

Enhancement of Seaweed Rhizoid and Blade Formations by the Chlorophyte *Codium fragile* Extract

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Living organisms can maintain or extend their territories by producing allelochemicals that influence the growth, survival, and reproduction of other organisms. To identify natural biostimulants of positive allelochemicals, we screened 18 common seaweed extracts for enhancement of rhizoid and blade production in a convenient *Porphyra suborbiculata* monospore assay. By addition of methanolic extract from the most potent green seaweed, *Codium fragile*, 100% and 50% enhancement doses reflecting the amount of *C. fragile* extract required to enhance rhizoid formation (in terms of number of spores with rhizoids per total spores tested) were approximately 100 and 50 µg/ml, respectively, in the *P. suborbiculata* monospore culture. The *C. fragile* extract quickly enhanced rhizoid formation, rhizoid numbers per rhizoid-holding spore, rhizoid length, blade formation (in number of spores with blade per total spores tested), and blade length from most monospores at early culture days. The extract enhanced rhizoid formation after 2 days of culture significantly, rhizoid numbers per rhizoid-holding spore after 3 days, rhizoid length after 3 days, blade formation after 2 days, and blade length after 1 day, respectively, from most monospores. The allelochemicals that enhanced favorite seaweed species may be efficacious for new seaweed management technologies, including the development of biostimulant agents based on natural products.

Key words : Allelochemicals, *Codium fragile*, enhancement, monospore, rhizoid

Introduction

The marine ecosystem is a community of living organisms with severe competition, cooperation, and regulation occurring between them. For competition or cooperation, some plants produce allelopathic substances—biochemicals that influence the growth, survival, and reproduction of other organisms—that facilitate growth of the producing or receiving organism [21]. Allelochemicals are either beneficial (positive allelopathy) or detrimental (negative allelopathy) to the target organisms and play an important role in plant induction or defense against herbivory [18]. Allelochemicals that suppress or eliminate competing plant species have received special attention due to their potential as natural her-

bicides in agriculture [9, 20]. This focus has shifted attention to alternative seaweed control technologies, such as algicidal or growth-stimulating agent development based on selective natural products. For example, the red seaweed *Ceramium rubrum* has anti-germination activity in *Sargassum muticum*, *Enteromorpha intestinalis*, and *Ulva lactuca* [8]. The green seaweed *Monostroma nitidum* produces a microalgal growth-enhancer levoglucosan [13]. The green seaweed *Monostroma arctium* has a positive effect on the growth and photosynthetic activity of *Porphyra yezoensis* in co-cultures, cultured medium filtrate, and dry powder assays [22]. Foliar application of eckol, from the brown seaweed *Ecklonia maxima*, enhances shoot and root length, leaf area and number, and aphid resistance capacity in cabbage plant [16]. For the development of environmentally friendly biostimulant products for seaweed growth, natural compounds from marine plant and animal sources are the best candidates.

The red seaweed *Porphyra suborbiculata* is a common wild seaweed that uses a discoidal holdfast to grow on rocks in the higher intertidal zone [1]. Monospores (blade archeospores) from juvenile blades can be produced year-round

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by adjusting culture conditions in the laboratory. Most monospores germinate to produce new juvenile blades, which themselves produce monospores under axenic culture conditions [2]. Thus, monospores of *P. suborbiculata* are used as a bioassay for rhizoid and blade formation. To search for natural biostimulant or seaweed growth-enhancing products, we prepared 18 common seaweed extracts and screened for enhancement of rhizoid and blade formation at the early stage using the monospore assay. Lead extracts were further optimized for treatment concentrations, and the effects during culturing with the most potent enhancer, *Codium fragile*, were also measured.

Materials and Methods

Seaweed, extraction, and reagents

Seaweed thalli collected from 18 different species on the coast of Korea were dried for 3-7 days at room temperature. Thalli were then ground to a powder for 5 min using a coffee grinder (HMF-340; Hanil Co., Seoul, Korea). For each 20 g sample, one liter methanol was used for extraction at room temperature for 24 hr. For a stock solution of each methanol-soluble fraction, 1 ml dimethyl sulfoxide (DMSO) was added to every 40 mg dried extract. Most of reagents used in this study were of analytical grade from Sigma- Aldrich Co., St. Louis, MO, USA.

