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Induction of terpene biosynthesis in dinoflagellate symbionts of Caribbean gorgonians

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Abstract

This report describes a series of experiments designed to determine if terpene biosynthesis is inducible in two families of marine terpenes, pseudopterosins from the gorgonian coral *Pseudopterogorgia elisabethae* and fuscol from*Eunicea fusca*. Since we have recently shown that terpene biosynthesis is not under the control of the invertebrate host, but rather occurs within a dinoflagellate preparation, we examined the terpene content of the dinoflagellate symbiont following a decrease in UV/vis radiation as well as in response to the addition of methyl jasmonate, salicylic acid and gibberellic acid. We demonstrated that pseudopterosin and fuscol biosynthesis can be markedly increased through the addition of the plant bioactive substances. We also demonstrated that, while the terpene content of *P. elisabethae* increases in response to decreased UV/vis light, this is due primarily to an increase in the concentration of the dinoflagellate rather than simply an induction of terpene biosynthesis.

Keywords: *Pseudopterogorgia elisabethae*; *Eunicea fusca*; Dinoflagellate; *Symbiodinium*; Terpene biosynthesis; Pseudopterosin; Fuscol; Induction

1. Introduction

Marine gorgonian corals have proven to be a prolific source of bioactive terpenoid compounds (Proksch et al., 2002). One such organism, *Eunicea fusca*, possesses diterpenes with anti-inflammatory potencies similar to that of the industry standard indomethicin. Fuscoside B (**1**) (Fig. 1) was shown to inhibit phorbol myristate acetate (PMA)-induced ear edema in murine models (Jacobson and Jacobs, 1992a). It inhibits leukocyte infiltration into PMA-treated tissue and selectively inhibits leukotriene C_4 biosynthesis with no effect on prostaglandin E_2 synthesis. Furthermore, it is not metabolized after prolonged exposure to cells (Jacobson and Jacobs, 1992a). Fuscol (**2**) (Fig. 1), the aglycone of fuscoside B, is a major metabolite of *E. fusca* (Gopichand and Schmitz, 1978) and exhibits similar anti-inflammatory activity (Jacobson and Jacobs, 1992a). The coral *Pseudopterogorgia elisabethae* is the source of a family of diterpene glycosides known as the pseudopterosins (e.g. **3**–**6** in Fig. 2) (Look et al., 1986; Roussis et al., 1990). These represent a second group of potent anti-inflammatory and analgesic agents which are used as anti-irritants in cosmetic applications (Roussis et al., 1990).

While there is a substantial body of literature describing the structural diversity of bioactive terpenes of marine origin, little is known about their biosynthetic origin, their ecological role or factors controlling their level of expression. In terrestrial organisms, the induction of terpene biosynthesis has been examined in multiple systems. For instance, methyl jasmonate (MeJA) and salicylic acid (SA) are known to stimulate a prophylactic defense and resistance mechanism in uninfested plants during insect wounding on neighboring plants (Farmer and Ryan, 1990; Shulaev et al., 1997). These defense activities have been shown to produce mainly terpenoid products (Piel et al., 1998).

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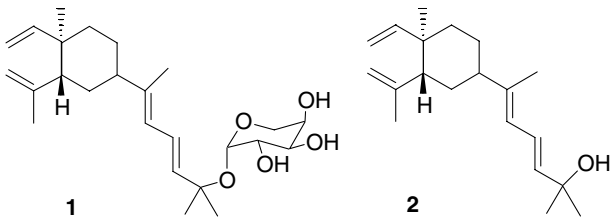
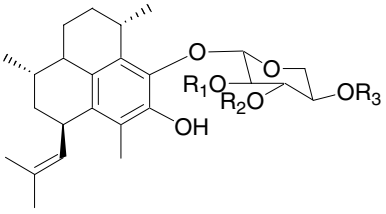


Fig. 1. Fuscoside B (**1**) and fuscol (**2**).



3 PsA R_1=R_2=R_3=H
4 PsB R_1=Ac, R_2=R_3=H
5 PsC R_2=Ac, R_1=R_3=H
6 PsD R_3=Ac, R_1=R_2=H

Fig. 2. Pseudopterosins A–D (**3**–**6**).

