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RESEARCH

Ecological analysis and culture methods for freshwater green algae *Prasiola japonica*

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Background: *Prasiola japonica* is a freshwater green algae species that can only be seen in Korea and Japan. The various conditions necessary for its growth and reproduction have not been fully elucidated. Therefore, in this study, we aimed to investigate conditions related to the growth and reproduction of *P. japonica* for the purpose of conserving and producing this species. We first examined differences in growth according to various conditions in different habitats to understand the growth environment of *P. japonica*.
Results: The experimental results revealed that the optimal temperature for growth and reproduction of *P. japonica* was between 10°C–15°C, and the optimal light intensity was 1,000–1,500 lux. Furthermore, when Provasoli enriched seawater with iodine (PESI) agar was used, the growth of *P. japonica* was found to be at least 1.5–8 times greater than that of the general Sohan Valley water sample, and it also showed 2–4.5 faster growth rate to reach 30 μm. These results emphasize the importance of PESI agar in the culture of *P. japonica*, and are expected to be helpful in suggesting ways to utilize and conserve *P. japonica* resources.
Conclusions: Through these research findings, we suggest new methods for conserving and producing *P. japonica*, highlight the importance of preserving the *P. japonica* ecosystem, and explore ways to utilize *P. japonica* resources. This research promotes the understanding and protection of *P. japonica* resources in Korea and beyond, and underscores the need for further research and conservation efforts.

Keywords: cultivation method; life cycle; mass production; *Prasiola japonica*; species conservation

Introduction

Prasiola japonica is a freshwater green algae species that belongs to the Chlorophyta phylum, Trebouxiophyceae class, Prasiolales order, and Prasiolaceae family. It grows to about 7 cm in length and 2.5 cm in width and reproduces through both sexual and asexual reproduction. Originally called *P. japonica*, it is now commonly referred to as indigenous freshwater algae (Kim et al. 2015; Takeda et al. 1967). The chloroplasts of the unicellular stage of the algae are distinctly visible, giving it a green color, and have a size of about 6–8 μm. The mature cells, on the other hand, mostly lack chloroplasts and do not have a visible color, with a size of about 2–4 μm. *Prasiola japonica* thrives in a low-temperature, weakly alkaline environment with fast-flowing streams and can attach to rocks and other surfaces. It contains high levels of vitamins and minerals (Seo et al. 2013) and has been evaluated as a potential candidate for developing anti-tumor and anti-inflammatory drugs (Lee et al. 2021). It is also used as a raw material in various foods, additives, medicines, and cosmetics (Park et al. 2018, 2022; Rahmawati et al. 2021). In Korea, *P. japonica* was first reported by Japanese scientist Okada during the Japanese colonial period in 1938 (Okada 1938). In 1967 (Takeda et al. 1967), during an ecological survey of the Chodang Cave conducted by the Samcheok-gun Elementary Education Council, *P. japonica* was found growing in the Chodang Cave's Sohan Valley; since then, Sohan Valley has been reported as its only natural habitat in Korea (Park et al. 1970).

The genus *Prasiola*, which includes both freshwater and marine green algae, has a wide global distribution, with approximately 65 species found in areas ranging from the Arctic and Antarctic to tropical and temperate climates. Of the 61 reported species, there are 34 surviving specimens, and they are distributed across a range of environments from marine to freshwater habitats. A total of six species have been reported in China, Japan, and South Korea, with two species being reported as variations (Guiry and Guiry 2008). China has reported four species, including *P. japonica*, *P. yunnaica, P. subareolata,* and *P. formosana*, while Japan has reported only *P. japonica*. North Korea and Myanmar have reported the *P. formosana* variation (Kim et al. 2015; Naw and Hara 2002).

In Korea, *P. japonica* is only found growing in the Sohan Valley of Samcheok city, with an annual harvest of only 4–5 kg. In 2012, the Sohan Valley was designated as an ecological conservation area (Gangwon-do Notice No. 2012-307), strictly prohibiting the collection of *P. japonica*. To conserve the natural population of *P. japonica* and address the issue of supply, research on its cultivation has been conducted. This study aimed to establish the conditions for separating and culturing haploid and diploid cells of *P. japonica*, as well as providing information on the cultivation of other *Prasiola* species.

