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Effects of storage temperature on the physiological characteristics and vegetative propagation of desiccation-tolerant mosses

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Abstract. Mosses, as major components of later successional biological soil crusts (biocrusts), play many critical roles in arid and semiarid ecosystems. Recently, some species of desiccation-tolerant mosses have been artificially cultured with the aim of accelerating the recovery of biocrusts. Revealing the factors that influence the vegetative propagation of mosses, which is an important reproductive mode of mosses in dry habitats, will benefit the restoration of moss crusts. In this study, three air-dried desiccation-tolerant mosses (*Barbula unguiculata*, *Didymodon vinealis*, and *Didymodon tectorum*) were hermetically sealed and stored at five temperature levels (0, 4, 17, 25, and 30 °C) for 40 days. Then, the vegetative propagation and physiological characteristics of the three mosses were investigated to determine the influence of storage temperature on the vegetative propagation of desiccation-tolerant mosses and the mechanism. The results showed that the vegetative propagation of the three mosses varied with temperature. The most variation in vegetative propagation among storage temperatures was observed in *D. tectorum*, followed by the variation observed in *B. unguiculata*. In contrast, no significant difference in propagation among temperatures was found in *D. vinealis*. The regenerative capacity of the three mosses increased with increasing temperature from 0 to 17 °C, accompanied by a decrease in malondialdehyde (MDA) content, and decreased thereafter. As the temperature increased, the chlorophyll and soluble protein contents increased in *B. unguiculata* but decreased in *D. vinealis* and *D. tectorum*. As to storage, the MDA and soluble sugar contents increased after storage. The MDA content of the three mosses increased at each of the investigated temperatures by more than 50 % from the initial values, and the soluble sugar content became higher than before in the three mosses. The integrity of cells and cell membranes is likely the most important factor influencing the vegetative propagation of desiccation-tolerant mosses. A 40-day storage period caused cell injury. Our results suggest that storage temperature can enhance or suppress such injury and change the regenerative capacity of the three mosses. The data indicate that the suitable storage temperature is 4 °C for *B. unguiculata* and 17 °C for both *D. vinealis* and *D. tectorum*.

1 Introduction

Biological soil crusts (biocrusts) are composed of microscopic (cyanobacteria, algae, fungi, and bacteria) and macroscopic (lichens, mosses) poikilohydric organisms (Belnap et al., 2016). Biocrusts are widely distributed in arid and semiarid ecosystems and play important roles in soil surface stabilization, soil fertility enhancement, and soil hydrology regulation (Belnap and Lange, 2003). As major components of later successional biocrusts, mosses exert much stronger ecological functions than cyanobacteria (Seppelt et al., 2016; Gao et al., 2017; Lan et al., 2012). Thus, some researchers suggest artificially culturing moss biocrusts on degraded soil surfaces to accelerate the recovery of degraded arid and semiarid ecosystems (Belnap and Eldridge, 2003; Zhao et al., 2016). Recently, some mosses have been investigated by culturing gametophytes (Jones and Rosentreter, 2006; Xiao et al., 2015). However, cultivation research on moss crusts remains tentative, potentially due to the lack of knowledge regarding the vegetative propagation of mosses.

Vegetative propagation is an important reproduction mode of bryophytes (hornworts, liverworts, and mosses) in dry habitats, and gametophyte fragments may serve as the dominant inoculum in mosses (Mishler, 1988; Tian et al., 2005). To date, several moss cultivation experiments have been conducted in which gametophyte fragments are used to establish new colonies in the laboratory and field (Cleavitt, 2002; Jones and Rosentreter, 2006; Xiao et al., 2015). All of these experiments have demonstrated that artificial cultivation can accelerate the succession process of moss crusts. For example, Antoninka et al. (2016) found that the coverage and biomass of mosses on an artificially inoculated soil surface increased more rapidly than they did on uninoculated soil. Some researchers have suggested that inoculation material should be mass-produced by vegetative regeneration with rapid development (Jones and Rosentreter, 2006; Mishler, 1988) because of the need for moss biocrusts to inoculate large areas. The factors that influence the tissue cultivation of mosses have been investigated for many years (Duckett et al., 2004; Hoffman, 1966; Sabovljevic et al., 2003); however, the mechanism of moss regeneration remains unclear.

After mosses regenerate protonema and gametophytes suffer desiccation stress, desiccation tolerance (DT) has a critical influence on their survival and restoration abilities (Proctor et al., 2007). Adult gametophytes of some species can recover physiological activities and generate new shoots after being stored for more than 10 years in a desiccated state (Stark et al., 2017; Keever, 1957). Desiccation-tolerant mosses can suspend metabolism and maintain cell integrity during dry periods (Mansour and Hallet, 1981; Platt et al., 1994); then, within a few minutes to a few hours after being rehydrated, they can resume cellular activity and return to a normal, hydrated state (Platt et al., 1994; Pressel et al., 2006). However, the decline and disappearance of the regenerative capacity of *Syntrichia ruralis* showed that long-term desiccation can cause irreversible damage, despite viability differences among individuals (Stark et al., 2017). It remains unclear why the potential for vegetative propagation in mosses can be altered by storage and why recovery ability following drought-induced dormancy varies among moss species. The lack of knowledge in these areas has impeded the study of moss cultivation.

