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From spore germination to gametophyte development: the culture, propagation and anatomical protonemal structure of *Takakia lepidozioides* (Bryophyta) in Tibet Plateau

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Abstract – *Takakia lepidozioides* is a rare and potentially relictual species of a genus that diverged early in the evolution history of mosses. Plants of *Takakia* grow very slowly in the wild and are almost too small (only ~ 5 mm high) to be genetically manipulated. We collected *Takakia* plants with mature spore-bearing capsules from the Nyingchi region at about 4,000 meters elevation in the Tibetan Plateau. We analyzed the pH and inorganic elements of the soil below the population to then optimize the composition of the Beneck medium that is generally suitable for moss culture. We explored the effects of several factors on spore germination and gametophyte growth and established the conditions for efficient spore germination and rapid gametophyte propagation. The characterization of spore germination and early gametophyte development revealed that the sporeling of *Takakia* is thalloid as in *Sphagnum* and *Andreaea*. These results provide the foundation to facilitate genetic, physiological and biochemical research with *T. lepidozioides* and also developmental studies, which may contribute to our understanding of the morphological transformations in the early diversification of mosses.

***Takakia lepidozioides* / spore / protonema / gametophyte / propagation / Tibetan Plateau**

INTRODUCTION

Takakia S. Hatt. & Inoue is characterized by traits that are unique among mosses, such as the divided vegetative leaf and the spiral line of dehiscence of the sporangium (Schuster, 1997; Renzaglia, 1997). The genus is deeply rooted in the moss tree of life and may be sister to all other mosses or share a unique ancestor with *Sphagnum* L. (e.g., Newton *et al.*, 2000; Qiu *et al.*, 2006; Chang & Graham, 2011). *Takakia* comprises two species: *T. lepidozioides* S. Hatt. & Inoue and *T. ceratophylla* (Mitt.) Grolle. *Takakia ceratophylla* was originally described by Mitten (1861) as a hepatic (i.e., *Lepidozia ceratophylla* Mitt.). The species remained in obscurity until Dr. Takaki discovered plants, for which Hattori and Inoue (1958) erected the genus *Takakia*. The affinities of *Takakia* to liverworts and mosses remained ambiguous (Inoue, 1961; Mizutani, 1967; Mizutani, 1972; Crandall-Stotler,

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1986; Murray, 1988) until the discovery of antheridia and sporophytes firmly established it as a moss (Smith & Davison, 1993). This hypothesis was subsequently supported by phylogenetic inferences from DNA sequences (Newton *et al.*, 2000; Qiu *et al.*, 2006; Chang & Graham, 2011; Volkmar & Knoop, 2010).

Takakia is known from Western North America and South and Eastern Asia. It is reported from Japan (Hattori & Inoue, 1958; Schofield, 1972), Gary Mandan of Indonesia (Hattori, 1963), Sikkim (Grolle, 1963), Nepal (Hattori *et al.*, 1973), the Himalayan region of China (Aleutian Island, Alaska of USA, coastal British Columbia and Western Islands of Canada (Persson, 1958; Sharp & Hattori, 1967; Smith, 1978; Hong, 1987). In China, *T. lepidozioides* has been collected from fabri forests at altitude of about 4000 m in Galongla Pass, Tibet Plateau (Wu *et al.*, 1983) and *T. ceratophylla* has been collected from Deqin County, Yunnan Province (Higuchi & Zhang, 1998). The eastern Himalayas of Tibet Plateau, China may be the current distribution center of *Takakia* (Hattori *et al.*, 1974).

Wild plants of *Takakia* are short and have little biomass, which severely limits the in-depth study on its genetics, physiology and biochemistry. In 1979, *T. ceratophylla in vitro* cultures were established and calluses were obtained, however, growth was very slow (Crandall-Stotler & Bozzola, 1988). Therefore, establishing an efficient sterile culture system for *Takakia* is of great significance. The aim of this work is to determine the optimal conditions (light, temperature, medium, pH, nutrients and so on) for culturing *T. lepidozioides.* Based on the composition and pH of soils in the original habitat and the previous reports, we established the optimal media for spore germination and protonemal development. Moreover, we also investigated the characterization of spore germination and early gametophyte development, which reveals that the sporeling of *Takakia* is thalloid as in *Sphagnum* and *Andreaea*.

MATERIALS AND METHODS

Materials. *Takakia lepidozioides* plants bearing mature spore capsules, soil and water samples were collected in September and October in 2007, in shady hillside areas at about 4,000 m elevation, between Bomi and Medog County, Nyingchi Region, Tibet, China.

Analysis of soil samples. Soil samples were passed through a sieve selecting for particles less than 2 mm in size, and the particles smaller than 2 mm were air-dried and 10 g were then added to 25 mL de-CO_2 water in a 50 mL beaker and stirred for 1 min. The solution was then kept at room temperature for 30 min, and the supernatant was collected to measure soil pH using a PHB-4 portable acidimeter. Soil composition including ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), copper (Cu), manganese (Mn), zinc (Zn), iron (Fe) and boron (B) were determined using conventional methods (Vaisanen *et al.*, 2000).

Growing medium. Sterile cultures from spores (see Ono *et al.*, 1988) were initiated on sterile Knop, Beneck, BCD, and MS media (Lal, 1984). Mature capsules were washed several times in distilled water, sterilized with 75% ethanol for 60 s and 0.05% $HgCl_2$ for 50 s, and rinsed three times with sterile water. Capsules were placed in a centrifuge tube and crushed in a vertical flow bench. The scattered spores

were suspended in 5 mL sterile water, diluted to desired concentration and uniformly spread on media.

