BD Biosciences

Technical Bulletin #463

Use of BD Falcon™ Cell Culture Inserts to Evaluate Allelopathic Effects Among Marine Phytoplankters *In Vitro*

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Introduction

Molisch introduced the term allelopathy to refer to biochemical interactions between all types of plants, including microorganisms.[1] Rice defined allelopathy as any direct or indirect harmful effect by one plant, including microorganisms, on another through production of chemical compounds that escape into the environment.[2] Later, Rice recanted his earlier definition because apparently most allelopathic compounds have both inhibitory and stimulatory effects on growth.[3] Despite recent studies and reviews of the allelopathic interactions among marine and freshwater phytoplankton,[4,5] no satisfactory explanations have been provided to explain why certain phytoplankton species are able to completely dominate a phytoplankton community. Thus, we need to improve our understanding of the inhibitory and stimulatory interactions among many phytoplankton species in order to elucidate the role of allelopathy in algal succession and the outbreak mechanisms of algal blooms.

The diatom *Skeletonema costatum* and the flagellate *Olisthodiscus luteus (=Heterosigma akashiwo)* form alternating blooms in Narragansett Bay (Rhode Island, USA).[6] In the fishing port of Hakozaki (Hakata Bay, Fukuoka, Japan), Honjo et al. found that several kinds of centric diatom disappeared temporarily during the period of *Heterosigma* sp. red tide.[7] We examined allelopathic interactions between axenic strains of *H. akashiwo* and *S. costatum* by way of bi-algal culture experiments under non-contact conditions using BD Falcon Cell Culture Inserts.

Materials and Methods

Algal Species and Culture Conditions

Axenic strains of *S. costatum* (NIES-324) and *H. akashiwo* (NIES-10) were obtained from the National Institute for Environmental Studies (NIES, Japan). These strains were tested for bacterial contamination using the fluorochrome 4', 6-diamidino-2-phenylindole (DAPI) (Cat. No. 340-07971 from Dojindo Laboratories, Kumamoto, Japan) staining method (Porter & Feig 1980); all were verified as axenic. Cultures were aseptically maintained in 200 ml Erlenmeyer flasks containing 100 ml of modified SWM-3 medium[8,9] (***Table 1***) with a salinity of 25 at 25°C under 228 (±5) μmol m^{-2} s^{-1} of cool-white fluorescent illumination on a 12:12 h light:dark cycle. The modified SWM-3 medium was autoclaved (121°C, 15 min) and buffered to avoid pH effects. Irradiance in the incubator was measured with a Quantum Scalar Laboratory Irradiance Sensor (QSL-2100/2101; Biospherical Instruments).

Table 1
Components of modified SWM-3 medium

Component	Qty.
SWM-3 medium	
Seawater	1000 ml
$NaNO_3$ (WAKO, Osaka, Japan)	170 mg
$NaH_2PO_4 \cdot 2H_2O$ (WAKO, Osaka, Japan)	15.6 mg
Na_2EDTA (DOJINDO, Kumamoto, Japan)	11.16 mg
Fe-EDTA (WAKO, Osaka, Japan)	0.84 mg
$NaSiO_3 \cdot 9H_2O$ (WAKO, Osaka, Japan)	56.8 mg
P1-metal mixture solution[1]	10 ml
P2-metal mixture solution	5 ml
Vitamin mixture solution[3]	5 ml
Tris (hydroxymethyl) aminomethane (WAKO, Osaka, Japan)	400 mg
[1]P1-metal mixture solution	
Distilled water	1000 ml
EDTA (DOJINDO, Kumamoto, Japan)	1 g
$MnCl_2 \cdot 4H_2O$ (WAKO, Osaka, Japan)	0.69 g
$CoCl_2 \cdot 6H_2O$ (WAKO, Osaka, Japan)	2.38 mg
$ZnCl_2$ (WAKO, Osaka, Japan)	54.8 mg
H_2SeO_3 (WAKO, Osaka, Japan)	173 μg
[2]P2-metal mixture solution	
Distilled water	1000 ml
H_3BO_3 (WAKO, Osaka, Japan)	6.18 g
[3]Vitamin mixture solution	
Distilled water	500 ml
Vitamin B_{12} (WAKO, Osaka, Japan)	100 μg
Vitamin B_1 (WAKO, Osaka, Japan)	1 g
Biotin (WAKO, Osaka, Japan)	100 μg