Spore collection

To obtain monospores, juvenile blades of *P. suborbiculata*, collected from Cheongsapo (35°9'46.47" N, 129°11'43.76" E), Busan, Korea, were sonicated (28 kHz) twice for 1 min in autoclaved seawater, and immersed in 1% betadine for 2 min to eliminate epiphytes. For each 24-well plate, five excised

tissue pieces (each 5×5 mm²) were cultured in 1 ml Provasoli's enriched seawater (PES) [15]. The blades were incubated at 18°C with 40 µmol/m²/s light intensity on a 12 hr light:12hr dark cycle to obtain monospores.

Monospore culture and bioassay

Spore germination assays (for rhizoid and blade formation) were performed by adding approximately 100 - 200 monospores to a 200-µl aliquot of PES in a 96-well plate, which was placed in the dark at 18°C for 1 day. After non-settled spores were removed by centrifugation (1,500 rpm, 15 min) in an inverted position, 200 µl fresh PES was added to each well with 1 µl extract (200 µg/ml final concentration). DMSO inhibited spore germination by a minimum at 0.5% (data not shown). Spore cultures were placed at 18°C and 80 µmol/m²/s light intensity on a 12L:12D cycle for 1 week to facilitate spore development [3]. After 1 week, rhizoid formation (number of spores that produced rhizoids per total spores tested), number of rhizoids per rhizoid-holding spore, rhizoid length, blade formation (number of spores that produced blades per total spores tested), and blade length were measured using a microscope (200×). Lengths of rhizoid and blade were measured by a haemocytometer. The relative rate (%) of rhizoid or blade formation was determined by the following formula: (S/T) ×100, where S = number of spores that produced rhizoids or blades, and T = total spores tested.

Statistical analysis

The experiments were repeated at least three times. Mean differences between extract and control assays were compared using Student's *t*-test.

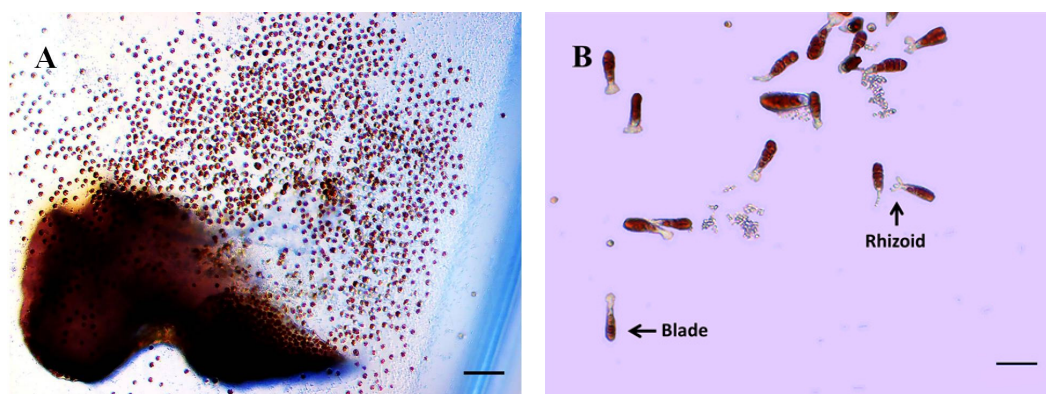


Fig. 1. Micrographs of monospores released from the juvenile blade of *Porphyra suborbiculata* (A) and their rhizoids and blades formed from monospores (B). The bars in A and B indicate 100 µm, respectively.

Results

To search for positive allelopathic or growth-enhancing agents in seaweeds, common seaweed extracts were tested for their ability to enhance rhizoid and blade production in a *P. suborbiculata* monospore assay. The 18 seaweed species tested included green seaweed (*Codium fragile*, *Monostroma nitidum*, *Ulva linza*, *Ulva pertusa*), brown seaweed (*Ecklonia cava*, *Eisenia bicyclis*, *Hizikia fusiformis*, *Ishige sinicola*, *Saccharina japonica*, *Sargassum fulvellum*, *Sargassum hemiphyllum*, *Sargassum hornei*, *Sargassum thunbergii*, *Scytosiphon lomentaria*, *Undaria pinnatifida*), and red seaweed (*Chondrus ocellatus*, *Corallina pilulifera*, *Pachymeniopsis elliptica*). Most seaweed extracts at 200 µg/ml suppressed the rhizoid and blade production (Table 1). Among the seaweed extracts tested, *C. fragile* exhibited significant enhancement, with 63% of monospores producing rhizoids compared with 41% of the control PES ($p<0.01$). Rhizoid numbers per rhizoid-holding spore also increased. In *C. ocellatus* and *C. fragile*, rhizoid length was enhanced significantly, with average rhizoid lengths of 40.2 and 34.6 µm, respectively, compared with 19.5

