



Thallus sectioning as an efficient monospore release method in *Pyropia yezoensis* (Bangiales, Rhodophyta)

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Abstract

The red seaweed *Pyropia yezoensis* is a marine crop of important economic value and is widely cultivated in the coastal areas of northern China. The current seedling-raising system relies on the germination of conchospores, a process not fast enough to meet the increasing demand from farmers. In this study, we developed a monospore-dependent seedling method based on the asexual reproduction of the *Pyropia* thallus. The *Pyropia* thallus was physically sectioned into small pieces (microthalli) that were cultivated at 15 °C. The algal cells in the microthalli became morphologically condensed, underwent cell division, and then developed into monospores on the 5th day. The monospores were able to attach to the seeding rope in 24 h and germinated into healthy thalli. To optimize the efficiency of monospore release, we tested the effect of temperature and the size and original positions of the microthalli as well as the age of the mother thallus. Microthalli with a size of 30–50 cells from the middle and apex of 21-day-old thalli cultivated at 15 °C yielded the optimum production of monospores. Theoretically, in this thallus-to-thallus seeding strategy, 0.1 g of thallus could produce at least 10⁷ monospores, with the same number of offspring thallus seedlings. Taking all of these advantages together, including the high efficiency, short time, low cost and easy operation, this physical sectioning method could serve as a promising seed source especially for new cultivars with superior traits in *Pyropia* farming and rapid replenishment of seedlings when thalli undergo disastrous diseases.

Keywords *Pyropia yezoensis* · Monospores · Sectioning · Asexual reproduction

Introduction

Pyropia yezoensis (previously *Porphyra yezoensis*) belongs to the red algal order Bangiales. It is one of the most economically important marine crops (Cao et al. 2016) in Far East countries including South Korea, Japan, and China. The annual production of *Pyropia* (both *P. yezoensis* and *P. haitanensis*) has

reached approximately 1.8 million tonnes, with an estimated value of approximately US\$ 1.5 billion (<http://www.fao.org/fishery> 2017). *Pyropia yezoensis* has been widely cultivated in densely concentrated sea farms in China since the 1990s and becomes one of the largest seaweed industries in recent decades. According to genetic and cytological analyses, it has been demonstrated that *Pyropia* has a complex life cycle with two heteromorphic generations, reciprocally alternating between the macroscopic gametophytic thallus (n) and microscopic filamentous conchocelis ($2n$) phases (Saga and Kitade 2002; Blouin et al. 2011). Conchocelis inhabit the microholes of shells and release conchospores, which undergo meiosis and then develop into gametophytic thalli (Krishnamurthy 1969). The blades of *P. yezoensis* are reported to be monoecious. Both sperm and carpospores can be produced from one individual thallus. The fertilization of carpospores by sperm can result in the formation of zygospores, which germinate into conchocelis.

The breeding strategy at existing sea farms is mainly based on the above sexual life cycle. In late April or early May, depending on the water temperature, farmers collect either

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zygospores from fertilized thalli or free conchocelis and inoculate them into mollusk shells. The resulting shell conchocelis will swell to form conchosporangial branches. It takes more than 4 months for the shell conchocelis to grow, swell, and finally release conchospores in October. The conchospores quickly attach to seeding ropes and develop into gametophytic seedlings within 1 week. One month later, the first round of mature thalli can be harvested and then regrown until the following April for the next round. During the whole cultivation process, nearly half the time is spent inoculating and growing conchocelis. In addition to the process being time-consuming, space-demanding, and expensive, some diseases, such as yellow spots, can occur in shell conchocelis and lead to severe economic loss (Ryu et al. 2001). Therefore, new strategies for seedling collection that circumvent the conchocelis stage are urgently needed.