Bi-algal culture experiments under non-contact conditions

For these experiments, we used BD Falcon™ Cell Culture Inserts with a 3 μm pore (Cat. No. 353091) loaded into BD Falcon 6-well Companion Plates (Cat. No. 353502) (***Figure 1***). *H. akashiwo* cells in stationary phase (5 to 6 x 10^5 cells ml^{-1}) were diluted to densities of 10^2 and 10^4 cells ml^{-1} with modified SWM-3 medium, and 5 ml was added into the outer chambers of the well plates. *S. costatum* cells in stationary phase (10 to 12 x 10^5 cells ml^{-1}) were then diluted to a density of 10^2 cells ml^{-1} with modified SWM-3 medium, and 3 ml of the cell suspension was added to the inner chambers of the well plates. Similarly, *S. costatum* cells in stationary phase (10 to 12 x 10^5 cells ml^{-1}) were suspended into modified SWM-3 medium at densities of 10^2 and 10^4 cells ml^{-1}, and 5 ml was inoculated into the outer chambers of the well plates. *H. akashiwo* cells in stationary phase (5 to 6 x 10^5 cells ml^{-1}) were then suspended at a density of 10^2 cells ml^{-1} in modified SWM-3 medium, and 3 ml of the cell suspension was inoculated into each inner chamber. Three replicate wells were used for each treatment. On day 8 of the incubation, if necessary, were diluted 10 to 50 times with fresh modified SWM-3 medium before counting.

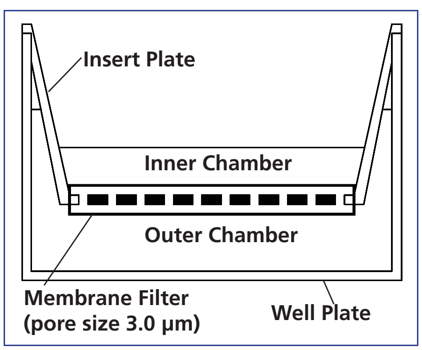

Figure 1
Cross section of well plate with a BD Falcon Cell Culture Insert

Results

Growth of *S. costatum* (initial cell density: 10^2 cells ml^{-1}) in bi-algal cultures with *H. akashiwo* (initial cell density: 10^2 or 10^4 cells ml^{-1}) under the non-contact conditions provided by cell culture inserts was substantially inhibited (16% of the control) by *H. akashiwo* when initial cell density was 10^4 cells ml^{-1} (***Figure 2A***).[9] In contrast, growth of *H. akashiwo* was not inhibited by *S. costatum* under these conditions. Growth inhibition of *H. akashiwo* (initial cell density: 10^2 cells ml^{-1}) in bi-algal cultures with *S. costatum* (initial cell density: 10^2 or 10^4 cells ml^{-1}) under the same non-contact conditions was dependent on the cell density of *S. costatum* (***Figure 2B***).[9] In contrast, growth of *S. costatum* was not inhibited by *H. akashiwo* under these conditions.

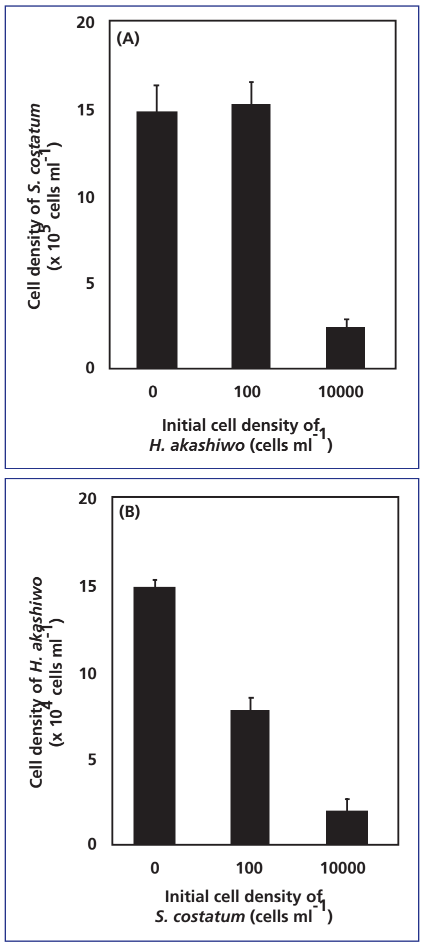

Figure 2. Bi-algal cultures under non-contact conditions. (**A**) Effects of *H. akashiwo* (initial cell density: 10^2 and 10^4 cells ml^{-1}) on growth of *S. costatum* (initial cell density: 10^2 cells ml^{-1}). (**B**) Effects of *S. costatum* (initial cell density: 10^2 and 10^4 cells ml^{-1}) on growth of *H. akashiwo* (initial cell density: 10^2 cells ml^{-1}). Data are mean ± MAD (cells ml^{-1}) of triplicate measurements.

Discussion

Previously, we investigated the growth interaction between *C. polykrikoides* and *A. sanguinea* by way of bi-algal culture experiments and bi-algal culture experiments under non-contact conditions using BD Falcon Cell Culture Inserts, and indicated that growth inhibition and the formation of morphologically abnormal cells of *A. sanguinea* would be induced by constant cell contact with *C. polykrikoides*.[10] In this study, our experiments with bi-algal cultures under non-contact conditions (Figure 2)[9] indicated that substances secreted or released by *S. costatum* and *H. akashiwo* inhibited the growth of the other species. This result successfully demonstrates that allelopathy is involved in the growth interactions between *S. costatum* and *H. akashiwo*. Hence, use of BD Falcon Cell Culture Inserts makes it possible to distinguish between allelopathic effects and effects that result from cell contact among marine phytoplankters.

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