J Appl Phycol (2007) 19:27–32
DOI 10.1007/s10811-006-9107-x

ORIGINAL ARTICLE

Effects of germanium dioxide, an inhibitor of diatom growth, on the microscopic laboratory cultivation stage of the kelp, *Laminaria saccharina*

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Received: 25 May 2006 / Accepted: 1 June 2006 / Published online: 29 November 2006

Abstract The kelp, *Laminaria saccharina*, is an economically and biologically valuable seaweed used in integrated multi-trophic aquaculture. The development of the initial microscopic phase of the life cycle of this species is carried out in the laboratory. A treatment of germanium dioxide (GeO_2) can be applied to algal laboratory cultures to inhibit the growth of contaminating diatoms. Previous studies reported inhibitory effects also affecting the macroscopic stage of the life cycle of *L. saccharina*, the sporophyte, but the effects on the microscopic gametophytic life stage are unknown. To determine the effects, cultures of *L. saccharina* were treated with varying concentrations of GeO_2 and the resulting lengths and surface area of the juvenile sporophytes produced were measured. It was determined that GeO_2 follows a typical dose-effect pattern, increasing the growth rate of juvenile sporophytes until a critical point at which an inhibitory effect was observed. To obtain juvenile sporophytes ready for deployment to aquaculture sites in the shortest time and to successfully reduce diatom contamination, it was determined that a concentration between 0.10 and 0.50 mL of GeO_2 per litre of seawater, applied at day 8, was the most efficient.

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Keywords Diatom inhibition · Gametophyte · Germanium dioxide · Integrated aquaculture · *Laminaria saccharina* · Multi-trophic

Introduction

Laminaria saccharina (Linnaeus) Lamouroux is currently being cultivated as the inorganic extractive component of an integrated multi-trophic aquaculture system along with *Salmo salar* Linnaeus (fed component) and *Mytilus edulis* Linnaeus (organic extractive component) in the Bay of Fundy, Canada (Chopin et al., 2004). *Laminaria saccharina* has an alternation of generation life cycle between two distinct phases: the macroscopic, diploid sporophyte, and the microscopic, haploid gametophyte. In the seedstock production stage, spores extracted from sporophytes are cultured in laboratory conditions until the life cycle is completed to produce juvenile kelp sporophytes, at which time they are deployed to the aquaculture sites (Merrill & Gillingham, 1991). During the microscopic stage of production, there is a possibility of contamination, especially by other algae. The most serious effect of fouling by algae is the potential overgrowth and elimination of the kelp germlings, most typically by diatoms (Merrill & Gillingham, 1991). A treatment of germanium dioxide (GeO_2) has been recommended, but its effects have not been clearly documented.

Lewin (1966) reported that GeO_2 is a specific inhibitor of diatom growth and interferes in some way

with the processes of silica frustule formation. The effect of GeO_2 on the main algae in culture has had very little investigation and is poorly understood. Several early studies on the effect of this compound concluded that GeO_2 inhibited the growth of mature Chlorophyta and Phaeophyta species; however, the Rhodophyta species under study showed no growth inhibition (Chen et al., 1969, 1970; McLachlan et al., 1971; Hopkin & Kain, 1978; Markham & Hagmeier, 1982). Markham and Hagmeier (1982) conducted studies on macroscopic *L. saccharina* sporophytes using the "hole punch" method and concluded that *L. saccharina* did show inhibition of growth from GeO_2, but suggested that GeO_2 could still be used without serious damage in most cultures of Phaeophyta at concentrations not exceeding 0.01–0.04 mL of a saturated solution of GeO_2 per litre. Merrill and Gillingham (1991) recommended using a treatment of 1–2 mL of a saturated solution of GeO_2 per litre during the microscopic gametophyte development of *Nereocystis luetkeana* (approximately at day 10). However, they did not mention any consequences regarding the effects this chemical would have on kelp growth. Since it was known that germanium potentially replaced silica atoms in compounds, the GeO_2 treatment may have been assumed to have no detrimental effects on kelp, since silica is not a major or essential element for *L. saccharina* and most macroalgae (Fritsch, 1971).

