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Treatment potential of a synergistic botanical pesticide combination for rotifer extermination during outdoor mass cultivation of *Spirulina platensis*

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A B S T R A C T

Rotifer-control effects of a synergistic celangulin/toosendanin (CA/TSN) combination at a 1:9 ratio, and its safety for *Spirulina platensis* growth, were studied. Two additions of 0.003–0.006 mg/L of the combination inhibited rotifer reproduction within 3 days, and showed no toxicity toward algal biomass and phycocyanin levels. A comparison between the CA/TSN and NH_4HCO_3 treatments indicated that 200 mg/L NH_4HCO_3 produced more acute toxicity based on ammonia intoxication, while 0.003 mg/L CA/TSN showed lasting effects by sterilizing rotifers. In the NH_4HCO_3 treatment, rotifers still had slow reproduction, partially owing to ammonia volatilization. Neither the NH_4HCO_3 nor CA/TSN treatment changed algal filament morphology; however, the NH_4HCO_3 treatment initially inhibited algal growth more significantly. *S. platensis* treated by the CA/TSN combination reached higher biomass in the same time frame. Given its effectiveness, together with projected farm-earnings increase, the CA/TSN combination showed excellent potential and economic feasibility for rotifer extermination in outdoor mass cultures of *S. platensis*.

1. Introduction

The cyanobacterium *Spirulina* has long been cultivated as a dietary supplement for humans and animals, including aquatic animals in aquaculture. It is gaining increasing attention because of its diverse potential products, such as phycobiliproteins (phycocyanin and allophycocyanin), pigments, amino acids, γ-linolenic acid and other high-value chemicals [1,2]. *Spirulina* can enhance the immune system. Even small amounts of *Spirulina* supplement aid both the humoral and cellular components of the immune system [3,4]. To realize this alga's potential value fully requires the successful commercial production of cyanobacteria by large-scale intensive cultivation. The raceway pond with a suspended algal culture is the most common approach to large-scale cultivation. However, infection of raceways with biological contaminants is a significant constraint in mass cultivation and impedes the industrial process [5]. The contaminating organisms generate stress, cellular disruption and ultimately death of the algal culture. Among the biological contaminants, which include bacteria, other microalgae and zooplankton, the competing algae can be avoided or eliminated by the alkaline cultivation conditions needed by *Spirulina*. However, these conditions have no impact on grazing species. The large-scale cultures are still susceptible to grazing by zooplankton, especially rotifers, which can rapidly reduce the algal concentration to low levels and can actually clear the entire culture within just a few days [6].

A number of methods have been investigated for rotifer extermination during *Spirulina* cultivation, with limited success. Small rotifers in algal cultures can be exterminated by adding the large rotifer *Asplanchna* (>700 μm), which feeds on the other rotifers. The growth of *Asplanchna* then can be inhibited by 100 mg/L Vitamin E [7]. A pH of 9.0 and 5 klx light intensity are recommended for rapid cell growth in *Spirulina* cultures [8], allowing new growth to exceed the biomass lost by grazing. However, as *Spirulina* is commercially grown in large volumes, by large-scale, outdoor cultivation under natural light conditions, these methods are impractical. Discovering specific and efficient chemicals has long been expected to be the solution for mitigating or eliminating biological contamination. Treatment agents such as sodium hypochlorite and copper sulfate are used to disinfect the microalgal culture ponds, aiming at the prevention of rotifer contamination. For now, ammonium bicarbonate is considered to be the most effective agent for exterminating rotifers. Méndez and Uribe [9] exterminated the rotifer *Brachionus* sp. by the addition of 100–150 mg/L of ammonium bicarbonate to a contaminated outdoor mass culture of *Arthrospira* sp. The ammonium toxicity for rotifers is related to the non-ionized ammonia, which can quickly exterminate majority of the rotifers. However,

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various degrees of growth inhibition and damage to the *Spirulina* caused by the ammonia are also observed [10].

Certain pesticides derived from plants have acute and chronic toxicity to rotifers, and specifically celangulin (CA) and toosendanin (TSN) have been found to be good alternatives for controlling rotifers, due to their distinctive insecticidal mechanisms, rapid degradation and ecological acceptability [11]. With advantages of decreased insecticide resistance, increased efficacy and reduced dosage, the synergistic CA/TSN combination at the ratio 1:9 showed great rotifer-control effects in marine *Chlorella* and *Nannochloropsis* cultures under laboratory conditions [12]. However, *Spirulina* is a genus of cyanobacteria, whose tolerance to pesticides differs from that of the green algae [13–15]. We are still lacking a comprehensive understanding of the effects of CA/TSN treatment on *Spirulina* cultivation, including any effects on the phycocyanin pigment levels and amount of biomass at harvest. Although the CA/TSN combination has been shown to exterminate large numbers of *Brachionus* species, the treatment protocol, including the dosage amounts, time and frequency of CA/TSN application and cost for attaining rotifer control, would be expected to be species-dependent, which cannot be ignored, especially in mass algal cultures for commercial production. In addition, outdoor algal cultivation conditions are more variable and less controlled as compared with cultivation in the laboratory, which would inevitably influence the rotifer-control efficiency. Thus, the present work first studied the possible use and optimum dosage of CA/TSN for exterminating rotifers in *Spirulina* outdoor cultures. Based on the positive results, we further compared the CA/TSN treatment with the conventional ammonium bicarbonate treatment for rotifer extermination, aiming to do a comprehensive evaluation for the practical application of CA/TSN during outdoor mass cultivation of *Spirulina*.