µm for the control ($p<0.01$). Regarding germinated spores, *E. bicyclis* extract enhanced blade formation significantly, with 27% of monospores germinating to juvenile blades in 1 week compared with 14% for the control ($p<0.05$). *C. fragile* showed blade formation similarly to the control. *E. cava*, *U. linza*, and *U. pertusa* extracts enhanced blade growth significantly, to an average of 14.3, 10.9, 11.9 µm, respectively, compared with 7.1 µm for the control ($p<0.01$). *C. fragile* increased blade length a little. Thus, the *C. fragile* extract was selected for further evaluation, based on overall enhancing activities of rhizoid and blade formation.

Various concentrations of the *C. fragile* extract were added to the monospore culture to determine enhancement activity. The enhancement dose 100 (ED₁₀₀) is expressed as the concentration of *C. fragile* extract required to produce rhizoids maximally from monospores after 7 days of culture. The enhancement dose 50 (ED₅₀) is the extract concentration required to increase rhizoid production in 50% of monospores after 7 days. The ED₁₀₀ and ED₅₀ values reflecting the amount of *C. fragile* extract required to enhance rhizoid formation (in terms of number of spores with rhizoids per total spores

Table 1. Comparison of various seaweed extracts for enhancing activities based on rhizoid and blade productions of *Porphyra suborbiculata* monospores

Code of species	Rhizoid formation (%)	No. of rhizoids / rhizoid-holding spore	Rhizoid length (µm)	Blade formation (%)	Blade length (µm)
1	11±9	1.0±0.1	40.2±3.2**	7±7	0.0±0.0
2	63±8**	1.2±0.0*	34.6±2.3**	11±3	9.5±2.5
3	6±4	1.0±0.0	2.7±0.4	15±2	6.9±0.3
4	0±0	0.0±0.0	0.0±0.0	2±0	14.3±0.2**
5	10±4	1.0±0.0	4.5±0.3	27±5*	7.9±2.2
6	0±0	0.0±0.0	0.0±0.0	0±0	0.0±0.0
7	13±6	1.1±0.1	8.5±1.1	5±1	8.8±2.7
8	20±2	1.2±0.1	14.5±0.3	12±3	11.7±3.0
9	14±3	1.0±0.1	7.4±3.8	14±5	3.7±4.3
10	14±4	1.0±0.0	8.1±0.7	8±2	7.3±0.4
11	1±0	1.0±0.3	2.1±1.1	15±5	4.5±0.5
12	11±4	1.0±0.0	12.5±1.0	10±6	0.0±0.0
13	0±0	0.0±0.0	0.0±0.0	0±0	0.0±0.0
14	3±1	1.0±0.3	4.5±2.2	11±3	0.0±0.0
15	10±2	1.0±0.2	7.9±1.3	17±7	3.3±4.3
16	0±0	0.0±0.0	0.0±0.0	18±3	10.9±0.5**
17	0±0	0.0±0.0	0.0±0.0	10±2	11.9±1.1**
18	0±0	0.0±0.0	0.0±0.0	0±0	0.0±0.0
PES	41±5	1.1±0.0	19.5±2.1	14±4	7.1±0.4

Monospores were cultured in each extract (200 µg/ml) for a week. Seaweed number 1, *Chondrus ocellatus*; 2, *Codium fragile*; 3, *Corallina pilulifera*; 4, *Ecklonia cava*; 5, *Eisenia bicyclis*; 6, *Hizikia fusiformis*; 7, *Ishige sinicola*; 8, *Monostroma nitidum*; 9, *Pachymeniopsis elliptica*; 10, *Saccharina japonica*; 11, *Sargassum fulvellum*; 12, *Sargassum hemiphyllum*; 13, *Sargassum hornei*; 14, *Sargassum thunbergii*; 15, *Scytosiphon lomentaria*; 16, *Ulva linza*; 17, *Ulva pertusa*; 18, *Undaria pinnatifida*; PES, Provasoli's enriched seawater. Values are expressed as means ± SE (n>3). * $p<0.05$ and ** $p<0.01$ as compared with PES control by student *t*-test.

