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Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*

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Abstract

A spectrophotometric nitroblue tetrazolium (NBT) reduction assay was used to demonstrate the production of superoxide anions (O_2^-) by haemocytes of the white shrimp *Penaeus vannamei*. It was found that haemocytes, without receiving an experimental stimulant, showed a rather high background activity. Therefore, optimal parameters (number of haemocytes, type of incubation medium, type and concentration of stimulants) were first established, in order to obtain a reliable and reproducible quantitative assay. With this optimized assay, and using specific inhibitors, it was shown that it is indeed the production of O_2^- that was measured. Activities varied strongly among individual shrimp specimens. Live bacteria, among these *Vibrio* strains, induced O_2^- production in the haemocytes, in a dose-dependent manner. Whereas *Vibrio anguillarum* and a probiotic strain of *V. alginolyticus* evoked clear reactions, a pathogenic strain of *V. harveyi* failed to elicit O_2^- production in the haemocytes. It is discussed that this may explain this strain's capability of evading the host's oxidative microbicidal activity, which would be a virulence factor in these bacteria. Heat-killed bacteria hardly induced NBT reduction in the cells. The fungicide propiconazole or Tilt®, found as a pollutant in the aquatic environment where the shrimp are reared, was tested for its effect on NBT reduction by the haemocytes. In haemocytes that did not

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receive an experimental stimulant, Tilt® induced the reduction of NBT in a dose-dependent manner. In experimentally stimulated haemocytes, however, Tilt® strongly reduced the reaction upon the stimulant PMA. Probable explanations for these seemingly controversial effects of Tilt® are discussed, as are possible consequences of this sort of pollutants for shrimp aquaculture. This easy to perform and relatively cheap and simple quantitative assay for measuring the activity of an oxidative microbicidal mechanism in shrimp haemocytes, appears quite reliable and may therefore prove to be a valuable tool for monitoring shrimp health and immunologic status.

Keywords: Crustacea; Shrimp; *Penaeus vannamei*; Superoxide anion; NBT assay; Immune check-up

1. Introduction

Research in penaeid shrimp immunology has only recently become a priority because of the dramatically increasing impact of — mainly infectious — diseases on sustainability and economic durability of shrimp aquaculture. Crustacean immune responses are based on both cellular and humoral components, which cooperate to eliminate potentially infectious microorganisms. The first immune process is the recognition of microorganisms, and it is mediated by plasma proteins and by the haemocytes (Söderhäll and Cerenius, 1998). Phagocytosis, which represents an important way to eliminate microorganisms or foreign particles, has been well described and documented in invertebrates (Bayne, 1990). Whereas much attention has been devoted to recognition and opsonization processes, comparatively little is known about post-phagocytic events and the role of intracellular killing mechanisms against pathogens.

In mammalian phagocytic cells, the oxygen-dependent defence mechanism consists in the generation of reactive oxygen intermediates (ROIs) with powerful microbicidal activity (Babior, 1984). The phenomenon known as respiratory burst can be elicited, upon suitable stimulation, by soluble components such as phorbol myristate acetate (PMA), lectins, lipopolysaccharides (LPS) or by particulate phagocytic stimuli such as zymosan (yeast cell wall). The stimulation leads to increased consumption of oxygen of which the reduction, catalyzed by a membrane-bound enzyme, NAD(P)H-oxidase, gives rise to superoxide (O_2^-). Starting from superoxide, a number of reactions lead to the production of hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical (OH) and numerous other reactive products.

In invertebrates, this microbicidal system has been first demonstrated in gastropods (Dikkeboom et al., 1987; Adema et al., 1991). Then, it was shown in several marine bivalves (Larson et al., 1989; Bachère et al., 1991a), with a special attention to the interaction of the oxidative metabolism with specific intracellular parasites. Adaptations for avoiding or bypassing the haemocytic oxidative microbicidal system have been demonstrated in several pathogens, using in vitro host–pathogen models with isolated specific pathogens (Hervio et al., 1989; Le Gall et al., 1991; Volety and Chu, 1995).

In crustaceans, the demonstration of respiratory burst is quite recent. Using the method of ferricytochrome-*C* reduction, Bell and Smith (1993) demonstrated the generation of superoxide anions by haemocytes of the decapod *Carcinus maenas*; working with separated haemocyte fractions, they showed that hyaline cells are responsible for this activity, whereas the semi-granular and granular haemocytes would not be involved in the production of ROIs. In the penaeid shrimp, *Penaeus monodon*, Song and Hsieh (1994) have used two different methods for the demonstration of oxygen metabolism, leading to different results. The authors were able to spectrophotometrically detect the generation of oxygen metabolites, based on nitroblue tetrazolium (NBT) reduction. However, using a chemiluminescence (CL) method, the same elicitors used failed to elicit haemocyte activation. In contrast, CL activity has been successfully demonstrated in *P. japonicus* haemocytes upon PMA or zymosan stimulation (Bachère et al., 1995). These different results probably underline the need of method standardization in research of crustacean haemocyte functioning in vitro.

The present study was undertaken in order to explore a method for its possible applicability in marine aquaculture, more in particular shrimp farming. The respiratory burst was chosen as a parameter encompassing several immunological activities, viz. recognition, phagocytosis and cytotoxicity. To this end, the production of ROIs in the haemocytes of the white shrimp, *P. vannamei* was investigated using a quantitative microplate assay of the reduction of NBT. The functional capability of the haemocytes to generate a respiratory burst upon stimulation with standard stimuli was assessed. In addition, the interaction of the oxidative intracellular killing mechanism with bacteria has been studied. Some of these bacteria belong to the Vibrionaceae. We used a strain of *Vibrio harveyi*, associated with shrimp larvae mortalities linked to the "Bolitas" syndrome in Ecuador (Robertson et al., 1998) and a *V. alginolyticus* strain, designated Ili (I. Morales, personal communication). For this strain, a probiotic effect for *P. vannamei* has been shown in the laboratory.

Shrimp aquaculture in Ecuador has been developed mainly in the Gulf of Guayaquil. This aquatic environment has received an important number of xenobiotics from human activities. Among these pollutants there is a variety of biochemical compounds for control of pests of agronomic plants. The literature indicates that environmental contaminants can lead to non-infectious diseases. Indeed there is evidence for oxidative stress-related pathologies from pollutants in marine organisms (Ahmad, 1995). We chose the fungicide propiconazole or Tilt®, used in banana production to control the black sigatoka disease, to study the effect of an environmental stressor on the respiratory burst.