In the life history of *Pyropia* there is also an asexual reproduction cycle. Cells in the margin of the *Pyropia* thallus tend to differentiate into monospores when they encounter environmental stimuli, such as cold temperature and rapid tides. Once released, monospores quickly attach to seeding ropes and develop into secondary seedlings (Duffield et al. 1972; Xu et al. 2003). The spontaneous accumulation of secondary seedlings from asexual monospores makes an essential contribution to the overall productivity. In this study, we developed a highly efficient method to artificially induce the asexual reproduction of the *Pyropia* thallus. Large amounts of monospores generated in this way could serve as “seeds” that can be used directly for seedling cultivation.

Materials and methods

Gametophytic thalli of *Pyropia yezoensis* strain RZ (PYL201306-440) were cultured in PES medium made with sterilized seawater under irradiance of $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a photoperiod of 12L:12D at 10°C and continuous aeration with filter-sterilized air. Fresh culture medium was changed every 3 days.

A *Pyropia* thallus of approximately 10 cm in length was first spread on a culture dish containing PES medium. A small piece of approximately 1 cm^2 in size was cut from the middle or apex of the thallus and was placed on a glass slide. Adding one drop of PES medium onto the glass slide keeps the thallus wet. Too much medium would make the materials splashing out the in sectioning. We then used a scalpel to section the piece of thallus into very small pieces containing about 30–50 cells, which are referred to as microthalli in the remainder of the text. A total of 96 microthalli were randomly selected under an inverted microscope (OLYMPUS CKX41) and pipetted into 96-well plates with one microthallus in each well. Three thalli were used as replicates for this experiment. Observations of the cell morphology and development were

performed daily under the inverted microscope. For each thallus replicate, the number of released monospores in each well was counted and averaged. Microthallus release was referred to as successful if the number of monospores exceeded 20, which is the general number of healthy algal cells in the microthallus. The release efficiency was calculated as the percentage of successfully released microthalli in the 96-well plates.

To test the effect of culturing temperature on the efficiency of monospore release, microthalli sectioned from the middle of the thallus were pipetted into a 96-well plate and put in a 10°C , 15°C and 20°C incubator. Three plates were prepared for each temperature. The number of monospores was counted at the sixth day after sectioning to calculate the release efficiency. To test the effect of the microthallus size, microthalli from the middle part of the mother thallus were sectioned into different sizes and cultured in the 15°C incubator. To determine the effect of original locations, microthalli from each specific location in a size of around 30 cells were also cultured in the 15°C incubator. Microthalli from different ages of the mother thallus were also processed as above. To investigate the adhesion of monospores to seeding ropes, microthalli were cultured in a glass beaker containing 100 mL PES medium with gentle shaking at 15°C . When monospores were released, which for most materials occurred on the 6th day as observed under a microscope, the culture was collected and filtered using a $0.45\text{-}\mu\text{m}$ filter to collect monospores. A given number of the monospores, referred to as M1, were transferred to another glass beaker with sterilized seeding ropes. After 24 h, the seeding ropes were removed and the number of monospores remaining in the beaker, referred to as M2, was counted. The monospore adhesion proportion was calculated by the following formula: $C = \frac{M1-M2}{M2}$.

Results

Monospores are released from microthalli

A *Pyropia* thallus was sectioned into microthalli as described in the “Materials and methods” section. On the 1st day, the microthalli were surrounded by the debris of broken cells. The intracellular space was clear and the cell contents were loosely distributed (Fig. 1a). On the 3rd day, the cell contents became compact and the cell boundaries were clearer. Irregular cell divisions were observed. The orientations and timings of the cell divisions were diverse, and thus one vegetative cell could split into two or more offset cells (Fig. 1b). On the 5th day, the number of cells in each microthallus significantly increased due to multiple cell divisions. Cells at the edge of the microthallus were released into the medium, while cells in

