Effect of Photon Flux Density and Temperature on the Production of Halogenated Monoterpenes by Plocamium cartilagineum (Plocamiaceae, Rhodophyta)

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The effect of different photon flux densities (PFD) and temperatures on the relative growth rate (RGR) and the concentration of three halogenated monoterpenes in samples of Plocamium cartilagineum L. (Dixon), a marine alga (Rhodophyceae), were studied. The highest RGR (22.8 ± 0.04 d⁻¹) was obtained at 15 °C and 41 µmol m⁻² s⁻¹ of PFD and the lowest (18.0 ± 0.12 d⁻¹) was obtained at 18 °C and 120 µmol m⁻² s⁻¹. The different temperatures and light used in assays did not affect significantly the production of organic compounds. The production of mertensene and violacene was not affected significantly. However, compound 1 reached the highest concentration at 15 °C and 65 µmol m⁻² s⁻¹. The relationship between growth and production of monoterpenes of P. cartilagineum and the effect of temperature and the PFD were analyzed.

Key words: Plocamium cartilagineum, Halogenated Monoterpenes

Introduction

It has been proposed that secondary metabolites present in algae have biological properties, such as antimicrobial, anti-fungal, antifouling, and antifeedant (Rovirosa et al., 1990; San-Martín et al., 1991). Moreover, it has been informed that some metabolites could play a therapeutic role (Noda et al., 1990; Depix et al., 1998).

Plocamium cartilagineum L. (Dixon) is a marine alga (Rhodophyceae) with a wide distribution, including the Antarctic. Eight monoterpenes have been isolated in samples collected at the coast of Chile (San-Martín and Rovirosa, 1985; Rovirosa et al., 1990). Two of them, mertensene and violacene, cause antifeedant effects against the aphids Rhopalosiphum padi, Myzus persicae and Schizaphis graminum, which are cereal pests (Argandoña et al., 2002). They also have deleterious effects on the moth of tomato, Tuta absoluta and Leptinotarsa decemlineata, and the potato scarab (Myndersen and Faulkner, 1974; Argandoña et al., 2000, 2002). It has been postulated that these metabolites should have a regulating competition role among organisms, including allelopathic effects (De Nys et al., 1991; Leone et al., 1995). Some compounds isolated from P. cartilagineum exhibited selective cytotoxicity against colon and cervical adenocarcinoma cells (De Inés et al., 2004).

It has been informed that the composition of monoterpenes changes as a function of the geographic localization and the season in which the samples were collected (San-Martín and Rovirosa, 1986; Capon et al., 1984; Blunt et al., 1985; König et al., 1990). The cause of this variation is unknown (Abreu and Galindo, 1998; Jongaramruong and Blackman, 2000; Rezanka and Dembitsky, 2001). Environmental conditions have an important role in the relationships between organisms because they may affect the concentration of secondary metabolites (Disch et al., 1998; Eisenreich et al., 2001). Light and temperature are important environmental factors in the survival, distribution and reproduction of algae (Yokoya and Oliveira, 1992; Lüning, 1994; Jiménez et al., 1998; Garza-Sánchez et al., 2000), also, in determining photosynthetic reaction rates. Other factors that influence the algal physiology are salinity and nutrients (Lobban and Harrison, 1997).

The purpose of this work was to evaluate the effect of temperature and photon flux density on the growth of P. cartilagineum and on its production of halogenated monoterpenes (mainly violacene, mertensene and compound 1) that have interesting bioactivities. The biomass of this alga is rather low, so this investigation represents the first effort to culture it artificially.
Experimental

Materials

Plocamium cartilagineum was collected in San Lorenzo cove, Chile (30° 20'10, 9°S). The samples were wrapped with moist paper and transported to the laboratory in thermo boxes at low temperatures. The material was cleaned with seawater and then cultivated in 181 aquariums with sterile micro-filtered seawater enriched with von Stosch media (Edward, 1970). Also 0.5 ppm of GeO₂ was added to prevent diatoms proliferation. The cultures were acclimated over 20 d inside chambers at 15 °C with a 12 h light:12 h dark photoperiod with PFD of 60 µmol m⁻² s⁻¹ and with constant ventilation. The water was changed every 2 d.

