Short-Term Effects on *Artemia salina* of Aponin and *Gomphosphaeria aponina* in Unialgal Cultures and in Mixed Cultures with *Gymnodinium breve*

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Abstract □ Previous investigations showed that the natural marine substance aponin, produced by the blue-green alga *Gomphosphaeria aponina*, was cytolytic toward Florida's red tide organism, *Gymnodinium breve*. As part of a study of the aponin–alga biological interactions, the effects of unialgal (*G. aponina*) and mixed cultures (*G. aponina* + *G. breve*) on the viability of *Artemia salina* were investigated. Two contrasting effects were observed in mixed cultures: survival of *A. salina* was promoted in low *G. aponina* populations, while enhanced toxicity of *G. breve* to *A. salina* occurred at higher culture populations. Unialgal *G. aponina* cultures exerted no adverse effects on *A. salina*. The apparent protective effect is thought to result from the observed change in *G. breve* morphology (motile to sessile); toxicity was the result of enhanced toxin release by cytolyzed *G. breve* cells. In dose–response studies, aponin exhibited no adverse effect on *A. salina* at concentrations (1 unit) that were deleterious to *G. breve*. However, at higher applied levels (4–6 units), mortality was substantial (>70%) after 48 hr of incubation. Probit analysis yielded an apparent LD_{50} of 2.3 units, where 1 unit was that amount required to cytolyze 50% of the *G. breve* cells (2000 cells/ml) after 20 hr of incubation.

Keyphrases □ Aponin—effect on *Artemia salina*, unialgal and mixed cultures with *Gomphosphaeria aponina* and *Gymnodinium breve*, potential biological control agent □ *Gymnodinium breve*—effect of aponin, *Gomphosphaeria aponina*, unialgal and mixed cultures, potential biological control □ *Artemia salina*—effect of aponin, bioassay method □ Biological control—*Gymnodinium breve*, effect of aponin and *Gomphosphaeria aponina* in unialgal and mixed cultures, *Artemia salina* bioassay

Interest in localized management measures for the ichthyotoxic blooms of Florida's red tide organism, *Gymnodinium breve* (an unarmored dinoflagellate), was rekindled with the isolation and partial purification of a biologically active natural marine product, aponin, from cultures of the blue-green alga, *Gomphosphaeria aponina* (1, 2). Earlier reports (3, 4) described aponin cytolysis of *G. breve* cells; presumably, this action results from either a direct lytic interaction with the cell membrane or a perturbation/inhibition of a vital cellular function, causing cell degeneration. The aponin effect on *G. breve* cells is rapid (<24 hr), irreversible, and dosage dependent. Further aponin purification is underway and should provide a better understanding of its structural, chemical, and biological properties.

BACKGROUND

Additional evidence for aponin cytolysis of *G. breve* was demonstrated in mixed culture studies with *G. breve* (4). *G. breve* cultures incubated with various amounts of log-phase *G. aponina* cells declined linearly with the number of blue-green alga cells present after 10 days of growth. This effect was not attributed to organism–organism competition for a growth-limiting nutrient; freeze-dried *G. aponina* cells also were cytolytic toward *G. breve* cells (4). The incubation period required prior to the initiation of *G. breve* degeneration (8–10 days) and the growth response of *G. aponina* were in good agreement with the time of maximum aponin elaboration (6–8 days) and inocula size–growth rate relationships observed in unialgal cultures of the blue-green alga (5).

In contrast to the neurologic (6) and hemolytic (7) properties exhibited

by components of the ichthyotoxin associated with *G. breve*, aponin has not been shown to possess any short-term (25 hr) ichthyotoxic activity when applied at high levels (about 15 units, or 7.5 times the amount required to cytolyze all *G. breve* cells) to several fish species. A 25-hr test period corresponds to 83 times the standard death time of fish exposed to *G. breve* toxin (8). An increase in the mean survival time of *Poecilia spenops* and *Mollienesia latipinna* was observed when aponin was incubated at low concentration with *G. breve* cells for 25 hr; enhanced ichthyotoxicity of *G. breve* cultures toward these species also was noted at higher aponin dosages, resulting from the anticipated *G. breve* cell degeneration and subsequent toxin release into the test vessels (2, 4).