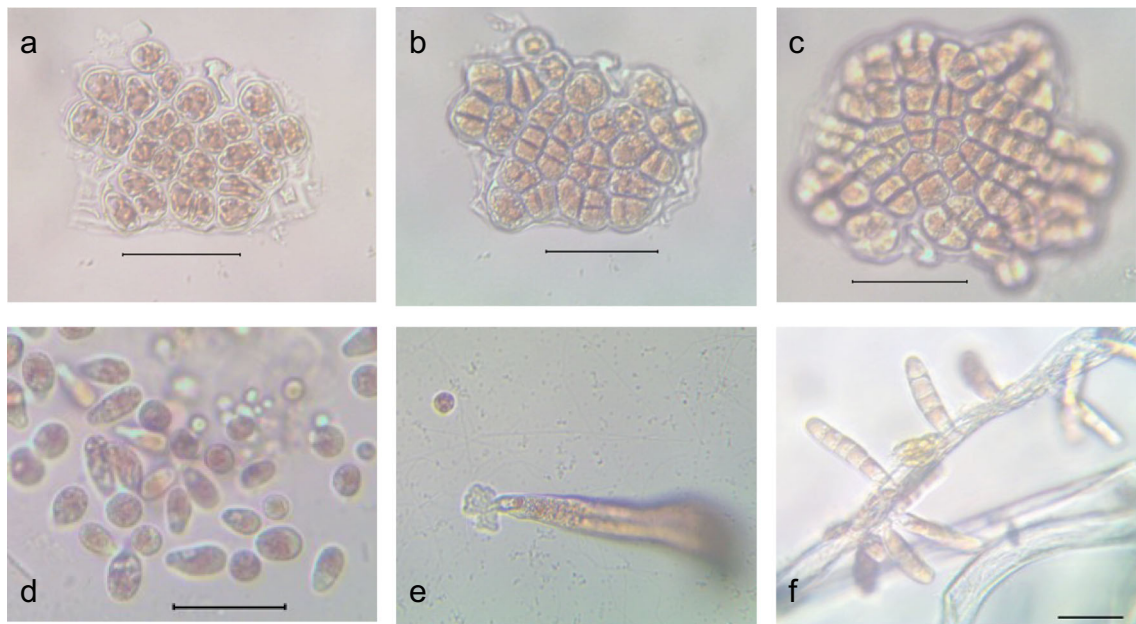


Fig. 1 Monospores released from microthalli. Morphological changes of a microthallus observed on the 1st day (a), the 3rd day (b), and the 5th day (c) after sectioning; d, unicellular monospores were released from

microthalli on the 6th day after sectioning; e, rhizoids were observed on the 12th day; f, monospore-germinated seedlings attached to seeding ropes

the central part remained linked to each other (Fig. 1c). On the 6th day, all algal cells were released. The released cells were initially round and soon became oval and exhibited cell polarity (Fig. 1d). On the 12th day, eight cells generated by three cell division events were linearly arranged in each thallus seedling. Additionally, we also observed several filamentous conchocelis in the culture. The ratio of conchocelis to monospore-germinated thalli was approximately 2:15. A few microthalli exhibited robust healing abilities and developed into intact thalli afterwards and several microthalli formed cell clusters resembling the calli of plants.

Since cells at the edge of an intact thallus can differentiate into monospores during early growth stages at sea farms, we compared the efficiency of spontaneous release to that of the sectioning method. An intact seedling was cultured under the same conditions as the microthalli. After 7 days, only five monospores were released from the intact thallus seeding, whereas the microthalli released an average of 63 monospores. The significantly increased efficiency of the mechanical sectioning method further underscored that the method could be a promising seedling-raising strategy for *Pyropia* cultivation.

The conchospores produced by shell conchocelis can adhere to a seedling rope immediately during the first 5 h after release. Successful adhesion is essential for proper morphogenesis and also protects the thallus from being carried away by ocean waves. Thus, adhesion efficiency is a main factor determining the number of seedlings collected during *Pyropia* cultivation. Rhizoids were clearly observed at the ends of monospore-derived seedlings on the 12th day, indicating that

holdfasts had possibly developed (Fig. 1e). To test the adhesion capability, we incubated seedling ropes in the monospore culture and found that 63.5% of the monospores could successfully attach to the seedling ropes after 24 h and 81.3% attached after 48 h (Fig. 1f).