2. Materials and methods

2.1. Reagents

Laminarin (L 9634; Sigma), extracted from *Laminaria digitata*, was prepared at 60 mg/ml in modified Hank's balanced salt solution (Gibco) containing 2.6 g/l HEPES, 85 mM NaCl, pH 7.2, 780 mosM (MHBSS).

LPS from *Escherichia coli* serotype 055:B5 (Sigma) were dissolved at 100 μg/ml in MHBSS.

A suspension of yeast cell walls (Zymosan A; Sigma; 40 mg/ml) in filter-sterilized seawater (SSW) was heated at 100°C for 30 min, washed twice and resuspended in SSW at a density of 1.43×10^8 particles/ml.

PMA (Sigma) was prepared in DMSO (dimethyl sulfoxide; Sigma) at a concentration of 10 mg/ml.

All suspensions and solutions were stored at −20°C and thawed just before use.

Superoxide dismutase (SOD; Sigma) stock solution was prepared to 13 500 U/ml in distilled water. *N*-ethylmaleimide (NEM; Sigma), used as an inhibitor of cytosolic cofactors leading to a deactivation of NADPH oxidase (Akard et al., 1988), was prepared as a 0.1-M stock solution in MHBSS. Iodoacetamide (Sigma), an inhibitor of the respiratory burst which is thought to block glycolysis (Pick et al., 1981) was prepared at 30 mM in MHBSS.

NBT (N 6876, Sigma) stock solution was prepared at 10 mg/ml in distilled water and kept at 4°C in the dark. From this stock solution, a NBT working solution was prepared in culture medium at 0.3% just before utilization. This solution was diluted three times in each well, giving a final concentration of NBT in the well of 0.1%.

Propiconazole (Tilt®, Ciba) was diluted in MHBSS with 12 mM Ca^{2+} and 26 mM Mg^{2+} at 3, 30 and 300 ppb.

2.2. Bacteria

V. harveyi, a pathogenic strain involved in the "Bolitas" syndrome in Ecuador, had been isolated in CENAIM (Ecuador). *E. coli* D31, a streptomycin-resistant mutant, was obtained from Dr. J. Hoffmann (IBMC, Strasbourg, France); *Micrococcus luteus* A270 was from the Pasteur Institute Collection (Paris, France); *V. alginolyticus* had been isolated in Ecuadorian hatcheries; this strain has a probiotic effect in shrimp larvae (I. Morales, personal communication). *V. anguillarum* V408 was obtained from the IFREMER laboratory of Palavas (France). *E. coli* D31 and *M. luteus* were grown at 37°C in LB medium containing 1% Bacto-tryptone (Difco), 0.5% Bacto-yeast (Difco) and 1% NaCl. The marine bacterial strains were grown in Marine Broth (Difco) at 25°C.

2.3. Shrimp and haemolymph collection

P. vannamei shrimps, originating from a local shrimp farm (OPUMAR) in Provincia del Guayas, Ecuador, were maintained in the laboratory in 500-l tanks with a constant flow of circulating seawater. Animals, at about 13 g, in intermoult stage were used in the experiments.

Haemolymph (about 300 μl per shrimp) was obtained from the ventral sinus located at the base of the first abdominal segment. The samples were collected using a 23-gauge needle and 1 ml syringe containing 300-μl pre-cooled (4°C) 10% sodium citrate solution in distilled water, used as anticoagulant. Haemolymph samples from 20 animals were pooled or kept individually, and the haemocytes were counted with a haemocytometer (Erma).

2.4. Superoxide assay: NBT reduction

General protocol: The reduction of NBT to insoluble blue formazan was used as a probe for superoxide generation, although it is not entirely specific for O_2^- (Auclair and Voisin, 1985).

A determined number of haemocytes (usually 5×10^5) were deposited in triplicate, in 100 μl final volume of medium (see below) in wells of a 96-well microtiterplate (Corning, Sigma M-0156) and incubated in humid conditions, during 30 min at room temperature for adherence of the haemocytes. The supernatants were then eliminated and replaced by 50 μl of medium, after which were added 50 μl of medium containing respiratory burst elicitors or not, and with or without inhibitors. Then, 50 μl of 0.3% NBT working solution in the appropriate medium were immediately distributed to the wells. Following a 2-h incubation, the supernatants were removed and the haemocytes were fixed by the addition of 200-μl absolute methanol, washed twice with 70% methanol, then dried. The formazan deposits were solubilized in 120 μl, 2 M KOH and 140 μl DMSO. After homogenization of the contents in the wells, the extinction was read at 620 nm in a Labsystem Multiskan spectrophotometer.

2.5. Medium screening

Shrimp haemocytes (5×10^5) were incubated in 50 μl of a mixture of haemolymph–anticoagulant and 50 μl of culture medium (using different culture media, see below). In the first assays it was tested, if the presence of the haemolymph plasma and of citrate during the NBT reaction has some effect on superoxide production. For this, 50 μl of the mixture hemolymph–anticoagulant culture medium was left in the well, and 50 μl of medium with or without 10 μg/ml PMA as the stimulus, and 50 μl of NBT in the medium were added.

At the same time, and with the same pool of haemocytes (5×10^5 per well), NBT reduction was performed in the same set of media in the absence of plasma and citrate, using the general protocol.

The media that were tested were: Dulbecco's phosphate-buffered saline (PBS, Sigma) $1\times$ and $2\times$, SSW, MHBSS without Ca^{2+} and Mg^{2+}, and MHBSS supplemented with different final concentrations of Ca^{2+} and Mg^{2+}, viz. MHBSS-1: 3 mM Ca^{2+} and 6.5 mM Mg^{2+}, MHBSS-2: 6 mM Ca^{2+} and 13 mM Mg^{2+} and MHBSS-3: 9 mM Ca^{2+} and 19.5 mM Mg^{2+}.

2.6. Elicitors

Various stimulants were assayed: laminarin at final concentrations of 1, 5 and 10 mg/ml, LPS used at 4, 20 and 40 μg/ml, zymosan at the ratio particles to haemocyte of 5/1, 10/1, 20/1, PMA at final concentrations of 1, 5 and 10 μg/ml. The incubation medium was MHBSS-2.

2.7. Haemocyte concentration

Shrimp haemocytes (1×10^6, 5×10^5 and 1×10^5) were distributed in microplate wells as previously described. They were stimulated with the different elicitors, PMA (10 μg/ml), laminarin (10 mg/ml) and zymosan (10/1) in MHBSS-2.