Aponin possesses nine significant characteristics that are favorable for its use as a biocontrol agent for Florida's red tide organism, including thermal stability and slow activity loss in alkaline solutions (base labile) (3). The problems of aponin specificity and of its effects on biological systems other than *G. breve* cells have been addressed only recently. This paper considers findings on short-term effects of aponin (cytolytic factor) and *G. aponina* on *Artemia salina* (brine shrimp) in unialgal and in mixed cultures with *G. breve*.

A. salina may not be widely used in biological assays, but ample precedent exists, including the assay of ciguatera (9), *G. breve* (10), fungal toxins (11), and insecticides (12). The importance of *A. salina* in tropical food chains and its commercial value as a potential food source in shrimp and fish mari-culture (13) render *A. salina* an important organism for consideration. However, *A. salina* is more tolerant to *G. breve* toxin than are fish (10), which may mitigate against its use in some bioassays. For example, one *A. salina* (6 mg) could tolerate more than 10 times the amount of *G. breve* toxin than a 60-mg *Gambusia affinis* fish could withstand (10). Such tolerance has been attributed to medium detoxification by chemical absorption or metabolic means and would suggest that a population effect should be considered in the interpretation of bioassay results (10).

EXPERIMENTAL

Organism Source and Culture—Unialgal *G. breve*[1] cultures were maintained as axenic stock cultures in artificial sea water medium enriched with modified Wilson's B-5 supplements (14). *G. aponina*, isolated as detailed previously (1), were maintained as bacteria-free unialgal stock cultures, as described elsewhere (15). *A. salina* (mean dimensions: length = 7 ± 0.8 mm and wet weight = 2 mg) were obtained through local sources, and the stock organisms were maintained in artificial sea water.

The crustacea were nourished periodically with a few drops of Baker's yeast solution and aerated continuously (~250 ml/min). Stock cultures of the three organisms were maintained under similar salinity (34‰), pH (7.8), and temperature (24 ± 1°) conditions and under continuous illumination (approximately 2000 lux) provided by 40-w cool-white fluorescent lamps with lead shielded cathodes (16).

Organism Enumeration—Microorganism cultures were routinely sampled, and populations were enumerated with an electronic particle counter[2] using the electronic and threshold settings reported elsewhere (17). Organisms also were examined microscopically with an inverted binocular microscope.

A. salina cultures were observed and counted visually. Individual crustacean mortality was defined as the cessation of all peleopod locomotor activity for no less than 5 min.

Isolation and Bioassay of Aponin—Aponin was isolated from the
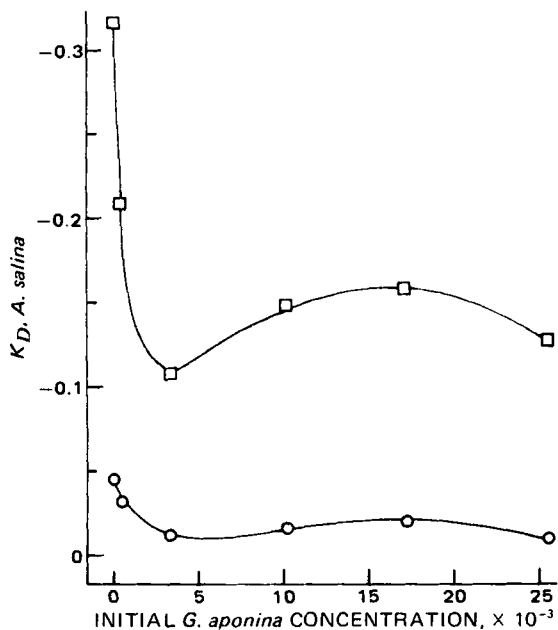

Figure 1—*Influence of various initial* G. aponina *populations in unialgal* (O) *and in mixed* (□) *culture with* G. breve *on* A. salina *mortality.*

10-day-old *G. aponina* cultures using the procedure of McCoy (2). *G. aponina* cells were cultured in a large-scale (20-liter) continuous culture system (18) and were harvested by continuous centrifugation using a Szent-Gyorgi apparatus[3]. The cell-free centrifugate was extracted for 24 hr with redistilled chloroform (50 ml/liter of centrifugate). The chloroform layer was then evaporated to dryness by rotary evaporation, and the residues were redissolved in a known chloroform volume.