Materials and Methods

Analysis of seasonal abundance of *P. japonica* and rainfall

To analyze environmental factors affecting the growth of *P. japonica*, we observed their distribution and habitat changes according to the season. We measured the distribution of *P. japonica* and rainfall along the entire river section (0.95 km) from the front of Sohan Cave to the end point (buoy) for five years. Measurements were conducted every 100 m (n = 9) and classified into five levels: none (0), little (0.1–1), average (1.1–2), considerable (2.1–3), and very considerable (3.1–4). Additionally, we analyzed rainfall under these conditions by installing a rainfall measurement device every 100 m (n = 9). The analysis was conducted using the mean value of the analyzed results. The results were schematized using the MeV program.

Collection of *P. japonica* samples and investigation of growth and maturity

To investigate the growth and maturity of *P. japonica*, samples of each *P. japonica* specimen found were collected from different habitat areas (upstream, midstream, downstream) of the Sohan Valley, which starts at the Sohan Cave between Geundeok-myeon and Hamangbang-ri in Samcheok-si. Collection was performed in August 2022, when growth was optimal. The collected samples were measured for maximum leaf length and average value to determine differences in *P. japonica* growth (n > 50). Additionally, each collected *P. japonica* was cleaned and examined for the presence of male and female reproductive cells using a BX53F2 microscope (Olympus, Tokyo, Japan) at magnifications of 100–400 ×, and the results were analyzed to determine *P. japonica* maturity.

Verification of conditions for reproduction and spore release

To investigate reproductive conditions for *P. japonica*, samples were collected in August 2022. The collected samples were ground and stored at −80°C for three months, then cultured again to observe the release of gametes, zygotes, and spores. To confirm the conditions for spore release in *P. japonica*, various temperature conditions (5, 10, 15, 20°C) were tested through static and turbulent cultures for 15 days. In the static culture, *P. japonica* was placed in a 3-L round flask filled with medium (water from Sohan River), and spore release was induced without aeration at 12°C. In the turbulent culture, *P. japonica* was placed in a 3-L round flask filled with medium (water from Sohan River), and a TechnoTakatsuki HP-100 bubble generator (Tokyo, Japan) was used to generate bubbles from the bottom to provide aeration, followed by spore release induction. After 15 days of cultivation, the quantity of spores released was observed under an Olympus BX53F2 microscope, and the amounts of released spores was measured and recorded in a Table 1, distinguishing between asexual spores, female gametes, and male gametes.

Confirmation of spore cultivation conditions

Spores released through asexual and sexual reproduction were used for static and shaken cultivation. Static cultivation was carried out using a temperature gradient incubator (MTI-202; EYELA, Tokyo, Japan). Spores were inocu-

Table 1 Spore formation under different conditions

Culture method	Temperature	Spore formation by asexual reproduction (spores/mL)	Spore formation by sexual reproduction (spores/mL)	Notes
Stationary	5°C	$< 3 \times 10^3$	$< 1.5 \times 10^3$	
	10°C	$> 5 \times 10^4$	$> 3 \times 10^4$	
	15°C	$> 5.5 \times 10^4$	$> 3.5 \times 10^4$	
	Over 20°C	-	-	Melts away without spore formation
Aeration	5°C	$< 2.5 \times 10^3$	$< 2.5 \times 10^3$	
	10°C	$> 3.5 \times 10^3$	$> 3.5 \times 10^3$	
	15°C	-	$> 5.5 \times 10^3$	
	20°C	-	-	Difference in maturation by sex

-: not applicable.

lated onto Petri dishes (90 mm × 15 mm) with cover glasses (24 mm × 60 mm), filled with culture medium (Sohan Valley water), and then cultivated at various temperatures (5, 10, 15, 20, 25°C). Shaken cultivation was carried out using a shaking incubator (HB-201SF, Bucheon, Hanback Science, Korea). Spores were inoculated onto petri dishes (90 mm × 15 mm) with cover glasses (24 mm × 60 mm), filled with culture medium (Sohan Valley water), and then cultivated at various temperatures (5, 10, 15, 20, 25°C) with agitation at 90 rpm. The culture medium was exchanged weekly during cultivation. To measure the growth rate of the spores under each set of conditions, leaf length (n = 30) of each individual was measured and growth rate was calculated based on the size of the initially inoculated spore. Additionally, spore growth trends were observed by capturing images of each condition using a microscope.

Optimal growth temperature for blade growth

Blade tissues of a certain size (approximately 2 cm in length and 0.5 cm in width) were collected and cultured in 3-L Erlenmeyer flasks hanging from a ring stand for 55 days at various temperatures (5, 10, 15°C) to determine the optimal temperature for blade growth. Provasoli enriched seawater with iodine (PESI) medium was used, and the medium was replaced every week. The length and width of the blades (n = 30) were measured when the medium was replaced to confirm the growth rate.