2.8. Inhibition assays

SOD was used at 75, 150 and 300 U/μl, NEM at 1, 3 and 5 mM and iodoacetamide at 1, 10 and 20 mM. The inhibitors were administered in 50 μl MHBSS with 12 mM Ca^{2+} and 26 mM Mg^{2+} containing 10 μg/ml PMA as the stimulant.

2.9. NBT reduction assay with bacteria

NBT reduction assays were performed, as previously described, using as phagocytic stimulant the different bacteria *V. harveyi*, *E. coli* D31, *M. luteus*, *V. alginolyticus* and *V. anguillarum*. The logarithmic-phase bacterial cultures were pelleted (5000 rpm, 15 min) and after resuspension in MHBSS with 12 mM Ca^{2+} and 26 mM Mg^{2+}, 50 μl of bacterial suspensions were added to the haemocytes, in triplicate. Both live and heat-killed bacteria were used.

2.10. NBT reduction assay in presence of propiconazole

NBT reduction assays were performed in presence of the fungicide propiconazole (Tilt®) at final concentrations of 1, 10 and 100 ppb in the wells. In order to increase the sensibility of the test, 1×10^6 cells were deposited per well. The control was a similar reaction without the antifungal agent.

The volume of haemolymph necessary to obtain 1×10^6 cells was incubated in presence of the same volume of MHBSS-2, for 30 min at room temperature. The supernatants were eliminated and replaced by 50 μl of MHBSS-2; immediately added were 50 μl of MHBSS 12 mM Ca^{2+} and 26 mM Mg^{2+} (with or without Tilt®) in presence or absence of 10 μg /ml PMA, and 50 μl of NBT working solution. After 2 h of incubation, the fixation and solubilization were performed following the general protocol.

2.11. Statistical analyses

All the measurements were made in triplicate. The results were analyzed using the Duncan new multiple range test ($p = 0.05$) at 95% confidence level with the Super Anova (S-E +) program.

3. Results

In order to assess the different parameters leading to an optimal superoxide assay in terms of reliability and sensitivity, not only the level of stimulated activity was considered (optical density (OD) of solubilized formazan deposits). Unstimulated haemocytes, in the absence of an inhibitor, always showed a considerable base activity.

Therefore, the haemocyte activity was expressed as the ratio of OD620 from the stimulated haemocytes to the OD 620 of control haemocytes.

Observation with an inverted microscope showed that the haemocytes had phagocytosed zymosan particles.

3.1. Optimization of superoxide assay

3.1.1. Culture medium screening

A first series of experiments was performed with 5×10^5 haemocytes/well stimulated with 10 μg/ml PMA and 50 μl mixture haemolymph–anticoagulant culture

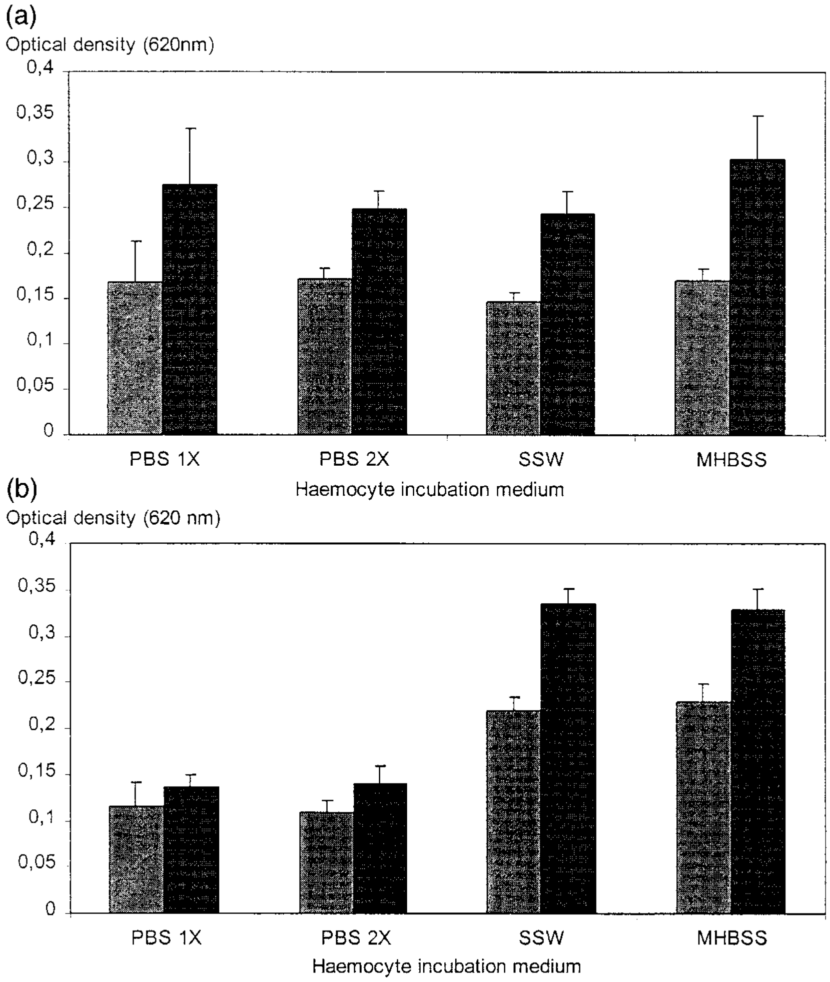

Fig. 1. NBT reduction in haemocytes of *P. vannamei* in response to different culture media. (a) With the plasma present in all steps of the assay. (b) Without plasma during NBT reduction. ▒: In the absence of stimulant. ■: In the presence of stimulant (10 μg/ml PMA). BS: Dulbecco's phosphate-buffered salt solution; SSW: sterile seawater; MHBSS: modified Hank's buffered salt solution.

medium (PBS 1 ×, PBS 2 ×, SSW and MHBSS) remaining during all the steps of the assays (Fig. 1a). MHBSS without divalent cations yielded the best responses in terms of relative production of O_2^-, corresponding to the OD ratio of 1.788 between PMA stimulated values and haemocyte base activity values (0.304 ± 0.048 and 0.170 ± 0.014, respectively). SSW produced the second best NBT reduction response with a ratio of 1.653 (0.243 ± 0.016 stimulated and 0.147 ± 0.010 base activities). Despite a relatively high base activity, a statistically significant ratio of 1.450 (0.248 ± 0.021 stimulated and 0.171 ± 0.012 base activities) was obtained when haemocytes were incubated in PBS 2 ×. When haemocytes were incubated in PBS 1 ×, the ratio of 1.627 (0.275 ± 0.061/0.169 ± 0.044) was not statistically significant ($p = 0.05$).