Cytolytic activity of the crude aponin extract was determined using a modification of the bioassay described previously (2). In triplicate, serial volumes of the crude preparation were placed in 15-ml test tubes, and the chloroform was removed in an evacuated oven (41°). Ten milliliters of well-mixed *G. breve* culture[4] were pipetted carefully into each assay tube, and the cultures were mixed gently for 1 hr. After 20 hr of incubation, the *G. breve* cells remaining were counted and viability was checked microscopically.

One (relative) unit of aponin activity was defined as that amount of material required to produce 50% mortality of *G. breve* cells, at an initial concentration of 2000 cells/ml, after 20 hr of incubation (2000 lux, 25 ± 2°).

Unialgal and Mixed Culture Studies—Six pairs of sterile culture vessels (500-ml Fleaker flasks fitted with foam stoppers and aeration ports) were prepared; one set was designated as the unialgal (U) cultures, and the other was designated as the mixed (M) cultures. Into each U flask was placed 400 ml of sterile artificial sea water (34‰) enriched with the modified B-5 supplements; 400 ml of *G. breve* culture (cell counts >1400/ml) were introduced into the M flasks. Equal volumes of log-phase *G. aponina* cell concentrate and B-5 medium were pipetted into corresponding pairs of vessels.

The final volume of each culture was adjusted to 450 ml with artificial sea water medium and then both organisms were counted. After 24 hr of incubation (constant illumination of ~2000 lux) and aeration (~6 ml/min), *A. salina* (25–30 organisms in 15 ml of medium) were introduced into each vessel. Survival of *A. salina* individuals was monitored continuously for the initial 24 hr and regularly (3–6 hr) thereafter. *G. breve* and *G. aponina* were sampled and enumerated periodically, and cell viability was observed.

Aponin Toxicity—The protocol to test the toxicity of aponin on *A. salina* was similar to the bioassay procedure described. In triplicate, serial aponin solution volumes (in chloroform) were pipetted into 50-ml test tubes. Solvent and medium controls also were prepared. After the solvent was evaporated, 35 ml of modified B-5 medium (filtered, sterilized arti-

ficial sea water, 34‰) were placed into each tube, and the solutions were mixed for 24 hr at 25°.

Following mixing, five healthy *A. salina* (in 5 ml of medium) were added gently to the cultures, which were illuminated (2000 lux) and aerated (6 ml/min) continuously. *A. salina* survival was monitored hourly for the first 12 hr and periodically (3–4 hr) thereafter. To establish dosage levels applied to the organisms, a bioassay of the same aponin preparation with *G. breve* was run concurrently.

RESULTS

Three approaches were used to assess aponin and *G. aponina* effects on *A. salina* survival: direct presentation (surviving *A. salina* as a function of time), kinetic treatment (calculation of the zero-order mortality constant, K_D, where $K_D = m$, the slope of the linear portion of the direct presentation plot), and probit transformation[5]. These approaches were used appropriately with three groups of studies.

First, preliminary unialgal (*G. aponina* + *A. salina*) studies (Fig. 1) revealed two interesting results. *A. salina* survival (adults and larvae) was promoted in the presence of *G. aponina* relative to controls (B-5 medium without *G. aponina* and mixed culture systems). A threefold (1.3–4.3×) reduction in mortality constant values, K_D, for unialgal cultures of *G. aponina* (flasks U_2–U_6) was noted relative to control values (U_1, artificial sea water only). In addition, a twofold reduction in K_D values was observed for mixed culture systems (flasks M_2–M_6) relative to *G. breve* controls (M_1, 1400 cells/ml initially). These results also were confirmed by statistically significant (t test, $p < 0.001$) differences in the percent brine shrimp surviving for 90 hr in unialgal (80%) *versus* mixed (30%) cultures.

No apparent increase in the mixed culture toxicity to *A. salina* was noted, as anticipated for cultures containing high initial *G. aponina* populations. However, the *G. breve* cells used were relatively old (early stationary phase). Although *G. breve* cell numbers were sufficient to cause ichthyotoxic mortality, reported as populations greater than 250,000 cells/liter (20), the toxicity of this particular culture may have been reduced by enzymatic or physicochemical toxin degradation or by reduced toxin biosynthesis during the stationary phase of culture growth (21).