Verification of growth medium conditions

To investigate the growth conditions according to the cultivation medium of the midget *P. japonica* spores, we cultured them for approximately one month using water from Sohan Stream in Sohan Valley and PESI medium. We also verified whether temperature had an impact by setting different temperature conditions (10, 15°C) for each group and measured the leaf lengths grown during the experiment.

Confirmation of growth stages of *P. japonica*

Prasiola japonica was cultivated using its spores and grown for approximately three months at 12°C under static conditions until reaching the leaf stage. Images were taken weekly using a microscope to show the growth stages of the *P. japonica*.

Statistical analysis of experimental results

All data in this study are expressed as mean ± standard deviation from at least three independent experiments. Kruskal–Wallis/Mann–Whitney U test was used to compare statistical differences between experimental and control groups, and a p-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (IBM Corp., Armonk, NY, USA).

Results

Analysis of seasonal abundance of *P. japonica* and rainfall

The distribution of *P. japonica* in Sohan Valley, Samcheok, was found to vary each year. In 2017, the highest abundance of *P. japonica* was observed in June, whereas in 2018, it was about two months later, in August. The abundance of *P. japonica* was significantly lower in 2019 and 2020 compared to other years. In 2021, the greatest abundance was observed in October, and in 2022, it was observed in August (Fig. 1A). An analysis of rainfall in Sohan Valley showed that the amount of rainfall varied each year, but it was found to be closely related to the seasonal variations in *P. japonica* abundance. For instance, in August 2018, the highest amount of rainfall was observed (400 mL) (Fig. 1B), and at the same time, the abundance of *P. japonica* showed the highest value. These results demonstrate the close relationship between rainfall and *P. japonica* abundance, indicating that rainfall has a significant impact on the growth and distribution of *P. japonica* (Fig. 1C).

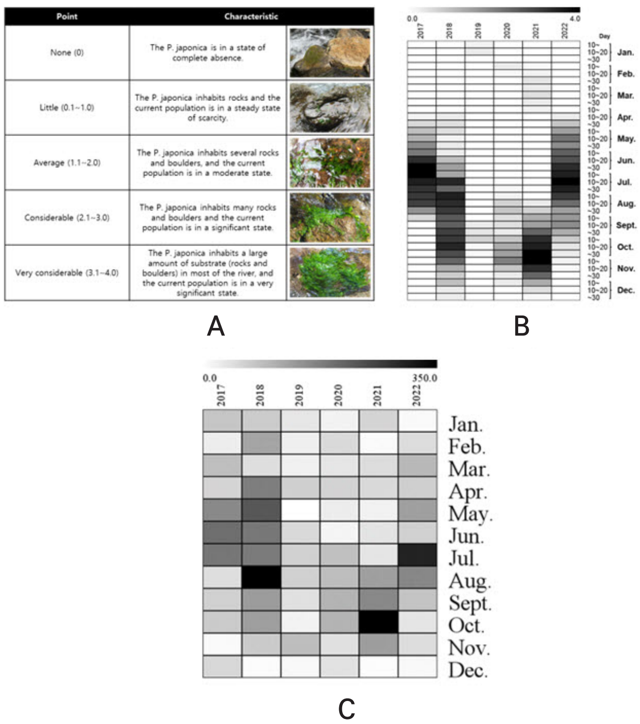

Fig. 1 The environmental factors to affect the growth of *Prasiola japonica*. (A) Five-stage classification to evaluate the growth level of *P. japonica* observed in habitat, Sohan Valley, Samcheok. According to spreading level of *P. japonica*, points from None to Very considerable stages are included 0 (white) to 3.1–4.0 (dark black). (B) Analysis results of habitable abundance for *P. japonica*. From 2017 to 2022, abundance of *P. japonica* was exhibited in July to October. (C) Analysis of rainfall (mm) in habitat of *P. japonica*, Sohan Valley. Level of rainfall showed similarity to the growth pattern of *P. japonica*. Rainfall level: 0 (white) to 350 (dark black). All data were expressed in gradient.