When plasma was removed and replaced by medium before incubation with the stimulus, SSW and MHBSS again produced the best results with ratios of 1.522 and 1.436, respectively. The haemocytes incubated in PBS 1 × and 2 × did not show any significant reaction to PMA (Fig. 1b).

To standardize the experimental conditions of the assay, it appeared necessary to use synthetic medium instead of SSW, of which the composition can be subject to seasonal and/or environmental variations. Therefore, the effect of MHBSS with different concentrations of Ca^{2+} and Mg^{2+} was tested: MHBSS without Ca^{2+} and Mg^{2+} (MHBSS), MHBSS with 3 mM Ca^{2+} and 6.5 mM Mg^{2+} final concentration (MHBSS-1), with 6 mM Ca^{2+} and 13 mM Mg^{2+} (MHBSS-2), with 9 mM Ca^{2+} and 19.5 mM Mg^{2+} (MHBSS-3). The haemocytes incubated in MHBSS-2 presented the best responses with a PMA-stimulated activity of 0.178 ± 0.026 OD versus a base activity of 0.089 ± 0.003 OD, giving a ratio of 2.0. MBSS-1 and MBSS-3 led to haemocyte responses of 1.526 and 1.314, respectively (Fig. 2). The haemocytes incubated in MHBSS without Ca^{2+} and Mg^{2+} showed the responses of 1.39.

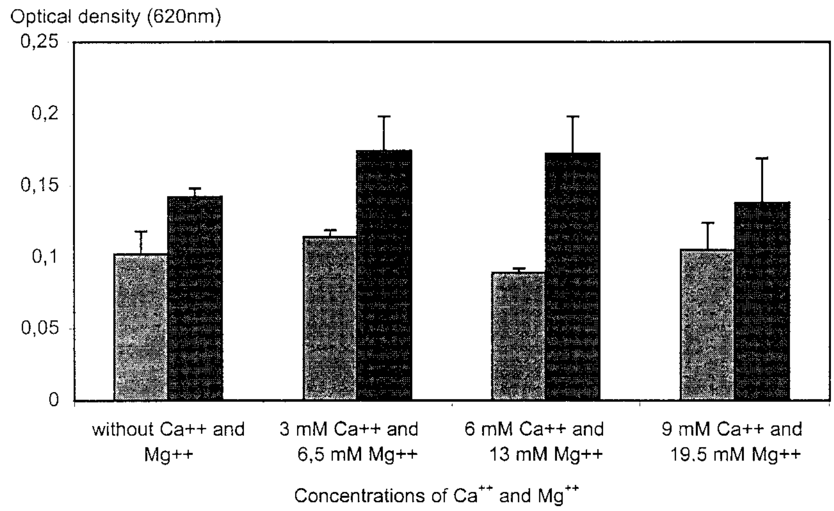

Fig. 2. NBT reduction of the haemocytes kept in MHBSS supplemented with different concentrations of Ca^{2+} and Mg^{2+}. ▒: In the absence of stimulant. ■: In the presence of stimulant (10 μg/ml PMA).

3.1.2. Elicitor nature and concentration

Equal concentrations of haemocytes (1×10^5) activated with different concentrations of stimulant produced a concentration-dependent increase in the NBT reduction up to final concentrations of 10 mg/ml laminarin, 40 μg/ml LPS and to 20/1 zymosan/haemocyte ratio, reaching, respectively, OD of 0.092 ± 0.002, 0.103 ± 0.002 and 0.105 ± 0.010. Haemocytes activated with PMA reached the maximum concentration-dependent activity at 5 μg/ml PMA (0.099 ± 0.011), the final concentration of 10 μg/ml inducing an identical level of activity (0.099 ± 0.011).

No significant differences in NBT reduction were observed between the highest values of the haemocytes activated with the different elicitors assayed ($p = 0.05$) (Fig. 3).

3.1.3. Haemocyte concentration

Different haemocyte concentrations were activated with optimal concentrations of PMA, zymosan and laminarin. The amount of formazan increased significantly with the number of haemocytes, with no significant differences ($p = 0.05$) between the elicitors used at the same number of haemocytes (Fig. 4).

3.1.4. Specific inhibition

3.1.4.1. Effect of SOD. The addition of SOD to haemocytes stimulated with 10 μg/ml PMA appeared to produce a inhibition of the NBT reduction. However, only at the SOD concentration of 300 U/ml, was the effect statistically significant (Fig. 5).

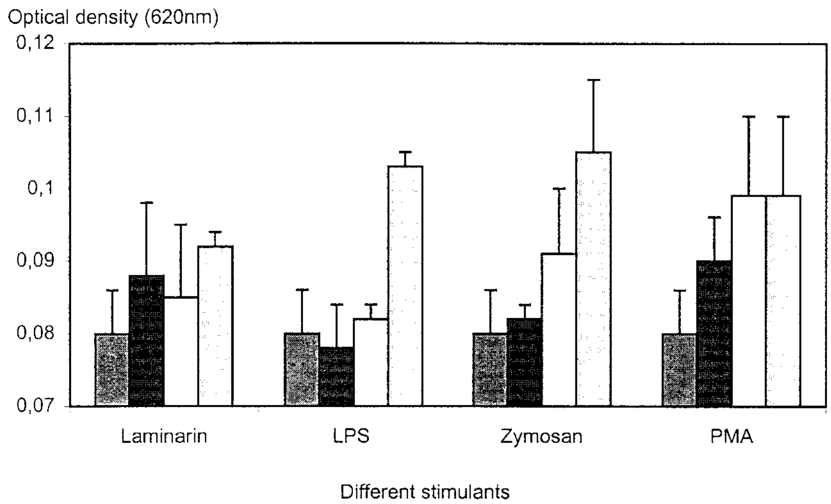

Fig. 3. Effect of different stimulants of the respiratory burst on haemocytes of *P. vannamei*, using three concentrations of stimulant described below. Darkly shaded bar: Without stimulant. Filled bar: 1 mg/ml laminarin, 4 μg/ml of LPS or 5 particles of zymosan/haemocyte, 1 μg/ml of PMA. Unshaded bar: 5 mg/ml laminarin, 20 μg/ml of LPS or 10 particles of zymosan/haemocyte, 5 μg/ml of PMA. Lightly shaded bar: 10 mg/ml laminarin, 40 μg/ml of LPS or 20 particles of zymosan/haemocyte, 10 μg/ml of PMA.