Spores were then only cultured on the Beneck medium at a density of 4 cm^{-2} at 18/14°C (light/dark) temperature and light (16/8 hours of light to dark, and intensity of 4,500 lx), reflecting the natural environmental conditions of *Takakia lepidozioides* at the sampling site. To find the optimal medium component, several glucose concentrations (0, 1, 2, 3, 4, and 5% w/v), pH values (4.7, 5.3, 5.8, 7.0), $FeSO_4 \cdot 7H_2O$ concentrations (5, 10, 15, 20 and 50 mg L^{-1}), $CaCl_2$ concentrations (30, 50, 100, 500 and 1000 mg L^{-1}), and NH_4NO_3 concentration (30, 50, 100, 500 and 1000 mg L^{-1}) were tested using the Beneck medium as base medium.

Protonema culture conditions. Several spore storage conditions (at 4°C, –18°C, and room temperature, for 2, 3, 4, 5, 6 and 12 months), spore densities (4, 12, 24 and 48 cm^{-2}), culture temperature (12/8°C, 18/14°C and 22/20°C, light/dark cycle of 16/8 h), light intensities (2500 lx, 4500 lx, 8500 lx and 18000 lx) were also tested using the Beneck medium.

Morphological observation. The sterile cultures of *Takakia lepidozioides* were sampled under a stereomicroscope at 60, 90, 120 and 150 days after spore germination, and plants were mounted on slides. The preparation were sealed with transparent nail polish, and observed and photographed using a stereomicroscope (Nikon SMZ800), a multifunctional microscope (Leica DMRE), and a confocal scanning electron microscope (Leica SP2). The obtained images were analyzed using SPOT4.0 software.

Data collection and statistical analyses. The spore germination rate and the length of the longest branches were recorded. Experiments were carried out in triplicate. For parametric data, an analysis of variance (ANOVA) was used. The level of significance was set at $\alpha = 0.05$ for all tests (Zar, 1998).

RESULTS

***In situ* soil characteristics.** The composition and pH of soil collected from the original habitat of *Takakia lepidozioides* were analyzed (Table 1). Compared to the average values of soil in China and the world (China National Environmental Monitoring Centre, 1990), this soil, which has a pH of 5.2, contains almost no NO_3^--N, very low level of NH_4^+-N, K, Zn, and Mg, moderate amount of Cu and Mn, but very high levels of Fe (81,030 μg g^{-1}) and Ca (23,750 μg g^{-1}) compared to the averages of 29,400 μg g^{-1} for Fe and 15,400 μg g^{-1} for Ca in China and in Knop and Benecke media, the two conventional inorganic media used for bryophytes cultures.

Table 1. The mineral composition of soil associated with *Takakia lepidozioides* (μg g^{-1})

	NH_4-N	*NO_3-N*	*K*	*Ca*	*Mg*	*Fe*	*Cu*	*Mn*	*Zn*
Soil sample	11.5	0	4010	23750	2740	81030	29	677	11.8
Mean in China*			18600	15400	7800	29400	20	482	67.7
Mean in the world*			14000	15000	5000	40000	30	1000	9

* The data presented here are from "*Background values of soil elements in China*" (China National Environmental Monitoring Centre, 1990).

Optimal culture medium. The Beneck medium is the most suitable for spore germination and protonemal growth of *Takakia lepidozioides*. Spores germinated on Knop, Beneck and BCD media, but not on MS medium (Figs 1 & 2). The germination rate was very high on Beneck medium, reaching 93.8% after 35 days in culture, but comparatively low on BCD and Knop media (i.e., 40.2% and 52.6% rates, respectively). Protonema of *T. lepidozioides* grew steadily on Knop, Beneck and BCD media. After five months of growth, the number of protonemal branches was highest on Beneck medium, and lowest on BCD medium. Therefore, the Beneck medium was selected for subsequent experiments seeking to optimize conditions for spore germination and gametophyte growth.

The spore germination and protonemal growth of *Takakia lepidozioides* requires no or very low concentrations of external glucose. Spores germinate on medium supplemented with 1% or less of glucose, but not on medium supplemented with 2-5% glucose. Germination rate was highest on medium without supplementing any glucose (Fig. 3). Similarly, the protonema grew rapidly on medium supplemented with 0% or 1% glucose, but extremely slow in medium supplemented with 2-5% glucose (Fig. 4).

