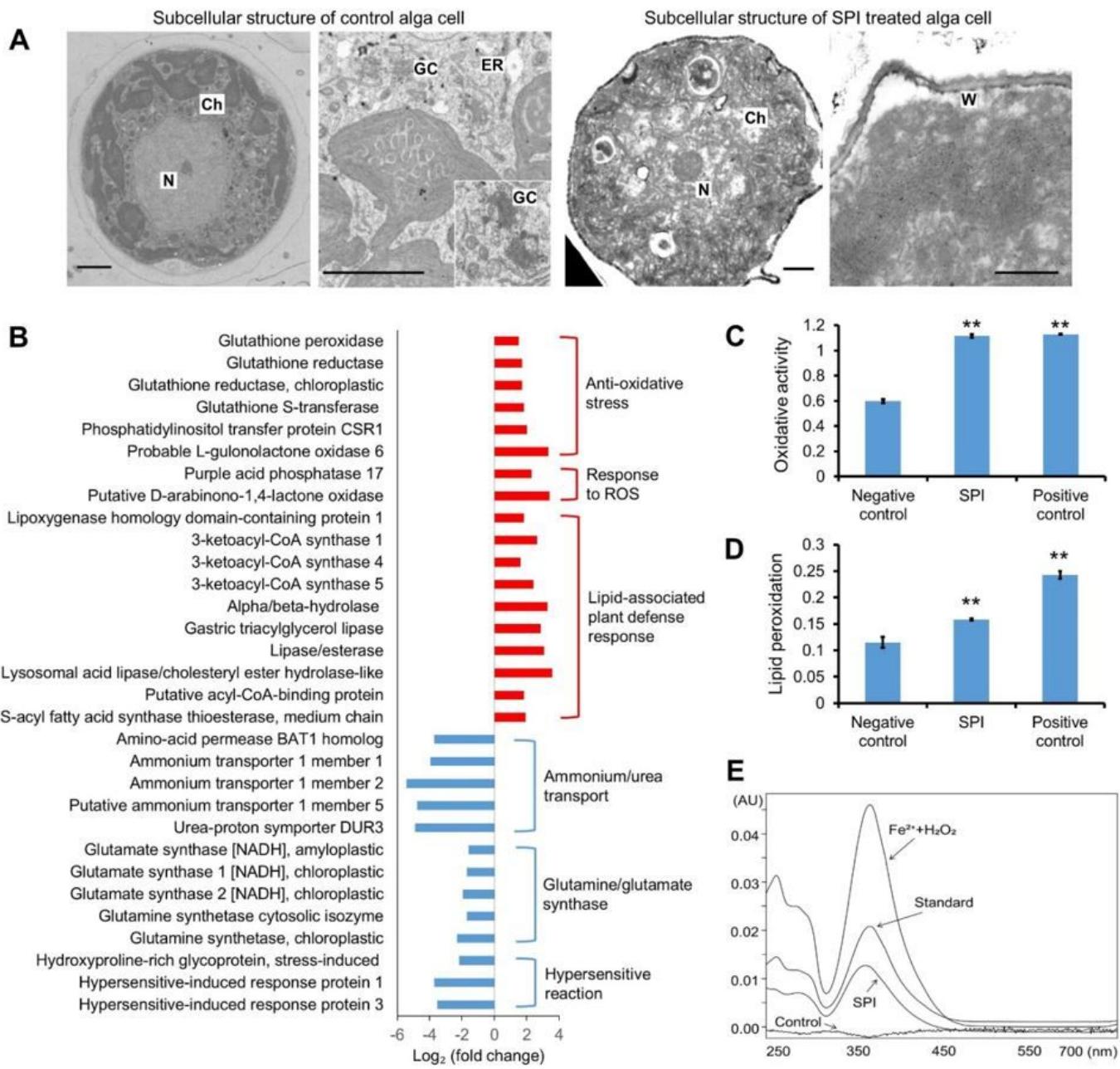


Figure 2

The SPI caused alga structure degradation and exhibited oxidative activities both in vivo and in vitro. (A) TEM observation on the algal cells treated with SPI. N, nuclear. W, cell wall. Ch, chloroplast. GC, Golgi complex. ER, endoplasmic reticulum. (B) RNA-seq analysis. (C) Oxidative activities of the SPI. (D) Lipid peroxidation in host algal cells treated with the SPI. (E) Hydroxyl radical detection. Bars=2 μ m.