Because of these conflicting results between the microscopic and macroscopic stages of different species of kelps and the wide range of GeO_2 concentrations used in the studies mentioned above (between 0.045 and 10 mg GeO_2 per litre), this study was undertaken to determine the effect of the diatom inhibitor, GeO_2, when applied to the microscopic gametophytic stage of *L. saccharina* in culture. This was achieved by comparing the effects of different concentrations of GeO_2 on the length and surface area of the juvenile sporophytes produced.

Materials and methods

Laboratory preparation of cultivation spools

The substrate used for cultivating the kelps was a 2.5 mm flat nylon twine, which was cut into 60 separate pieces, each measuring 14 m in length. The pieces of nylon twine were then soaked in boiling distilled water to remove the waxy film. The nylon twines were then spooled onto 60 PVC pipes (18 cm × 7.5 cm diameter), using a mechanical lathe. The spools were then soaked in sodium carbonate, followed by a distilled water rinse.

Seaweed collection and inoculation

Forty-four sexually mature *L. saccharina* sporophytes with large distinct sori were collected at Green's Point, Letite, New Brunswick, Canada (45°02′15″N; 66°53′30″W).

The sori were cut from the kelps, cleaned, and placed in a refrigerator overnight. They were then removed from the refrigerator and allowed to dry at room temperature for approximately 3 h. A spore release followed by re-immersion of the sori into filtered seawater at 11 °C, and stirring every 30 min for 4 h. The spore solution was then added to the inoculation chamber (200 L tank with gentle aeration) containing the spools and filled with seawater filtered to 0.2 μm. The chamber was covered and kept in darkness for 24 h.

The culture tanks (10 × 60 L white laundry tubs) were supplied with aeration tubes, a transparent Plexiglas cover, and two cool fluorescent lights (80–100 $\mu E\, m^{-2}\, s^{-1}$) set to a 18:6 L:D photoperiod regime. They were filled with 0.2 μm filtered seawater maintained at 11 ± 1 °C, a pH of 8, and a salinity of 33 ± 1 ppt. After the 24 h inoculation period, the spools were removed from the chamber and randomly placed in the culture tanks with gentle aeration.

Germanium dioxide treatment

A saturated solution of GeO_2 was prepared by adding 0.894 g of reagent grade, powdered GeO_2 (Acros Organics) to a 200 mL volumetric flask with de-ionised distilled water. It was then placed on a stirrer and allowed to mix overnight. The culture tanks randomly receive a treatment of 0.00, 0.02, 0.10, 0.50, or 1.00 mL of GeO_2 per litre of seawater on day 8 (two tanks for each treatment).

Sampling

Samples were taken twice a week once juvenile sporophytes were observed on the twines. Three randomly selected spools from each tank were sampled by plucking a small piece of the nylon twine using a pair

of forceps. The samples were then placed on three separate slides and viewed under a light microscope equipped with a digital camera. From each slide, five digital pictures were captured and transferred to a computer for analysis. The photo software program, Image J, was used to calculate the sporophyte length and surface area.

Statistical analyses

All statistical analyses were preformed with the Minitab^{TM} s14 statistical package. In order to pool the data from the two tanks with the same treatment, an Anderson – Darling normality test was conducted to determine if the data points were normally distributed. Once normality was determined, an F-test was conducted to observe if there was any difference in variance between the two tanks. All data sets were considered normal (Anderson–Darling test, $p > 0.05$) and treatment tanks were homogeneous (F-test, $p > 0.05$); consequently, the data from the tanks with the same treatment were pooled.

The effects of the different treatments on the sporophyte length and surface area were assessed for each sampling date by one-way ANOVA, holding sample date fixed and using either length or surface area as the response variable. A *posteriori* comparison among treatments was then made using a Tukey post-hoc multiple comparisons test ($\alpha = 0.05$). The influence of the length of time on the sporophyte length and surface area for each treatment was then assessed by another one-way ANOVA, holding the treatment fixed and using the length and surface area at each sample date as the response. A *posteriori* comparison among treatments was then made using a Tukey post-hoc multiple comparisons test ($\alpha = 0.05$).

