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Protective effect of microbial immunostimulants and antiviral plants against WSSV in *Litopenaeus vannamei* cultured under laboratory conditions

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ABSTRACT

The effect of a mixture of antiviral plants (*Echinacea purpurea* and *Uncaria tomentosa*) and microbial immunostimulants (*Pediococcus parvulus* and *Candida parapsilosis*) on growth performance, survival, white spot syndrome virus (WSSV) prevalence, and immune response of *L. vannamei* was evaluated under laboratory conditions. Powdered plants (PP) were included in the commercial feed pellet at 0.0, 1.0, 2.0, and 4.0 g kg feed^{-1} and microbial immunostimulants (MI) were included at 0.0, 2.1, 4.2, and 8.4 mg kg feed^{-1}. A bioassay was conducted for 21 days. Feed supplemented with PP and MI did not influence survival and shrimp growth. However, the prevalence of WSSV in shrimp with low viral load decreased from 100% in the control group to 0% in animals fed with the PP and MI mixture. Additives increased significantly the phenoloxidase activity but not hemocyte and superoxide anion amount. The PP and MI mixture, added to feed, has the potential to be used prophylactically as an antiviral against WSSV in cultured shrimp.

1. Introduction

Shrimp farming is a profitable industry due to the generated revenues and jobs, and has experienced a rapid development since 1970s (DeWalt et al., 2002). Along with the rapid growth of the shrimp industry, the emergence of various diseases caused by pathogens such as protozoa, bacteria, fungi, and viruses (Martínez-Córdova, 1992) has increased. Viruses are considered a limiting factor in the development of the shrimp industry (Lightner et al., 1996). In the state of Sinaloa, three viruses have been reported, the taura syndrome virus (TSV), the infectious, hypodermic, and hematopoietic virus (IHHNV), and the white spot syndrome virus (WSSV) (De la Rosa-Vélez, 2001; Unzueta-Bustamante, 2000).

The white spot disease caused by the WSSV is so far the most devastating disease reported for penaeid shrimps in many countries (Flegel, 1997; Jiang et al., 2006; Lightner, 1996), it can cause mass mortalities up to 100% (Sahul-Hameed et al., 2006) over a period of two to ten days after the appearance of the first signs (Jory and Dixon, 1999; Xu et al., 2006), especially when there are sudden environmental changes (Sánchez-Martínez et al., 2007). In this way, many organisms can be asymptomatic carriers of a pathogen and, in normal conditions, they are protected by defense mechanisms (Vargas-Albores et al., 1996).

L. vannamei presents two lines of defense. The first one is a physical barrier constituted by the cuticle, which prevents entrance of pathogens to the body. The second one, as in other shellfish, is based on cellular and humoral effectors, which combine to eliminate infectious microorganisms (Vargas-Albores et al., 1996). Shrimp hemocytes are the cellular effector that plays a central role on phagocytosis, melanization, encapsulation, cytotoxicity, and clotting (Sritunyalucksana et al., 1999). The humoral effectors are agglutinins, clotting proteins, hydrolytic enzymes, and antimicrobial peptides that are released upon lysis of hemocytes, which is induced by lipopolysaccharides (LPS), β-1,3-glucans, and peptidoglycans (Chisholm and Smith, 1995; Destoumieux et al., 2000; Muta and Iwanaga, 1996; Söderhäll et al., 1994).

In the last years, efforts have been made to study compound that prevent or reduce the incidence of diseases in cultured shrimps. MI are naturally occurring substances that have the ability to modulate the immune response to increase resistance to pathogens, particularly during stress periods (Bricknell and Dalmo, 2005). Regarding microbial immunostimulants, they are molecules from the cell wall of fungi (β-1, 3-glucans), Gram (+) bacteria (peptidoglycans), and Gram (−) bacteria (peptidoglycans and lipopolysaccharides). Also, the whole cell can be used as immunostimulant (Partida-Arangure et al., 2013; Sajeevan et al., 2009; Tsoni and Brown, 2008). Regarding plants, some have immunostimulant and/or antiviral activity because of their bioactive

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compounds that comprise flavonoids, alkaloids, tannins, coumarins, saponins, quinones, cardiac glycosides, phlobatannins, terpenoids, steroids, simarubalidans, limonoids, lactones, lignans, and melicianins (Çirak et al., 2007; Edeoga et al., 2005; Orzechowzki et al., 2002).