Terpene biosynthesis has been studied in the leaves of *Vitis vinifera* after treatment with MeJA, which caused a strong up-regulation of *de novo* terpene biosynthesis (Hampel et al., 2005). When MeJA was exogenously applied to plants it exerted various effects, including both inhibition and induction, in the form of morphological and physiological changes (Sembdner and Parthier, 1993). It is believed that MeJA plays an integral role in the intracellular signal-transduction cascade operative in the inducible defense mechanisms that plants have evolved against pathogens and by means of which plant cells counteract stressors in general (Creelman and Mullet, 1997). MeJA is presumed to interact with receptors in the cell which activate a signaling pathway resulting in changes in transcription, translation, and other responses (Creelman and Mullet, 1997). MeJA has also been shown to mimic fungal elicitation and mechanical wounding in the Norway Spruce with respect to terpenoid resin biosynthesis (Martin et al., 2002). In addition, Taxol® biosynthesis has been induced in *Taxus canadensis* cell cultures treated with MeJA (Ketchum et al., 1999a).

Other plant signaling factors such as gibberellins and SA have been used to induce the growth of trichomes, a response to insect damage in *Arabidopsis* (Traw and Bergelson, 2003). SA is known to up-regulate defense genes in plants when they undergo an attack by a predator. Although SA is considered to be a signaling molecule that induces genes related to defense responses, it has been shown to inhibit nod gene expression. Nod genes produce nod factors which are important in the signalling between the host plant and its roots. Studies involving nod genes indicate that the induction mechanism is quite different from that of the jasmonates in that SA inhibits nod gene expression and molecules from the jasmonate family induce such genes (Rosas et al., 1998). Gibberellins are a group of tertacyclic diterpenes which are essential regulators that influence growth and development processes during the plant life cycle. These processes include shoot elongation, the expansion of leaves, flowering and seed germination (Kende and Zeevaart, 1997). These hormones work with auxins to promote rapid elongation and division of stem tissue, as well as expansion activity (Wright, 1993). In fact, they are known to play an important role in breaking dormancy after inhibition of water by the seed coat and ultimately signal germination activities (Reinoso et al., 2002).

We recently reported the results from a series of experiments directed at examining the inducibility of terpene biosynthesis in the gorgonian *P. elisabethae* (Thornton and Kerr, 2002). In one study, colonies of *P. elisabethae* were shaded from UV/vis light and analysis of pre- and post-treatment coral clippings demonstrated that the blocking of this radiation increased the pseudopterosin content by approximately 100% (Thornton and Kerr, 2002). More recently, our lab has discovered that a dinoflagellate preparation from *P. elisabethae* and *Pseudopterogorgia bipinnata* possess the ability to biosynthesize diterpenes previously ascribed solely to the host coral (Mydlarz et al., 2003; Boehnlein et al., 2005). The dinoflagellate symbiont of gorgonians has been identified as belonging to the genus *Symbiodinium*. Given the historical success of the induction of secondary metabolites through elicitation with plant signaling molecules and given this newly discovered source of terpene biosynthesis, we have re-examined the inducibility of terpene biosynthesis in two gorgonian corals and, rather than exclusively examining the terpene content of the holobiont (coral and all associated microorganisms), we have described the terpene content of the isolated dinoflagellate preparation. Specifically, we investigated the effects of reduced UV/vis radiation and MeJA on pseudopterosin A–D (**3**–**6**) biosynthesis in the gorgonian holobiont and a dinoflagellate preparation, and the addition of MeJA, SA and gibberellic acid (GA) on fuscol (**2**) biosynthesis on the dinoflagellate preparation of *E. fusca*.

2. Results and discussion

We have previously reported that terpene biosynthesis is inducible in the *P. elisabethae* holobiont (Thornton and Kerr, 2002), and have subsequently demonstrated that a dinoflagellate preparation free of gorgonian cells is a source of pseudopterosin biosynthesis (Mydlarz et al., 2003). To more rigorously address the issue of terpene biosynthesis inducibility in these marine systems, we conducted a number of induction experiments and quantified the terpene content of isolated dinoflagellate preparations from treated and control organisms. In addition, the concentration of dinoflagellates in the gorgonian was measured in response to the stimuli.