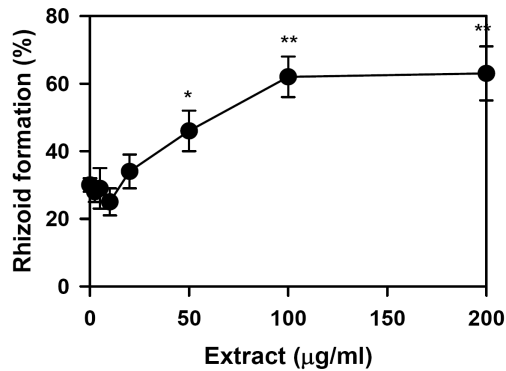


Fig. 2. Effects of different concentrations of *Codium fragile* extract on the rhizoid formation from *Porphyra suborbiculata* monospores. Enhancing activities of rhizoid formation were expressed as % of spores with rhizoid per total monospores tested. No addition of *C. fragile* extract is the value for PES control. * $p<0.05$ and ** $p<0.01$ as compared with PES control by student *t*-test.

tested) were approximately 100 and 50 µg/ml, respectively, in the monospore culture (Fig. 2). Next, growth of *P. suborbiculata* monospores was observed upon treatment with 100 µg/ml (approximate ED₁₀₀ for rhizoid formation) *C. fragile* extract for 10 days. The *C. fragile* extract quickly enhanced rhizoid formation (Fig. 3A), rhizoid numbers per rhizoid-holding spore (Fig. 3B), rhizoid length (Fig. 3C), blade formation (Fig. 3D), and blade length (Fig. 3E) from most spores at early culture days in the monospore assay. The extract enhanced rhizoid formation after 2 days culture significantly, rhizoid numbers per rhizoid-holding spore after 3 days, rhizoid length after 3 days, blade formation after 2 days, and blade length after 1 day, respectively, from most *P. suborbiculata* monospores. However, after 10 days of culture, monospores began to reach almost maximum in rhizoid and blade formation.