Investigations of DT in mosses have primarily focused on the mechanism and evolutionary history (Proctor et al., 2007; Oliver et al., 2000), with fewer investigations addressing DT in artificial cultivation. However, many studies suggest that DT research can help improve artificial cultivation methods. For example, the impact of desiccation stress on moss regeneration varies with drying time and storage temperature (Keever, 1957; Burch, 2003), and an understanding of these relationships may guide research on the regenerative mechanism of mosses upon desiccation and their asexual propagation. Furthermore, DT plays essential roles in moss regeneration in dry habitats, highlighting the potential value of investigating the relationships between the physiological characteristics of mosses and their vegetative propagation. Based on the above observations, it can be hypothesized that (1) dry storage impacts the vegetative propagation of desiccation-tolerant mosses, (2) changes in vegetative propagation after storage involve the influences of storage on the physiological characteristics of mosses, and (3) the degree to which storage affects vegetative propagation and physiological characteristics is related to the storage temperature.

In this study, three desiccation-tolerant mosses, *Barbula unguiculata*, *Didymodon vinealis*, and *Didymodon tectorum*, which are the dominant mosses in biocrust communities in the Loess Plateau region, were stored at five temperatures (0, 4, 17, 25, and 30 °C) for 40 days. Then, (1) the effect of storage temperature on the vegetative propagation of each moss and (2) the changes in physiological indices from before to after storage, including the contents of chlorophyll, soluble sugar, soluble protein, and malondialdehyde (MDA), were investigated to reveal the influences of storage temperature on the vegetative propagation of mosses and the mechanism.

2 Materials and methods

2.1 Study site and moss species

The study was conducted in Ansai Country, Shaanxi Provence, China (36°51′ N, 109°19′ E), which is located in the central part of the Loess Plateau. The elevation of the sampling plot varies from 1068 to 1309 m. The plot has a typical semiarid continental climate, with an average annual temperature of 8.8 °C, and its average temperature in January and July is −7.2 and 22.8 °C, respectively. The average annual precipitation is 500 mm, with 60 % or more of the precipitation falling between June and September (Zhang et al., 2011). For the month of November when the moss crusts were collected, the average monthly precipitation was 11.98 mm, and the average monthly temperature was 9.88 °C (high) to −3.64 °C (low) (Chinese Central Meteorological Station, 2017). Cyanobacteria and mosses dominate the biocrust communities in this region, and the coverage of moss-dominated biocrusts can reach approximately 80 % on north-facing slopes in the study region (Zhao et al., 2014).

The moss taxa used in the study were *Barbula unguiculata*, *Didymodon vinealis*, and *Didymodon tectorum*, which dominated the moss crusts in the plot. *B. unguiculata* dominated in woodland areas and was found in shaded areas and under vegetation coverage. *D. vinealis* was widely distributed in the study site among different water and light environments, and the species were collected from croplands that had been abandoned for more than 10 years. The dominant vegetation of the croplands was grasses; thus, most *D. vinealis* was exposed to sunlight in the winter. *D. tectorum* grew on side slopes and was occasionally collected from under the shade of vascular plants.

2.2 Experimental design

Some of the three moss crusts were used to measure initial values of physiological indices (chlorophyll content, soluble sugar content, soluble protein content, and MDA content) and germination parameters (gametophyte germination, gametophyte increment, and gametophyte vigor index) immediately following their transport to the laboratory. The rest of the moss crusts were stored at one of five temperature levels, i.e., 0, 4, 17, 25, and 30 °C. Each temperature was controlled within ±1 °C around the target. On the 41st day of storage, the moss crusts were removed, and the physiological indices and germination parameters described above were measured.

2.3 Moss crusts storage and mosses collection

The crusts of three species of mosses were collected from many colonies and then air-dried in the shade for 24–48 h; most of crust samples were dried in the field. Then, the samples were transported to the laboratory of the State Key Laboratory of Soil Erosion and Dry-land Farming on the Loess Plateau in Yangling, Shaanxi Province. Samples were stored in one of two refrigerators (at 0 or 4 °C) or one of three growth chambers (at 17, 25, and 30 °C). Before storage, the moss crusts had been placed in resealable plastic bags to prevent changes in water content. The samples were stored in the dark under light-blocking fabric. The water content measurements of the moss gametophytes were all less than 10 %, and the equilibrating relative humidity during storage was 55 %. After the 40-day dry period, subsamples of desiccated gametophytes were collected to measure the physiological indices and germination parameters.

2.4 Measurement of the physiological indices and germination parameters

2.4.1 Physiological indices

Living mature gametophytes of *B. unguiculata*, *D. vinealis*, and *D. tectorum* were collected from the moss crusts. Shortly after being rehydrated and washed with deionized water, the gametophytes were measured for the contents of chlorophyll, soluble sugar, soluble protein, and MDA. Approximately 0.1 g fresh mass of gametophytes was used to measure the contents of soluble sugar, soluble protein, and MDA in each replicate, whereas the measurements of chlorophyll content used approximately 0.05 g fresh mass per replicate. The four indicators were measured by using the following protocols with three replications.

The chlorophyll was extracted by 95 % (v/v) ethanol, and the solution was boiled at 85 °C for 5 min. After being centrifuged at 4000 rpm for 10 min, the chlorophyll in the supernatant was measured at absorbances of 665 and 649 nm with a spectrophotometer (UV-2300; Techcomp, Shanghai, China; Wellburn and Lichtenthaler, 1984).

After the soluble protein was extracted in ice-cold 50 mmol L^{-1} phosphate buffer (pH 7.8), the suspension was centrifuged at 8000 rpm for 30 min at 4 °C, and the supernatant was collected. The soluble protein was stained with Coomassie brilliant blue G-250, and the absorbance was read at 595 nm (Bradford, 1976).

MDA and soluble protein were extracted and centrifuged. Then, the supernatant was homogenized with 0.6 % (W/V) thiobarbituric acid dissolved by 1 mol L^{-1} NaOH and 10 % (W/V) trichloroacetic acid. The mixed solution was heated at 100 °C for 20 min, and then the absorbance was read at 450, 523, and 600 nm (Hodges et al., 1999). The Techcomp UV-2300 spectrophotometer was used to measure the absorbance of the MDA and soluble protein.