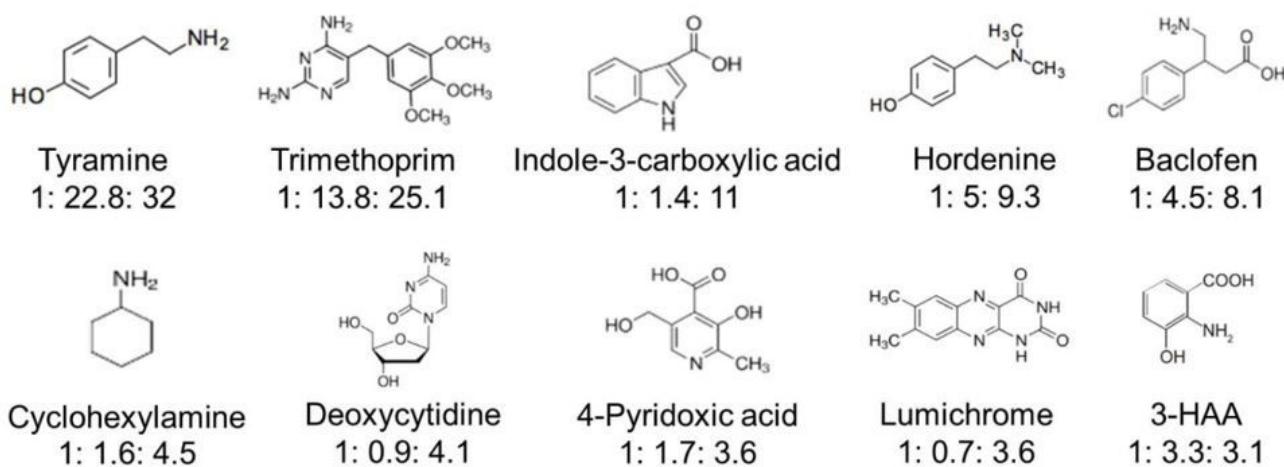
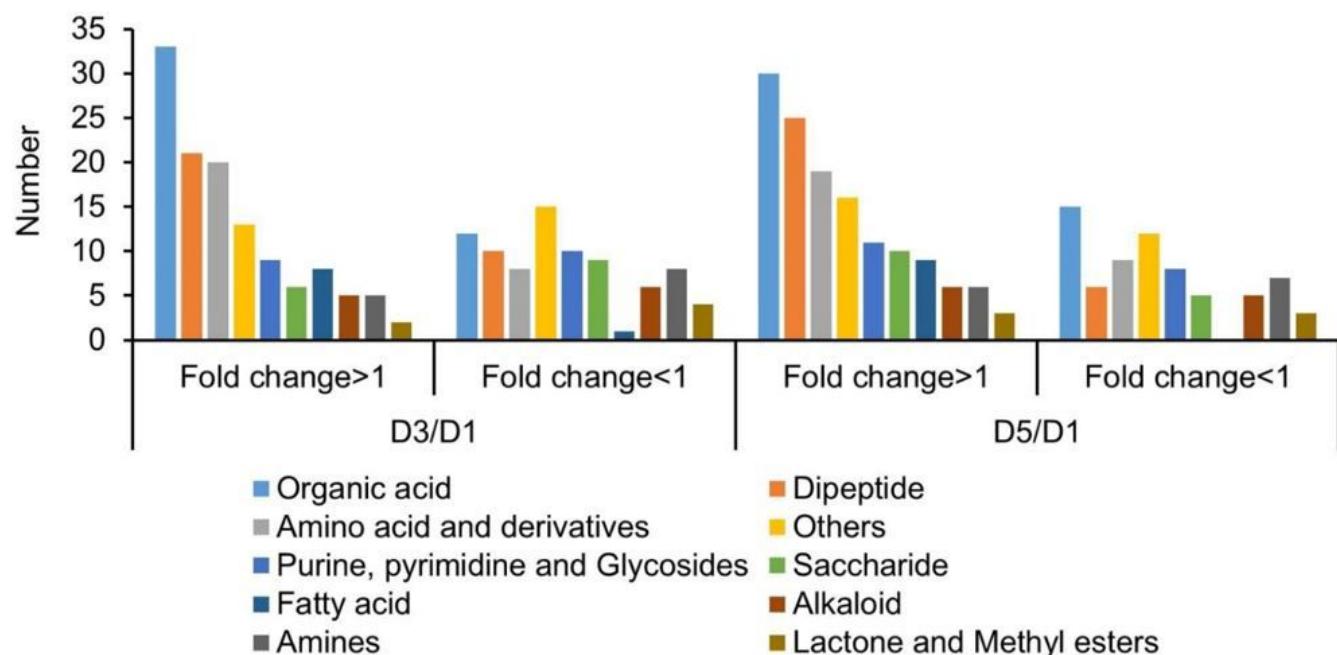