In contrast, at low initial *G. aponina* concentrations, a definite protective influence was exerted, as evidenced by the reduced *A. salina* mortality constants at these levels. This result may have been due in part to feeding of the brine shrimp on *G. aponina*, as suggested from inspection of fecal remains. Microscopic examination of the mixed cultures revealed sessile *G. breve* forms in cultures M_2–M_4 after 10 days; motile forms were observed in M_1 (*G. breve* only), and a few motile cells and cell debris were observed in cultures M_5 and M_6.

A second group of unialgal and mixed culture studies confirmed the original observations with a few notable exceptions (Table I). In these studies, log-phase *G. breve* cells (3 weeks old, 1900 cells/ml initially) were used. Enhanced *A. salina* mortality rates were observed in mixed cultures with high initial *G. aponina* populations, resulting from the expected cytolytic action exerted on *G. breve* cells. Furthermore, increased *A. salina* survival was noted at low initial *G. aponina* concentrations (cultures M_2 and M_3).

Aeration had no adverse effect on *G. breve* (viability or morphology), as evidenced by an increase in cell counts and size distributions. However, sessile *G. breve* forms were noted only in the latter mentioned cultures; cell debris and a few motile forms were found in M_4–M_6, and motile cells were observed in M_1. These differences in morphology also were reflected in culture population size distributions. Motile cells had morphologies as described previously (22), with cell volumes ranging from 3608 to 6220 μm^3, while sessile forms similar to those described previously (23) had volumes of 2240–4168 μm^3.

No significant deleterious short-term effect of unialgal *G. aponina* cultures on *A. salina* viability was found; *G. aponina* promoted brine shrimp survival, although the mortality rate increased slightly at intermediate initial cell populations. In addition, *G. aponina* cultures exerted no obvious effect on larval maturation or viability. Hatchlings were observed in the unialgal cultures and survived the duration of the experiment. In the mixed cultures, however, larvae did not persist for more than 3–4 days after hatching. Death of *A. salina* individuals in cultures containing *G. breve* followed a period of confused hyperactivity, disorientation, and contortions (curling) (10). These symptoms were not noted in the unialgal *G. aponia* cultures, where more subtle signs of death were

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[3] Centrifugation was carried out at 12,000×g in a SS-3 centrifuge (DuPont-Sorvall); the flow rate was 100 ml/min.
[4] A stock culture was swirled for 30 min at 120 rpm on a G-22 Gyrotory shaker bath (New Brunswick Scientific).

[5] The probit transformation converts a sigmoidal relationship (percent killed *versus* dose) to a linear one (probits *versus* log_e dose); a probit of 5 corresponds to 50% killed (19).

Table I—Unialgal and Mixed Culture Interactions with *A. salina*

Culture [a]	Initial[b] *G. aponina*	$-K_D \pm SD^c$	*A. salina* Surviving[d], %	Percent *G. breve* Decrease	Percent *G. aponina* Increase
Unialgal					
U_1	0	0.161 ± 0.007	8	—	—
U_2	474	0.085 ± 0.008	28	—	+484
U_3	1,836	0.078 ± 0.007	40	—	+958
U_4	5,629	0.107 ± 0.008	44	—	+365
U_5	8,637	0.122 ± 0.011	44	—	+295
U_6	14,068	0.059 ± 0.004	52	—	+167
Mixed					
M_1	0	2.08 ± 0.09	0	+26.3	—
M_2	474	1.36 ± 0.05	0	−3.4	—
M_3	1,836	1.87 ± 0.16	0	−37.8	—
M_4	5,629	1.85 ± 0.04	0	−69.5	—
M_5	8,629	2.07 ± 0.14	0	−51.5	—
M_6	14,068	2.73 ± 0.21	0	−72.8	—

[a] Unialgal (*G. aponina*) and mixed (*G. aponina* + *G. breve*, initial concentration of 1910 ± 80 cells/ml) cultures, maintained at a salinity of 34‰ and 25 ± 2° with continuous aeration (milliliters per minute) and illumination (2000 lux). [b] Serial dilution of log-phase cell concentrate in cells per 10-sec flow. [c] Zero-order rate constant of *A. salina* mortality in organisms per hour. [d] Duration of experiment was 246 hr.