Results

Diatom contamination was stopped in all the tanks treated with GeO_2. Over the length of the study (40 days), it was observed that the various GeO_2 treatments did not trigger any delay of the time at which the successive life stages of *L. saccharina* occurred (based on previous years' culture schedules). The effect was only observed on the size of the kelps at each developmental stage.

Sporophyte length as a function of germanium dioxide concentration

A significant difference was observed in the mean lengths of the sporophytes exposed to different concentrations of GeO_2 (Fig. 1).

On the final experimental day, day 40, the lengths of the sporophytes exposed to 0.50 and 0.10 mL GeO_2 per litre were not significantly different from each other but were both significantly longer ($p < 0.001$) than the sporophytes exposed to all other treatments. The lengths of the sporophytes treated with 1.00 and 0.00 mL GeO_2 per litre were also not significantly different from each other but were significantly shorter ($p < 0.001$) than those from all the other treatments.

The sporophytes grown under 1.0 mL GeO_2 per litre treatment were much more abundant than the ones that received no GeO_2 treatment.

Sporophyte surface area as a function of germanium dioxide concentration

On the final sampling date, day 40, the surface areas of the sporophytes receiving 0.02, 0.10, and 0.50 mL GeO_2 per litre treatments were not significantly different from each other, but were significantly larger ($p < 0.001$) than those exposed to 0.00 and 1.00 mL GeO_2 per litre treatments, for which the surface areas were not statistically different (Fig. 2).

Discussion

When applied to the gametophytic life stage of *L. saccharina*, GeO_2 was observed to have a significant effect on the length and surface area of the sporophytes produced. It, however, did not have an effect on the timing of the occurrence of the successive developmental stages of the life cycle of *L. saccharina* compared to what has been observed in previous years without GeO_2 treatments. A general dose effect occurred when an increase in GeO_2 concentration increased the growth rate of the sporophytes to a critical point at which GeO_2 had a severe inhibitory effect on sporophyte growth. This is observed as an increase in the length and surface area of the sporophytes receiving a treatment that increases from 0.00 to 0.02 to 0.10 and to 0.50 mL GeO_2 per litre, respectively. At the highest treatment of 1.00 mL GeO_2 per litre, GeO_2 had an inhibitory