The aim of this study was to evaluate the effect of the mixture of microbial immunostimulants and antiviral plants on the immune system and on the prevalence of WSSV in *L. vannamei* cultured under laboratory conditions.

2. Materials and methods

2.1. Animals

One batch of 170 apparently healthy shrimp was collected from a commercial farm (Acuícola Cuate Machado, Guasave, Sinaloa, Mexico) and transported to the lab facilities of CIIDIR Sinaloa in plastic containers (20 L) provided with sea water and oxygen. The collected shrimp had no signs of WSSV, IHHNV, and/or bacterial infections. However, experimental animals were screened for WSSV to determine prevalence.

2.2. Shrimp acclimation to culture conditions

Shrimp were acclimated to culture conditions for 2 days in 120-L indoor plastic tanks containing 80 L of filtered (20 μm) sea water (30‰) and continuous aeration in groups of 10 organisms per tank. There were no health issues during the acclimation period. Shrimp were fed twice daily at 09:00 and 17:00 h with commercial feed (Nutrimentos Acuícolas Azteca®, Guadalajara, Jalisco, Mexico, 35% protein). Feeding ratio was 5% of average body weight. Uneaten food and waste material were removed daily before feeding.

2.3. Microbial immunostimulants

Lactic acid bacteria (*P. parvulus*) and yeast (*C. parapsilosis*) used in this work as MI were originally isolated and characterized by Apún-Molina et al. (2009) and Luna-González et al. (2013). These microorganisms (heat killed) were tested by Flores-Miranda et al. (2011) with good results on the survival and immune response of *L. vannamei* challenged with *Vibrio sinaloensis*.

2.4. Preparation of experimental diet with microorganisms and plants

P. parvulus and *C. parapsilosis* were heat killed at 74 °C and then included in the commercial feed pellet (35% protein) at 0.0, 2.1, 4.2, and 8.4 mg kg feed^{-1} (80% *P. parvulus* and 20% *C. parapsilosis*). The amount of MI was based on the work of Flores-Miranda et al. (2011). Microorganisms were grown and washed as described in Apún-Molina et al. (2009). Cells from each microorganism were centrifuged at 12,000 *g*, dried in an oven (Felisa, Jalisco, Mexico) at 74 °C for 4 h, and weighed. The dried cell pellet was ground in a mortar. Powdered plants were included in the commercial feed pellet at 0.0, 1.0, 2.0, and 4.0 g kg feed^{-1} (83% *E. purpurea* and 17% *U. tomentosa*). The amount of PP was based on the work of Medina-Beltrán et al. (2012) who found a decrease of 91.7% of the prevalence of WSSV in *L. vannamei* infected with low viral load. Four grams of cellulose was included in the diet of the control shrimp group.

2.5. Experimental design

A bioassay was conducted to evaluate the effect of feed supplemented with PP and MI on cultured shrimp. Animals were maintained in an outside culture system in 120-L plastic tanks with 80 L filtered (20 μm) sea water and continuous aeration. Each treatment had three replicates with 10 shrimp per tank selected at random. Shrimp were fed with commercial feed (35% protein) twice daily at 09:00 and 16:00 h. Initially, animals were fed 6% of the mean body weight and adjusted thereafter according to the feeding response in each tank. Uneaten food and waste material were removed every 3 days before feeding, and 50% of the water was exchanged every 5 days. Physicochemical parameters like pH (HI 98127 pHep, Hanna Instruments, Woonsocket, RI, USA), salinity (Refractometer W/ATC 300011, Sper Scientific, Scottsdale, AZ, USA), dissolved oxygen, and temperature (YSI model 55 oxygen meter, Yellow Spring Instruments, Yellow Springs, OH, USA) were monitored every 2 days. At the beginning and the end of the bioassay, ammonium, nitrites, and nitrates were determined by the method of Strickland and Parsons (1968).