Soluble sugar was extracted by distilled water at 100 °C for 30 min. After being filtered and diluted, the extract was added to an anthrone–sulfuric acid solution. The mixed solution was used to measure the absorbance at 620 nm with a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan; Morris, 1948).

The fresh weight of gametophytes was measured shortly after rehydration, and dry weight was measured after oven drying to a constant weight at 70 °C (Schonfeld et al., 1988). The fresh and dry weights were used to calculate the four physiological indices on a dry basis.

2.4.2 Germination parameters

At the same time as the physiological indices was measured, some gametophytes of each of the three moss species were collected to measure the germination parameters. The loessial soil (uniform soil texture of Calciustepts) collected from the study region was used to culture the mosses. The soil was sieved through a 0.25 mm mesh and placed in each pore of a six-well plate; each pore had a diameter of 35 mm and a depth of 12 mm. Then, the soil water content was adjusted to 23 % (W/W) (the field water-holding capacity of the soil) by adding deionized water, and the surface was flattened before inoculation. Five inocula representing the top 2 mm of living mature gametophytes of the mosses were cut, rehydrated, washed, and placed in each well. Thirty inocula were placed in each six-well plate as one replication. Three six-well plates were established for each moss species. In total, 90 experimental inoculations were established for the measurement of germination parameters before and after storage at each of the five temperature levels for each moss species. Meanwhile, three six-well plates without inoculated mosses were set up as experimental controls for the effect of other propagules, such as spores, in the experimental soil. The six-well plates were wrapped tightly with transparent plastic film to retain the soil moisture. Next, they were placed into a growth chamber (AGC-D003N; Qiushi, Hangzhou, China) to incubate. The parameters of the growth chamber were set to a 12 h photoperiod (4500–5500 Lux), a constant temperature of 17 °C (±1 °C), and a relative humidity of 60–70 %.

During the incubation period, deionized water was supplied to maintain the soil moisture at 23 %. The new gametophytes were counted every 5 days beginning on the day they were found. Five observations were made over the subsequent 25 days. This paper reports the results of cultivation at the fifth observation. No new gametophytes were found in the blank six-well plates during the entire incubation period. It was difficult to distinguish protonemal germination between the underside of original inocula and the soil substrate; therefore, protonemal growth was not quantified.

By analogy with seed germination, the vegetative propagation of moss gametophytes was described by three germination parameters: gametophyte germination, gametophyte increment and the gametophyte vigor index. In this paper, gametophyte germination is defined as the percent of moss inocula that germinated. Gametophyte increment is the average number of new gametophytes per six-well plate. The gametophyte vigor index is analogous to the seed vigor index, which is calculated by multiplying the seed germination percentage by the length of the hypocotyl (Abdul-baki and Anderson, 1973). Here, the seed germination percentage and the length of hypocotyl were replaced by the gametophyte germination and gametophyte increment, respectively, and used to calculate the gametophyte vigor index. Thus, the germination parameters were calculated by using Eqs. (1)–(3).

$$\text{gametophyte germination} = \frac{\text{number of germinated inocula}}{\text{number of total inocula}} \times 100\,\% \quad (1)$$

$$\text{gametophyte increment} = \frac{\text{number of new gametophyte}}{\text{number of total inocula}} \quad (2)$$

$$\text{gametophyte vigor index} = \text{gametophyte germination} \times \text{gametophyte increment} \quad (3)$$

According to Eqs. (1)–(3), the gametophyte vigor index summarizes the vegetative propagation of the mosses.

2.5 Statistical analyses

The differences in physiological indices and germination parameters among treatments and mosses were tested using one-way analysis of variance (ANOVA) with Fisher's least significant difference post hoc test (LSD) at $P < 0.05$. The relationships between the physiological indices and germination parameters of the three moss species were quantified by calculating Pearson correlation coefficients. These statistical analyses were completed using SPSS 22.0.

The effects of physiological characteristics on vegetative propagation were analyzed by a gray incidence analysis in Microsoft Excel 2010 (Deng, 1982; Lin et al., 2009). The gray incidence degree between each of the reference sequences (physiological indices) and the compared sequence (gametophyte vigor index) was calculated by using Eqs. (4)–(6):

$$\Delta_i(k) = |y(k) - x_i(k)|, k = 1, 2, \ldots\ldots, n; i = 1, 2, 3, 4 \quad (4)$$

$$\xi_i(X_i, Y) = \frac{\min_i \min_k \Delta_i(k) + \rho \max_i \max_k \Delta_i(k)}{\Delta_i(k) + \rho \max_i \max_k \Delta_i(k)}, \quad k = 1, 2, \ldots\ldots, n; \quad i = 1, 2, 3, 4, \quad (5)$$

$$r_i = \frac{1}{n}\sum_{k=1}^{n} \xi_i(k), \quad k = 1, 2, \ldots\ldots, n; \quad i = 1, 2, 3, 4. \quad (6)$$

where $\Delta_i(k)$ and $\xi_i(X_i, Y)$ are the absolute difference and the gray relational coefficient, respectively, between X_i (physiological index i) and Y (gametophyte vigor index) at point k. The gray relational coefficient (r_i) is between the ith physiological index and its gametophyte vigor index when the distinguishing coefficient (ρ) is 0.5.

The gray incidence degree is the sum of the gray relational coefficients.