Table II—Effect of Crude Aponin on *A. salina* Viability

Crude Aponin Applied per 10 ml of Media, ml	Units of Aponin Applied[a]	Percent *G. breve* Mortality	K_D of *A. salina* (Organisms/hr)	Time for 100% Mortality, hr
0.00	0.02 ± 0.01	2.2 ± 1.2	−0.20 ± 0.02	113 ± 45
0.10	0.29 ± 0.02	35.2 ± 2.1	−0.17 ± 0.02	73 ± 21
0.20	0.57 ± 0.04	70.3 ± 4.6	−0.19 ± 0.01	77 ± 13
0.40	0.79 ± 0.01	96.0 ± 0.5	−0.25 ± 0.03	71 ± 19
0.80	1.6[b]	99.2 ± 1.8	−0.43 ± 0.03	39 ± 9
1.0 CHCl₃	0.04 ± 0.02	4.5 ± 1.8	−0.21 ± 0.03	140 ± 41

[a] One aponin unit (relative) is defined as that amount required to cause 50% mortality in *G. breve* culture of 2000 cells/ml: units = 2 × fraction cells killed × [cells per milliliter used/(2000 cells/ml)]. [b] Estimation based on volumetric dilution.

noted: a slow, prolonged loss of motor-related activity associated with the peleopods, which ceased at death.

A third group of studies, involving crude aponin and *A. salina* (Table II), revealed two significant results. First, aponin concentrations (up to 0.8 unit of aponin) had little adverse effect on either adults or larvae, at least as indicated by the mortality constant, K_D. Second, higher concentrations exhibited adverse effects. For example, the K_D for 1.6 units was 72% greater than the value for 0.8 unit, and the time for 100% mortality decreased by a similar amount (82%).

Little or no aponin effect on *A. salina* was noted at concentrations less than 1 unit, as compared with control organisms (artificial sea water enriched with the B-5 supplements, and chloroform), which survived without loss of any individuals up to 60 hr. *A. salina* mortality at higher aponin levels (4 and 6 units) was observed within 24 hr. Probit analysis performed on percent *A. salina* killed after 48 hr yielded a linear relation and gave an apparent aponin LD₅₀ for *A. salina* of 2.3 units (Fig. 2). Larvae survived the duration of the experiment (96 hr), except at the higher doses (4 and 6 units) where none survived. Aponin produced signs of death similar to those described for *G. aponina*.

DISCUSSION

The present study of the short-term effect on *A. salina* viability of the cytolytic factor aponin and of *G. aponina* in unialgal and mixed cultures with the toxic dinoflagellate *G. breve* suggested the following:

1. Although relatively high aponin concentrations accelerated brine shrimp (*A. salina*) mortality, these concentrations were at or beyond those levels required to cause complete cytolysis of *G. breve* cells. In addition, test conditions were unrealistically stressful in the sense of being static.

2. In unialgal cultures, the crustacea (adults and larvae) were relatively unaffected by the presence of, or variations in, *G. aponina* populations. In fact, *G. aponina* enhanced the *A. salina* viability, presumably due to grazing, as compared to control cultures.

3. In mixed cultures with *G. breve*, *G. aponina* exerted two contrasting effects on *A. salina* viability. At high blue-green alga concentrations, adult and larval *A. salina* mortality was accelerated, at rates comparable to or greater than the mortality rates of *G. breve* cultures. In contrast, at lower *G. aponina* populations, mortality was reduced.

The noted toxicity to *A. salina* of mixed *G. breve* cultures incubated with large numbers of *G. aponina* is attributed to the additional endo-