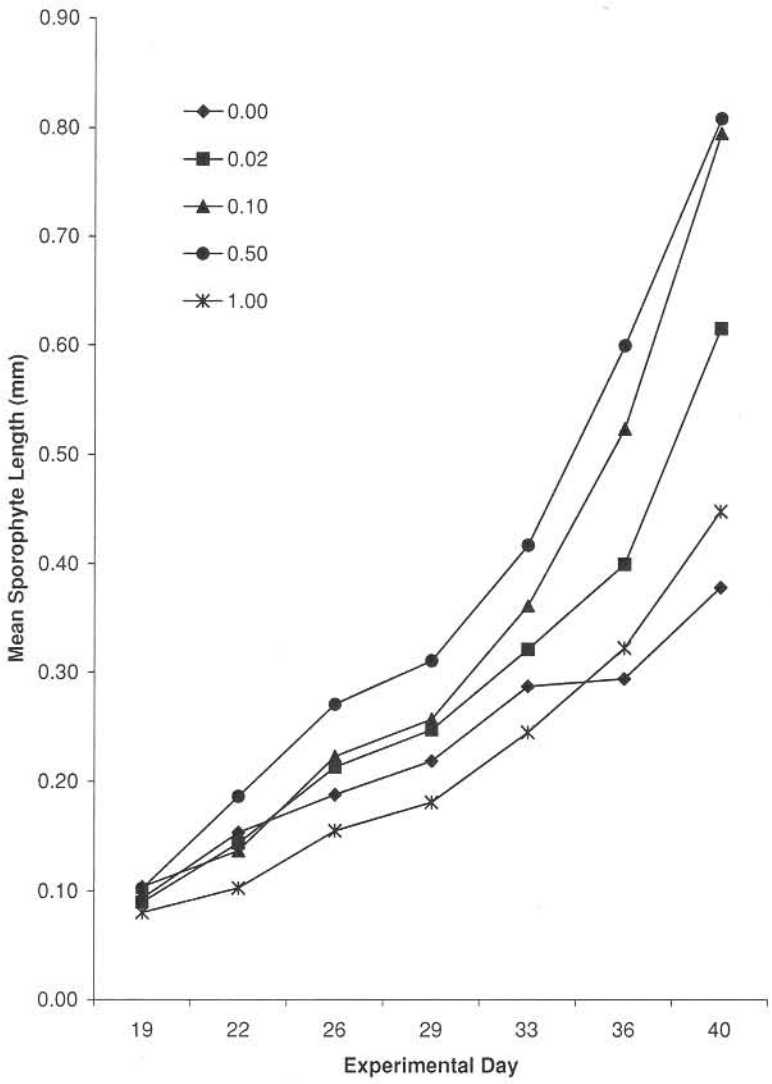

Fig. 1 Mean lengths of *Laminaria saccharina* sporophytes treated with different concentrations of germanium dioxide (millilitre of GeO_2 per litre seawater)

effect on the sporophyte growth. Concluding that the higher concentrations of the treatment removed more diatoms, and therefore caused an increase in growth, should be rejected since diatoms were eliminated in all the tanks with treatment. The biological reasoning of how and why GeO_2 enhances the sporophyte growth up to a critical point is unknown. There is no known reported use of germanium in any chemical or biological processes in *L. saccharina*. Goldschmidt (1937) postulated that the mechanism behind the inhibitory effects on diatoms is due to the replacement of silica by germanium in the final mineralization of the silica frustule. Such process is, however, not happening in kelp gametophytes. Graham and Wilcox (2000) stated that silica is not an essential element in kelps and is only required in the development of diatom frustules, silicoflagellate skeletons, synurophyte scales, stomatocyst walls, and the walls of the ulvophyte *Cladophora*. To understand the mechanisms of how the kelp growth is affected by GeO_2, further biological and molecular studies will have to be conducted.

For an effective treatment of diatom contamination, it was found that even the smallest concentration of GeO_2 ($0.02\,mL\,L^{-1}$), was able to block the growth of diatoms in the culture tanks. Diatoms were, in fact, observed again in small numbers in all the tanks that received a GeO_2 treatment on day 33; however, at this