3 Results

3.1 The initial measurement values of the mosses

The three moss species began to germinate new gametophytes from the original inocula at different times, whereas no gametophyte germinated in the control groups as of the final (fifth) observation. *B. unguiculata* germinated on the 11th day of inoculation, and the entire length of its cultivation period was 35 days. *D. vinealis* and *D. tectorum* each germinated on the sixth day, with a 30-day cultivation period. The initial values of the physiological indices and germination parameters of the three mosses are shown in Table 1. It can be seen that the four physiological indices and gametophyte germination of *D. vinealis* were significantly higher than those of the other two species. The largest values of gametophyte increment and gametophyte vigor index were found in *D. tectorum*, and the lowest germination parameter values were found in *B. unguiculata*. However, no significant differences in the contents of chlorophyll, soluble protein, and MDA between *D. tectorum* and *B. unguiculata* were found.

3.2 Effect of storage temperature on the vegetative propagation of mosses

The germination times of each of the three mosses after storage at each temperature did not differ significantly from the initial values, whereas controls still had no gametophyte. At the fifth observation, the gametophyte germination of each of the three species had changed from the initial value by no more than 20 % (Fig. 1a; Table 1). The highest gametophyte germination of *B. unguiculata* was 94.44 % at 17 °C. No significant difference was found between the maximum value and minimum value (75.56 % at 0 °C). In *D. vinealis*, gametophyte germination did not significantly differ among the storage temperatures and ranged from 95.56 % (0 °C) to

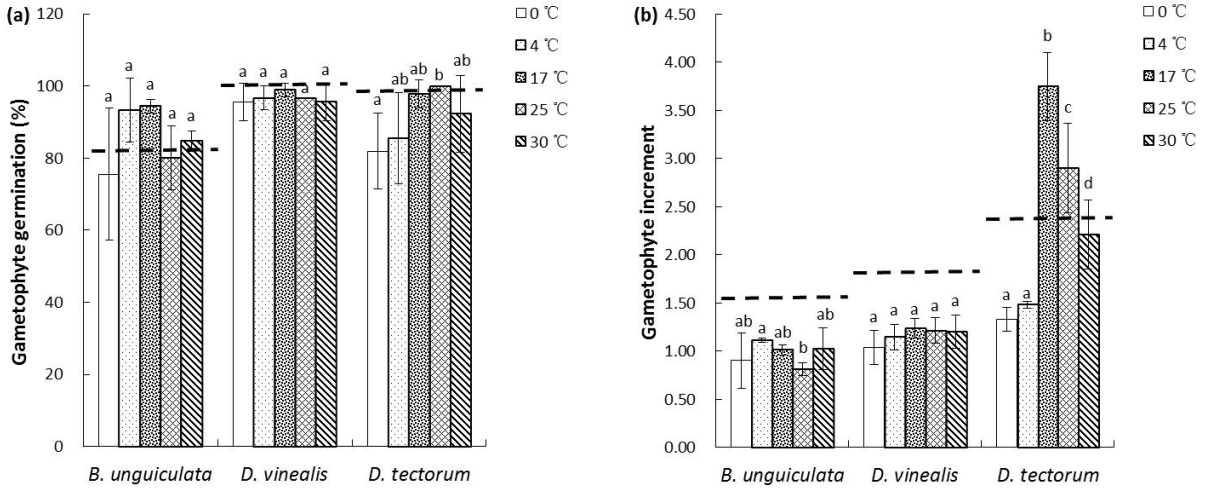

Figure 1. Data (average ±1 SE) for the three moss species on **(a)** gametophyte germination and **(b)** gametophyte increment after the 40-day storage period at each of the five temperatures. Different letters indicate significant differences ($P < 0.05$) among the five temperatures within the same species. Dotted lines represent the approximate values of the two germination parameters before storage for each species (the true values are shown in Table 1).

Table 1. Initial values of physiological indices and germination parameters in the three mosses.

Index	*B. unguiculata*	*D. vinealis*	*D. tectorum*
Chlorophyll content ($mg\,g^{-1}$)	1.53 ± 0.13a	3.33 ± 0.18b	2.19 ± 0.44a
Soluble sugar content ($mg\,g^{-1}$)	30.02 ± 3.67a	44.13 ± 3.41b	14.19 ± 1.77c
Soluble protein content ($mg\,g^{-1}$)	6.28 ± 1.40a	12.24 ± 0.26b	7.92 ± 0.46a
MDA content ($\mu mol\,g^{-1}$)	24.02 ± 0.47a	35.07 ± 3.12b	23.68 ± 0.50a
Gametophyte germination (%)	82.93 ± 10.00a	100.00 ± 0.00a	98.33 ± 2.36a
Gametophyte increment	1.54 ± 0.18a	1.82 ± 0.40ab	2.37 ± 0.05b
Gametophyte vigor index	1.28 ± 0.15a	1.82 ± 0.40ab	2.33 ± 0.05b

Data are average ±1 SE, and different letters indicate significant differences ($P < 0.05$) among the three species.

98.89 % (17 °C). The only significant difference in gametophyte germination was observed in *D. tectorum* and was between 81.92 and 100 % after storage at 0 and 25 °C, respectively.

The changes in gametophyte increment were all more than 20 % after storage except in *D. tectorum* at 30 °C, for which a slight decrease of 6.57 % was observed (Fig. 1b; Table 1). After storage, the largest gametophyte increment of *B. unguiculata* was 1.11 at 4 °C, whereas the smallest gametophyte increment was 0.81 at 25 °C. Except for a significant difference between 4 and 25 °C, no significant difference in gametophyte increment was found among the storage temperatures in *B. unguiculata*. Similarly, no significant difference in the gametophyte increment of *D. vinealis* was observed among the storage temperatures. The maximum and minimum gametophyte increments after storage were 1.03 and 1.23 at 0 and 17 °C, respectively, for *D. vinealis*. Larger differences in gametophyte increment among the storage temperatures were observed in *D. tectorum* except for the difference in gametophyte increment between 0 and 4 °C. The maximum gametophyte increment of *D. tectorum* was 3.74 at 17 °C after storage, and the minimum value was 1.32 at 0 °C.