Analysis of growth and maturity of *P. japonica* according to location

The growth of *P. japonica* varied geographically among the upper, middle, and lower sections of the Sohan River, with each section showing different maximum and average values. The upper section, near Sohan Cave, showed the lowest growth rate, with a maximum blade length of 2 cm and an average length of 0.5 cm. The lower section showed a moderate growth rate, with a maximum length of 6 cm and an average length of 4.1 cm. The middle section showed the highest growth rate, with a maximum length of 9 cm and an average length of 5.2 cm. There were significant differences in growth rate between each section, with the middle section showing a 4.5 to 10-fold higher growth rate than the upper section and the lower section showing a 3 to 8-fold higher growth rate than the upper section (Fig. 2A). The maturity of the *P. japonica* showed a similar pattern as the growth rate. In the upper section, the differentiation of spores and gametes was the least distinct, followed by the lower and middle sections, where the differentiation of spores and gametes became more distinct (Fig. 2B).

Spore production of *P. japonica*

Prasiola japonica was homogenized using a mixer and the homogenized sample was added to PESI agar media prepared using water from Sohan Valley. The media were stored at −80°C for three months before being cultured under constant conditions with a temperature of 13°C in a growth chamber on a glass plate (Fig. 3A). More than 80% of the samples survived on both PESI agar media prepared with Sohan Valley water (Fig. 3B). Moreover, the release of cells from the surviving frozen cells was confirmed. To confirm that the released cells were spores, a germination experiment was conducted using bamboo. The results showed that the released cells germinated and grew on bamboo, confirming that they were spores (Fig. 3C).

Measurement of spore release quantity according to culture conditions

Through controlled and batch cultivation at various temperatures, we investigated the conditions for spore release in *P. japonica*. We found that under controlled cultivation at 5°C, a low level of monospores was released along with gametes. Under the 10°C and 15°C controlled cultivation conditions, many monospores were released, as well as a similar mass release of gametes. However, at the 20°C controlled cultivation condition, the *P. japonica* disappeared without any spore release. In contrast to controlled cultivation, batch cultivation showed the release of both monospores and gametes at 5°C and 10°C, but only release of gametes was observed at 15°C due to the maturity of the monospores. Furthermore, at 20°C, no mature gametes were released (Table 1).

Growth of monospores and gametes according to temperature and light intensity

Monospores and gametes of *P. japonica* were cultured at various temperatures, and their leaf lengths were measured to confirm their growth rates. For monospores, the highest growth was observed at 10°C when cultured under a light/dark cycle at 5°C–25°C, and at 15°C, normal growth was observed until 42 days, after which growth stopped and death was confirmed. At temperatures above 20°C, slight growth was observed until 21 days, after which growth ceased and death was observed (Fig. 4A). For gametes cultured under the same conditions as monospores, growth stopped at 5°C without significant changes in size, but they remained in the form of spores without dying. At 10°C and 15°C, normal growth was observed, and the maximum growth rate was observed at 15°C. At temperatures above 20°C, growth patterns similar to those observed in mono-

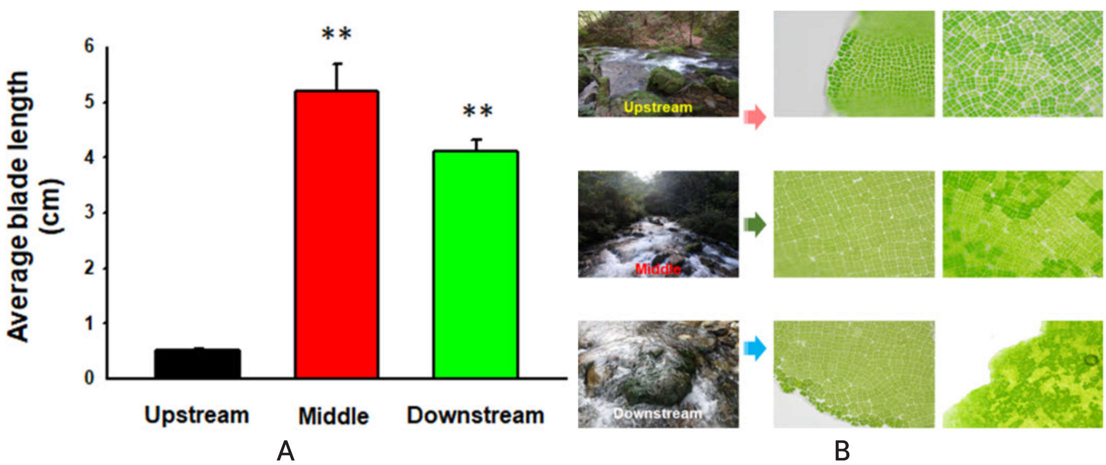

Fig. 2 Growth survey of *Prasiola japonica* in the native area. (A) Analysis of *P. japonica* blade length was conducted on 30 individuals of the species in the upstream, midstream, and downstream sections of the Sohan River. Statistical significance was evaluated using the Kruskal–Wallis/Mann–Whitney U test. ** $p < 0.01$ compared to the upstream section. (B) Maturation difference of *P. japonica* by habitat. Male gametes are indicated in light green, and female gametes are indicated in deep green.