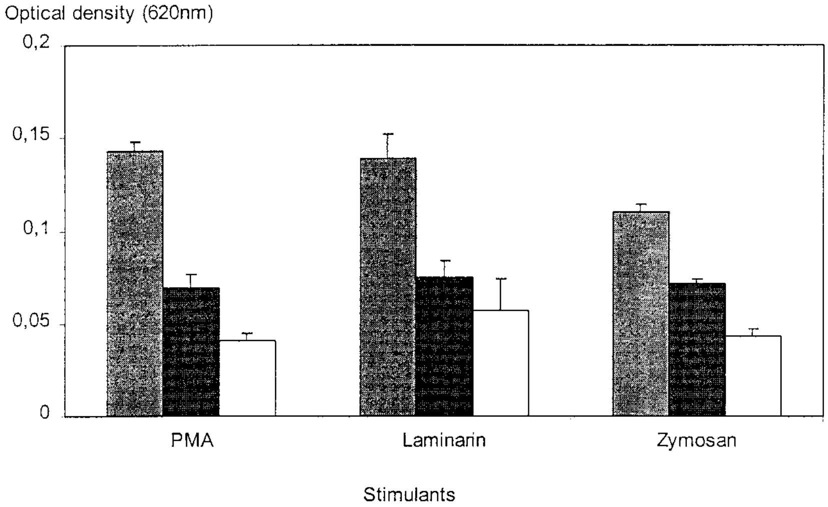

Fig. 4. Response to stimulation of the respiratory burst using different quantities of haemocytes. NBT reduction with ▩: 1×10^6, ■: 5×10^5, □: 1×10^5 haemocytes. The haemocytes were stimulated with 10 μg/ml of PMA, 10 mg/ml of laminarin or 20 particles of zymosan/haemocyte.

3.1.4.2. Effect of NEM and iodoacetamide. Adding of 1 mM and higher of NEM or 1 mM and higher of iodoacetamide, completely inhibited the haemocyte activity of NBT reduction (Fig. 5).

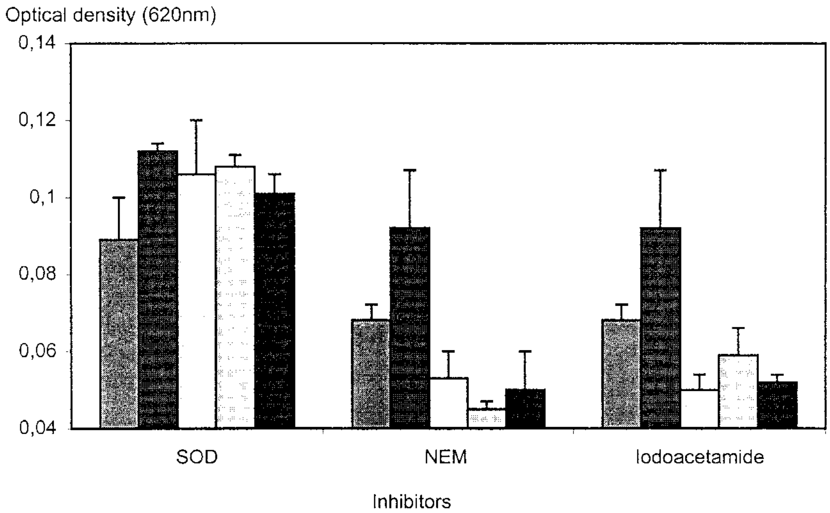

Fig. 5. Effect of different inhibitors of the respiratory burst on NBT reduction using haemocytes previously stimulated with 10 μg/ml of PMA. Darkly shaded bar: Without stimulant and inhibitor. Filled bar: Stimulated. Unshaded bar: Stimulated and inhibited with 75 U/μl of SOD, 1 mM of NEM, 1 mM of iodoacetamide. Lightly shaded bar: Stimulated and inhibited with 150 U/μl of SOD, 3 mM of NEM, 10 mM of iodoacetamide. Filled bar: Stimulated, and inhibited with 300 U/μl of SOD, 5 mM of NEM, 20 mM of iodoacetamide.

3.1.5. Application of optimized NBT reduction assay

3.1.5.1. Individual variability in shrimp haemocyte superoxide anion production. Quantitative analysis of the production of O_2^- was performed with individual shrimps. The NBT reduction responses obtained with equal numbers of haemocytes revealed a high degree of individual variability, 2 out of 10 animals not showing any response at all to stimulation by PMA. A variability was also identified in the number of haemocytes per milliliter of haemolymph ($29.17 \pm 7.56 \times 10^6$) (Fig. 6).

3.1.5.2. Bacterial phagocytosis and effect on haemocyte superoxide anion production. Different bacterial strains were assayed for their capacity to induce haemocyte production of O_2^-. Different bacteria to haemocyte ratios were tested, 10/1, 20/1 and 40/1. Live bacteria of all strains assayed, except *V. harveyi*, were able to induce the production of superoxide anion in the haemocytes with the higher activities recorded for the ratio 40 bacteria to one haemocyte. However, the reduction resulting for all the bacteria at 10/1 and 20/1 were not significantly different ($p = 0.05$) from the control (Fig. 7a). No activity was recorded with heat-killed *M. luteus*, *E. coli* D31 and *V. harveyi* at any of the bacteria/haemocyte ratios used. Significant activity ($p = 0.05$) of the haemocytes was detected with heat-killed *V. alginolyticus* only at the ratio 40/1. Heat-killed *V. anguillarum* lead to haemocyte production of superoxide anion in a dose dependent manner (Fig. 7b), but only at the ratio 40/1 was the difference statistically significant.

Microscopic observation showed that bacteria had been engulfed by the haemocytes. This holds true for both live and heat-killed bacteria. Although *V. harveyi* did not elicit a response in the haemocytes, it had been phagocytosed.