2. Materials and methods

2.1. Cyanobacterium and rotifers

Spirulina platensis (=*Arthrospira platensis*) was cultivated in modified Zarrouk medium [16], containing the following constituents (per L): 5.0 g $NaHCO_3$, 0.85 g $NaNO_3$, 0.70 g NaCl, 0.37 g KCl, 0.24 g KH_2PO_4, 0.10 g $MgSO_4 \cdot 7H_2O$, 0.06 g $(NH_2)_2CO$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.06 EDTA–$2Na \cdot 2H_2O$ and 0.08 g NH_4HCO_3. The rotifer *Brachionus calyciflorus* was isolated from an infected mass-scale outdoor culture of *S. platensis*. The isolated rotifers were then continuously cultivated in fresh water (pH 8.8 ± 0.5) at 30 ± 2 °C, and fed with *S. platensis* daily.

2.2. Pesticide agents for rotifer extermination

The botanical pesticides celangulin (CA) and toosendanin (TSN) were prepared as stock solutions in distilled water, with concentrations of 10 mg/L and 0.5 mg/L, respectively. The synergistic combination of CA/TSN in the 1:9 ratio (1 mg/L) was obtained by mixing the stock solutions of CA and TSN and distilled water at a volume ratio of 45:98:357. The synergistic combination should be used right after it is ready. The ammonium bicarbonate was chemically pure.

2.3. Safety evaluation of the synergistic CA/TSN combination for S. platensis cultivation

All the experiments were performed outdoors at the Dongtai City Spirulina Bio-engineering Co., Ltd. In this experiment, 10 L of *S. platensis* at the exponential growth stage was inoculated into 50 L culture medium in round plastic jars measuring 50 × 40 cm (H × D). The *B. calyciflorus* rotifers were added into the algal culture medium (initial pH 9.1) to reach an initial population density of 21–28 individuals/mL. After acclimation for one day, the synergistic combination of CA/TSN was added into the algae-rotifer culture at day 2 and day 4. A rotifer-free algal culture was used as control. The algal medium was aerated at an airflow rate of 500 L/h. Each treatment and control had three replicates. The experiment was performed outdoors, under natural light intensity and photoperiod. The water temperature remained in a range of 27–34 °C during *Spirulina* cultivation.

2.4. Comparison of the CA/TSN combination and ammonium bicarbonate for rotifer extermination in S. platensis mass cultures

Rotifer-infected *S. platensis* cultures under outdoor mass cultivation were used for this comparison experiment. The population density of rotifers was approximately 50 individuals/mL. The experiment was carried out in ponds measuring 5.4 × 1.8 × 0.26 m (L × W × H), containing 2.5×10^3 L of infected algal culture. The algal medium was aerated via 16 airstones, each with an airflow rate of 500 L/h. The experiment was performed outdoors under natural light and temperature conditions. Either the synergistic CA/TSN combination or the ammonium bicarbonate was added at day 2. Three samples were taken from each treatment for the daily measurements.

2.5. Population density and egg ratio of rotifers

The population density of rotifers was counted using a 1-mL zooplankton counter under the dissection microscope at 20× magnification. The ratio of eggs to female rotifers was calculated as: Egg ratio = N_e / N_f, where N_e and N_f were the total number of eggs and number of female rotifers respectively.

2.6. Biomass, chlorophyll and carotenoid analysis

The cell dry weight (CDW, g/L) was determined by correlating the $OD_{560\ nm}$ with the linear equation: $CDW = -6.94E-4 + 0.42OD_{560\ nm}$ ($r^2 = 0.9996$) [17]. Chlorophyll and carotenoid contents of *S. platensis* were estimated after methanol extraction [18].

2.7. Phycocyanin determination and morphological observation of filaments

Phycocyanin was extracted by repeated freezing and thawing of the fresh biomass in 50 mM sodium phosphate buffer, pH 6.8, and measured based on the dry weight using the method of Patel et al. [19]. Morphological features of algal cells, including the length and width of filaments, diameter of the helix and the helix pitch, were determined by direct measurement using a microscope. Twenty filaments were taken randomly and measured.

2.8. Data analysis

Statistical evaluation of data was performed using SPSS 16.0 software (SPSS Inc.: Chicago, USA). Statistically significant differences between the treatments were determined by ANOVA with Dunnett's test at the $P < 0.05$ level.

3. Results and discussion

3.1. Safety evaluation of the CA/TSN combination for S. platensis cultivation

The population density of *B. calyciflorus* exhibited sharp increases, while the egg ratio reached a maximum on day 2, and then decreased gradually during the cultivation without addition of the CA/TSN combination (Fig. 1). Low food availability resulting from the rapidly increased numbers of rotifers was responsible for the early decline of the egg ratio [20]. With addition of 0.003 or 0.006 mg/L CA/TSN on day 2, the numbers of rotifers declined by more than half, and the egg ratio sharply decreased to zero in 24 h. This indicated that the CA/TSN combination affected rotifer reproduction more drastically than it did in animal survival, which was attributed to the increased metabolic costs associated