To identify the algae, keys described by South and Adams (1979), Womersley (1994), and Hoffman and Santelices (1997) were used. Samples were deposited at Museo Nacional de Historia Natural de Chile Santiago, Chile. Cultures of Plocamium cartilagineum were subjected to three PFD: 41 ± 4; 65 ± 2; 120 ± 2 µmol m⁻² s⁻¹, combined with three temperatures: 11 ± 1; 15 ± 1; 18 ± 1 °C. For each combination of PFD and temperature, 3 flasks of 300 ml were used containing 5 g of algae (fresh weight) and 250 ml sterilized micro-filtered seawater, enriched with von Stosch media. GeO₂ was also added. The flasks were maintained under constant ventilation. The photoperiod used for all assays was 12 h:12 h (L:D). The PFD was obtained from 8 tubes of cold fluorescent light of 40 W each. The PFD gradient was obtained using a white diamond paper as a filter over the samples (Acuña, 2000). The cultures were inside of an incubator on a table with aluminum surface on which a thermal gradient was constructed (Edding et al., 1987; Acuña, 2000). The PFDs were measured with a Licor model Li-250 and the temperature with a digital thermometer (Miniterm HI-8753 HANNA). Instruments were located inside the incubator. The experiment lasted 16 d. The water was changed every 2 d. The RGR of algae was quantified using the equation proposed by Bischoff-Bäsmann and Wiencke (1996): RGR = ln (Xf/Xi) × 100/Δt, where Xi and Xf are the fresh weight at the beginning and end of the assay, respectively; Δt is the time period (see Table I).

Chemical analysis

Samples of each treatment were dried on absorbent paper in plastic trays inside the laboratory at room temperature, preventing the direct exposition to solar light and high temperature. The dried samples were weighed and kept in plastic boxes. Then, they were extracted exhaustively with a dichloromethane/methanol (9:1 v/v) solution during 24 h. Each solution was concentrated and the extract obtained was purified by flash chromatography on silica gel using 5% dichloromethane in hexane as eluent. This first purification gave the total monoterpenes extracts. The TLC plates were developed with mixtures of petroleum ether and ethyl acetate. The spray reagent for TLC was H₂SO₄/MeOH (1:9 v/v). Violacene, mertensene and compound 1 (Fig. 1) were isolated, purified by further medium pressure chromatography and identified by comparison with authentic samples and by spectroscopic methods (San-Martín et al., 1991). 2.5 µl of each extract was injected into a gas chromatograph (Perkin-Elmer Sigma 3 B) with an analytical capillary column (25 m long, 0.25 µm in diameter) of methyl silicone. Pure compounds obtained previously were injected for comparison. The compounds were identified by their retention times. The content of organic compounds was calculated using the relation: (extract weight obtained by the procedure described previously/dry weight of algae sample) × 100. All values in the Tables represent the mean ± S. E. of 5 samples.

Statistical analysis

This assay corresponds to a balanced orthogonal pattern of two factors and 3 treatments by factor with 3 replicas per treatment. For statistical analysis a two way analysis variance was used. To see the significant differences between replicas and between treatments of PFD and temperature and combination of both a Tukey test was used. The analysis shows that variances were homocedastics (P = 0.134), and belong to a population with normal distribution (P = 0.435).

Results and Discussion

Table I shows the effect of temperature and PFD on the P. cartilagineum growth. The relative growth rate of the algae decreased in all assays reaching a lowest value in the treatment at 18 °C and 119.6 µmol m⁻² s⁻¹ (RGR = 18.0 ± 0.12 d⁻¹). The algae growth at 11 °C and different irradiances was not significantly different. The highest growth of algae was at 15 °C and 41 µmol m⁻² s⁻¹,
Table I. Relative growth rate (RGR) of P. cartilagineum under different PFD and temperature conditions.

<table>
<thead>
<tr>
<th>PFD [µmol m⁻² s⁻¹]</th>
<th>Temperature [°C]</th>
<th>RGR [d⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>11</td>
<td>21.0 ± 0.08</td>
</tr>
<tr>
<td>41</td>
<td>15</td>
<td>22.8 ± 0.04</td>
</tr>
<tr>
<td>41</td>
<td>18</td>
<td>20.2 ± 0.05</td>
</tr>
<tr>
<td>65</td>
<td>11</td>
<td>20.8 ± 0.06</td>
</tr>
<tr>
<td>65</td>
<td>15</td>
<td>21.5 ± 0.04</td>
</tr>
<tr>
<td>65</td>
<td>18</td>
<td>19.9 ± 0.06</td>
</tr>
<tr>
<td>120</td>
<td>11</td>
<td>21.6 ± 0.06</td>
</tr>
<tr>
<td>120</td>
<td>15</td>
<td>20.5 ± 0.06</td>
</tr>
<tr>
<td>120</td>
<td>18</td>
<td>18.0 ± 0.12</td>
</tr>
</tbody>
</table>