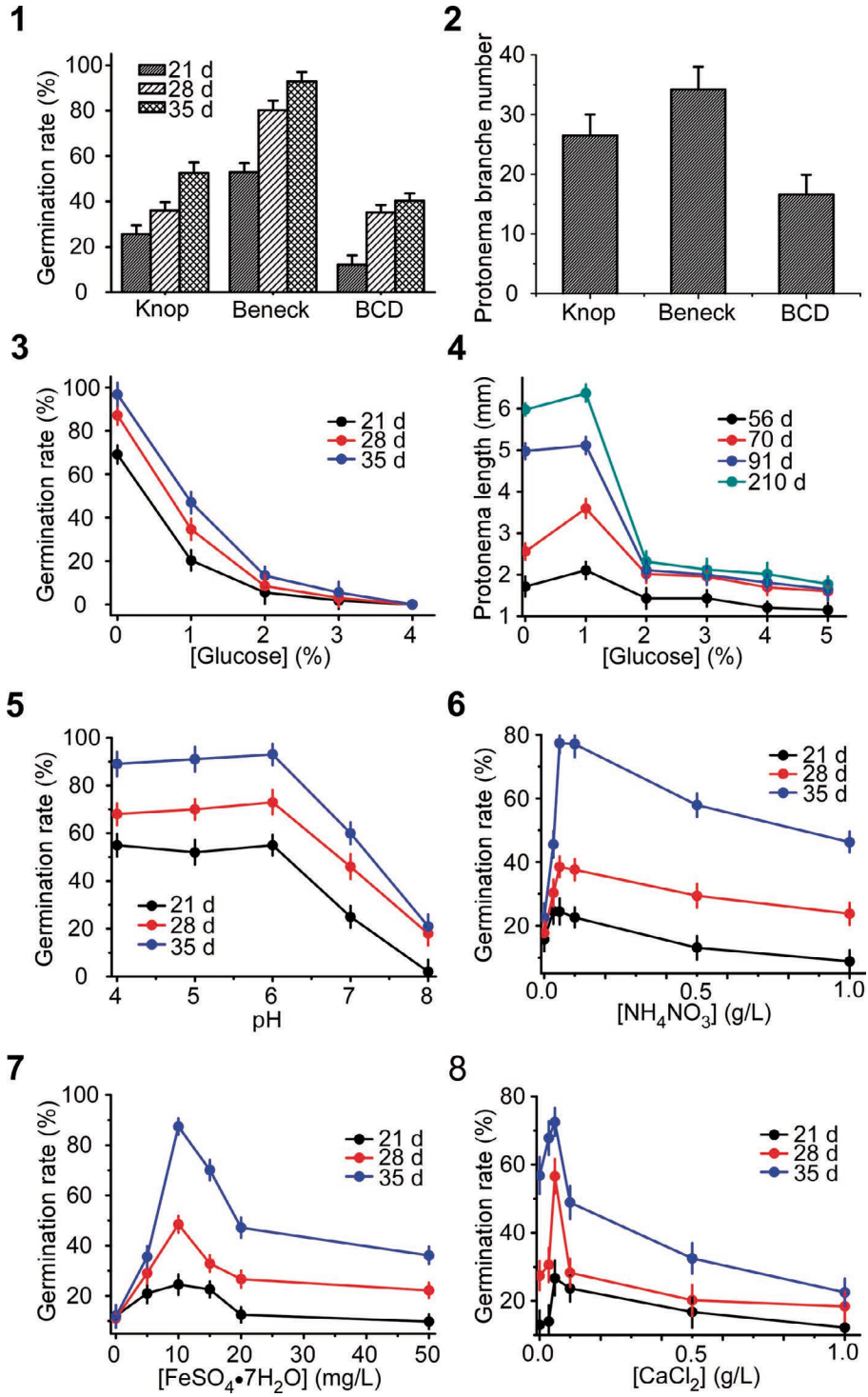
The optimum pH range for spore germination of *Takakia lepidozioides* is between 5.3 and 5.8. After 21 days in culture, spores started to germinate. The germination rate reached 59%, the highest level, on medium with a pH of 5.8 (Fig. 5). After 35 days, the germination rate reached 92% and 99% on medium with a pH of 5.3 and 5.8, respectively, significantly higher than that of 64% on medium with a pH 7.0. This is consistent with the observed in situ acidity of the soil of the sampled *T. lepidozioides* population.

A certain amount of NH_4^+-N is required for spore germination. When no NH_4NO_3 is supplemented, the germination rate varied between 16-23% at 21, 28 or 35 days of culture (Fig. 6). On medium supplemented with 30-1000 mg L^{-1} NH_4NO_3, the germination rate was 9-25% at 21 days of culture, 24-39% at 28 days, and 46-77% at 35 days. The ANOVA analysis indicated that supplementing the basic medium with 100 mg L^{-1} NH_4NO_3 provided the most suitable condition for spore germination (results not shown).

A certain amount of Fe^{2+} is essential for spore germination. Spores could germinate when no Fe^{2+} is added to the medium (Fig. 7), but the germination rate was less than 10%. On medium supplemented with 5-50 mg L^{-1} $FeSO_4 \cdot 7H_2O$, the germination rate was 10-20% at 21 days of culture, 22-49% at 28 days and 36-87% at 35 days. The ANOVA analysis revealed that supplementing the basic medium with 10 mg L^{-1} $FeSO_4 \cdot 7H_2O$ provided the most suitable conditions for spore germination (results not shown).

Similarly, a high spore germination rate requires supplemental Ca^{2+}. Without supplementing $CaCl_2$, spores could germinate, but the germination rate was only 10.2% (Fig. 8). On the medium supplemented with 30-1000 mg L^{-1} $CaCl_2$, the germination rate was 12-27% at 21 days of culture, 18-57% at 28 days and 23-73% at 35 days. Based on the ANOVA analysis supplementing 50 mg L^{-1} $CaCl_2$ appears optimal for spore germination.

