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Antifungal treatments in artificial incubation of crayfish eggs (*Pacifastacus leniusculus*, Astacidae): Searching for alternatives to formaldehyde

J.M. Carral[(1)], A. González[(1)], J.D. Celada[(1)], M. Sáez-Royuela[(1)], P.M. Melendre[(1)], R. González[(1)], V. García[(1)]

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ABSTRACT

Key-words: *antifungal treatment, artificial incubation, astacid crayfish*

Considering the concerns about the safety of the use of formaldehyde as antifungal agent, the effects of three alternative chemicals (potassium sorbate, copper hydroxide and magnesium chloride) were tested in the artificial incubation of signal crayfish (*Pacifastacus leniusculus*) eggs. Eight treatments were performed during 15 min every other day: formaldehyde at 3000 ppm (control), potassium sorbate at 5000 and 10 000 ppm, copper hydroxide at 40, 60, 80 and 200 ppm, and magnesium chloride at 10 000 ppm. Eggs were incubated in a flow through system at a density of 20 eggs·cm^{-2}. After 61 days of incubation, the highest efficiencies were obtained with 200 ppm of copper hydroxide (77.6% of survivors to stage 2) with no significant differences from the control (74.3%). Lower concentrations of copper hydroxide resulted in high egg mortality whereas potassium sorbate and magnesium chloride were ineffective to avoid fungal growth and total egg mortality took place. From the obtained results, copper hydroxide baths at 200 ppm could be considered as a good alternative to formaldehyde.

RÉSUMÉ

Traitements antifongiques lors d'incubations artificielles d'œufs d'écrevisse (*Pacifastacus leniusculus*, Astacidae) : recherche d'alternatives au formaldéhyde

Mots-clés : *traitement antifongique, incubation artificielle, écrevisse astacidé*

Considérant les préoccupations sur l'emploi sécurisé du formaldéhyde comme antifongique, les effets de trois alternatives chimiques (sorbate de potassium, hydroxyde de cuivre et chlorure de magnésium) ont été testés en incubation artificielle d'œufs d'écrevisse signal (*Pacifastacus leniusculus*). Huit traitements différents ont été effectués pendant 15 minutes chaque jour : formaldéhyde à 3000 ppm (contrôle), sorbate de potassium à 5000 et 10 000 ppm, hydroxyde de cuivre à 40, 60, 80 et 200 ppm, et chlorure de magnésium à 10 000 ppm. Les œufs ont été incubés à une densité de 20 œufs·cm^{-2} dans un système d'eau courante. Après 61 jours d'incubation, les plus fortes efficacités ont été obtenues avec 200 ppm d'hydroxyde de cuivre (77.6 % de survivants au stade 2) sans différence avec le contrôle (74.3 %). Les plus faibles concentrations en hydroxyde de cuivre ont entraîné une plus forte mortalité des œufs alors que le sorbate de potassium et l'hydroxyde de cuivre étaient inefficaces à éviter la croissance

(1) Dpto. de Producción Animal, Universidad de León, Campus de Vegazana s/n, 24071 León, Spain, jmcarl@unileon.es

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fongique et une mortalité totale des œufs intervenait. À partir des résultats obtenus, des bains d'hydroxyde de cuivre à 200 ppm peuvent être considérés comme une bonne alternative au formaldéhyde.

INTRODUCTION

In astacid crayfish, advances in artificial incubation techniques (AI) provide reliable options for its application under culture conditions (see review by González *et al.*, 2009). Depending on stripping time and temperature, artificial incubation covers long periods, over 100 days (Pérez *et al.*, 1998), during which nonviable eggs can be invaded by fungi, usually *Saprolegnia* spp. and other Oomycetes (Edgerton *et al.*, 2002). Their hyphae can spread to the surrounding healthy ones (Celada *et al.*, 2004; Melendre *et al.*, 2006; Sáez-Royuela *et al.*, 2009) causing an important decrease of efficiency rates. Periodical removal of dead eggs has shown positive effect on the hatching rate (Carral *et al.*, 2004; Policar *et al.*, 2006) but it is laborious and manipulation can cause damages in healthy ones, giving rise to new losses (Sáez-Royuela *et al.*, 2009). Thus, the use of antifungal treatments would be advisable, and several studies have been addressed to find effective chemicals.