RGR = ln (X_f/X_i) x 100/∆t, were X_f and X_i are the final and initial fresh weight of algae, respectively; ∆t = 16 d. Each value of RGR represents the mean ± S. E. of 9 samples.

reaching a RGR of 22.8 ± 0.04 d⁻¹. These results suggest that temperature was the factor that affected more significantly the RGR of P. cartilagineum (P < 0.001). Light intensity and temperature affected significantly the growth of P. cartilagineum. The lowest RGR was obtained under 18 °C. Under 11 °C and 15 °C, no significant differences were detected. These temperatures were similar to those in the collection sites during winter and beginning of spring when the algae population increased. This suggests that growth could be conditioned by a seasonal pattern. Kain (1989) had found a similar behavior, and reported that the algae died at temperatures higher than 20 °C. Lüning (1994) suggested that P. cartilagineum migrated to the American continent from the Antarctic, expanding to the austral zone and to the south of South America. This is in agreement with the results obtained in relation with the decrease in growth at 18 °C.

Table II shows significant differences in the production of compound 1 (Fig. 1) showing that temperature is the factor with more influence (P > 0.05). The production of each compound in treatments at 15 °C was significantly higher than at 11 °C and 18 °C. The production of compound 1 in alga population cultivated under 41 µmol m⁻² s⁻¹ did not vary significantly at the different temperatures. The highest production (1.76 ± 0.2 x 10⁻⁷ g) was obtained in samples of treatment at 15 °C and 65 µmol m⁻² s⁻¹. The differences in production between the treatments at 11 °C and 15 °C were significant (P > 0.05). The production of mertensene was one order of magnitude lower than the production of compound 1 (Table II). The highest value was obtained with the combinations 65 µmol m⁻² s⁻¹ and 15 °C and 41 µmol m⁻² s⁻¹ and 18 °C. Only a tendency to an increase in the production of mertensene could be seen in the

Table II. Production of three monoterpenes [Compound 1 (C. 1), mertensene and violacene] in P. cartilagineum under different conditions of PFD and temperature.

<table>
<thead>
<tr>
<th>PFD [µmol m⁻² s⁻¹]</th>
<th>11 °C</th>
<th>15 °C</th>
<th>18 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. 1</td>
<td>Mertensene</td>
<td>Violacene</td>
</tr>
<tr>
<td>41</td>
<td>1.03 ± 0.15</td>
<td>0.41 ± 0.10</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>65</td>
<td>1.26 ± 0.12</td>
<td>0.73 ± 0.18</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>120</td>
<td>1.14 ± 0.16</td>
<td>0.54 ± 0.08</td>
<td>0.01 ± 0.06</td>
</tr>
</tbody>
</table>

The monoterpenic concentration is about 10⁻⁷ g per 100 g dry weight. Each value represents the mean ± S. E. of 5 samples.
treatments at 41 µmol m⁻² s⁻¹ as a function of temperature. In treatments at 18 °C, there was a tendency to decreased mertensene production as a function of PFD increase. However, the statistical test did not show significant differences between treatments (P > 0.05). The production of violacene was two orders of magnitude lower than the production of mertensene and three orders of magnitude lower than that of compound 1. The highest production was detected in the treatment maintained at 41 µmol m⁻² s⁻¹ and 18 °C and 120 µmol m⁻² s⁻¹ and 15 °C reaching values of 0.100 ± 0.03 × 10⁻⁷ g and 0.107 ± 0.04 × 10⁻⁷ g. At a PDF of 41 µmol m⁻² s⁻¹, the violacene production showed a tendency to increase as a function of the temperature.

The results obtained from the chemical analysis showed that the concentration of compound 1 was affected significantly by temperature. However, the production of organic compounds was not affected by PDF and temperature used in this experiment. Compound 1 showed as significant variation in its production when P. cartilagineum was subjected to different PFD and temperature conditions. However, the temperature was the factor with more effect. With respect to mertensene and violacene production, the results showed only general tendencies that were statistically not significant. The production of violacene was one order of magnitude lower than of mertensene. It is important to emphasized that violacene is biologically more active than mertensene and compound 1 (Argandoña et al., 2002, 2000). San-Martín and Rovira (1986) had detected mertensene and violacene as major compounds in some samples of P. cartilagineum. However in other cases, other monoterpenes were the most abundant. Our results showed that at least PFD and temperature affected significantly the production of compound 1 and showed only a tendency in mertensene and violacene production. It is possible that other factors such as the reproductive stage of the algae (Capon et al., 1984; Blunt et al., 1985), interaction with other organisms (Cronin and Hay, 1996) and the presence of different ecotypes (Acuña, 2000) are involved.