The gametophyte vigor index of the three moss species showed significant changes over the 40-day storage period (Table 2). The largest changes in gametophyte vigor index after storage were observed in *D. tectorum*, with the index ranging from a 53.36 % decrease (0 °C) from the initial value to a 57.32 % increase (17 °C). No significant difference in the gametophyte vigor index among the five temperatures was observed in *D. vinealis*. However, the index values were all significantly lower than the initial value (before storage), representing decreases of 32.86 % (17 °C) to 45.65 % (0 °C). After storage, the gametophyte vigor index values of *B. unguiculata* decreased the least by 18.81 % at 4 °C and the most by 49.20 % at 25 °C, representing changes between those of *D. vinealis* and *D. tectorum*.

After the 40-day storage at the five temperatures, the highest gametophyte germination percentages of *B. unguiculata* and *D. vinealis* were at 17 °C, whereas the highest percent-

Table 2. Gametophyte vigor index of the three mosses under treatments.

Treatment	*B. unguiculata*	*D. vinealis*	*D. tectorum*
Initial value	1.28 ± 0.15a	1.82 ± 0.40a	2.33 ± 0.05a
0 °C	0.68 ± 0.22b	0.99 ± 0.17b	1.09 ± 0.10b
4 °C	1.04 ± 0.02ac	1.11 ± 0.13b	1.26 ± 0.03b
17 °C	0.95 ± 0.05c	1.22 ± 0.10b	3.66 ± 0.35c
25 °C	0.65 ± 0.06b	1.17 ± 0.13b	2.90 ± 0.46a
30 °C	0.86 ± 0.18bc	1.15 ± 0.17b	2.04 ± 0.33a

Data are average ± 1 SE, and different letters indicate significant differences ($P < 0.05$) among treatments within the same species.

age in *D. tectorum* was at 25 °C. The highest gametophyte increment of *B. unguiculata* was at 4 °C. The highest gametophyte increment values in *D. vinealis* and *D. tectorum* were both at 17 °C, as observed for the gametophyte vigor index values of these two species.

3.3 Effects of storage temperature on the physiological indices of mosses

As shown in Table 1 and Fig. 2a, the chlorophyll content of *B. unguiculata* increased after storage at four of the five temperatures, i.e., all but 0 °C. The chlorophyll content of *B. unguiculata* showed an increasing trend with increasing storage temperature, with the maximum increase of 73.08 % observed at 30 °C. The smallest change in chlorophyll content was observed in *D. vinealis*, which showed a maximum decrease of 17.89 % at 4 °C and a minimum decrease of 2.39 % at 17 °C. The chlorophyll content of *D. tectorum* after storage was decreased by 31.51 % at 17 °C and increased by 18.50 % at 25 °C, yielding the highest and lowest content values, respectively.

A similar increasing trend with temperature was found for soluble sugar content (Fig. 2b). The soluble sugar content was consistently higher after storage than before except in *B. unguiculata*, in which sugar content was decreased by 56.52 and 40.47 % at 0 and 4 °C, respectively (Fig. 2b; Table 1). The soluble sugar content of *D. vinealis* showed less variation than the other species. No significant difference was found between the minimum and maximum increases, which were 9.92 % at 0 °C and 23.14 % at 25 °C, respectively. The greatest changes in soluble sugar content, with greater than 65 % increases at all storage temperatures, occurred in *D. tectorum*.

MDA content showed greater variation than sugar content, increasing by more than 50 % in all stored gametophytes (Fig. 2d; Table 1). The MDA content of both *B. unguiculata* and *D. tectorum* decreased as the temperature increased from 0 to 17 °C; the minimum value of MDA content (at 17 °C) was 1.70 times and 2.06 times the initial value, respectively. However, the MDA content of *D. vinealis* was 1.54 to 2.98 times the initial value after storage and continuously decreased with increasing temperature.

Some temperatures caused the soluble protein content to change significantly (Fig. 2c; Table 1). The soluble protein content of *B. unguiculata* increased abruptly from a 31.79 % decrease from the initial value to a 40.06 % increase with increasing temperature. In contrast, soluble protein showed the opposite trend in *D. vinealis* and *D. tectorum*. Both species presented a maximum increase at 0 °C, which was 16.64 % in *D. vinealis* and 23.65 % in *D. tectorum*. The lowest soluble protein content of *D. vinealis* and *D. tectorum* represented a decrease of 16.00 % at 25 °C and a decrease of 21.38 % at 30 °C, respectively.

Our results indicated that the sharpest changes in chlorophyll content and soluble protein content with increasing temperature were observed in *B. unguiculata*; furthermore, soluble sugar content and MDA content changed more rapidly with increasing temperature in this species than in *D. vinealis* and *D. tectorum* (Fig. 2a–d; Table 1). *D. vinealis* showed slower changes in chlorophyll, soluble sugar, and soluble protein contents with increasing temperature than the other two species. MDA content, however, varied widely with temperature. The largest increases in soluble sugar content and MDA content after 40 days of storage were observed in *D. tectorum*. In all three moss species, the greatest changes were observed in MDA content, followed by soluble sugar content (Fig. 2b and d; Table 1).