toxin released from cytolyzed *G. breve* cells. Similar enhancements of *G. breve* toxicity were reported for two fish species when incubated with large doses (15–30 units) of aponin (2, 4). However, the apparent protective effect (increased survival) exerted by *G. aponina* toward *A. salina* when incubated in low populations with the dinoflagellate is more complex and not well defined. *A. salina* grazing on the blue-green algae was observed and would account for the decline in mortality rates of indi-
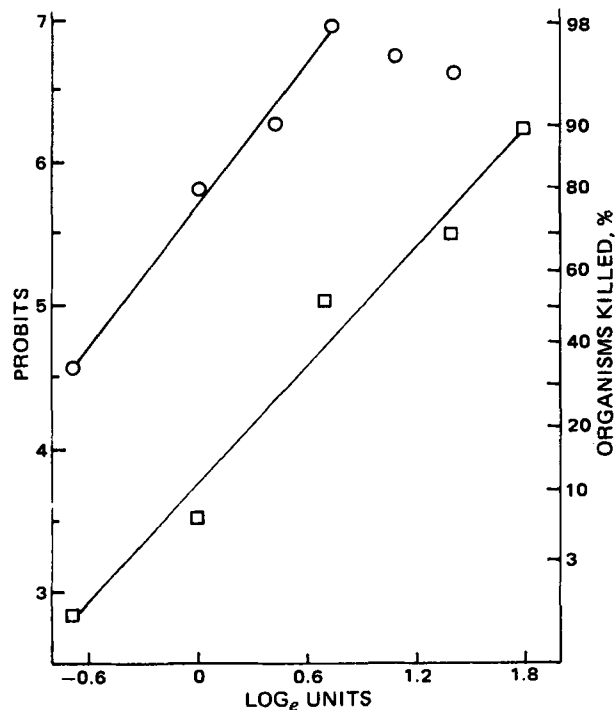

Figure 2—*Effects of various amounts (in relative units) of crude aponin on the probit of percent* A. salina *(O) and* G. breve *(□) mortality after 48 and 20 hr of incubation, respectively. Corresponding percent killed is given.*

viduals observed in unialgal cultures compared to controls. Feeding in mixed cultures also occurred, although ingestions of *G. breve* cells would enhance mortality of *A. salina* individuals, as was observed when compared with unialgal systems. However, feeding might not be directly responsible for the protective effect inasmuch as increases in the survival times of two fish species were noted when *G. breve* cells were incubated with small amounts (<1 unit) of aponin (4).

The appearance of sessile *G. breve* forms in certain mixed cultures would indicate that their presence might be related to the increased *A. salina* survival. Little is known about the nature and toxicity of the sessile or "resting" *G. breve* stage. In laboratory studies, high light intensities and spurious radiation from fluorescent lamps (16), as well as agitation and swirling (23), will initiate their formation. Agitation (aeration and periodic swirling) had no effect on *G. breve* viability or morphology in these experiments. Moreover, the fluorescent lamp cathodes were shielded, and illumination intensities were considerably less (2000 lux) than those thought to initiate sessile formation [10,000 lux (12)].

If such subtle external perturbations as those mentioned induce sessile formation, then other phenomena might also initiate such a change. One factor to consider is competition between organisms for a vital nutrient such as iron, which would result in rapid growth and dominance of one species (15). Equally important is biochemical "conditioning" of the medium by elaborated substances of one organism that are deleterious to the other. At low *G. aponina* populations, sufficient aponin may not be present to cause cytolysis, but perhaps enough is present to initiate a change in *G. breve* morphology. If, indeed, the *G. breve* sessile phase is a protective one or an intermediate of the encysted form, then it is reasonable that toxin release by *G. breve* would be reduced. Recent reports (24) demonstrated that benthic cysts of a New England red tide organism, *Gonyaulax tameranesis*, were highly toxic to shellfish and crustacea. However, preliminary fish assays (5) indicated that in laboratory cultures the sessile *G. breve* form exhibited less toxicity to *Gambusia affinis* than did motile organisms of the same age and concentration.

CONCLUSION

The cytolytic factor, aponin, did not adversely affect or significantly increase the mortality of adult and larval *A. salina* incubated at aponin concentrations (1 unit) that cause *G. breve* cytolysis. However, at application levels (4–6 units) considerably higher than those that might realistically be used for red tide control, >70% mortality was observed after 48 hr.

In addition, two contrasting effects on *A. salina* were observed in mixed cultures of the dinoflagellate and the blue-green alga, *G. aponina*: (*a*) survival was promoted at low *G. aponina* populations, a result of the presence of sessile *G. breve* forms; and (*b*) enhanced mortality was observed at high *G. aponina* populations, caused by toxin released from cytolyzed *G. breve* cells. Unialgal *G. aponina* cultures exerted no adverse effect on adult and larval *A. salina*, as compared to controls.

Despite the obvious need for an environmentally and economically practical control measure for the red tide (25), it is too early to judge the utility of aponin without additional scrutiny of its broad biological implications. Nonetheless, these studies indicated its potential use as a biocontrol agent.

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