Figure 3

Identification of the metabolites with putative functions in causing oxidative stresses in the host algal cells through comparative metabolomics analysis. The formula of each metabolites was referred to Sigma-Aldrich and KEGG data-base (<https://www.genome.jp/kegg/>), the relative content of each metabolites from D1: D3: D5 was also indicated below.

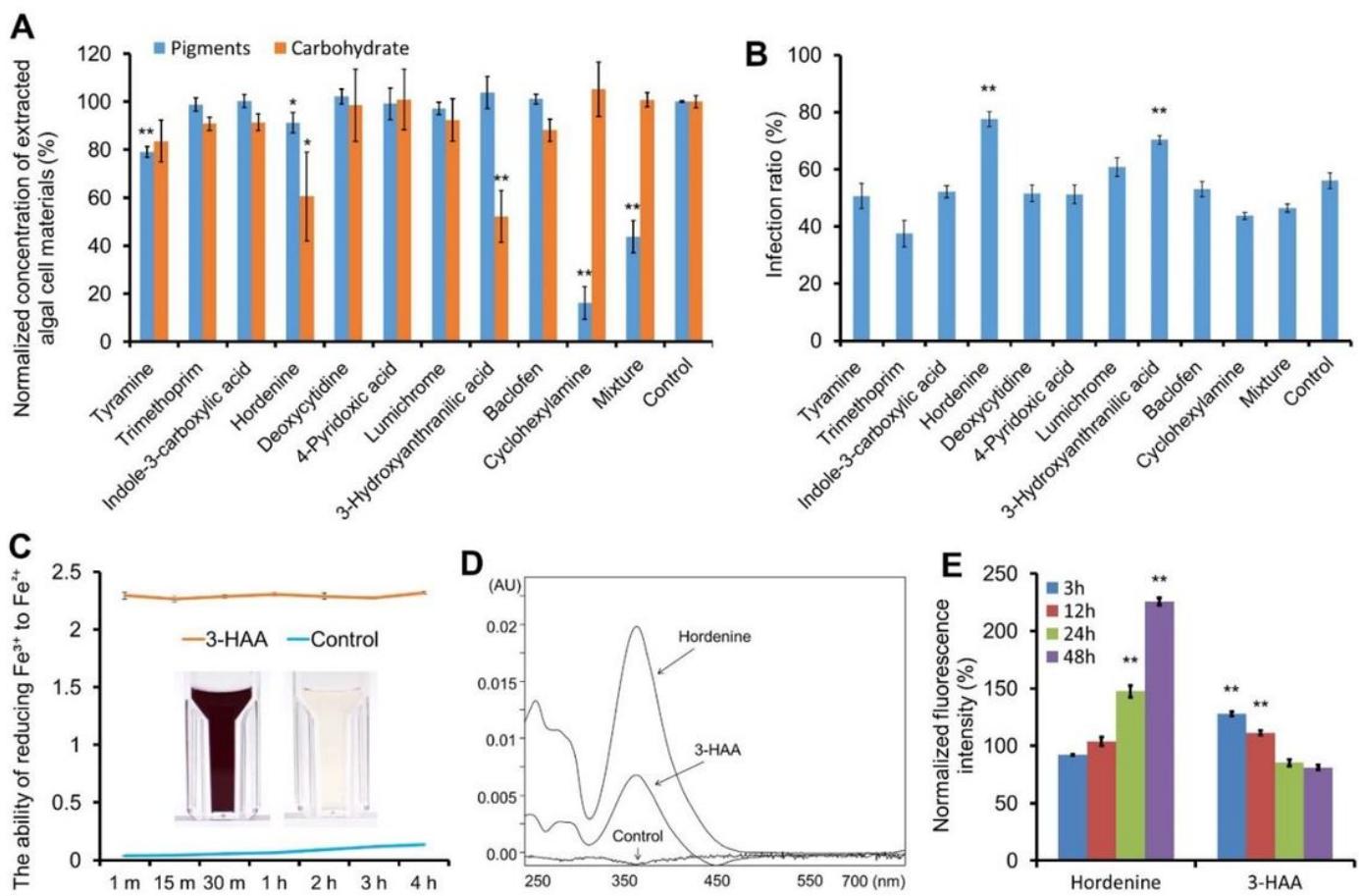