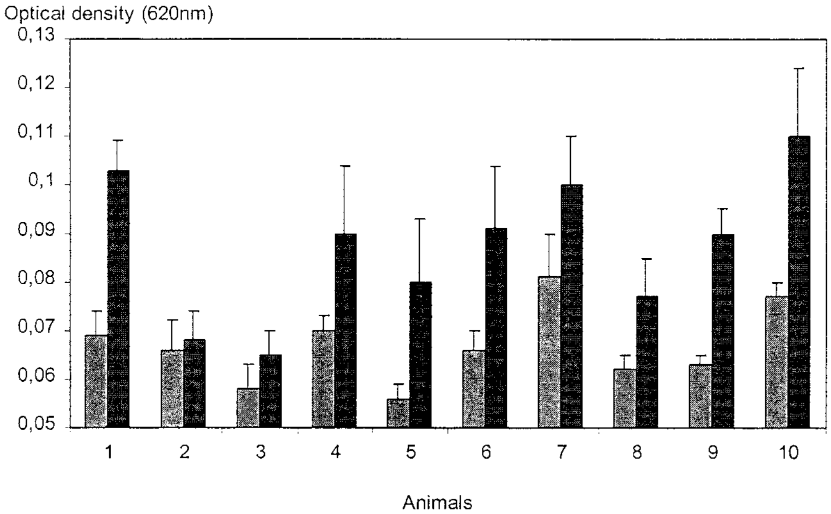

Fig. 6. Variations of NBT reduction amongst 10 different shrimps (5×10^5 haemocytes). ▨: Without stimulant. ■: In the presence of 10 μg/ml of PMA.

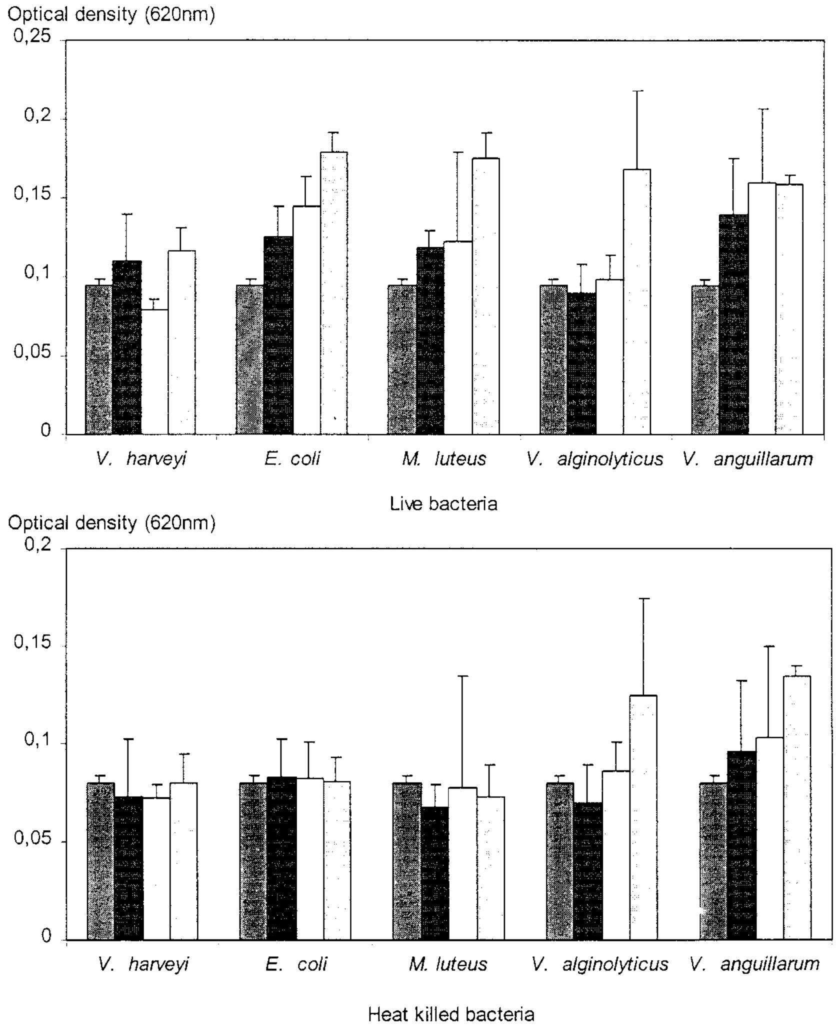

Fig. 7. NBT reduction in 5×10^5 haemocytes of *P. vannamei*, in response to stimulation with different bacterial strains, using live bacteria (a) and heat-killed bacteria (b). Three different concentrations of bacteria were used, as indicated below. Darkly shaded bar: Without stimulant. Filled bar: 10 bacteria/haemocyte for each strain. Unshaded bar: 20 bacteria/haemocyte for each strain. Lightly shaded bar: 40 bacteria/haemocyte for each strain.

3.1.6. Effect of the environmental stressor propiconazole (Tilt®) on superoxide anion production

We performed NBT reduction assays in the presence of the antifungal agent propiconazole at final concentrations of 1, 10 and 100 ppb (in vitro exposure). Fig. 8 shows a significant increase of the base activity in unstimulated cells, with increasing propiconazole concentrations. However, when the haemocytes were stimulated with PMA, we observed a significant decrease of O_2^- generation at 1 ppb of propiconazole.

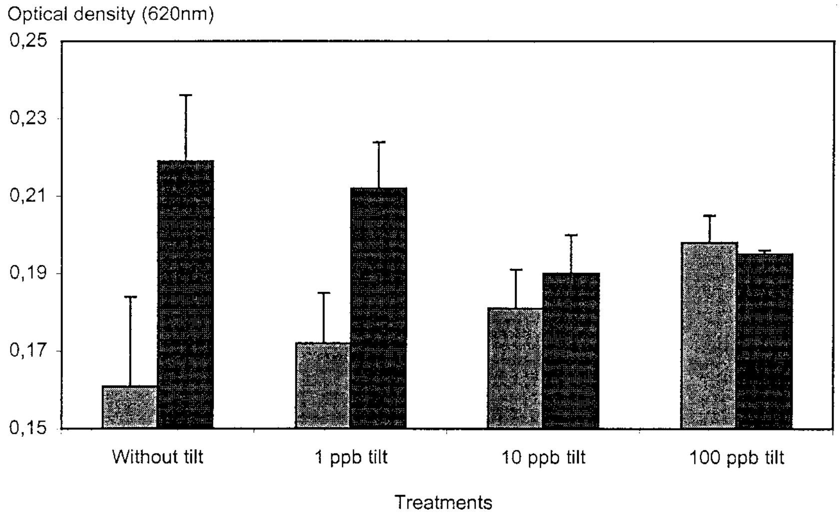

Fig. 8. NBT reduction by 1×10^6 haemocytes in direct contact with the fungicide Tilt®. ▒: In the absence of stimulant. ■: In the presence of 10 μg/ml of PMA.