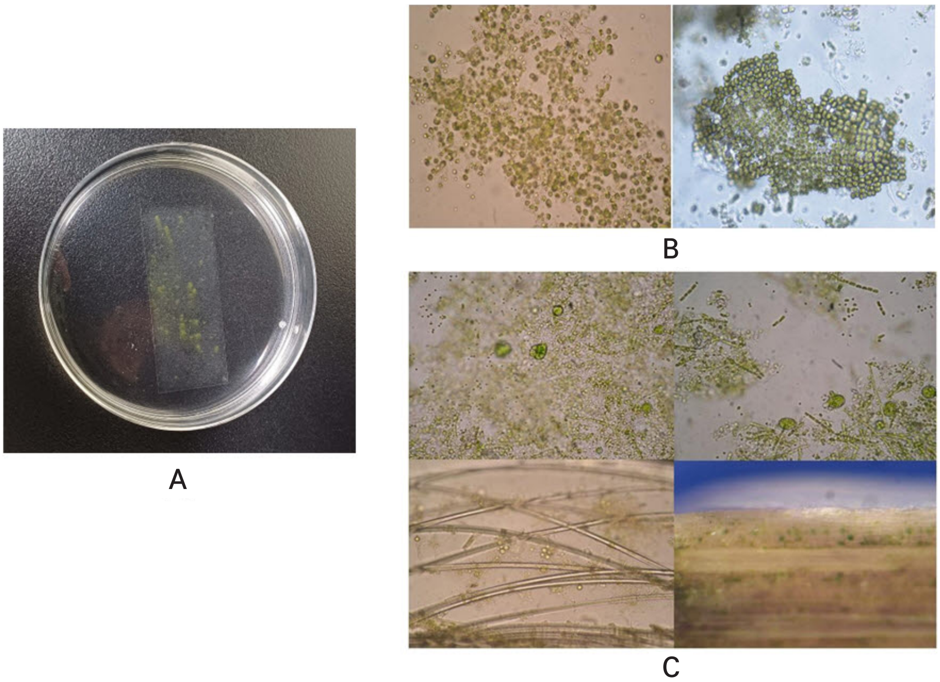

Fig. 3 *Prasiola japonica* survival characteristics. (A) Spore germination method in petri dish with cover glasses; (B) Morphology of cryopreserved cells prepared after separation and culture; (C) Morphological shapes of germinated cells (top) and spores of *P. japonica* germinated on bamboo (bottom) obtained after grinding the *P. japonica* preserved at –80°C for three months.