Figs 1-8. Responses of *Takakia lepidozioides* to culture conditions. **1-2.** The effect of medium components on spore germination and protonemal development, respectively. Spores could not germinate on MS medium and hence MS medium is not included in Figs 1 & 2). **3-4.** The effect of glucose concentration on spore germination and development of protonema, respectively. **5-8.** The effect of pH, $NH4^+$ concentration, F^{e2+} concentration and Ca^{2+} concentration, respectively, on spore germination. All values are mean ± SD, with $n \geq 150$. Error bars represent SD. ▶


1
Germination rate (%)
21 d
28 d
35 d
Knop
Beneck
BCD
2
Protonema branche number
Knop
Beneck
BCD
3
Germination rate (%)
21 d
28 d
35 d
[Glucose] (%)
4
Protonema length (mm)
56 d
70 d
91 d
210 d
[Glucose] (%)
5
Germination rate (%)
21 d
28 d
35 d
pH
6
Germination rate (%)
21 d
28 d
35 d
[NH4NO3] (g/L)
7
Germination rate (%)
21 d
28 d
35 d
[FeSO4•7H2O] (mg/L)
8
Germination rate (%)
21 d
28 d
35 d
[CaCl2] (g/L)

In summary, the optimal medium for spore germination and protonema growth of *Takakia lepidozioides* was the Beneck medium supplemented with 100 mg L^{-1} NH_4NO_3, 50 mg L^{-1} $CaCl_2$, 10 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 100 mg L^{-1} KH_2PO_4, 60 mg L^{-1} $MnCl_2$ and 100 mg L^{-1} $MgSO_4$, pH5.3-5.8.

Optimal storage, temperature and light conditions. After optimizing the culture medium, the effects of spore storage and culture conditions such as spore density, temperature and light intensity on spore germination rate and protonemal growth were explored (Figs 9-16).

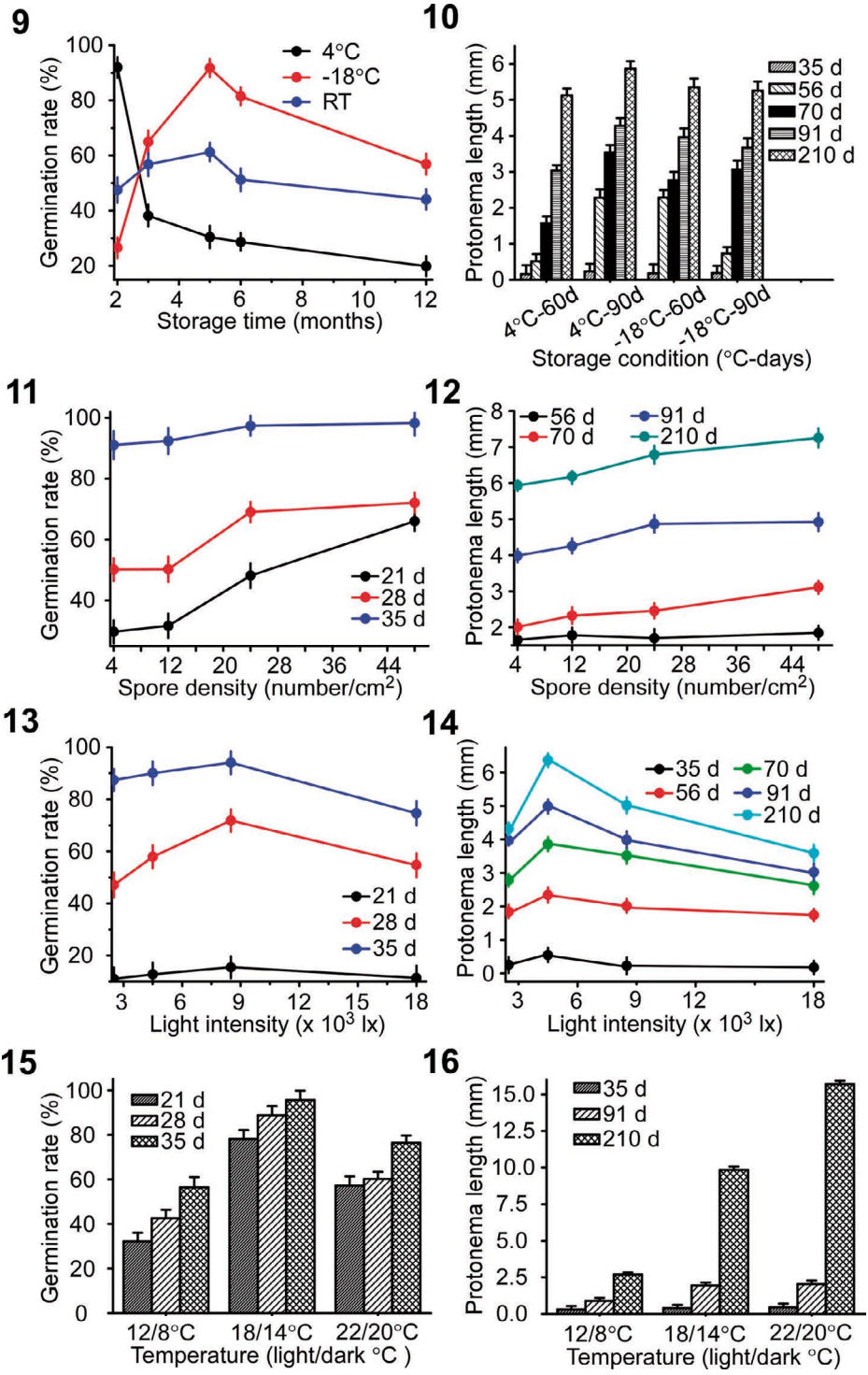
Firstly, we studied the effect of spore storage conditions on germination and protonema growth. Fresh spore capsules were stored at 4°C, –18°C or room temperature for different periods and spores were then sampled to initiate cultures on the optimized medium as described above. The germination rate of spores stored at room temperature (RT) varied with storage time, showing a maximum rate of 61% after 5 month of storage (Fig. 9). In addition, spore capsules stored at room temperature easily broke open, preventing easy retrieval of spores following storage. For capsule stored at –18°C, the spore germination rate increased with storage time, reaching maximum of 92% at 5 months and then decreased gradually to 56.89% at 12 months of storage. The germination rate following storage at 4°C increased with storage time, reaching a peak of 92% at 2 months and then gradually decreased to 38% at 3 months and 20% at 12 months. Based on the ANOVA analysis, the optimal spore storage conditions were 4°C for 1-2 months or –18°C for 3-6 months (results not shown). Growth in terms of the longest protonemal branches from spores stored for 60 or 90 days at –4˚C or –18˚C was not significantly different (Fig. 10).