The bioassay was conducted for 21 days with shrimp weighing 3.6 ± 0.58 g. The bioassay consisted of four treatments: I) Control group, shrimp fed with commercial feed + cellulose (4.0 g kg feed^{-1}); II) shrimp fed with commercial feed + PP (1.0 g kg feed^{-1}) + MI (2.1 mg kg feed^{-1}); III) shrimp fed with commercial feed + PP (2.0 g kg feed^{-1}) + MI (4.2 mg kg feed^{-1}); IV) shrimp fed with commercial feed + PP (4.0 g kg feed^{-1}) + MI (8.4 mg kg feed^{-1}). Shrimp were fed with feed plus additives, except for the days (7 and 10) when animals were fed with 2 g per tank of shrimp paste (muscle and gills) with low WSSV load (nested PCR). At the end of the bioassay, survival and weight of shrimp were determined. Shrimp were analyzed separately for WSSV by single or nested PCR. In addition, hemolymph was extracted for the immune system study.

During the bioassay, the water temperature ranged from 28.8 ± 2.7 to 29.0 ± 2.7 °C, dissolved oxygen from 5.03 ± 0.5 to 5.3 ± 0.7 mg L^{-1}, salinity from 30.4 ± 0.9 to 31.0 ± 1.5‰, pH from 8.1 ± 0.13 to 8.2 ± 0.15, nitrites from 0.01 ± 0.01 to 0.04 ± 0.01 mg L^{-1}, nitrates ranged from 0.63 ± 0.24 to 0.74 ± 0.14 mg L^{-1}, and ammonium from 0.55 ± 0.62 to 0.95 ± 0.25 mg L^{-1}. The physicochemical parameters were within acceptable ranges for shrimp culture (Boyd and Tucker, 1998).

Specific growth rate (SGR) was determined using the equation:

$$SGR = (\ln Wt - \ln W_0) \times 100/t$$

where t is the culture period in days, ln W_0 is the natural logarithm of the weight of the shrimp at the beginning of the bioassay and ln Wt is the natural logarithm of the weight of the shrimp at day t (W_0 and Wt are in grams) (Ziaei-Nejad et al., 2006).

2.6. Prevalence of WSSV

Genomic DNA was extracted from gill lamellas and pleopods (50–100 mg) with the DNAzol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. Quantification and quality assessment of DNA were performed in a Pearl nanophotometer (Implen, Inc. Westlake Village, CA, USA). DNA quality was also determined by agarose gel electrophoresis.

WSSV prevalence was determined in 12 shrimp per treatment (four per tank). Detection of WSSV was performed by single and nested PCR, using the primers WSSV out-1/WSSV out-2 and WSSV in-1/WSSV in-2 (Kimura et al., 1996), which amplified genome fragments of 982 and 570 bp, respectively. When negative samples were found, they were tested with an internal control that amplified a 298 bp segment of shrimp Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA using the primers GAPDH298F and GAPDH298R by one-step PCR (Tang and Lightner, 2001).

2.7. Hemolymph extraction

Hemolymph was sampled from 15 intermolt shrimp per treatment. Hemolymph (200 μL) of individual shrimp was withdrawn from the pleopod base of the first abdominal segment with a sterile 1-mL syringe (25 G × 13 mm needle). Before hemolymph extraction, the syringe was loaded with 400 μL of a precooled (4 °C) solution (SIC-EDTA,

Na2) (450 mM NaCl, 10 mM KCl, 10 mM Hepes, and 10 mM EDTA Na2, pH 7.3) used as an anticoagulant (Vargas-Albores et al., 1993).

2.8. Hemocyte count

Fifty microliters of the anticoagulant–hemolymph mixture from 2 shrimp per tank (six per treatment) was diluted in 150 μL of formaldehyde solution (6%) and, then, the sample was placed on a hemocytometer (Neubauer) to count total hemocytes using a compound microscope. The remainder of the hemolymph was stored individually in Eppendorf tubes and kept on ice for separation of plasma and hemocytes.