Colonies of *P. elisabethae* were selected at random on a reef at Sweetings Cay, Bahamas. Eight colonies were affixed to a substrate on the ocean floor under a 1 m^3 acrylic sheet that filtered UV/vis radiation per our previous report (Thornton and Kerr, 2002). While there was some degree of indirect light, these samples received significantly less than ambient light. An additional eight colonies were likewise affixed to a substrate and not shaded. This second set of gorgonians served as a control group. Clippings were taken from each coral prior to the experiment and will be referred to as pre-treatment samples. An additional clipping was also taken from each coral following a 72-hour period (post-treatment samples). The dinoflagellates were purified from the coral samples by homogenization of the coral tissue and centrifugation over a discontinuous Percoll™ gradient. Dinoflagellate cell density per gram of coral increased by 57% as a result of the decreased radiation levels in the UV/vis light shading experiments and the data found to be statistically significant based on the 95% confidence interval with $p < 0.05$. The pseudopterosin content of the isolated dinoflagellates was found to increase by 10% over the treatment period. This value was also found to be within the 95% confidence interval for statistical significance with $p < 0.05$. It seems reasonable to postulate that the coral's nutrient requirements remain constant and therefore this increase in dinoflagellates is the result of decreased photosynthetic activity of the individual cells in response to the reduction in light levels, although this observation requires additional experiments to validate. Our previous publication indicated that lower UV/vis radiation levels resulted in a significant induction of pseudopterosin biosynthesis (100%) within the host coral (Thornton and Kerr, 2002). These new data demonstrate that there is only a very slight increase in pseudopterosin content (10%) of the dinoflagellate and that the observed increase in pseudopterosin content of the holobiont is primarily due to an increase in the concentration of dinoflagellate.

The second set of experiments was conducted in order to investigate the inducibility of pseudopterosin biosynthesis in response to the addition of MeJA. In this case, colonies of *P. elisabethae* were collected, placed in 50 gallon opaque containers and allowed to acclimate for 4 weeks. At this time, the pseudopterosin content was determined and found to be uniform for all individuals. Nine colonies of the gorgonian were treated with 150 μM MeJA for 5 days. The use of this concentration of MeJA was based on the amount of MeJA employed in Ketchum et al. (1999a) work in which a dose–response study indicated that treatment of *Taxus* cultures with concentrations of MeJA above 200 μM MeJA caused a sharp decline in paclitaxel elicitation. For each coral, the pseudopterosin content of the isolated dinoflagellate preparation from pre-treatment and post-treatment clippings was measured. The pseudopterosin content of the dinoflagellate cells was found to increase by 94% in response to the addition of MeJA. This data was determined to be statistically significant by a 95% confidence interval with $p < 0.05$. In the past it has been demonstrated that treatment of plants and cell cultures with MeJA has induced terpene biosynthesis significantly (Kaukinen et al., 1996; Mirjalili and Linden, 1996; Yukimune et al., 1996; Bohlmann et al., 1998a; Hefner et al., 1998; Ketchum et al., 1999a,b; Lapointe et al., 2001; Martin et al., 2002). Our experiments were designed to examine the effect of various plant growth factors on terpene biosynthesis in two marine systems. When the *P. elisabethae* holobiont was treated with MeJA the concentration of terpenes increased significantly (by an average of 94%) thus indicating that pseudopterosin biosynthesis is inducible in response to such plant signaling factors. Interestingly, when the dinoflagellates were first isolated from *P. elisabethae* and then treated with MeJA, no increase in pseudopterosin content was observed. We assume that this is a reflection of the poor health of the isolated dinoflagellate cells from this gorgonian. The concentration of dinoflagellates was also quantified after treatment with MeJA and no statistical difference was found.

To follow up on the observation of the large increase in pseudopterosin content of the dinoflagellate in response to the addition of MeJA treatment, we expanded our analysis to include additional known inducers of terpene biosynthesis in terrestrial systems. Thus, a series of experiments with MeJA and SA were performed on the isolated *Symbiodinium* sp. from *P. elisabethae*. In this series of experiments, the dinoflagellate symbionts from this coral was isolated, maintained in FSW and treated with one of the plant signaling factors with the goal of examining terpene content of the control and treated algae in the absence of the gorgonian host.