3.4 Relationships between physiological characteristics and the vegetative propagation of mosses

After analyzing the correlations between the physiological indices and germination parameters of the desiccation-tolerant mosses, a significant correlation ($P < 0.01$) was found between each physiological index except for chlorophyll content and MDA content (Table 3). Gametophyte germination was significantly correlated ($P < 0.05$) with soluble protein content and highly significantly correlated ($P < 0.01$) with both chlorophyll content and soluble sugar content. MDA content was significantly negatively correlated ($P < 0.05$) with both gametophyte increment and gametophyte vigor index.

At a distinguishing coefficient of 0.5, the gray incidence degrees between the physiological indices (X1: chlorophyll content; X2: soluble sugar content; X3: soluble protein content; X4: MDA content) and the gametophyte vigor index in the three moss species were (1) X4 > X1 > X2 = X3 in *B. unguiculata*, (2) X3 > X4 > X2 > X1 in *D. vinealis*, and (3) X4 > X3 > X1 > X2 in *D. tectorum* (Table 4).

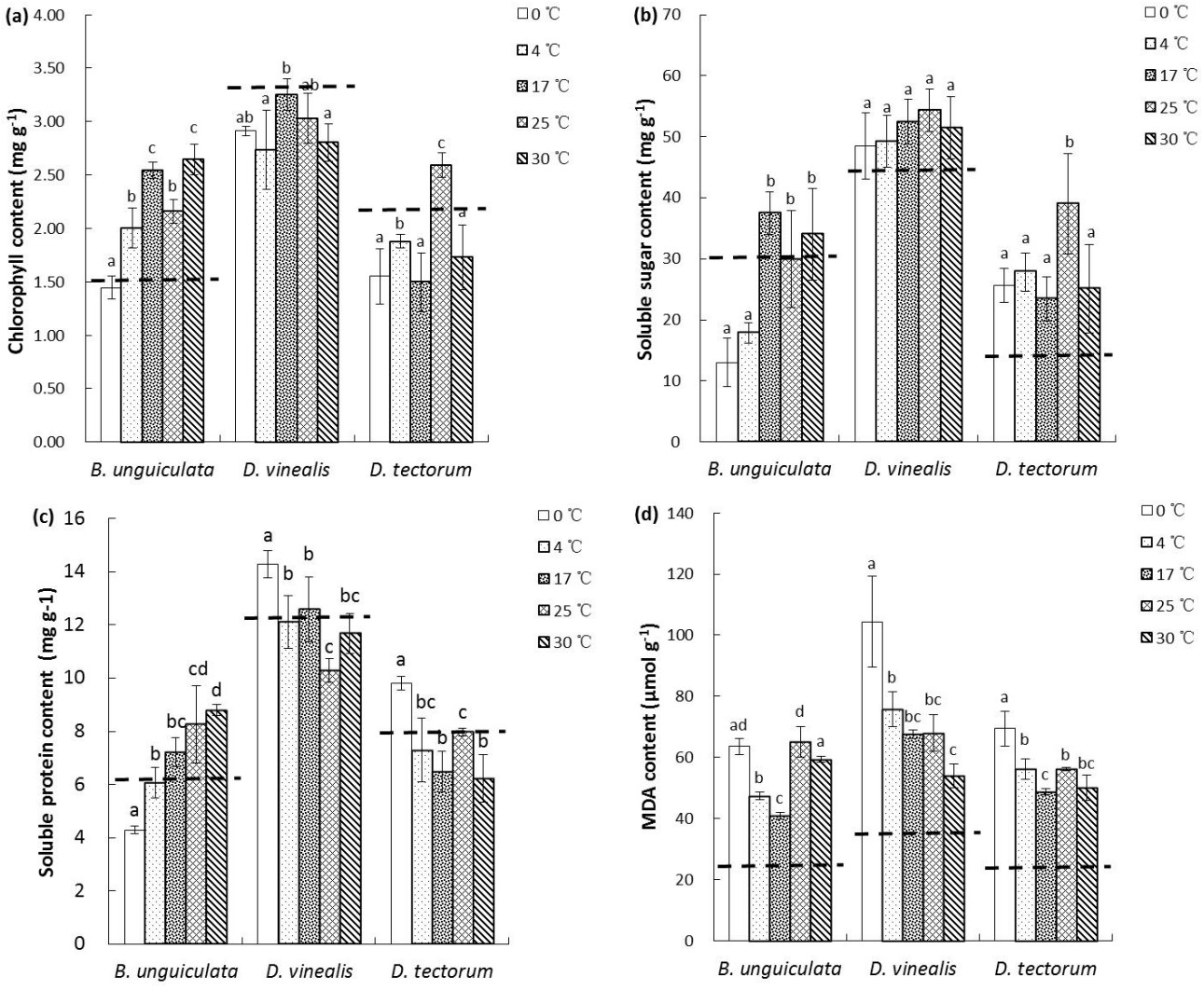

Figure 2. (a–d) Data (average ± 1 SE) for the three moss species on **(a)** chlorophyll content, **(b)** soluble sugar content, **(c)** soluble protein content, and **(d)** MDA content after the 40-day storage period at each of the five temperatures. Different letters indicate significant differences ($P < 0.05$) among the five temperatures within the same species. Dotted lines represent the approximate values of the two germination parameters before storage for each species (the true values are shown in Table 1).

Table 3. Correlation coefficients between physiological indices and germination parameters across all mosses and treatments.

Variables	Chlorophyll	Sugar	Protein	MDA	Germination	Increment
Sugar	0.762**					
Protein	0.747**	0.781**				
MDA	0.220	0.402**	0.510**			
Germination	0.473**	0.414**	0.313*	−0.022		
Increment	−0.239	−0.187	−0.249	−0.344*	0.388**	
Vigor index	−0.158	−0.122	−0.191	−0.328*	0.441**	0.995**

Chlorophyll: chlorophyll content; sugar: soluble sugar content; protein: soluble protein content; MDA: MDA content; germination: gametophyte germination; increment: gametophyte increment; vigor index: gametophyte vigor index. The * symbol indicates a significant correlation at $P < 0.05$; ** indicates a significant correlation at $P < 0.01$.