The cellular response to stimulant drops completely to the level of unstimulated cells at 10 and 100 ppb of propiconazole.

4. Discussion

The NBT reduction assay is widely used to detect the production of superoxide anions within phagocytic cells. The measurement of O_2^- is considered to be an accurate method for estimating the cell's capability to generate a respiratory burst. Compared to other methods such as CL, this assay can be assumed less sensitive because detecting only intracellular O_2^- activity. The method of CL permits to measure, both intracellularly and extracellularly, the whole of the different ROIs generated upon stimulation of the cell. Luminol-enhanced CL is thought to detect primarily the cellular myeloperoxidase–hydrogen peroxide–halide system (DeChatelet et al., 1982), whereas lucigenin-enhanced CL would rather measure the production of the superoxide anion (Gyllenhammar, 1987). Measurements must be conducted with an automatic luminometer or with a scintillation counter, and this provides quantitative analyses and kinetics of the respiratory burst activities. However, the NBT reduction assay presents some advantages. The method can be performed in microplates, allowing the study of 96 samples at a time, and providing both qualitative and quantitative analyses. Moreover, the process of phagocytosis and the intracellular O_2^- production can be followed by microscopic examination. Also, the quantification of superoxide anion production is rendered possible by spectrophotometric analyses of solubilized formazan deposits (Secombes, 1990). The method is fast, simple, and relatively cheap because a luminometer or scintillation counter is not needed. On top of that, cells do not have to be separated from plasma;

handling steps, which can have deleterious effect on haemocyte functionality, are consequently reduced. This represents a great advantage for shrimp haemocytes, which are very labile and difficult to manipulate in vitro.

This study shows in vitro production of the superoxide anion by *P. vannamei* haemocytes, submitted to respiratory burst stimulants. The specificity of the response has been verified by inhibition experiments with SOD, which catalyses the dismutation of O_2^- into hydrogen peroxide. The involvement of O_2^- has also been verified by using NEM which is known as an inhibitor of some cytosolic cofactors involved in the activation of the enzyme generating O_2^-, NADPH oxidase, as well as iodoacetamide, an inhibitor of the respiratory burst (Pick et al., 1981). The first aim of the work was to optimize the parameters of the assay (NBT reduction) in order to increase sensitivity when working with low numbers of haemocytes. This is particularly important for further applications of the method in analyses of respiratory burst activities of individual shrimps, and of juvenile shrimps with limited haemolymph volumes and haemocyte numbers.

For optimizing the NBT assay, we paid attention to the choice of culture medium in which the haemocytes were held before and during the production of intracellular O_2^-. We have also considered the differences between the NBT reduction values of unstimulated haemocytes and those obtained following stimulation. The haemocyte base activities correspond to spontaneous O_2^- production, which may reveal a normal physiological cell activity. To a certain degree, O_2^- production can be related to an activation of the haemocytes during the isolation procedure, or when the haemocytes adhere to the plastic as observed for instance for bivalve haemocytes (Pipe, 1992). Avoiding activation of the haemocytes during handling steps is essential until when incubating them in suitable medium for specific respiratory burst stimulation. For that, it is advisable to use an anticoagulant solution that maintains the haemocytes in a quiescent, non-stimulable state. A spontaneous activation can also result from the effect of the medium used for incubating the haemocytes, as shown in vertebrate blood cells (Bowman et al., 1985) and, in our study, when incubating haemocytes in Dulbecco's PBS. High base activity values may reveal stressful or injurious situations due to in vitro factors. However, in another respect, high base activity can also reflect a physiological state of the shrimp, representing inflammatory or stressful reactions. In any case, subsequent stimulation with a specific trigger of the respiratory burst could be impaired, ineffective or unreliable. We have shown that the reaction media substantially influenced the production of O_2^- and subsequent NBT reduction. It is well established that calcium (Ca^{2+}) and magnesium (Mg^{2+}) are required to initiate phagocytosis and respiratory burst in vertebrate blood cells (Roschger et al., 1990) and marine bivalve haemocytes (Fisher and Newell, 1986; Bachère et al., 1991b). Seawater has been widely used for in vitro assays when marine invertebrate haemocytes functionality is studied because it may provide the haemocytes with cations or other essential elements for their successful stimulation and activity. However, natural seawater not having a constant composition, for development of standardized assays the use of synthetic medium of determined composition is more advisable. Comparison of O_2^- production obtained using sterile seawater and MHBSS, complemented with suitable concentrations of Ca^{2+} (6 mM) and Mg^{2+} (13 mM), showed that the latter satisfies the functional needs of the haemocytes.

P. vannamei haemocytes can be stimulated by PMA, zymosan, LPS and laminarin, which lead to similar productions of O_2^-. In contrast, in adult *P. monodon*, Song and Hsieh (1994) obtained differences in the stimulative effects of various elicitors. In their experiments, β-glucan had the stronger effect followed by zymosan and PMA, leading, respectively, to 2.5, 2 and 1.3 times greater activity than unstimulated control cells. Apart from possible interspecies differences, the differences observed in levels of activity between *P. monodon* and, in our experiments, *P. vannamei*, could be partly related to a difference of age. The *P. monodon* specimens used by Song and Hsieh (1994) were adults weighing 30 to 40 g, whereas the *P. vannamei* used in the present study were juvenile animals of about 10 to 13 g. It can be assumed that the haemocyte defence capabilities would be related to age-dependent maturation processes. Little is known about the ontogeny of the immune system in shrimp, and this would merit further attention. The establishment of the defence system could be studied, taking into account possible changes in susceptibility to various pathogens, in relation to the shrimp's age.

A relatively high degree of variability in the production of O_2^- has been observed between individual animals. A number of factors may influence the NBT reduction activity of individual shrimps, such as their physiological or immune state. Indeed, the generation of O_2^- has been considered as adapted for the evaluation of the effect of chemical or biological compounds which can modulate the phagocytic capabilities of mammalian cells (Fromtling and Abruzzo, 1985) or marine bivalve haemocytes (Larson et al., 1989; Bachère et al., 1991b; Morvan et al., 1994). The quantification of NBT reduction and the ability of haemocytes to produce ROIs can be considered as functional tests for the evaluation of immune potential at a cellular and organismal level. Further studies will be necessary to analyze the individual variabilities in relation to physiological or infectious parameters, as well as environmental stressful conditions, taking also into account the haemograms. Individual variabilities in NBT reduction may be related to differential compositions of haemocyte subpopulations. Indeed, using the method of ferricytochrome-*C* reduction, separated hyaline cells of *C. maenas* were shown to generate O_2^-, whereas the stimulation of semi-granular and granular populations did not result in a significant reduction of ferricytochrome-*C* (Bell and Smith, 1993). The establishment of methods for separating shrimp haemocytes (Rodriguez et al., 1995) will allow to analyze the microbicidal oxidative function of the different cell subpopulations in shrimp.