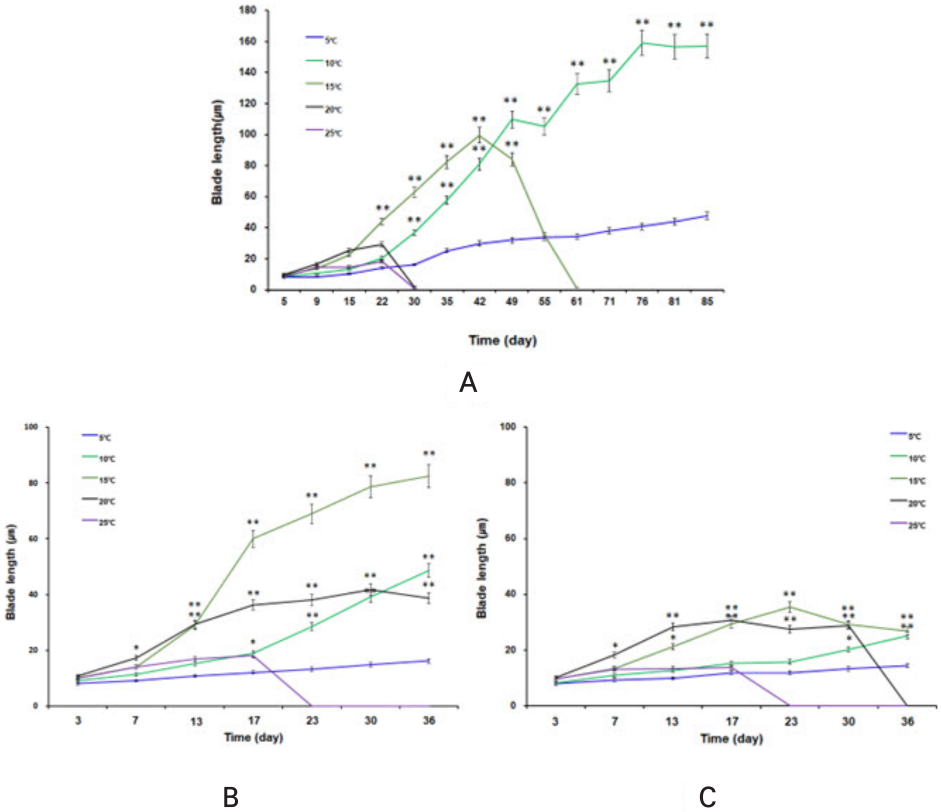

Fig. 4 Spore germination and growth patterns observed under different temperatures and illumination strength. (A) Growth curves of monospores by temperature. (B, C) Growth curve of gamates under illumination at 1,000 lux (B) and 2,500 lux (C). Statistical significance was evaluated using the Kruskal–Wallis/Mann–Whitney U test. *$p < 0.05$ and **$p < 0.01$, compared with control group (the 5°C Sohan Valley water group).

spores were observed. At 20°C, slight growth was observed, but growth stopped for the most part after 21 days, and no growth was observed at 25°C after 21 days (Fig. 4B). When monospores and gametes were cultured together under the above conditions without separation, the maximum growth rate was observed at 15°C, and growth did not occur or died out below 5°C or above 20°C. Based on these results, the best growth trend for monospores and gametes of *P. japonica* was observed at temperatures around 10°C to 15°C (Fig. 4C).

Analysis of leaf growth

Prasiola japonica leaf blades (approximately 2 cm in length and 0.5 cm in width) were selected and cultured under different temperature conditions to observe the growth pattern of young *P. japonica* individuals. Unlike the spores, growth did not occur in leaf blades at temperatures below 10°C, and only occurred at 15°C. Leaf growth was proportional to both length and width, and under the 15°C conditions, growth continued for about 40 days without spore release. Afterward, spore release occurred through asexual reproduction (Fig. 5A, B). Therefore, we confirmed that the optimal temperature for the growth and maturation of *P. japonica* is similar to the temperature range (approximately 15°C) required for the growth of spores.

Analysis of growth rate of *P. japonica* depending on culture media

We investigated the growth patterns of *P. japonica* gametes depending on different culture media. In each experiment, we compared the growth patterns of Sohan Valley water (natural habitat of *P. japonica*) and PESI medium at 10°C and 15°C. There was a statistically significant growth difference for gametes at 10°C in PESI medium compared to Sohan Valley water, with over 10-fold (26 days, $p < 0.01$) growth difference confirmed. At 15°C, we confirmed a statistically significant growth difference of approximately 8-fold (26 days, $p < 0.01$) between PESI medium and Sohan Valley water (Fig. 6A). Based on these results, we could expect faster growth of *P. japonica* using PESI medium rather than Sohan Valley water for cultivation and breeding.

Analysis of growth stages in *P. japonica*

Distinct characteristics of *P. japonica* were observed at each growth stage, as shown in Figure 6. During the initial occurrence of monospores and gametes, cells undergo division and produce two daughter cells from one mother cell (Day 1–4). While cells typically divide into 2n, it has been confirmed that *P. japonica* grows in a seemingly random pattern of 2, 3, and 4 divisions (Day 7–23). Once three germ tubes have formed (Day 25), leaflet growth occurs up to approximately 100 μm, followed by simultaneous growth of the leaflet and blade width. Thereafter, continuous growth occurs as the generated germ tubes maintain the shape of roots, and visible *P. japonica* can be confirmed with the naked eye after approximately 90 days of cultivation (Fig. 7).