2.9. Superoxide anion

The superoxide anion was quantified using the methodology of Song and Hsieh (1994). Samples (100 μL) of anticoagulant–hemolymph from 2 shrimp per tank (six per treatment) were centrifuged at 800 *g* for 10 min at 4 °C and the plasma was discarded. Hemocyte pellet was washed three times with SIC-EDTA buffer and stained with 100 μL of nitro blue tetrazolium (NBT) solution (0.3%) for 30 min at 37 °C. The reaction was completed through the elimination of the NBT solution by centrifugation and the addition of 100 μL of absolute methanol to the cell pellet. After three washings with 70% methanol, hemocytes were air dried for 30 min and 140 μL of DMSO and 120 μL KOH (2 M) were added to dissolve the cytoplasmic formazan. The optical density of the dissolved formazan was read at 630 nm in a Thermo Spectronic Genesys 2 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.10. Phenoloxidase activity (PO) in HLS

Hemolymph from 4 shrimp per tank (12 shrimp per treatment) was centrifuged at 800 for 10 min at 4 °C and the plasma was discarded. Hemocytes pellet was washed twice with cacodylate buffer (10 mM, pH 7), resuspended in the same buffer, and subjected to freeze thaw. Finally, the samples were centrifuged at 14,000 *g* for 10 min at 4 °C to obtain the hemocytes lysate supernatant (HLS). Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) following the procedures of Hernández-López et al. (1996).

2.11. Protein determination

Protein concentration in HLS was determined according to the method described by Bradford (1976), using bovine serum albumin (BSA) from Sigma as standard.

2.12. Statistical analysis of the results

One-way analysis of variance (ANOVA) was applied to determine the differences in SGR, total hemocytes, FO activity, and superoxide anion. Results of survival were arcsine-transformed according to Daniel (1997). If significant differences were found in the ANOVA, a Tukey's HSD test was used to identify these differences at $P < 0.05$.

3. Results

3.1. Effect of PP and MI on survival, SGR, and WSSV prevalence

Feed additives were not harmful to the shrimp because the survival was 100% in all treatments and animals were apparently healthy. Also, no significant differences in SGR were found ($P > 0.05$), which means that, at least, additives did not affect shrimp growth (Table 1).

The results showed a clear protective effect of plants and MI against WSSV. The initial WSSV prevalence of shrimp collected from Acuícola Cuate Machado was 100% but with low viral load (nested PCR). At the end of the bioassay, the WSSV prevalence (nested PCR) of shrimp from the control group was 100%, whereas, in treatment II, only 8.3% were positive for WSSV (nested PCR). Prevalence of WSSV in treatments III and IV was 0% (Table 1).

Table 1
Survival, specific growth rate, and WSSV prevalence in *L. vannamei* fed commercial feed with powdered plants and microbial immunostimulants.

Treatments	Shrimp survival (%)	WSSV prevalence (%)	SGR (% d^{-1})
I	100	100	3.17 ± 0.33
II	100	8.3	4.14 ± 0.15
III	100	0	3.84 ± 0.55
IV	100	0	3.46 ± 0.13

I) Control group, shrimp fed with commercial feed + cellulose (4.0 g kg $feed^{-1}$); II) shrimp fed with commercial feed + PP (1.0 g kg $feed^{-1}$) + MI (2.1 mg kg $feed^{-1}$); III) shrimp fed with commercial feed + PP (2.0 g kg $feed^{-1}$) + MI (4.2 mg kg $feed^{-1}$); IV) shrimp fed with commercial feed + PP (4.0 g kg $feed^{-1}$) + MI (8.4 mg kg $feed^{-1}$). SGR = Specific grow rate. PP = Powdered plants. MI = Microbial immunostimulants.

3.2. Effect of PP and MI on shrimp immune response

Powdered plants and MI did not increase the total hemocyte number in juvenile shrimp since no significant differences ($P > 0.05$) were found among treatments (Table 2).

Generation of superoxide anion (absorbance 630 nm) in hemocytes was not affected by the feed additives, since the results showed no significant differences among treatments ($P > 0.05$) (Table 2).

