Culture-based preservation of *Marchantia polymorpha* gemmalings and thalli without encapsulation, drying, or freezing

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**Abstract** Ongoing research has generated many important lines of the model liverwort *Marchantia polymorpha*, including mutants and transgenic lines. To maintain these lines, researchers typically spend a lot of time and effort periodically replanting thalli (e.g., every month). To avoid this routine maintenance, researchers have developed methods for cryopreservation of dried and frozen gemmae. In this study, we developed a culture-based method for preserving gemmalings and thalli without encapsulation, drying, or freezing. The method requires only tissue culture on agar medium supplemented with sucrose in the dark at regular temperature (22°C). These culture conditions severely inhibit growth of gemmalings and thalli; however, these tissues remained alive after more than 1 year of storage. Survival rate of tissues using this method was 100% in all tests. This method thus enables preservation of gemmaling and thallus cultures on medium under regular temperature conditions, thereby relieving researchers of labor-intensive routine maintenance.

**Key words:** liverwort, Marchantiophyta, preservation, transgenic plant.

*Marchantia polymorpha* is a model liverwort, and previous studies have generated many valuable *M. polymorpha* germplasm lines, such as mutants and transformants. Because *M. polymorpha* is a dioecious liverwort, crosses of male and female strains generate spores with diverse genetic information, and *M. polymorpha* spores with the same genetic information as their parents cannot be maintained. Therefore, valuable germplasm lines must be maintained without crossing by each laboratory. Many researchers spend a lot of time and effort maintaining gemplasm by periodic routine replanting of thalli (e.g., every month). To alleviate this routine maintenance, cryopreservation methods for *M. polymorpha* gemmae have been developed. Wu et al. (2015) reported cryopreservation of unencapsulated gemmae in liquid nitrogen (−196°C), and Tanaka et al. (2016) reported cryopreservation of encapsulated gemmae in liquid nitrogen (−196°C) and in a deep freezer (−80°C). Recently, we also reported cryopreservation of unencapsulated gemmae in liquid nitrogen (−196°C), in a deep freezer (−80°C), and in a non-frost-free freezer (−20°C) (Takahashi and Kodama 2020). Although these cryopreservation methods have high recovery rates and enable long-term storage (at least several months), they require drying and freezing of the gemmae. Here, we report a culture-based preservation method for *M. polymorpha* gemmalings and thalli without encapsulation, drying, or freezing procedures.

Thalli of a wild-type strain (female BC3-38 strain) of *Marchantia polymorpha* were cultured asexually on half-strength B5 (1/2 B5) agar medium under 75 µmol photons m⁻² s⁻¹ continuous white light (FL40SW; NEC Corporation, Tokyo, Japan) in a culture room at 22°C. Four transgenic lines (TG#060-1, TG#066-5, TG#164-3, and TG#253-6) were also cultured asexually under the same conditions (Fujii et al. 2020; Kimura and Kodama 2016; Sakata et al. 2019; Takahashi and Kodama 2020). White light intensity was measured using an LI-250 A light meter (LI-COR Biosciences, Lincoln, NE, USA). Gemmalings and thalli were preserved on 1/2 B5 agar medium supplemented with 1% (w/v) sucrose in Petri dishes (ϕ90×20 mm, 1-8549-04; AS ONE, Tokyo, Japan). Preserved gemmalings and thalli were recovered using 1/2 B5 agar medium (without sucrose) in Rectangular Petri dishes (AW2000, Eiken Chemical, Tokyo, Japan).

To preserve gemmalings and thalli, we collected the tissues using tweezers under a laminar flow hood: gemmae from gemma cups and pieces of thalli approximately 5×5 mm containing meristemetic regions from 1-month-old mature thalli, respectively. Although these cryopreservation methods have high recovery rates and enable long-term storage (at least several months), they require drying and freezing of the gemmae. Here, we report a culture-based preservation method for *M. polymorpha* gemmalings and thalli without encapsulation, drying, or freezing procedures.

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tape (No. 21; Nitto Denko, Japan) was used to seal the Petri dish. For preservation, sealed Petri dishes were incubated under dark conditions in a culture room at 22°C. Gemmalings and thalli grew long and thin under these conditions (Figure 1A, B). When we cultured the preserved gemmalings and thalli for 2 weeks (Supplementary Videos S3 and S4), we observed a 100% recovery rate (Figure 1C, D; Tables 1 and 2).

To optimize preservation conditions, we compared three sealing materials: microporous tape (adhesive), Parafilm (non-adhesive), and vinyl tape (adhesive). Note that microporous tape is typically used for culture of *M. polymorpha* tissues and that Parafilm and vinyl tape are more airtight than microporous tape. The tissues used in this comparison were cultured on preservation medium for 6 months. To check whether tissues regenerated after preservation, the preserved tissues were transferred onto

**Table 1.** Recovery of wild-type gemmalings after preservation for 6 months with various sealing materials.

| Sealing material | Experiment 1* | Experiment 2* | Experiment 3* |
|------------------|---------------|---------------|---------------|
| Microporous tape | 1/10          | 1/10          | 0/10          |
| Parafilm        | 10/10         | 10/10         | 10/10         |
| Vinyl tape      | 10/10         | 10/10         | 10/10         |

*The denominator and numerator indicate the preserved and recovered numbers of gemmalings, respectively.