The *Symbiodinium* isolated from *P. elisabethae* were divided into three replicates of 5.0×10^7 cells and were used for treatment with MeJA (50 μM) and SA (50 μM) for a period of 24 h. A lower concentration of MeJA was used with this experiment because the isolated cells were deemed to be more fragile than the intact holobiont. No significant change in pseudopterosin content was observed and the experiment was repeated and modified for a period of 48 h. In this set of experiments, six replicates of 6.0×10^7 cells were used for each of the treatments and the controls. Following a treatment period of 48 h, the cells were lyophilized and the pseudopterosin content determined by HPLC. No significant change (using Dunnett's method of statistical analyisis, $p \leqslant 0.05$) in pseudopterosin content was observed for any of the plant signaling factors at 50, 100 μM MeJA or the 50, 100 and 200 μM SA (see Fig. 3). In this experiment, multiple concentrations of MeJA were used based on the previous experiment. SA was also added in various concentrations to examine its usefulness in inducing terpene biosynthesis. Due to the limited amounts of biomass, the 200 μM MeJA treatment was not performed.

At this time we expanded our investigation to include the more readily accessible gorogonian *E. fusca*, as well as an additional plant growth regulator (GA). *Symbiodiniuim*

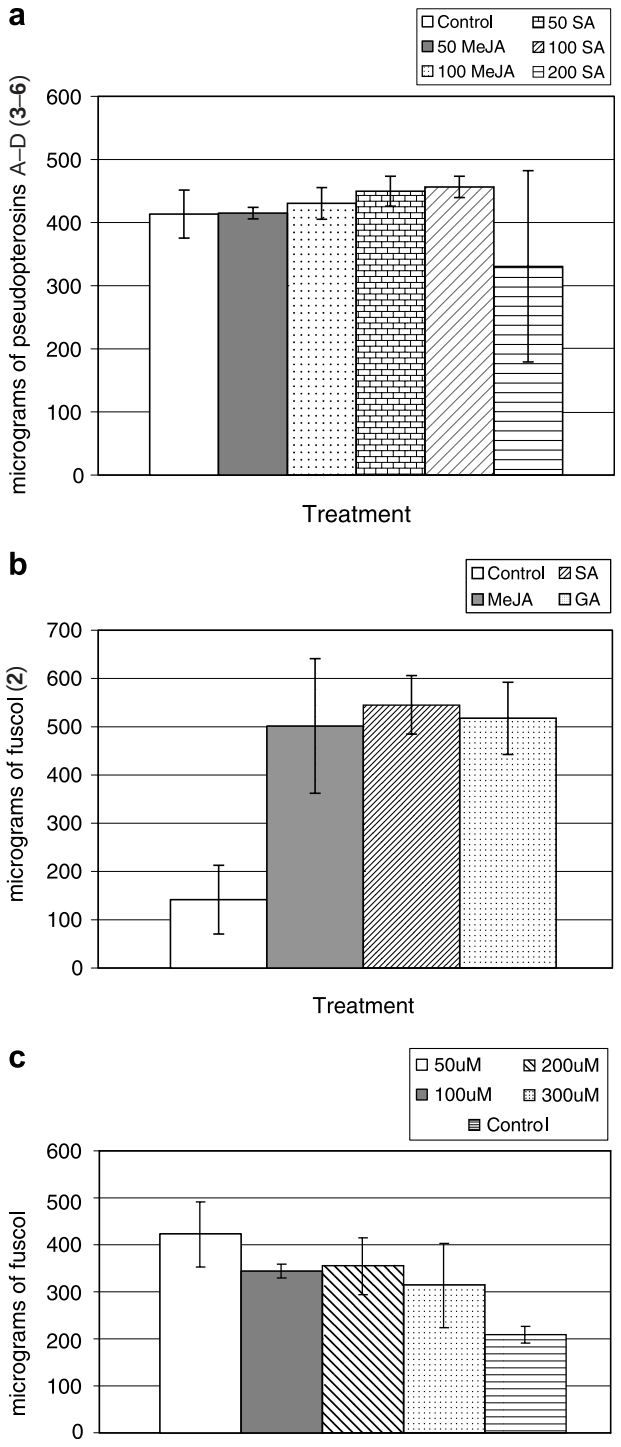


Fig. 3. (a) Treatment of *Symbiodinium* sp. from *P. elisabethae* with plant signaling molecules. (b) Treatment of *Symbiodinium* sp. from *E. fusca* with plant signaling molecules. (c) Dose–response study with *Symbiodinium* sp. from *E. fusca* treated with salicyclic acid.