The possible involvement of shrimp haemocyte ROIs in the elimination of bacteria was investigated. We used the Gram-positive bacterium *M. luteus*, and several Gram-negative bacteria *E. coli* D31 and *Vibrio* strains, as *V. harveyi*, pathogen for *P. vannamei* (Robertson et al., 1998), *V. alginolyticus*, named Ili (I. Morales, personal communication), observed in the shrimp microflora, and *V. anguillarum* considered potentially harmful for shrimps (Lightner, 1983). Under the experimental conditions all the strains except *V. harveyi*, added to the haemocytes as phagocytosis elicitors, when used alive were able to induce the generation of O_2^-. The response of the haemocytes was dependent on the concentration of bacteria, especially as shown with *E. coli*. However, in a general way, this capacity disappeared when the bacteria were previously heat-killed. Only the killed *V. alginolyticus* and *V. anguillarum*, at the bacteria-to-haemocyte ratio of 40/1, were able to induce a significant activity in the haemocytes.

The loss of inductive potential of the heat-killed bacteria is probably caused by heat-induced modifications of some membrane components, involved in their recognition by the haemocytes.

If considering studies of the interactions between shrimp and possible pathogens and probiotics, the NBT reduction assay appears to be an interesting tool. The fact that the *V. harveyi* strain, alive and heat-killed, cannot stimulate the production of O_2^- in a pool of haemolymph of *P. vannamei*, can be explained by its ability to avoid this immune barrier. This may represent part of this strain's pathogenicity. In contrast, both heat-killed and live *V. alginolyticus*, Ili strain, with probiotic effects in larvae of *P. vannamei*, are able to stimulate haemocyte O_2^- production using the same pool of haemolymph. On the other hand, it is interesting to note that in another experiment, using haemolymph from different individual shrimps (*P. vannamei*) and the same strain of *V. harveyi* (data not shown), there were a few shrimps with a rather high ratio of stimulation for production of O_2^-. These results may imply the utility of this tool for the selection strategy.

To know the immune and physiological conditions of shrimps is important information for the management of shrimp aquaculture. For measuring these conditions, the shrimp farmers used different criteria (salinity stresses, activity of the shrimps, origin of the shrimps, etc.) with relative success at the production level. In this context, the immune check-up may represent a welcome and effective criterion that may help increase success in shrimp production. For measuring of the shrimp's immunological activity state and potential, simple quantitative assays such as the NBT reduction assay or other tools can be developed. This information can provide the shrimp producers the criteria of susceptibility or deficiencies at resistance level of the animals, and represent an alternative in health management of the shrimp aquaculture.

In order to see if the fungicide propiconazole (Tilt®) has an action upon O_2^- generation, we performed a NBT reduction assay in the presence of this compound. The data obtained from unstimulated haemocytes in this in vitro assay suggest that propiconazole enhances the release of ROIs, by a sort of inflammatory reaction (effect on base activity). These findings might be explained by biochemical events of detoxification because, during phase I of the biotransformation of lipophilic chemicals by the P-450 monooxigenase system, reactive oxygen species as well as free radicals derived from the xenobiotics can be generated (Bainy et al., 1996). It is well known that free radicals including reactive oxygen species may react with several biomolecules, viz. nucleic acids, lipids, carbohydrates and proteins, and in doing so damage the cells. These perturbations are believed to underlie tissue injuries (De Groot, 1994) and physiological disorders (Kim, 1997. Another effect of this Tilt®-caused generation of oxygen radicals, is the drain of the cellular pool of reducing agents (NAD, NADPH, GSH). This impairs the metabolic processes (Di Giulio et al., 1995), including the important antimicrobial mechanism, the respiratory burst. This fact is in accordance with the dose-related decrease in O_2^- generation by PMA-stimulated haemocytes exposed to the fungicide. If propiconazole exert the same effects in vivo as we measured in vitro, this could have the consequence that antimicrobial defences would be impaired. Experiments testing the effect of in vivo administration of propiconazole are presently being conducted. On the other hand, in vivo-administered immunostimulants and vaccines (e.g., Sung et al., 1994; Teunissen et al., 1998) probably enhance antimicrobial defences, as they confer protection against bacterial infections. This may be due to their stimulating the ROI producing mechanism. Clearly, studies need to be done into the in vivo effects of immunostimulatory and of immunoinhibitory substances on both the respiratory burst and antimicrobial defences in aquacultured crustacea. Such studies have not yet been done. In fishes however, it was shown that increased O_2^- production, induced in vivo with polysaccharides, is related with the increment of survival rate after bacterial infections (Wang and Wang, 1997).

Moreover, propiconazole is one of the ergosterol biosynthesis inhibiting fungicides (EBIFs). In fungi, these compounds act on sterol 14-*a*-dimethylase, a microsomal cytochrome *P*450-dependent enzyme system. In vertebrates, the EBIFs have complex patterns of induction, suppression and inhibition of cytochrome *P*450 isozymes according to both the fungicide and the species (Van den Bossche et al., 1995). In crustaceans, there are no data in this respect, but taking into account the geographic situation of an important number of shrimp farms, it may be of interest to study the potentially toxic interaction between propiconazole (and other environmental stressors like other pesticides, and antibiotics used in aquaculture) present in the shrimp-farming environments.

In conclusion, the present study shows that the spectrophotometric measurement of NBT reduction is a reliable method to demonstrate in a quantitative way the production of superoxide by haemocytes of *P. vannamei*. Standardized conditions (number of haemocytes, incubation medium, type and concentration of stimulus) were determined. With the optimized assay, it could be demonstrated that there is an important variability in the activity among individual shrimps, that live bacteria — with the exception of the pathogenic *V. harveyi* — do induce the production of superoxide, and that the fungicide Tilt® enhances the background activity of haemocytes but reduces PMA-induced activity. We believe that this method may prove a useful tool to measure the health condition and immunological status of individual shrimps.

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