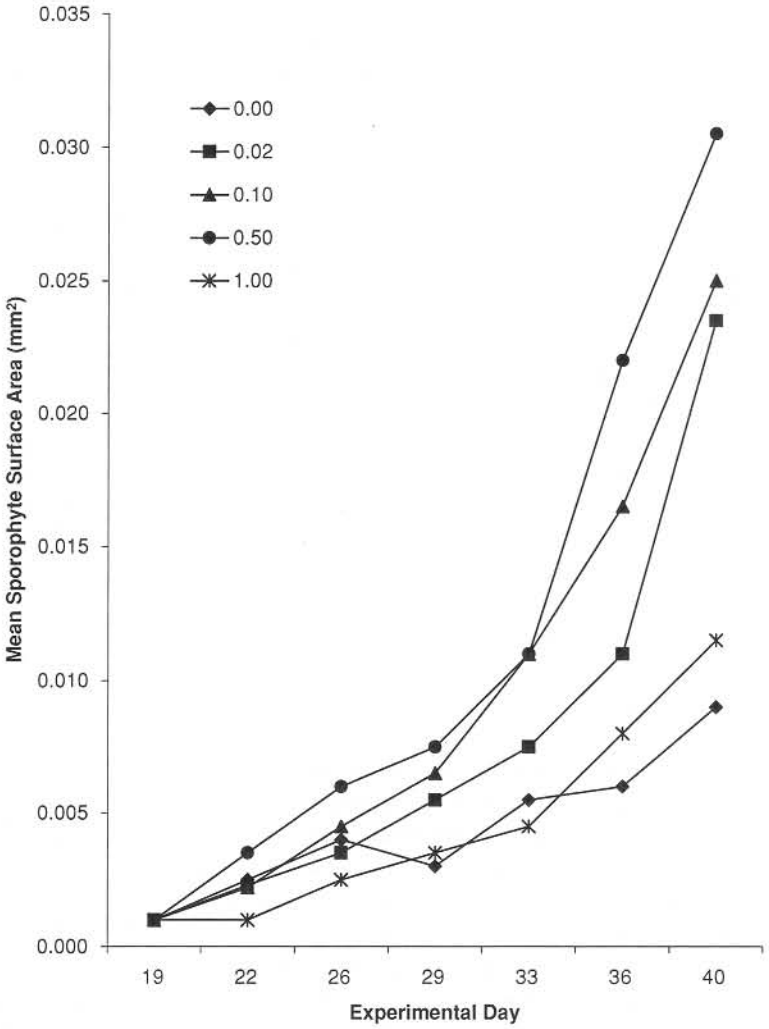

Fig. 2 Mean surface area of *Laminaria saccharina* sporophytes treated with different concentrations of germanium dioxide (millilitre of GeO_2 per litre seawater)

point, the sporophytes already had a secure hold on the inoculation substrate. Therefore, there was no competition for space. It is during the kelp gametophytic stage of growth that diatoms are a serious threat to the culture (Merrill & Gillingham, 1991).

The sporophyte lengths and surface areas obtained at 0.00 and 1.00 mL GeO_2 per litre treatments were not significantly different. This information could be misleading when comparing whether it is better to apply no treatment or a full treatment of GeO_2. The sampling methods used did not consider the difference in the sporophyte coverage of the spools. However, it was observed that there was very patchy and low coverage on the spools in the tanks that received 0.00 mL GeO_2 per litre, while all the other tanks had much more sporophyte coverage on the substrate. Substrate coverage could be taken into consideration by using destructive sampling techniques to calculate the percent coverage; however, this would disrupt the integrity of the spools and preclude repeating sampling over the 40 days of cultivation.

The study was not extended beyond 40 days, as the sporophytes treated with 0.10 and 0.50 mL GeO_2 per litre reached a length of approximately 0.8 mm, and previous culture experiments indicated that when *L. saccharina* sporophytes reach a size between 0.5 and 1 mm it is time to transfer them to the sea for the grow-out phase in proximity to salmon cages (Chopin et al., 2004). The cultivation strategy of the early stages of *L. saccharina* is to reduce the laboratory phase to a minimum and transfer the spools to the aquaculture sites as soon as possible. Once the sporophytes are transferred, they develop into large mature sporophytes within 6 months.

Merrill and Gillingham (1991) reported that obtaining 0.5 mm long sporophytes may take up to 60 days in *Nereocystis luetkeana*. Results from this study show that this length was reached by sporophytes of *L. saccharina* treated with 0.50 and 0.10 mL GeO_2 per litre in approximately 34 and 35 days. The sporophytes from 0.02 mL GeO_2 per litre treatment reached the deployment size at about day 38, while the sporophytes exposed to 0.00 and 1.00 mL GeO_2 per litre treatments did not reach the length for deployment during the course of this study.

In conclusion, GeO_2 not only inhibits the diatom contamination in tanks at a critical time of gametophytic development in *L. saccharina* but also has a significant effect on the growth of the early stages of development of the non-targeted species, *L. saccharina*. This effect is positive until a critical point is reached, between 0.50 and 1.00 mL of GeO_2 per litre. At this point, GeO_2 has a negative inhibitory effect on the laboratory growth of *L. saccharina*. The results of this study suggest that a treatment of GeO_2 between 0.10 and 0.50 mL GeO_2 per litre should be added to the industrial cultivations of *L. saccharina* to both inhibit the contamination of diatoms and reduce the laboratory cultivation time by producing sporophytes of appropriate size for deployment in the shortest time period. Moreover, it is worth noting that our recommended treatment concentrations are much lower than any previously indicated: between 0.10 and 0.50 mL of a saturated solution of GeO_2 per litre of seawater (or between 0.0004 and 0.0022 mg GeO_2 per litre of seawater). This treatment is also economically very reasonable. In the current configuration of our production system, we need 1.07 g of GeO_2 to treat the spools, which will produce 60 t of kelps. The present price of a 10 g bottle of powdered, reagent grade GeO_2 is US$93.40. Consequently, it costs only US$9.99 for the production of 60 t of kelp, which makes this minimal, and biologically- and cost-effective use of GeO_2 a very affordable treatment in routine commercial scale aquaculture operations.

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