Discussion

This study investigated growth and cultivation conditions of *P. japonica*, demonstrating differences in growth under various temperature and light conditions. Similarly, other algae have their own unique growth conditions including temperature (Singh and Singh 2015), flow rate (Goldman et al. 1975), light intensity (Sorokin and Krauss 1958), UV radiation (Poppe et al. 2003), and basic environmental factors (Adenan et al. 2013; Andersen 2005; Borowitzka 2016), as well as metal elements (Kim et al. 2021). In the case of *Enteromorpha intestinalis*, an edible seaweed commonly used in Korea, various factors such as light intensity (De Silva and Burrows 1973), temperature (Kim and Lee 1996), and season (Pringle 1986) were found to affect its growth, while *Ecklonia cava*, a type of kelp, was shown to have different optimal growth conditions such as temperature (Serisawa et al. 2002), nitrogen source (Gao et al. 2016; Lee et al. 2017), season (Choi et al. 2020), and flow rate (Kim et al. 2017).

The purpose of this study was to identify the growth conditions of *P. japonica* for the purpose of conserving individuals and for mass production through cultivation (Ko et al. 2020; Shen et al. 2009). The results of the experiment showed that the growth of spores and thalli of *P. japonica* exhibited distinct differences depending on the tempera-

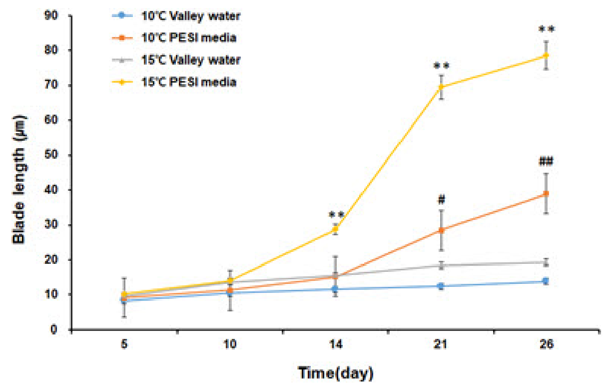

Fig. 6 Profiles of Growth curves of *Prasiola japonica* in different media. Statistical significance was evaluated using the Kruskal–Wallis/Mann–Whitney U test. $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ compared with the 10°C Sohan Valley water group; and $^{**}p < 0.01$ compared with the 15°C Sohan Valley water group.

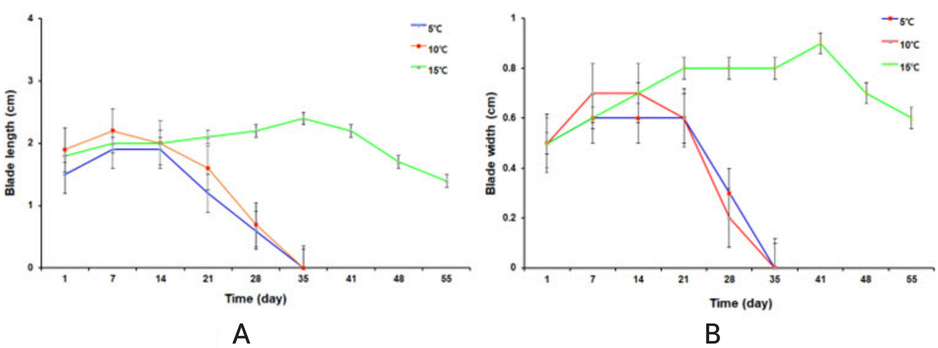

Fig. 5 Profiles of growth curves of *Prasiola japonica* at different temperatures. (A, B) Growth curves for blade length (A) or width (B). Statistical significance was evaluated using the Kruskal–Wallis/Mann–Whitney U test.