Regarding the effect of spore density, germination rates were not significantly different after 21 days of culture between 4 cm^{-2} (4 spores per cm^2) and 12 cm^{-2} inoculation groups, and between 24 cm^{-2} and 48 cm^{-2} groups, but significantly different between 4 cm^{-2} and 24 cm^{-2} groups or between 4 cm^{-2} and 48 cm^{-2} groups (ANOVA results not shown). However, these differences decreased after 28 to 35 days of culture among all inoculation groups (Fig. 11). As for the protonemal growth, it was not affected by spores density at inoculation (Fig. 12). It should be mentioned that high density culture are not suitable for morphological observation.

Increases in light intensity resulted in an increase and then decrease in spore germination rate, with a maximum of 92.6% germination under 8,500 lx (Fig. 13), the optimal irradiance based on ANOVA (results not shown). Protonemal growth responded similarly to increases in light intensity, with an initial increase but with a maximum at 4,500 lx, the optimal irradiance based on ANOVA (results not shown), before declining gradually with increasing light intensity.

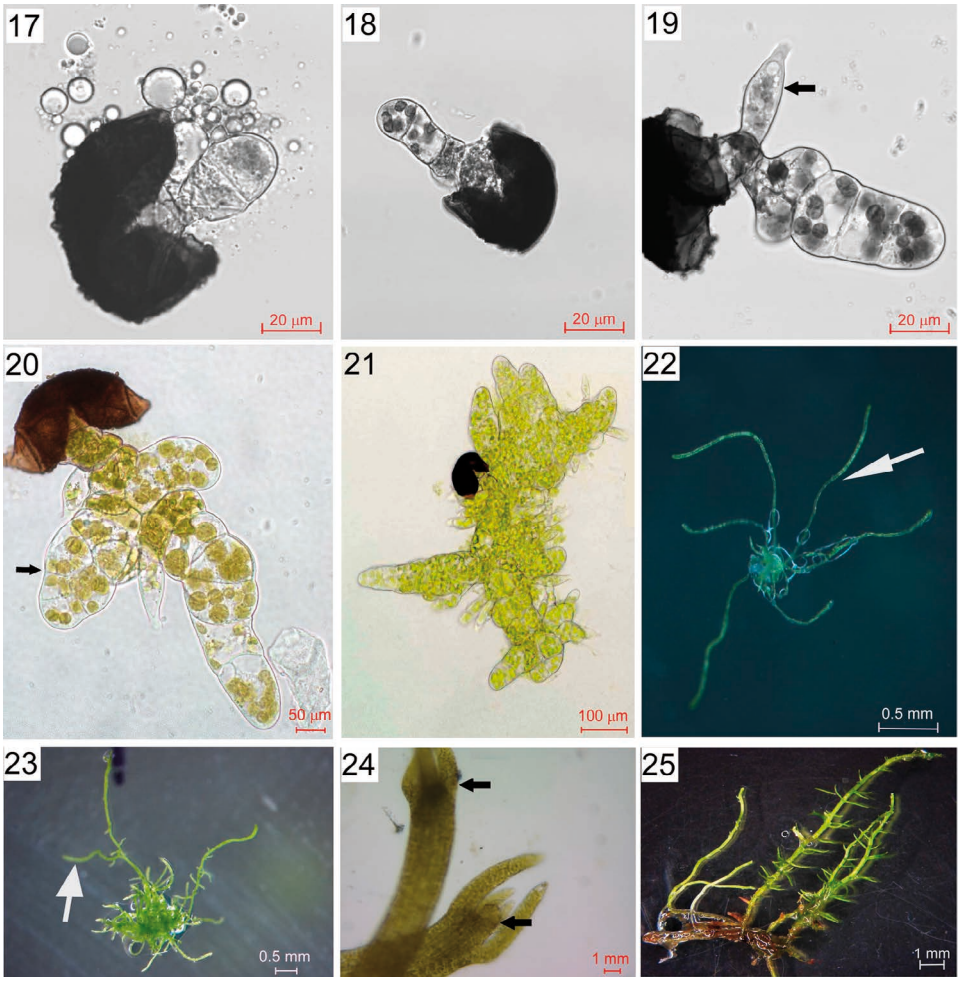
Finally, spore germination rates and protonemal growth were both affected by temperature. After 35 days in culture, the germination rate was 96% significantly higher at 18/14°C (day/night) than that of 56% at 12/8°C or 76% at 22/20°C (Fig. 15), with the latter two not leading to significantly distinct responses (ANOVA results not shown). By contrast, protonemal growth, after 91 days in culture, was highest (i.e., maximum length of 2.05 mm) under a 22/20°C regime and significantly different from that at 12/8°C, but not from that at 18/14°C. After 210 days in culture,