sp. from *E. fusca* were isolated and 2×10^7 cells treated with 100 μM MeJA, SA or GA. The use of this concentration of plant bioactive substances as a starting point for these studies was somewhat arbitrary but was based on Ketchum et al. (1999a) reported success with 100 μM MeJA elicitations. There were three replicates of each treatment and an additional three replicates were untreated and served as a control. Following the treatment period of 48 h, the cells were lyophilized and the fuscol content determined by HPLC analysis. As is summarized in Fig. 3b, all three plant bioactive substances gave rise to a substantial increase in fuscol (**2**) content. The fuscol (**2**) content of the treated cells was approximately 350% greater than that of the control cells. Statistical analysis by Dunnet's method indicated the MeJA, SA and GA treatments were all significantly different from the control with $p \leqslant 0.05$ (Fig. 3b). With regards to fuscol (**2**) biosynthesis, the addition of MeJA, SA and GA were evaluated as potential inducers of terpene biosynthesis in the isolated dinoflagellates. In this system, all three plant bioactive substances proved to be equally effective in the induction of fuscol (**2**) biosynthesis.

Given the positive results from the previous experiment with *E. fusca*, a dose–response study was designed around the plant signaling factor SA (Fig. 3c). The *Symbiodinium* sp. were isolated as mentioned above and three replicates each of 2.0×10^7 cells were used for the control (no treatment), 50 μM, 100 μM and 200 μM SA. The results indicated that although each treatment caused a statistically significant (Dunnet's method, $p \leqslant < 0.05$) induction of fuscol biosynthesis, the treatments were not statistically significant from each others. A dose–response study with lower concentrations of plant bioactive substances may shed light on the minimal concentration necessary to illicit an induction if fuscol biosynthesis, however, it is noteworthy that this increase in terpene content is significant and reproducible with different amounts of plant bioactive substances.

3. Conclusions

The data reported in this manuscript indicate that, under appropriate conditions, terpene biosynthesis can be induced within a dinoflagellate preparation of *P. elisabethae* and *E. fusca*. It is clear that an understanding of the organism responsible for the biosynthetic pathway is required to adequately address questions such as the inducibility of biosynthesis in the complex assemblages that often characterize marine invertebrates. It is important to note that our dinoflagellate preparations are not free of bacteria or fungi and thus we cannot rule out the role of such associated microbes in this set of experiments. We are, however, confident that pseudopterosin A–D (**3**–**6**) and fuscol (**2**) biosynthesis proceeds in the absence of gorgonian cells. It appears that despite the similarities in *Symbiodinium* sp. from *P. elisabethae* and *E. fusca*, there is an inherent difference between the inducibility of terpene biosynthesis in symbionts from *E. fusca* and in the symbionts of *P. elisabethae*. This may be a factor of the specific conditions employed in our experiments, such as concentrations of plant bioactive substances, but nevertheless there is clearly a difference between the factors influencing

terpene biosynthesis in the two systems evaluated. Further experiments are needed to address these issues.

It now appears that terpene biosynthesis in marine systems can be influenced by the same factors that have been shown to affect terpene chemistry in terrestrial plants. In one application this induction of terpene biosynthesis has led to the successful identification of terpene biosynthetic genes using differential display. These data will be reported separately.

4. Experimental

4.1. General chromatographic procedures

TLC analysis was performed using precoated silica gel UV_{254} plates (Whatman) with a mobile phase of hexanes:EtOAc (60:40). The spots were visualized by spraying the plate with 10% H_2SO_4 in MeOH and charring plate in an oven.

4.2. Dinoflagellate isolation

Coral tissue (typically 30 g) was homogenized in a Waring blender using 100 ml deionized H_2O. The homogenate was then filtered through cheesecloth. The dinoflagellate symbionts were pelleted by centrifugation at $900 \times g$ and washed 10 times with 45 ml of 0.2 μ filtered seawater (FSW). The algal symbionts were further purified by a discontinuous Percoll™ gradient, twice. The cells were then rinsed with FSW twice, re-suspended in FSW, with cells counted by hemocytometer using light microscopy.

4.3. Extraction and purification of pseudopterosins from dinoflagellates

The dried dinoflagellate preparation was extracted with MeOH:H_2O (9:1), followed by partitioning with hexanes to remove the less polar metabolites. The MeOH:H_2O layer was adjusted to a 1:1 ratio and partitioned with CH_2Cl_2. The organic solvent was removed *in vacuo* and the sample analyzed by normal phase HPLC using a Vydac semi preparative column with hexanes:EtOAc (60:40) as the mobile phase and a Perkin–Elmer LC-235 Diode array detector at a wavelength of 283 nm. Integration of peak areas was used to quantify the amounts of pseudopterosins A–D (**3**–**6**). Standard samples of the pseudopterosins (**3**–**6**) were isolated from *P. elisabethae* and structures confirmed by spectroscopic analysis.