Effect of temperature on thallus monospore release

As the thallus cultivation temperature in the sea is approximately 10 °C, we compared the release efficiency of monospores at 10, 15, and 20 °C. Microthalli, sectioned from the middle of the thallus and containing an average of 30 cells, were incubated at 10, 15, or 20 °C. At 15 °C, the microthalli began to release monospores on the 5th day, whereas at 10 and 20 °C, release began on the 6th day. On the 12th day, we counted the total number of monospore-germinated thalli. An average of 55–120 thalli were collected from the 15 °C culture, whereas only 30–50 thalli were collected from the 10 °C culture (Table 1). As cell division events were observed during the formation of the monosporangium, we inferred that the higher temperature might have induced cell division and led to the increased number of monospores. At 20 °C, less

Table 1 Effects of temperature on monospore release

Temperature	10 °C	15 °C	20 °C
Release day	6th day	5th day	6th day
Ratio of conchospores to monospores	1:15	2:15	7:15
Approx. number of monospores	30–50	55–120	20–30

than 40% of microthalli could successfully release 20–30 monospores on the 12th day. Interestingly, the ratio of filamentous conchocelis to monospores at 10 °C was only 1:15, which was much lower than the ratio at 15 and 20 °C (Table 1).

Effect of microthallus size on *P. yezoensis* monospore release

To investigate the significant difference in the release efficiency between the intact thallus and the sectioned microthallus, we collected four groups of microthalli, with the tiny, small, medium, and large groups comprising microthalli with less than 5, 15–25, 25–35, and more than 35 cells, respectively. All tiny microthalli formed irregular cell clusters or died. In the other three groups, monospore release started on the 5th day and completed on the 7th day. For small microthalli, 84.38% successfully released monospores and 6.3% died, while the others repaired their physical injuries and subsequently developed into individual thalli (Fig. 2a). The same percentage of medium microthalli released monospores, though no death was observed. However, for large microthalli, only 59.4% released, and 3.1% died.

Effect of original microthallus location on the monospore-release efficiency

To determine the effect of the vertical location of the microthallus within the mother thallus on the release efficiency, we collected microthalli from the base, middle, and apex of the mother thallus and investigated their developmental courses. On the 7th day after sectioning, up to 96.9 and 88.5% of microthalli from the middle and apex, respectively, were able to release monospores, whereas the same was true for only 2.1% from the base (Fig. 2b). Additionally, the death rate of the base microthalli on the 7th day was the highest (13.5%). We also tested the variation of the release efficiency for microthalli from the margin and center regions. Both

regions exhibited similar high efficiencies, specifically, 93.7 and 95.8% for the center and margin, respectively.

Effect of the age of the mother thallus on release efficiency

The age of each *Pyropia* thallus was denoted as the number of days after the generation of conchospores. Thalli 14, 18, 21, and 28 days of age were sectioned into microthalli to release monospores. The length of the 7-day-old thallus was only a few millimeters, so the entire thallus was sectioned. For longer thalli, the middle regions were used in this experiment. For 18-day-, 21-day-, and 28-day-old thalli, approximately 82–87% of the resulting microthalli successfully released monospores. The percentage for the 14-day-old thalli was slightly lower. The death rates of the microthalli exhibited a downward trend that correlated with increasing age and the death rate of the 14-day-old thalli reached 14.1% (Fig. 2c).