For many years malachite green has been the most effective fungicide used in aquaculture but since 1991 and 1997 is not permitted for aquaculture in USA (Schereier *et al.*, 1996) and EU (Celada *et al.*, 2004) respectively, because of its teratogenic and carcinogenic effects (Andersen *et al.*, 2006; Sapkota *et al.*, 2008). After a series of trials testing chemicals used in other aquatic species, Celada *et al.* (2004) and Melendre *et al.* (2006) reported formaldehyde as the most effective antifungal. Although formaldehyde is approved for use in fish culture in USA and EU, there is concern about user safety because of its suspected carcinogenicity and its potential adverse effects on the aquatic environment (Arndt *et al.*, 2001; Gieseker *et al.*, 2006).

Considering the convenience of antifungal agents alternative to formaldehyde, the aim of this study was to obtain data on the possible effectiveness of other chemicals. Three were selected: potassium sorbate (E202) is used to inhibit molds and yeasts in many foods for food preservation and extension of shelf-life (Dorko *et al.*, 1997), copper hydroxide ($Cu(OH)_2$) is commonly used to prevent some fungal diseases of fruit and vegetable crops, and magnesium chloride ($MgCl_2$) was proposed by Rantamäki *et al.* (1992) to prevent transmission of the crayfish plague fungus (*Aphanomyces astaci*).

MATERIALS AND METHODS

Eggs from 70 berried *Pacifastacus leniusculus* females from a crayfish farm were detached in the laboratory by sliding blunt forceps smoothly from the base to the tip of the pleopods. Prior to artificial incubation, embryonic phases were identified following Celada *et al.* (1985, 1987) being at phase IX (embryo with naupliar appendages).

A total of 6400 eggs were pooled and artificially incubated in the experimental devices described by Carral *et al.* (1992) and Sáez-Royuela *et al.* (2009) up to stage 2 juvenile production. Artesian well water was supplied in open system at a flow rate of 0.5 $L{\cdot}min^{-1}$ (12.5 $mL{\cdot}cm^{-2}{\cdot}min^{-1}$). The parameters of incoming water quality were: pH 7.9, hardness 5.2 °dH (calcium: 32.3 $mg{\cdot}L^{-1}$), dissolved oxygen about 8 $mg{\cdot}L^{-1}$, nitrite $<$ 0.015 $mg{\cdot}L^{-1}$, ammonium $<$ 0.02 $mg{\cdot}L^{-1}$, total dissolved solids 110.5 $mg{\cdot}L^{-1}$, and total suspended solids $<$ 0.5 $mg{\cdot}L^{-1}$. Temperature was 10 ± 1 °C until eggs reached the eye stage (phase XIII). Thereafter, it was raised to 15.5 ± 1 °C up to final stage 2 juvenile production.

Chemicals used were formaldehyde (Proquiman S.L.) at 3000 ppm, magnesium chloride ($MgCl_2$, Sigma-Aldrich) at 10 000 ppm, potasium sorbate (E202, Coralim Aditivos) at 5000 and 10 000 ppm, and copper hydroxide ($Cu(OH)_2$, Funguran-OH 50 WP, Nufarm España S.A.)

Table I
Survival rates in artificial incubation receiving eight antifungal treatments. Values followed by differing letters were significantly different ($P < 0.05$) from the others in the same column. SEM = standard error of mean.

Tableau I
Taux de survie en incubation artificielle selon huit traitements antifongiques. Les valeurs suivies de différentes lettres sont significativement différentes ($P < 0,05$) des autres de la même colonne. SEM = erreur-standard de la moyenne.

Chemical agent	Concentration (ppm)	Application form	Stage 1 (% ± SEM)	Stage 2 (% ± SEM)
Formaldehyde (control)	3000	Peristaltic pumps	83.7 ± 0.4[a]	74.3 ± 2.8[a]
Potassium sorbate	5000	Peristaltic pumps	0	0
	10 000		0	0
Copper hydroxide	40	Static baths	0	0
	60		0	0
	80		10.3 ± 3.1[b]	2.5 ± 1.5[b]
	200		86.2 ± 1.5[a]	77.6 ± 5.6[a]
Magnesium chloride	10 000	Peristaltic pumps	0	0

at 40, 60, 80 and 200 ppm. Treatments were administered during 15 min every other day up to the beginning of hatchings. Solutions of formaldehyde, potasium sorbate and magnesium chloride were discharged to the incoming water flow using peristaltic pumps. Because its low solubility in water, copper hydroxide treatments were performed in static baths.