Shrimp fed diets supplemented with additives showed higher fenoloxidase activity than shrimp fed control diet. There were significant differences in the phenoloxidase activity of HLS (proPO, absorbance 492 nm) between treatment IV and treatments I ($P = 0.0176$) and II ($P = 0.0003$) (Table 2).

Shrimp fed diets supplemented with PP and MI showed higher protein in HLS than shrimp fed control diet. There were significant differences in protein concentration (Table 2) of HLS between treatment III and treatments I ($P = 0.006$) and II ($P = 0.010$). Also, significant differences were found between treatment IV and treatments I ($P = 0.002$) and II ($P = 0.004$).

4. Discussion

There are no reports on the effect of a mixture of MI and PP on the immune system and WSSV prevalence in *L. vannamei* or another penaeid shrimp.

In the bioassay, PP and MI did not affect weight and survival of shrimp as compared with the control group. These results are consistent with those of Medina-Beltrán et al. (2012) who fed shrimp (WSSV positive) with the same powdered plants only. Considerations about plants are important because they have antinutritional factors that can affect food intake and nutrient utilization (Soetan, 2008).

The prevalence of WSSV, in shrimp with a low viral load, decreased from 100% in the control group to 0% in animals fed during 21 days with the mixture of PP (2.0–4.0 g kg $feed^{-1}$) and MI (4.2–8.4 mg kg $feed^{-1}$). However, it is important to note that with the lowest concentrations of additives (1.0 g PP kg $feed^{-1}$ + 2.1 mg MI kg $feed^{-1}$) prevalence decreased from 100% to 8.3%. These results are better than those obtained by Medina-Beltrán et al. (2012) who worked with the same plants (4 g kg $feed^{-1}$) in a bioassay that lasted 30 days, in which prevalence of WSSV in shrimp infected with a low viral load decreased from 100% to 8.3%. In other works, interesting results against WSSV have been obtained in *Penaeus monodon* and *L. vannamei* with *Aegle marmelos*, *Cynodon dactylon*, *Eclipta alba*, *E. pupurea*, *Ocimum sanctum*, *Picrorhiza kurooa*, *Sargassum hemiphyllum* var. chinense, *Tinospora cordifolia*, and *U. tomentosa* (Citarasu et al., 2006; Huynh et al., 2011; Peraza-Gómez et al., 2009). In this study, the decrease in the prevalence of WSSV could be due to antiviral substances from the plants, such as pentacyclic oxindole alkaloids, echinacein, and chicoric acid (Binns et al., 2002;

Table 2
Superoxide anion, total hemocyte count, phenoloxidase activity, and protein in *L. vannamei* fed commercial feed with powdered plants and microbial immunostimulants.

Treatments	THC ($\times 10^6$)	Superoxide anion (Abs 630 nm)	Phenoloxidase (Abs 492 nm)	Protein (mg mL^{-1})
I	8.2 ± 1.7	0.47 ± 0.11	0.71 ± 0.09^{b}	0.22 ± 0.05^{a}
II	10.5 ± 2.3	0.48 ± 0.09	0.65 ± 0.07^{b}	0.22 ± 0.09^{a}
III	9.3 ± 2.2	0.41 ± 0.08	0.73 ± 0.09^{ab}	0.31 ± 0.09^{b}
IV	7.0 ± 1.4	0.49 ± 0.06	0.82 ± 0.06^{a}	0.32 ± 0.10^{b}

I) Control group, shrimp fed with commercial feed + cellulose (4.0 g kg $feed^{-1}$); II) shrimp fed with commercial feed + PP (1.0 g kg $feed^{-1}$) + MI (2.1 mg kg $feed^{-1}$); III) shrimp fed with commercial feed + PP (2.0 g kg $feed^{-1}$) + MI (4.2 mg kg $feed^{-1}$); IV) shrimp fed with commercial feed + PP (4.0 g kg $feed^{-1}$) + MI (8.4 mg kg $feed^{-1}$). The error bars = mean ± standard error. Different superscripts indicate significant differences ($P < 0.05$). THC = Total hemocyte count.