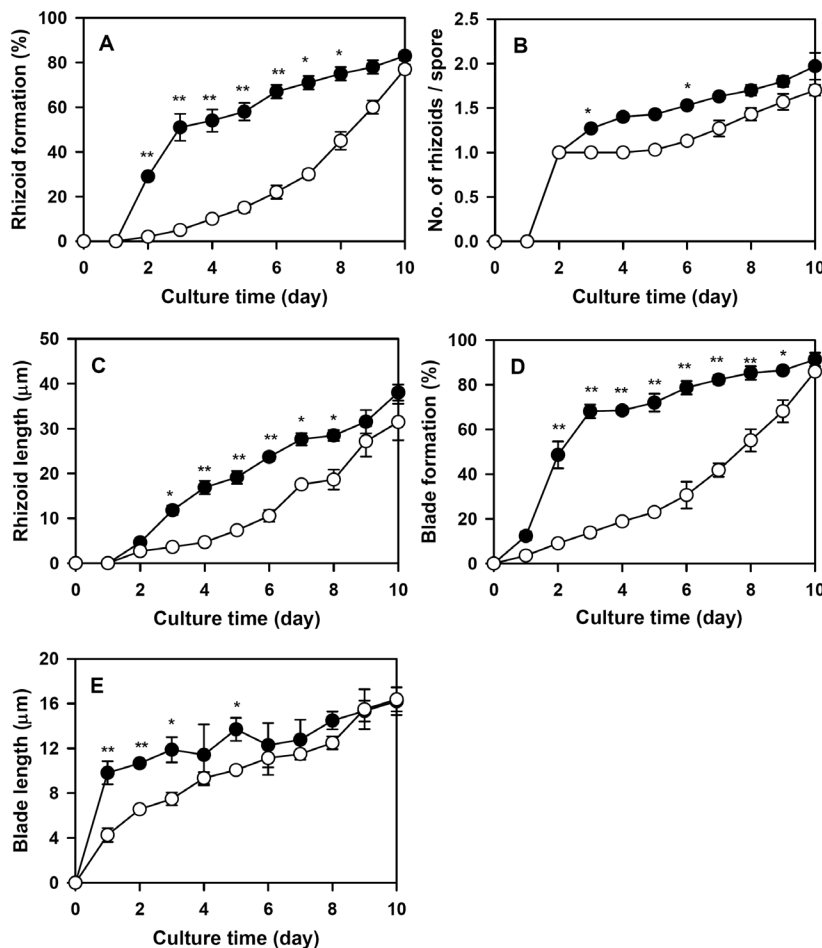


Fig. 3. Effects of *Codium fragile* extract on the productions of rhizoids and blade from *Porphyra suborbiculata* monospores during 10 days of culture. Enhancing activities were measured using rhizoid formation (% of spores with rhizoids / total spores tested; A), number of rhizoids / rhizoid-holding spore (B), rhizoid length (C), blade formation (% of spores with blade / total spores tested; D), and blade length (E). ●; *C. fragile* extract (100 µg/ml). ○; PES control. * $p<0.05$ and ** $p<0.01$ as compared with control by student *t*-test.

Discussion

Plant interactions in ecosystems are known to be mediated by plant active compounds, referred to as allelochemicals, which are receiving increasing attention in the context of sustainable plant management [4]. It should be paid to these chemical interactions for the development of new biostimulants. To search for such positive allelopathic agents in seaweed, common seaweed extracts were tested for their ability to enhance rhizoid and blade production in a monospore assay. Among the seaweed species tested, *C. fragile* showed the strongest enhancement activities. *C. fragile* is an edible and abundant aquaculturable green seaweed. The amount of *C. fragile* produced by farming in 2013 amounted to 2,045 t (wet weight), with an additional 132 t (wet weight) collected from natural populations in Korea [11]. Currently, it occurs in temperate regions worldwide as an invasive species [6]. In our previous study [10], *C. fragile* extracts showed *in vivo* anti-inflammatory, antipyretic, and analgesic activities. Siphonaxanthin having anti-angiogenic effect [7] and a sulfated galactan having immunostimulating effect [12] were also isolated from *C. fragile*.

The *C. fragile* extract quickly enhanced rhizoid formation, rhizoid numbers, rhizoid length, blade formation, and blade length at the early stages of the *P. suborbiculata* monospore germination. Rhodophyte *Gracilaria gracilis* tips and chlorophyta *U. lactuca* growth were increased by addition of a commercial kelp *E. maxima* extract [17]. The *E. maxima* triggered rooting in cucumber [14] and tomato plants [5]. Enhancing shoot length, root length, leaf area, and leaf number in cabbage plant was caused by the phlorotannin eckol compound from the *E. maxima* [16]. Some seaweeds contain plant growth hormones (such as auxin and cytokinin), and the additions to media increased growth of *Gracilaria vermiculophylla* [19, 23]. Even though active compounds from the *C. fragile* is not proved yet, the *C. fragile* extract demonstrated a positive effect on the rhizoid and blade formation of *P. suborbiculata* monospores. Thus, there is potential for using the *C. fragile* or its extract in the seedling of commercial *Porphyra* mariculture. Such positive allelochemicals that enhanced favorite seaweed species may be efficacious for new seaweed management technologies, including the development of biostimulant agents based on marine natural products.

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초록 : 녹조류 청각 추출물에 의한 해조류 가근 및 유엽형성 촉진

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많은 생물체들은 다른 생물체들의 성장, 생존, 재생에 영향을 미치는 allelochemical 물질들을 생성함으로써 자신들의 영역을 유지 확장할 수 있다. 천연 생물촉진활성을 가진 allelochemicals을 찾기 위하여 18종의 혼한 해조류를 대상으로 실험실에서 편리하게 배양가능한 등근돌김의 중성포자들로부터 가근 및 유엽의 형성 촉진효과를 탐색하였다. 그중 가장 활성이 높은 녹조류 청각의 추출물은 약 100 및 50 µg/ml 농도에서 최대 및 반 정도의 중성포자로부터 가근 형성 촉진효과를 나타내었다. 또한 청각 추출물은 중성포자 배양 초기에 가근형성, 가근갯수, 가근길이, 유엽형성, 유엽길이 등의 성장을 촉진시켰다. 이 같은 다른 해조류의 성장을 촉진시키는 allelochemicals 들은 천연 해조류 촉진제의 개발 및 유용 해조류 종들의 유지관리 등에도 활용될 가능성을 지닌다.