4.4. Extraction and purification of fuscol from dinoflagellates

Dried dinoflagellate cells were extracted with MeOH:H_2O (9:1) and partitioned with hexanes. The organic layer was examined by reversed phase HPLC using a semi preparative C18 column with MeOH:H_2O (70:30) as the mobile phase and a Perkin–Elmer LC-235 Diode array detector at a wavelength of 240 nm. Integration of peak areas was used to determine the amounts of fuscol (**2**). A standard sample of fuscol (**2**) was isolated from *E. fusca* and its structure confirmed by spectroscopic analysis.

4.5. UVlvis radiation blocking of P. elisabethae

P. elisabethae was collected at a depth of 10 m from Sweetings Cay, Bahamas, in May 2002. Individual colonies were attached to a stainless steel bolt which was secured to a paving stone at the site of collection. A total of eight such colonies were placed under a $1\ m^3$ UV blocking acrylic sheet. An additional eight colonies were placed under $1\ m^3$ UV transparent acrylic sheets to serve as a control group. Pre-treatment clippings were taken from each specimen at the time of installation under the acrylic sheets and post-treatment clippings taken at 72 h. All clippings were flash frozen in Falcon™ tubes for subsequent dinoflagellate isolation and chemical analysis. Pre-treatment and post-treatment samples were thawed and removed from Falcon tubes. Branchlets were trimmed from each sample, weighed and dinoflagellates isolated as described above. Pseudopterosins were purified and quantified as described above.

4.6. Treatment of P. elisabethae colonies with methyl jasmonate

P. elisabethae colonies were collected from Sweetings Cay, Bahamas, May 2002, by SCUBA and allowed to equilibrate in plastic tubs for one month. Clippings of 15–20 cm from nine colonies were removed prior to the addition of 150 μM methyl jasmonate and post-treatment clippings taken at 5 days. The dinoflagellates were isolated and pseudopterosins purified per the previous descriptions.

4.7. Treatment of dinoflagellates isolated from P. elisabethae with methyl jasmonate and salicylic acid

The coral was collected from Sweetings Cay, Bahamas during May of 2003 by SCUBA and dinoflagellates isolated by our general isolation method described above. Incubations of dinoflagellates for 24 h were performed with 5.0×10^7 cells with three replicates of 50 μM MeJA, three of 50 μM SA and three sets untreated that served as controls. The dinoflagellates were isolated and pseudopterosins purified per the previous descriptions.

The above experiment with purified dinoflagellates was repeated with the incubations maintained for 48 h. Purified dinoflagellate cells were allowed to equilibrate in T-flasks with filtered sea water for 24 h before treatment. Six replicates of 6.0×10^7 cells were used for each of the following treatments and control (no additive): 50 and 100 μM MeJA, 50, 100 and 200 μM SA. The dinoflagellates were isolated and pseudopterosins purified per the previous descriptions.

4.8. Treatment of dinoflagellates from E. fusca with methyl jasmonate, salicylic acid and gibberellic acid

E. fusca was collected off Long Key, in the Florida Keys during January of 2004 by SCUBA. Dinoflagellates were isolated as described above and the cells allowed to equilibrate in BD Flacon T-flasks™ for 24 h before treatment. Three replicates of 2.0×10^7 cells were used for each of the following treatments and control: 100 μM methyl jasmonate, 100 μM salicylic acid, and 100 μM gibberellic acid (gibberellin A_3, $C_{19}H_{22}O_6$). The dinoflagellates were maintained for 48 h and then fuscol isolated and quantified as described above.

4.9. Dose–response study of dinoflagellates from E. fusca with salicylic acid

E. fusca was collected off Long Key, in the Florida Keys during January of 2004 by SCUBA. Dinoflagellates were isolated as described above and the cells allowed to equilibrate in BD Flacon T-flasks™ for 24 h before treatment. Three replicates of 2.0×10^7 cells were used for each of the following treatments and a control: 50 μM salicyclic acid, 100 μM salicylic acid, and 200 μM salicyclic acid. The dinoflagellates were maintained for 48 h and then fuscol isolated and quantified as described above.

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