Figs 9-16. Responses of *Takakia lepidozioides* to culture conditions. **9.** The effect of storage time under different temperatures (RT = room temperature) on spore germination. **10.** The effect of storage conditions on growth. **11-12.** The effect of spore density on spore germination and growth of protonema. **13-14.** The effect of light intensity on spore germination and growth of protonema. **15-16.** The effect of temperature on spore germination and growth of protonema. All values are mean ± SD, with $n \geq 150$. Error bars represent SD. ▶


9
Germination rate (%)
Storage time (months)
4°C
-18°C
RT
10
Protonema length (mm)
35 d
56 d
70 d
91 d
210 d
4°C-60d
4°C-90d
-18°C-60d
-18°C-90d
Storage condition (°C-days)
11
Germination rate (%)
Spore density (number/cm2)
21 d
28 d
35 d
12
Protonema length (mm)
56 d
70 d
91 d
210 d
Spore density (number/cm2)
13
Germination rate (%)
Light intensity (x 10³ lx)
21 d
28 d
35 d
14
Protonema length (mm)
35 d
56 d
70 d
91 d
210 d
Light intensity (x 10³ lx)
15
Germination rate (%)
21 d
28 d
35 d
12/8°C
18/14°C
22/20°C
Temperature (light/dark °C)
16
Protonema length (mm)
35 d
91 d
210 d
12/8°C
18/14°C
22/20°C
Temperature (light/dark °C)

the protonemal branches were dramatically longer than after 91 days (Fig. 16), reaching maximum of 15.7 mm at 22/20°C, 2.70 mm at 12/8°C and 9.85 mm at 18/14°C, hence the optimal temperature regime is 22/20°C.

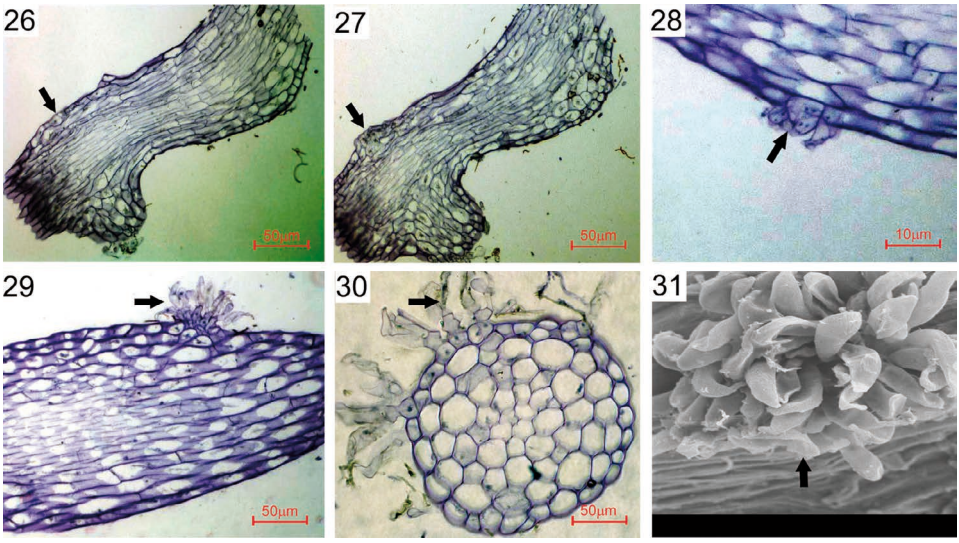
Taking together, the optimal culture conditions for *Takakia lepidozioides* from Tibet were as follows: 1) spores stored at –18°C for 3-6 months or at 4°C for 1-2 months; 2) spores inoculated at a density of 4 cm^{-2}, and germinated under 16 hours of light at 8,500 lx at 18°C and 8 hours of darkness at 14°C; 3) protonema grown under a daily regime of 16 hours of light at 4,500 lx at 22°C and 8 hours of darkness at 20°C.



Figs 17-25. Spore germination and cormus development of *Takakia lepidozioides*. **17.** Two-celled protonemata at 20 days after spore germination. **18.** Protonema with lens-shaped chloroplasts at 35 days after germination. **19.** Chloronemal filament with mucilage hairs (arrow) at 45 days after spore germination. **20.** Caulonemal filament (arrow) with some spindle-shaped chloroplasts. **21.** Thalloid protonema. **22-23.** Gametophyte with rhizomes (leafless horizontal stolon) (arrow) at 4-6 month after spore germination. **24.** Young buds growing from rhizome (leafless horizontal stolon) (arrow). **25.** Cormus at 10 months after spore germination.

Morphology and ultrastructure. About 21 days after absorbing water, the spores of *Takakia lepidozioides* started germinating unipolarly to form uniseriate chloronemal filaments, which were composed of 4 or 5 short, cylindrical cells in the size of 15.48 μm long and 17.86 μm wide (Figs 17-19). and a beaked mucilage hair (papilla) emerging from the base of these filaments (Fig. 19). Subsequently, the basal cells of these filaments divided transversely and vertically to produce the caulonema, followed with the continuous cell division, more caulonemata were generated that led to the formation of thalloid protonemata (Figs 20-21). Some cells on the thalloid protonemata's surface gradually developed into cylindrical rhizomes (leafless horizontal stolon) with multiple rows of cells about 5.89 μm long and 6.69 μm wide, on average respectively; however, no leaves appeared at this stage (Fig. 22). Then, the newly formed rhizomes continued to develop and produced multi-levels of branch clusters, which further thickened. The apical cells of some branches began to enlarge and differentiate to form buds with 3 or 4 cylindrical lobed leaves, which eventually developed to a cormus prototype (Figs 23-25). In this developmental process, several mucilage hairs appeared but not rhizoids.