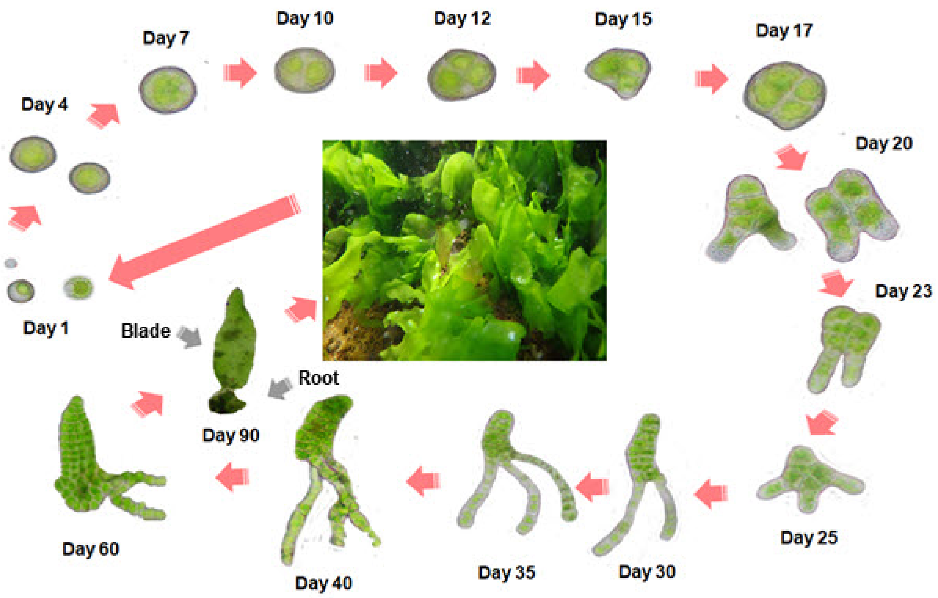

Fig. 7 Biological life cycle of *Prasiola japonica*. Morphological shapes and changes are indicated in each day.

ture. Similar results were obtained in other experiments with different culture media, cultivation methods, monospores, and gametes. Female gametes are dark green because of the high concentration of chlorophyll, while male gametes are light green since it has low concentration of chlorophyll. These results are similar to the variations in water temperature and growth of *P. japonica* in different sections of the Sohan Creek, indicating that water temperature is a key factor regulating the growth of *P. japonica*. A representative seaweed with similar growth conditions is the kelp *Saccharina sculpera* (Kim et al. 2021; Sato et al. 2020; Yoo et al. 2018).

We confirmed that the survival rate of *P. japonica* varies depending on the cultivation conditions and is closely related to the growth characteristics and survival strategies of the *P. japonica* population. When comparing with the growth rate at the upstream, it showed up to 4.5 to 10 times faster growth rate at the midstream, and at the downstream, it showed 3 to 8 times faster growth rate, which was confirmed to be due to differences in environmental conditions at each location. In the vicinity of Sohan cave, located upstream, growth is poor due to the low temperature (6°C–7°C) of the water discharged from the cave, and growth is most active midstream, where a temperature of 12°C–14°C is maintained throughout the year. Downstream there are large annual temperature fluctuations, and growth varies depending on the season. The abundance of the *P. japonica* in Sohan valley was varied throughout the year. Since the water temperature was constant during the seasons at the same point of the valley, we could conclude that another factor is the main cause of regulating the growth rate of *P. japonica*. Among those factors, monthly rainfall was correlated with monthly abundance of the *P. japonica* within 6 years. Thus, it is assumed that the rainfall is the other main factor that controls the growth of *P. japonica*. However, it is not elucidated as to how the rainfall can affect the growth rate of *P. japonica* with current data. Somehow, simple hydration or the change of flow rate that we could not checked in this paper might be considerable factors. Since *P. japonica* only inhabited near the weakly alkaline environment of the Sohan valley, replenishment of the mineral within water through rainfall might be another putative explanation affecting the growth of *P. japonica*. Therefore, further studies are still in need to elucidate for understanding growth pattern of *P. japonica*.

In indoor cultivation, the best growth was observed in the range of 10°C–15°C, and it was confirmed that spores are released after some growth, and growth and reproductive processes do not occur below 5°C, so it is assumed that the growth of *P. japonica* requires considerable energetic cost up to a certain size under appropriate conditions, and then energy is converted into reproduction.

Conclusions

Korea has four distinct seasons, and the conditions such as flow rate, flow velocity, and light intensity vary significantly from season to season. For this reason, the growth of *P. japonica* cannot always take place under optimal conditions, and the production volume decreases sharply every year, with a production volume of about 4–5 kg/year in 2021. Since this study only identified cultivation and growth conditions according to temperature, additional research on other environmental conditions is needed to identify better cultivation conditions. Our study indicates that the optimal temperature for growth and reproduction of *P. japonica* was between 10°C–15°C, and the optimal light intensity was 1,000–1,500 lux. Furthermore, it was found that PESI agar can increase the growth rate of *P. ja-*

ponica 1.5–8 times greater than Sohan Valley water. Therefore, long-term experiments should be conducted on domestic *P. japonica* to identify various factors related to growth conditions and growth inhibitors, and continuous monitoring should be performed to expand knowledge of this organism's life cycle.

Abbreviations

PESI: Provasoli enriched seawater with iodine

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Not applicable.

Authors' contributions

Dong Sam kim planned, collected, analyzed the data and conceptualized the article. Dong Seon kim wrote the original draft, revised the article and visualized the data. JYC supervised, planned and reviewed the final version of the article. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated during this study are included in this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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