Hudson and Vimalanathan, 2011; Hudson et al., 2005; Lee et al., 2010; Pietta et al., 1998; Reis et al., 2008; Robinson, 1998; Wagner et al., 1985).

Invertebrates, such as penaeid shrimp, possess an innate immune system consisting of cellular (hemocytes) and humoral (lysozymes, prophenoloxidase-activating cascade, lectins, lysosomal hydrolytic enzymes, and antimicrobial peptides) elements (Cerenius et al., 2008; Chisholm and Smith, 1995; Destoumieux et al., 2000; Lin et al., 2006; Muta and Iwanaga, 1996; Söderhäll et al., 1994; Sritunyalucksana et al., 1999) that are very important against pathogens. Hemocytes are reactive cells that are the main defense in invertebrates and they are responsible for adhesion, phagocytosis, nodule formation, encapsulation, cytotoxicity, and hemolymph clotting mechanism (Cerenius et al., 2008; Lin et al., 2006; Sequeira et al., 1996; Sritunyalucksana et al., 1999). Our results indicate that the additives did not increase the hemocyte number and are consistent with those reported for *L. vannamei* infected with WSSV and fed with *E. purpurea*, *U. tomentosa*, and *O. sanctum* (Medina-Beltrán et al., 2012; Peraza-Gómez et al., 2011). However, the work of Huynh et al. (2011) reports a significant increase of hemocytes in *L. vannamei* immersed in seawater containing the powder or extract of *S. hemiphyllum var. chinense*. Regarding the microbial immunostimulants used in this work, Flores-Miranda et al. (2011) reported a significant increase in hemocytes of *L. vanname* challenged with *Vibrio sinaloensis* strains.

The superoxide anion is a reactive molecule originated during phagocytosis as a product of the hemocytes' respiratory burst and plays a key role in microbicidal activity (Bell and Smith, 1993). Production of the oxidant molecule did not increase with the additives tested maybe due to the antioxidant effect of molecules (flavonoids, tannins, etc.) of the plants tested (Çirak et al., 2007; Edeoga et al., 2005; Orzechowzki et al., 2002). However, Citarasu et al. (2006) reported that production of intracellular superoxide anion was significantly enhanced by herbal immunostimulants (*C. dactylon*, *A. marmelos*, *T. cordifolia*, *P. kurooa*, and *E. alba*) in *P. monodon*. In the same way, immunostimulants (sodium alginate, chitin, and chitosan) can also increase significantly the respiratory burst in hemocytes of *L. vannamei* (Cheng et al., 2004; Wang and Cheng, 2005).

The prophenoloxidase cascade has a key role in melanization, but also stimulates phagocytosis, nodule formation, encapsulation, and hemocyte locomotion (Söderhäll et al., 1994; Yeh et al., 2009). In this study, a significant increase in PO activity was found in HLS of shrimp fed with PP and MI as compared with control shrimp. Similar results were found by Huynh et al. (2011), who reported that PO activity in *L. vannamei* immersed in seawater containing the powder or extract of *S. hemiphyllum* var. chinense was significantly higher than those of control shrimp. Conversely, in the same species, Peraza-Gómez et al. (2011) did not find significant differences between shrimp fed with plants (*E. purpurea*, *U. tomentosa*, and *O. sanctum*) and the control group. Regarding immunostimulants, phenoloxidase activity can be increased significantly when *L. vannamei* is fed with sodium alginate (Cheng et al., 2004), chitin and chitosan (Wang and Cheng, 2005), and inulin (Luna-González et al., 2012).

Protein concentration in HLS was significantly higher in treatments with the higher concentration of additives when compared with the control group. Downs et al. (2001), in grass shrimp (*Palaemonetes pugio*) and Campa-Córdova et al. (2005), in *L. vannamei*, reported that the increase of protein content in the hemolymph can be due to an increase in some immunoproteins. Therefore, further studies about the effect of PP and MI on shrimp immune system are needed, like the expression of immune related genes by RT-qPCR.

In conclusion, the mixture of PP and MI, added to feed, has the potential to be used prophylactically as antiviral (WSSV) in cultured shrimp.

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