Each treatment was performed by quadruplicate at an egg density of 20 cm^{-2} (one complete layer, eggs contacting to others). Throughout incubation eggs were daily checked by careful ocular inspection and any presence of fungal growth was noted. Dead eggs were not removed. The number of stage 1 (after hatching) and stage 2 (after first moult) juveniles was quantified. Collection of stage 2 juveniles was carried out daily following the recommendation of Melendre *et al.* (2007), because final survival is reduced the longer they remain in the incubators. Survival rates were calculated as the percentage of juveniles from the initial number of eggs.

Prior statistical analysis arc-sine transformation of percentages was made. Results were examined by analysis of variance (one-way ANOVA) using the SPSS 16.0 computer program (SPSS Inc., Chicago, USA). Mean comparison was tested using the Duncan's test. The significance level was $P < 0.05$.

RESULTS

Artificial incubation lasted for 61 days. Hatchings began on day 43, stage 2 juveniles were obtained from day 51 and the first moult period lasted for 10 days.

Survival rates in each treatment are presented in Table I. The highest survival rate to stage 2 (77.6%) was obtained with copper hydroxide at 200 ppm for 15 minutes every other day, with no significant difference from the formaldehyde control (74.3%). In both cases no fungal growth was observed. However, on eggs treated with copper hydroxide at 40 and 60 ppm fungi were observed from day 14 onwards, whereas at 80 ppm from day 19. In the later case, a low production of stage 2 juveniles was obtained (2.5%).

Earlier fungal growth was observed in eggs treated with potassium sorbate and magnesium chloride, from day 5 onwards, affecting all the eggs and causing total mortality.

DISCUSSION

Copper hydroxide is a widely used fungicide-bactericide approved for both organic and conventional agricultural production of vegetable crops for control of diseases (Rice *et al.*, 2007).

Although there are no references of its use in aquatic species it was the only one, among the chemicals tested as alternative to formaldehyde, effective to prevent fungal growth on crayfish eggs. As it has been proved with other antifungal tested (Celada *et al.*, 2004; Melendre *et al.*, 2006; Policar *et al.*, 2006; Sáez-Royuela *et al.*, 2009), artificial incubation of astacid crayfish eggs calls for higher concentrations than those recommended for other species. In the case of copper hydroxide, the lower concentrations tested did not avoid the presence of fungus which were observed after 14–19 days of incubation, resulting in poor survival to stage 2 juveniles (2.5% at 80 ppm) or total mortality (at 40 and 60 ppm). However at 200 ppm, juvenile production was high (77.6%) and similar to the obtained with formaldehyde.

Because their low water solubility, copper complexes, including $Cu(OH)_2$, tend to precipitate and thus administration by means of peristaltic pumps does not guarantee the concentration received on eggs, as Melendre *et al.* (2006) pointed out for the copper sulphate. Thus, the application of these chemicals should be restricted to static baths leading to additional man work throughout the AI period.

Considering that *Saprolegnia* spp. and the genus *Aphanomyces* belong to the same family (Diéguez-Uribeondo *et al.*, 2006), magnesium chloride was tested. The concentration used was the proposed by Rantamäki *et al.* (1992) as effective to reduce the sporulation process of *A. astaci*, but it did not prevent the fungal growth on eggs and total mortality was registered. The same occurs with potassium sorbate (E202) widely used to inhibit molds and yeasts in many foods, even when it was applied at higher concentrations. Considering this, both agents should be rejected as antifungal in astacid artificial incubation, at least at the concentrations tested.

Other antifungal agents effective on artificial incubation of crayfish eggs, such as formaldehyde, have also disinfection effects allowing producing specific virus-free juveniles (Edgerton and Owens, 1997) or faster growth juveniles (González *et al.*, 2009). Considering the potent biocidal properties of cooper, alone or in complexes, which determine their wide current usages as antibacterial, antifungal and antiviral (Borkow and Gabbay, 2009), it would be expected a similar performance.

To sum up, copper hydroxide at 200 ppm administered in 15 min static baths every other day can be considered as effective alternative to formaldehyde. Further research on accurate concentration of this copper compound and others commonly used in aquatic species, such as copper sulphate, should be developed.

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