Table 4. Gray incidence degree between physiological indices and the gametophyte vigor index across all treatments.

Reference sequences	*B. unguiculata*	*D. vinealis*	*D. tectorum*
Chlorophyll content (X1)	0.60 ± 0.20	0.55 ± 0.27	0.66 ± 0.21
Soluble sugar content (X2)	0.57 ± 0.20	0.62 ± 0.23	0.62 ± 0.17
Soluble protein content (X3)	0.57 ± 0.22	0.74 ± 0.28	0.70 ± 0.25
MDA content (X4)	0.77 ± 0.20	0.73 ± 0.22	0.76 ± 0.27

4 Discussion

4.1 Effects of storage temperature on the vegetative propagation of mosses

For more than a century, researchers have studied many aspects of mosses, such as inocula, pretreatment (e.g., storage and sterilization), culture methods, and culture conditions (Duckett et al., 2004; Hoffman, 1966). Some of these studies have implied that the physiological characteristics of moss gametophytes are closely related to the success of artificial cultivation; for example, pretreatment with sucrose and/or abscisic acid can improve the viability of mosses by increasing DT (Burch and Wilkinson, 2002). In line with previous studies, this study found that gametophyte regeneration within the same species after desiccation varied among different temperatures (Fig. 1a and b; Table 2), which is likely related to species-specific DT. The regenerative capacity of mosses can be summarily described by the gametophyte vigor index on the basis of Eqs. (1)–(3) and Table 3. The gametophyte vigor index most sensitive to storage temperature was that of *D. tectorum*, whereas that of *D. vinealis* varied little with storage temperature, with no significant differences among temperatures (Table 2). Thus, the effect of storage temperature on regenerative capacity was strongest in *D. tectorum* and weakest in *D. vinealis*.

The 40-day storage period adversely affected regeneration in most gametophytes (Fig. 1a and b; Table 1); however, some gametophytes of *D. tectorum* stored at 17 and 25 °C produced more new shoots than before. It is not clear whether this enhanced regeneration was associated with the low-temperature tolerance of *D. tectorum*. *D. tectorum* possibly suffered low-temperature stress in early winter. Furthermore, higher temperatures (e.g., 30 °C) injured the gametophytes of *D. tectorum*, as did the lower temperatures of 0 and 4 °C. These findings suggest that extreme temperatures are unsuitable for storing this moss species. Further studies are warranted on the impact of the storage environment on desiccation-tolerant mosses. For example, Burch (2003) found that the survival and regeneration of dehydrated protonemata were reduced after cryopreservation due to damage caused by intracellular ice crystals. The desiccation time can also affect the restorability of vegetative propagation in desiccation-tolerant mosses and their physiological characteristics (Keever, 1957; Proctor, 2001). Environmental changes or variation in the dormancy period of cells might influence the restoration results after rehydration.

4.2 Effects of storage temperature on the physiological characteristics of mosses

MDA, an important product of membrane lipid peroxidation, increased in all mosses over the storage period. This finding indicated that the 40-day storage period caused cell damage (Fig. 2d; Table 1). Accordingly, the soluble sugar content increased to protect the membranes and proteins in the dried gametophytes (Fig. 2b; Table 1). Sugars are the main substance used to stabilize protein structures in desiccation-tolerant cells (Hoekstra et al., 2001). However, the soluble sugar content of *B. unguiculata* stored at 0 and 4 °C was decreased relative to the initial value. This result might have been due to the low temperatures preventing the conversion from starch to soluble sugar (Pressel et al., 2006). When mosses suffered oxidative damage, the increases in chlorophyll content and soluble protein content in some gametophytes were related to the recovery ability of desiccation-tolerant cells (Fig. 2a and c; Table 1). In previous studies, the chlorophyll content of mosses increased during desiccation, and their photosynthetic capacity recovered rapidly after rewetting (Alpert, 1988; Csintalan et al., 1999). Similarly, protein synthesis recovered after rehydration (Oliver, 1991) since cellular recovery is an important part of DT (Proctor et al., 2007).

The recovery of photosynthesis and protein synthesis in *B. unguiculata* was facilitated by higher temperatures (not more than 30 °C; Fig. 2a and c). This finding is inconsistent with the pattern in other mosses, in which viability tends to be lower at increased temperatures (Hearnshaw and Proctor, 1982). However, the increasing trend of MDA content from 17 to 30 °C suggests that more extensive membrane damage may be caused by storage temperatures above 30 °C (Fig. 2d). The adverse effects of the higher temperatures in *D. vinealis* and *D. tectorum* were clearly reflected by the slower recovery of photosynthesis and protein synthesis (Fig. 2a and c). The changes in the MDA content in *D. vinealis* suggested more rapid repair of cell membrane with increasing temperature; however, the species possibly had stronger tolerance under the protection of abundant sugars when the recovery of photosynthesis and protein synthesis was slower (Fig. 2a–d).

The responses of the physiological characteristics of the three species to temperature reflected species variation in restoration ability over a short rehydration time. Because the rewetting periods were longer than 30 days in the cultivation, the vegetative propagation results can be considered as reflecting the long-term recovery of mosses. Thus, the long-term effect of cell recovery during short-term rehydration can be explained by the relationships between the physiological characteristics and vegetative propagation of desiccation-tolerant mosses.