Discussion

Since the 1970s, many researches have been devoted to developing a highly efficient monospore-release method to propagate seedlings. Researchers have used cellulase, pectinase and enzymes from the digestive gland of shellfish or mixtures of these enzymes to isolate protoplasts that could then develop into monospores (Polne-Fuller and Gibor 1984; Waaland et al. 1990; Chen et al. 1994). However, protoplasts are sensitive to enzymatic digestion. Due to the uneven exposure of algal cells to enzyme, a large proportion of single cells generated in this way die or form cell clusters when they are overdigested. Researchers found that oxidative stress, such as the addition of H₂O₂ to the medium, could stimulate asexual reproduction and apogamy in *P. yezoensis* (Takahashi and Mikami 2017). Approximately 154–2240 monospore germlings were produced from one thallus. In this study, we developed a physical sectioning method to induce asexual

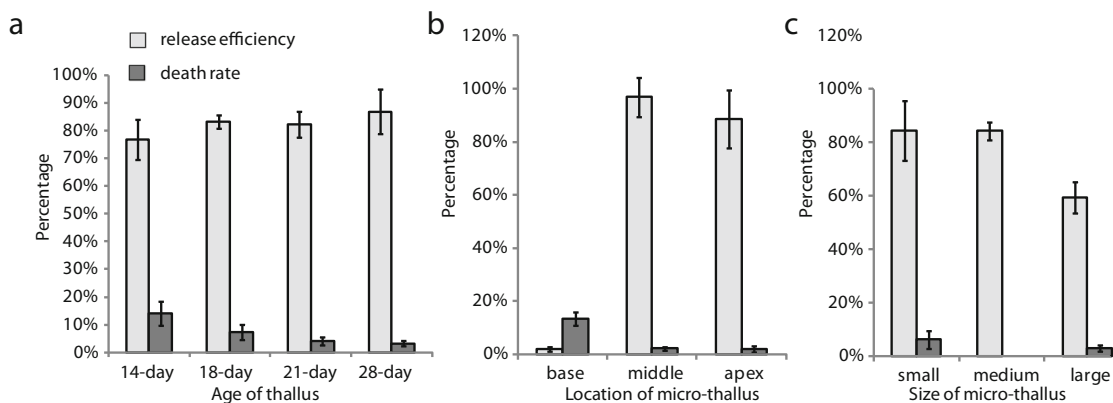


Fig. 2 Effect of several factors on the monospore-release efficiency. **a**, microthallus size; **b**, original microthallus location within the mother thallus; **c**, age of the mother thallus

reproduction of the *Pyropia* thallus and investigated the influence of a series of parameters, including the culture temperature, size and original location, as well as the age of the mother thallus, on the monospore-release efficiency. Under the optimized conditions, namely, using the center region of a 20- to 30-day-old thallus to prepare microthalli containing 20–30 cells and culturing them at 15 °C, we could collect, on average, 1.5 times more monospores than the original number of cells that would germinate into seedlings afterwards. Theoretically, 0.1 g thallus, if subjected in its entirety to sectioning and culturing using the above parameters, might provide at least 10^7 monospores in total and thus produce the same amount of offspring thallus seedlings.

Compared with traditional aquaculture mode based on the sexual production via shell conchocelis cultivation, our method demands minimal investment of instruments and space for efficient sectioning and is easy for farmers to adopt. Moreover, the whole propagation cycle took only 6 days, which is dramatically faster than conchospore-dependent seedling production at traditional *Pyropia* farms. Besides, in traditional aquaculture mode, *P. yezoensis* suffers from several lethal diseases, which still lack effective treatments, including white spot disease (Guan et al. 2013) infecting sporophytic shell conchocelis, as well as the red rot disease (Mo et al. 2016), and Olpidiosis disease (Klochkova et al. 2016) that infect gametophytic thallus. These diseases have caused severe economic loss in the *Pyropia* industry (Park et al. 2006). Our monospore-based seeding strategy completely circumvents the sporophytic phase and thus might avoid potential outbreak of shell conchocelis disease. Additionally, in the case *Pyropia* thalli had suffered from the above disastrous diseases when the conchocelis-based seeding period is already over, this physical sectioning method could replenish large amounts of seedlings in a short time to minimize culture, and consequently economic losses. Therefore, considering these advantages, the sectioning method may serve as an alternative *P. yezoensis* propagation strategy and provide important technical support for innovation in aquaculture. We also notice that there are several problems to be solved in order to transfer this lab-based sectioning method to a larger scale suitable for the industry. For example, the productivity of manual sectioning using a scalpel is low and only suitable for lab-scale experiments. Automated sectioning equipment, e.g., blender, needs to be tested to produce large amounts of monospores sufficient for *Pyropia* farming. According to the culture cycle in *Pyropia* farming, monospores would be collected at the end of the farming season (usually early May in China) when large amounts of thallus are still available and would then be used as seeds in seedling season (usually October to November). Therefore, the technology of monospore preservation is an urgent need.