Mucilage hairs were formed throughout the development following germination and occurred mainly above the branching site and branches of the cormus (Fig. 19). During the development of the protonema, 5-10 beaked mucilage hairs with an apical opening and secreting mucilage often clustered together (Figs 26-31). Rod-like mucilage hairs were not found. Scanning electron and light microscopy revealed that 50-100 mucilage hairs often clustered together on cormus branches (Figs 28-31). Their apical cells were cylindrical, organized in a single row originating from stem epidermal cells. At the early development stage, multiple rectangular stem epidermal cells of 10.87 × 2.85 μm anticlinally divided in two 4.26 × 5.20 μm basal cells with a darkly stained nucleolus and concentrated cytoplasm. The two basal cells subsequently divided and formed four mucilage cells projecting from the stem surface. When the cells reached 3.35 × 4.86 μm in size,



Figs 26-31. The colleter development of *Takakia lepidozioides*. **26.** Radix cell of the colleter. **27.** Transverse division of radix cells. **28.** Vertical division of radix cells. **29.** Top cell of the colleter. **30.** Cross section of stem. **31.** Scanning Electron Microscope (SEM) images of the colleter.

they divided periclinally once and formed eight mucilage cells cluster on the surface of the epidermis. These cells close to the outer layer divided periclinally once, and formed many uniseriate cylindrical hairs with apical cells 35.33 × 11.39 μm in size. The tip of the apical cells gradually became thinner. When the mucilage cells reached maturity, their tip cracked to secrete mucilage. Scanning microscopy showed that secreted mucilage accumulated around the apical mucous cells and forming a mesh-like structure.

DISCUSSION

Extant species of *Takakia* arose from an ancestor that is deeply rooted in the evolutionary history of mosses, sister to either all other mosses or only *Sphagnum* (Newton *et al.*, 2000; Qiu *et al.*, 2006; Volkmar & Knoop, 2010; Chang & Graham, 2011), and may thus be critical to further our understanding of the evolution of developmental processes in the moss gametophyte and sporophyte. So far, an efficient sterile culture system for observation of developmental processes has not been established in *Takakia*. Here we show that the integration of in situ abiotic factors (i.e., soil chemistry) in the optimization of the culture medium followed by experimentation on the effect of storage, temperature and light regimes on germination and growth provide for the first time, the in vitro conditions that optimize germination rate and early vegetative growth that will allow for critical ontogenetic studies.

In the Tibetan Plateau *Takakia lepidozioides* occurs between 3,200-4,100 m elevation, on bryophyte covered podzolic soil, in damp or foggy habitat, within a year-round hygric vegetation. We found that *T. lepidozioides* grows on acidic soil with high levels of Fe (Table 1), and not surprisingly the optimal variant of the Beneck medium had a low pH and high iron content. Optimal in vitro germination rate was observed at a pH of 5.3-5.8, and with a concentration of 10 mg L^{-1} $FeSO_4 \cdot 7H_2O$.

Takakia lepidozioides grows in the Hengduan Mountains, a typical alpine karst landform in the eastern Tibetan Plateau. The calcareous soil is developed from juvenile weathering crust of carbonate rocks and is rich in calcium. It has been reported that calcium enhances germination in *Orthotrichum cupulatum* (Vaarama & Tarén, 1963) and promotes protonema production in *Stereophyllum radiculosum* (Olarinmoye *et al.*, 1981), but depresses germination in *Dicranella cerviculata* (Vaarama & Tarén, 1963). Our results showed that 30-50 mg L^{-1} $CaCl_2$ is suitable for spore germination in *T. lepidozioides.*

Similar to vascular plants, bryophytes acquire and utilize nitrogen in the form of NO_3-N and NH_4-N, and unlike other land plants, many of them can also directly absorb and process amino acids and other organic matters, such as urea, as nitrogen sources (Sanville, 1988; Simola, 1975). Brown (1982) suggested that the pH or alkalinity affects availability of N for plants, with NO_3^- being more available in neutral or alkaline soils and NH_4^+ in acidic soils and water. The in situ soil of our *Takakia lepidozioides* sample was acidic and lacked NO_3-N, but held NH_4-N, which is thus likely the sole source of N for *T. lepidozioides*. Moreover, NH_4^+-N could reduce root pH, thus improve the effectiveness of Fe (Romheld & Marschner, 1986).

Spore germination of bryophytes, as a physical process, can occur in nutrient free medium (Olesen & Mogensen, 1978), whereas most bryophytes, such as *Bryum argenteum*, require light to germinate (Silva *et al.*, 2010). Spores of some

species would germinate only in light or in darkness + sucrose, indicating that the need for light is a need for energy. Sood (1976) found that 1.5% sucrose was optimum for germination, but that 4.8% was inhibitory for *Pogonatum aloides*, which does not germinate in the dark. Similarly, the spores of *Takakia lepidozioides* only germinated in low-glucose (i.e., < 4%) and glucose-free media in light conditions, suggesting that external glucose is not limiting spore germination rate in *T. lepidozioides*.