**Table 2.** Recovery of wild-type thalli after preservation for 6 months with vinyl tape.

| Sealing material | Experiment 1* | Experiment 2* | Experiment 3* |
|------------------|---------------|---------------|---------------|
| Vinyl tape       | 10/10         | 10/10         | 10/10         |

*The denominator and numerator indicate the preserved and recovered numbers of thalli, respectively.

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Figure 1. Morphology and recovery of gemmalings and thalli in culture-based preservation. (A) Morphology of gemmalings after 6 months of preservation in a 90-mm Petri dish sealed with vinyl tape (white color). (B) Morphology of thalli after 6 months of preservation in a 90-mm Petri dish sealed with vinyl tape (black color). (C) Recovery of gemmalings preserved for 6 months. A photograph shows the recovered 10 tissues. Scale bar indicates 10 mm. (D) Recovery of the thalli preserved for 6 months. A photograph shows the recovered 10 tissues. Scale bar indicates 10 mm.

Figure 2. Comparison of sealing materials. (A) Photographs of gemmalings after preservation for 2, 4, and 6 months with microporous tape. (B) Photographs of gemmalings after preservation for 2, 4, and 6 months with Parafilm. (C) Photographs of gemmalings after preservation for 2, 4, and 6 months with vinyl tape. (D) Photographs of gemmalings after preservation for 1 year with vinyl tape. (E) Recovery of gemmalings after preservation for 1 year with vinyl tape. A photograph shows the three recovered tissues. (A–E) Scale bars indicate 10 mm.
new agar medium and cultured for 2 weeks.

When microporous tape was used, gemmalings stored for 2 or 4 months regenerated (Figure 2A). However, most of the gemmalings stored for 6 months did not regenerate, even though they are greenish (Figure 2A; Table 1). Because the agar medium had dried out after 6 months, most gemmalings appeared to have died from dehydration stress. By contrast, when gemmalings were stored with Parafilm or vinyl tape, the agar medium remained moist after 6 months and the stored gemmalings regenerated (Figure 2B, C; Table 1). We recovered gemmalings stored for 1 year on plates sealed with vinyl tape (Figure 2D, E).

In our cryopreservation method for unencapsulated gemmae, recovery rates of four transgenic lines (TG#060-1, TG#066-5, TG#164-3, and TG#253-6) varied (Takahashi and Kodama 2020). We therefore tested culture-based preservation of the same transgenic lines for 6 months. The results showed 100% recovery of all transgenic lines as well as wild-type lines (Figure 3A–D; Table 3). To check whether the transgene is expressed after preservation and recovery, we analyzed Citrine fluorescence in the TG#253-6 line expressing Citrine fluorescent protein. When we observed gemmae from the TG#253-6 thalli after preservation and recovery, the fluorescence can be observed in the gemmae as well as in gemmae from the TG#253-6 thalli with no preservation (Figure 3E). These results suggest that the culture-based preservation method can be used for various mutants and transformants.

Maintaining germplasm lines of *M. polymorpha* typically requires a great deal of time and effort. By
preserving gemmalings and thalli on agar medium supplemented with 1% sucrose, we kept these tissues alive long-term and recovered them with 100% efficiency. Unlike previous cryopreservation methods (Takahashi and Kodama 2020; Tanaka et al. 2016; Wu et al. 2015), our culture-based method preserved not only gemmalings but also thalli. Because no photosynthesis occurs under dark conditions, tissue growth was inhibited; however, viability of the tissues was maintained through sucrose supplementation. Sealing with vinyl tape inhibited water volatilization of the agar medium in the Petri dishes, conserving the water content of the agar medium and the tissues. Remarkably, *M. polymorpha* tissues remain alive even under hermetic conditions seemingly by exchanging limited oxygen and carbon dioxide gases. Parafilm and vinyl tape can both be used for culture-based preservation, but we recommend using vinyl tape. Since Parafilm must be stretched when used, it is difficult to adjust the seal. Care must be taken, also, during culture-based preservation after the sealing. Cryopreservation methods are conducted under low-temperature conditions (−196°C, −80°C, or −20°C), which restrict the growth of microbial contaminants such as bacteria and fungi (Takahashi and Kodama 2020; Tanaka et al. 2016; Wu et al. 2015). Culture-based preservation can be performed under regular temperature conditions (22°C), which allow microbial contaminants to grow. If culture-based preservation is employed, periodic checks for contamination should be performed.

We developed a culture-based preservation method for *M. polymorpha* gemmalings and thalli without encapsulation, drying, or freezing procedures. Using the culture-based method, we achieved 100% recovery of all lines and demonstrated long-term storage (e.g., 1 year). This culture-based method can preserve both gemmalings and thalli and can be performed at regular temperature conditions (22°C). This method will eliminate the need for labor-intensive routine maintenance of *M. polymorpha*.

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| Transgenic line | Experiment 1* | Experiment 2* | Experiment 3* |
|----------------|--------------|--------------|--------------|
| TG#060-1      | 10/10        | 10/10        | 10/10        |
| TG#066-5      | 10/10        | 10/10        | 10/10        |
| TG#164-3      | 10/10        | 10/10        | 10/10        |
| TG#253-6      | 10/10        | 10/10        | 10/10        |

*The denominator and numerator indicate the preserved and recovered numbers of gemmalings, respectively.