4.3 Relationships between physiological characteristics and the vegetative propagation of mosses

Before storage, the four physiological indices of gametophytes showed significant differences between *D. vinealis* and *D. tectorum*. However, no significant differences between the two species were observed in regard to the three germination parameters (Table 1). Mosses of similar fertility showed significant differences in physiological characteristics. Species differences in DT led to larger differences in vegetative propagation among species than before, as evidenced by the values of the gametophyte vigor indices within the same treatment (Tables 1 and 2). Therefore, the recovery ability of dried mosses with respect to development and regeneration might be more informative for screening suitable inocula than using fresh mosses in dry habitats. Many studies have indicated that desiccation-tolerant mosses can recover from drying once they are rehydrated (Csintalan et al., 1999; Pressel et al., 2006). However, long periods of desiccation would impede the reuse of moss specimens and the restoration of dried biocrusts. This study showed that cells were subjected to oxidative damage after the 40-day desiccation period (Fig. 2d; Table 1). Over this period, the regenerative capacity of the three species declined (Table 2), which suggested that membrane integrity and/or other factors affected the vegetative propagation of the desiccation-tolerant mosses.

Based on the correlation coefficients among the physiological indices and germination parameters of desiccation-tolerant mosses (Table 3), gametophyte germination was significantly and positively correlated with chlorophyll content, soluble sugar content, and soluble protein content. In addition, gametophyte increment and gametophyte vigor index were significantly and negatively correlated with MDA content. These findings are in accordance with the observations that metabolic repair is favorable to the germination of new gametophytes and that long-term recovery is more dependent on cell integrity than metabolic repair. Therefore, to quantitatively compare the effects of the four physiological indices on vegetative propagation, the gray incidence degree between the physiological indices and the gametophyte vigor index for each of the three moss species was calculated by using Eqs. (4)–(6). As shown in Table 4, the effect of MDA content on the gametophyte vigor index was the strongest in *B. unguiculata* and *D. tectorum*, and the incidence degree of MDA (0.73) in *D. vinealis* was similar to the maximum (0.74). In all three mosses, MDA content increased as storage temperature decreased from 17 to 0 °C. Smaller gametophyte vigor index values were observed for *D. vinealis* and *D. tectorum* at 0 and 4 °C than at 25 and 30 °C (Fig. 2d; Table 2). This result indicated that the greater membrane damage incurred at low temperatures caused the decline in regenerative capacity. In addition, the higher gametophyte vigor index values of *D. tectorum* at 17 and 25 °C than before storage were possibly related to the reduced formation of intracellular ice crystals at these temperatures during the storage period (Burch, 2003), which facilitated more rapid recovery upon rehydration (Table 2). However, the number of negative effects on physiological characteristics increased with increasing temperature (Fig. 2a–c). The high temperatures were unfavorable to the recovery of the mosses (Hearnshaw and Proctor, 1982). When cells suffered damage under desiccation and temperature stress, the protection provided by additional sugars was important for maintaining cell integrity in the dry state (Fig. 2d; Table 1). *D. vinealis* showed no significant difference in regenerative capacity among temperatures, potentially because the level of cellular protection was equivalent among the different temperatures.

Researchers have summarized the recovery mechanisms of mosses upon rehydration, such as the rapid recovery of photosynthesis, respiration, and protein synthesis within minutes to hours (Proctor et al., 2007). However, recovery of the carbon balance, cell cycle, and the cytoskeleton require more than 24 h (Alpert and Oechel, 1985; Mansour and Hallet, 1981; Pressel et al., 2006). Based on these results, it has been speculated that cell integrity is more difficult to recover than physiological reactions and that cell integrity greatly limits the recovery and regenerative capacity of desiccation-tolerant mosses. Over long-term desiccation, the cumulative damage affects cell function and integrity (Proctor, 2001); different temperatures might enhance or suppress such cell damage. Thus, the effects of temperature on the ecology of DT in bryophytes warrant investigation, especially during the dry season in semiarid and arid areas. The greater sensitivity of *D. tectorum* observed here might provide insight into why this species is not a widely distributed species, such as *D. vinealis*, in the study region. Furthermore, the ecological niche requirements of different mosses in both dry and wet periods will influence the choice of moss inocula for artificial cultivation and biocrust restoration. Field studies are needed to better understand the ecological requirements of dried mosses. Furthermore, a precise description of microclimates and the application of quantitative methods would be helpful.

5 Conclusions

The conducted experiment explored the effect of storage temperature on the vegetative propagation of desiccation-tolerant mosses and influencing factors. The results indicated that the decline in regenerative capacity in mosses observed following storage was related to cell damage caused by dehydration during storage. The storage temperature during dehydration influenced the vegetative propagation of mosses through changes in moss cell activity. Further analysis showed that the factor with the strongest effect on vegetative propagation was membrane damage. During storage, soluble sugars increased to protect the cells, highlighting the important role of cell integrity in influencing the physiological characteristics and vegetative propagation of desiccation-tolerant mosses. In this study, the optimal storage temperature of *D. vinealis* and *D. tectorum* was 17 °C, whereas the optimal temperature for *B. unguiculata* was 4 °C. Different responses to temperature among the three moss species were associated with species differences in DT. These findings can potentially guide future research on suitable storage methods for inoculation material to improve the artificial cultivation of moss biocrusts.

In general, the properties of inoculation material are key factors affecting the development and recovery of moss biocrusts, such as species, physiological features, and/or other factors. The results provide insight into the factors that influence the vegetative propagation of desiccation-tolerant mosses and highlight the potential applicability of a rapid experimental approach for screening suitable inocula.

Data availability. Currently, data can only be accessed in the form of Excel sheets via contact with the corresponding author.

Competing interests. The authors declare that they have no conflict of interest.

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