Asexual reproduction can generate large numbers of offspring within a short period of time, while sexual reproduction

usually takes a long time and introduces more mutations. Importantly, the asexual reproduction is receiving continuously increasing attention in plant breeding, as it is able to fix the superior phenotype that had been selected for, e.g., the trait of high yield, in its offspring, in addition to its value in reducing time and expense of breeding. Vegetative propagation is widely applied in crops that are poor in flowering and seeding, such as *Allium sativum* and *Solanum tuberosum*. Another form of asexual reproduction, apomixis, is considered to be a potential revolution in plant breeding. Attempts have been made to introduce apomixis into sexual crop species (van Dijk et al. 2016).

Besides, plants, especially some lower plants and fungi, tend to adopt asexual reproduction when encountering rapid environmental changes (Hand and Koltunow 2014). Diaspores and fragmented propagules transformed from vegetative cells for propagation were recently reported in another red alga *Dipterocladia* and were likely an adaptive strategy to harsh conditions (Hwang and Boo 2019). The sexual-aseexual switch involves multiple cellular processes including cell de-differentiation, induction of cell totipotency, and cell re-differentiation. Research on plants and fungi has revealed that some proteins, such as fungal opsins, can sense environmental stresses and trigger asexual reproduction (Rodriguez-Romero et al. 2010; Wang et al. 2018), which might explain why only cells from the edge of the thallus can form monospores when subjected to strong sea flows. When the thallus is sectioned into small pieces, stress signals could be transduced to cells in the center, leading to the complete transformation towards asexual reproduction. Our study revealed that when the size of the microthallus exceeded 50 cells, the monospore-release efficiency significantly decreased. Much work has been done to develop methods to stimulate the production of asexual monospores (Waaland et al. 1990; Takahashi and Mikami 2017). Several genes that are preferentially expressed during asexual reproduction have been identified (Kitade et al. 2008). Furthermore, it has been found that Ca^{2+} influx and phosphoinositide signaling are essential for the development of monospores (Kitade et al. 2008; Li et al. 2009). However, the signal transduction pathway linking environmental stress to the resulting cell differentiation and the related regulatory mechanisms remain elusive. Thus, further studies are needed to improve our understanding of the molecular mechanisms and to provide insights into the key genetic signatures involved.

During the release of monospores from sectioned microthallus, some filamentous conchocelis were generated at a low proportion. The appearance of conchocelis was also observed in H_2O_2 -induced monospore release (Takahashi and Mikami 2017). This study inferred that the conchocelis derived from the apogamy of carpospores which were differentiated from released spores. In our work, the other possible origin of conchocelis lied in the fertilization of carpospores by

sperm, as the formation of spermatangia and succeeding discharge of sperm were observed. The two reproduction pathways are hard to distinguish morphologically as *P. yezoensis* is monoecious and further molecular study, such as the identification of key genetic markers of apogamy, would provide necessary insights into this issue.

Author contributions YXM and DMW conceived the study. LT and NCC performed the experiments. DMW, MC, XWG, and RC analyzed the results. DMW, NCC, and LT wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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