Figure 4

Effects of the screened metabolites on the algal cellular composition and infection process. (A-B) Activity verification of the screened metabolites. (C) 3-HAA reduced Fe³⁺ to Fe²⁺. (D) Hydroxyl radical production. (E) H₂O₂ level inside cells.

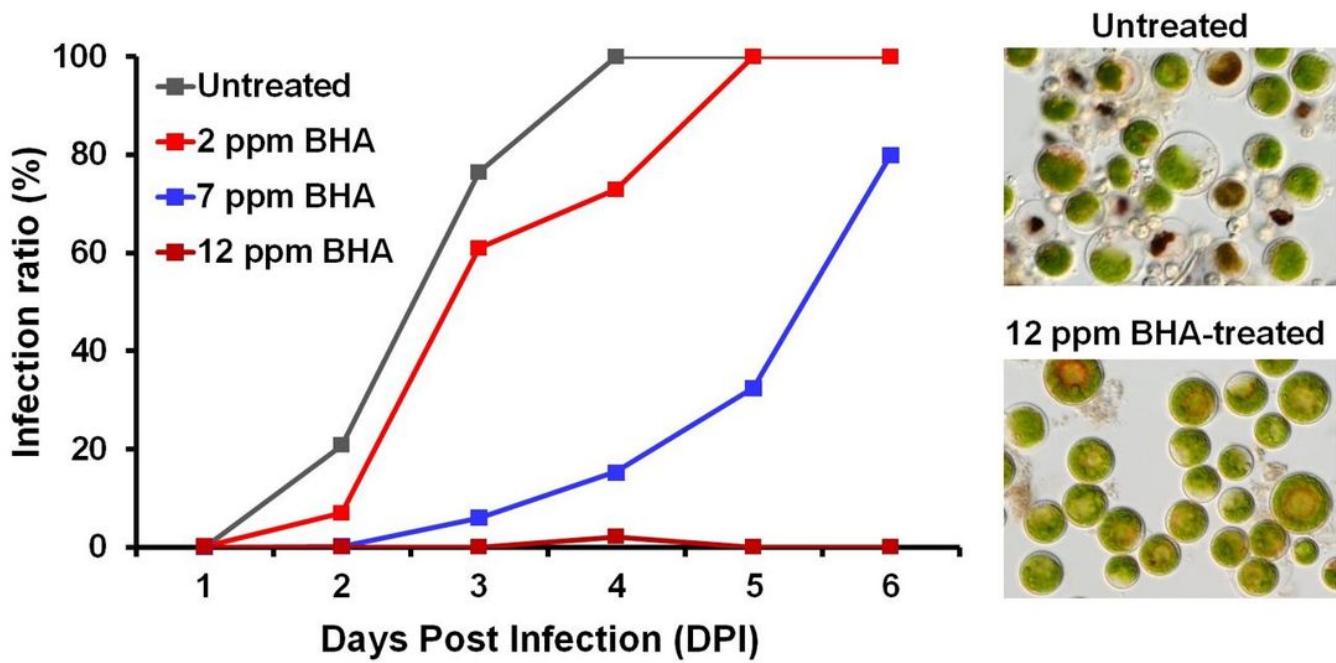


Figure 5

The inhibition effect of BHA to the fungal infection at different final concentrations.