Different species of bryophytes have different suitable medium (Lal, 1984; Rowntree, 2006). Spore germination rates and protonemal growth of *Takakia lepidozioides* were higher on Beneck medium. Spores did not germinate on MS medium even after 60 days of culture, possibly due to excessive nutrients absorption leading to excessive osmotic pressure in MS medium. Based on all these experiments, which in part sought to assess the effect of in situ soil characteristics on the growth of *T. lepidozioides*, we established that the Beneck medium complemented with Fe^{2+} and adjusted at an acidic pH provided the optimal medium for in vitro culture of Tibetan *T. lepidozioides*.

Storage conditions of spores have a significant impact on spore germination rate and protonemal development of bryophytes (Burch, 2003). In the Tibetan plateau, *Takakia lepidozioides* grows at elevations above 3,200 m, and is not readily accessible. Hence cultures should ideally be initiated from stored spores, and optimal storage conditions should thus be identified. Storage duration and humidity directly affect spore viability and germination rate. Spores of many bryophytes are dormant upon dispersal, with dormancy relieved in vitro by refrigeration and hormones (Ohta & Hirose, 1982; Takeda & Katoh, 1981). Mature spore capsules of *T. lepidozioides* can be stored at 4°C for 1-2 months or at –18°C for 3-6 months without jeopardizing spore germination capacity.

Temperature and light intensity are the main key factors affecting spore germination and protonemal growth (Hohe & Reski, 2005). Longton and Greene (1969) found that germination rate in *Pleurozium schreberi* steadily increased between 5° and 20°C, a normal temperature range for spring and autumn. However, in *Sphaerocarpos texanus* the best germination occurred when the spores were subsequently placed at 16/10°C (typical temperate spring temperatures) (McLetchie, 1999). Although spores can germinate under very low light intensities, Chopra and Rahbar (1982) showed that those of *Bartramidula bartramioides* germinated best at 3500-4000 lx of continuous light in the lab. Meanwhile, high light intensity can promote protonemal growth, as in *Microdus*, *Hymenostylium*, and *Campylopus* (Mehta, 1988). Here, we simulated circadian temperature and intensity and time of illumination in Tibet Plateau and obtained good results (Figs 13-16).

Spores of *Takakia lepidozioides*, like the vast majority of bryophytes, are dispersed by wind, resulting in their random distribution and hence various densities of spores on the substrate. In vitro, spore germination rates and protonemal growth increased with spore density, although not necessarily significantly so. Our results showed that early spore germination, not protonemal growth is affected by culture density.

Takakia lepidozioides has a well developed protonema, and its protonemata are able to produce multiple gametophytes as is typical for mosses. Interestingly, the protonemata of *Takakia* start as filaments, then become thalloid finally. Generally, true moss spores germinate and form filamentous protonemata, but those of the Sphagnopsida and Andreaeopsida also form thalloid protonemata (Shaw *et al*., 2003). Based on the Nehira (1983), therefore, the sporelings of *T. lepidozioides* should be classified as the *Sphagnum*-type. Thalloid protonemal morphology feature

of *Takakia* is shared with the Sphagnopsida and Andreaeopsida and is probably a plesiomorphic character within the Bryophyta (Mishler and Churchill, 1985). In addition, previous studies showed that the protonema form is plastic and can be modified by environment or culture conditions (Alcalde *et al*., 1996). For example, low temperature, submersion, low light intensity and other environmental factors could delay or prevent caulonema formation, thereby affecting cormus development (Bopp, 1976). In this study, we tried to eliminate the influence of environmental factors and demonstrated that the thalloid protonemal morphology was constant and unrelated to the culture conditions in *T. lepidozioides*.

The connecting rhizome (leafless horizontal stolon) is another notable feature during *Takakia* gametophyte development. A large number of rhizomes (horizontal stolon) were formed before the development of buds and gametophytes in *T. lepidozioides*, a pattern similar to *Haplomitrium rotundifolium* (Calobryales; Yang, 1967) and very different from other mosses. Due to the lack of rhizoids in *T. lepidozioides*, the rhizomes may fulfill the functions of anchorage and of water and nutrient acquisition (Raven & Edwards, 2001). Furthermore, at the early stages of evolution of the root, rhizoids were capable of being induced generally on rhizomatous axes when in proximity to the soil (Kenrick & Strullu-Derrien, 2014), therefore, the appearance of rhizomes but lack of rhizoids might indicate the primitiveness of *Takakia*.

Mosses have many specialized structures, some of which have physiological functions. For example, leaf axillary hairs secrete mucilage for juvenile leaves, thus preventing dehydration (Buck & Goffinet, 2000). The mucilage hairs (papillae) on *Takakia* cluster in the leaf axils, leaf, and some branches (Inoue, 1982). The mucilage is secreted from the tip of the beaked mucilage cells. As in most plants, the mucilaginous secretion is generally thought to play a protective role. As *T. lepidozioides* does not tolerate desiccation well, the beaked mucilage cells may help maintain moisture.

The sporeling development of *Takakia* in culture can be characterized by a thalloid stage, the development of mucilage hairs (papillae) and rhizomes and the lack of rhizoids, which are features shared with *Haplomitrium*. Both genera are early diverging in their respective lineage (i.e., mosses [Chang & Graham, 2011] and liverworts [Volkmar *et al.*, 2012]) and these features may potentially represent plesiomorphies for early land plants.

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