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Successful tissue culture of the medicinal moss *Rhodobryum giganteum* and factors influencing proliferation of its protonemata

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Rhodobryum giganteum is a rare Chinese traditional medicinal moss species and has special therapy value in heart diseases. In the present study, we successfully induced gametophores and protonemata of *R. giganteum in vitro* from its new shoots, which were disinfected with 0.1% $HgCl_2$ for eight minutes. We also tested the factors influencing the growth of its protonemata and found that: (1) modified Knop medium is able to facilitate the elongation of the protonemata *in vitro* and prolong their growth time *in vitro*, whereas MS medium is able to facilitate the branching of the protonemata, and the protonemata grew slowly in modified White medium, accompanied by the development of gametophores; (2) cutting of the protonemata increases protonemal proliferation *in vitro*; (3) 2,4-D facilitates the induction of protonemata from leaves at 0.5 mg l^{-1}, but inhibits the induction and quickens the aging process of the protonemata at 2.0 mg l^{-1}.

Key words: cutting, hormone, 2,4-D, Knop medium, MS medium, *Rhodobryum giganteum*, sterilization, White medium

Introduction

In recent years, with the progress in phytochemistry and pharmacology, biologically active compounds with novel structures have been found in bryophytes. Thus more attention has been paid to the utilization of bryophytes as medicinal resources (Xia *et al*. 2001, Zhu *et al*. 2002, Wang & Lou 2003, Stephen *et al*. 2004).

The moss *Rhodobryum giganteum* (Bryophyta: Bryaceae) usually grows in forests with moist humus or on thin, damp soil layer on rocks. The species is distributed in Japan, Korea, southern regions of tropical and subtropical Asia, Hawaii, Madagascar and South Africa (Noguchi 1988, Zhang *et al*. 2007). Previous studies on *R. giganteum* involved phytochemistry, pharmacology and clinical applications (Gao *et al*. 2004). From *R. giganteum*, flavonoids, volatile compounds, organic acids, phenolic compounds, amino acids, terpenes (including ursolic acid), and highly unsaturated fatty acids (including docosapentaenoic acid) are documented (Zhang & Zhang 1992, Zhang *et al*. 1998, Qiao *et al*.

2004a, 2004b). As a Chinese traditional medicinal plant, *R. giganteum* has great therapeutic value, especially for cardiovascular diseases (Yan *et al*. 1998a), and it is considered a unique Chinese traditional medicine plant for coronary disease (Gao *et al*. 2004). Yan *et al*. (1998b), Lei and Dong (1999) and Wu *et al*. (2004) reported cardiovascular drugs containing active compounds of *R. giganteum*.

However, because of the excess utilization of *R. giganteum*, its specialized habitat and sporadic distribution pattern, as well as its slow growth rate, *R. giganteum* in the wild is quite rare, and some species of *Rhodobryum* have been considered endangered in China (Cao *et al*. 2006). In the present work, our aim is to provide technological support to establish a convenient and effective resource regeneration system for *R. giganteum*.

Material and methods

Explant sterilization and protonemal proliferation

We collected the gametophores of *R. giganteum* from the west Tianmu Mountain of Zhejiang province, China (30°19´N, 119°25´E, alt. 1178 m a.s.l.), and kept them alive in a culture cabinet (Hangzhou Qianjiang Instrumentation Equipment Company, model ZRX) at 25 °C and 12 h light (ca. 200 μmol m^{-2} s^{-1})/12 h dark, with a relative humidity of 80%. Young shoots developed after a one-month culture in the cabinet. We cut young shoots into ~1-cm long fragments and sterilized them in a clean bench (Shanghai Shangjing Decontamination Equipment Ltd. Model: CDA-1080-2) as follows: (1) we washed 15 about 1-cm-long shoots with distilled water to clear the visible superficial dust, then washed three times in sterile distilled water before sterilizing; (2) we divided the 15 shoots into three groups and sterilized them with 0.1% $HgCl_2$ for 5 min, 8 min and 10 min, respectively; (3) after sterilization, we washed the shoots five times for one minute with sterile distilled water to eliminate the remaining $HgCl_2$; (4) we transferred the sterilized shoots onto a disinfected plate and cut each shoot into two fragments of similar lengths; (5) we inoculated each fragment in a plastic Erlenmeyer flask (Shanghai Jiafeng Gardening Ltd., model ZP8-300) with 45 ml of aseptic modified Knop medium (Chen *et al*. 2006) (autoclaved at 121 °C for 20 min); (6) we put the flasks in the sterile culture room at 25 °C, 12 hours light (ca. 200 μmol m^{-2} s^{-1})/12 hours dark with a relative humidity of 60%.

The sterile fragments of the gametophores in the media formed a radiating occupation of protonemata (we call it protonematal colony) with the new-born protonematal colony about 1 cm in diameter after a one-month culture. Then we divided the colony into four parts, and cultured each part using modified Knop medium. After a two-month culture, we cultured the protonemata again for one month for further experiments.

Basic culture medium selection

Before the experiment, we picked four groups of protonemata of almost the same size in asepsis condition, got rid of the agar in the protonemata, and divided each group into six colonies, respectively, then dried the six colonies of one group of protonemata at 40 °C for three hours and weighed them to get the initial dry mass of these colonies (expressed as mean ± SE). We used the other three groups of protonemata in the selection experiment of basic culture media.

We prepared three types of media including modified Knop medium (Chen *et al*. 2006), modified White medium with bivalent iron NaFeEDTA instead (White 1963), and basic MS medium (Murashige & Skoog 1962), with pH 5.8 and sucrose (5 g l^{-1}) and agar (5 g l^{-1}), inoculated one piece of protonemata (each about 0.5 mg dry mass) into a flask with 45 ml of medium (autoclaved at 121 °C for 20 min), and put the flasks into the sterile culture room at 25 °C, 12 hours light (ca. 200 μmol m^{-2} s^{-1})/12 hours dark with a relative humidity of 60%. For each type of medium, we cultured six flasks of protonemata. We recorded the diameter of the colony of protonemata monthly for the first two months (each with six duplications), and also weighed dried protonemata from three random duplications of each treatment after a two-month culture (these three duplicates were then discarded), and finally we recorded diameters and dry mass of the

remaining three duplications after a three-month culture.

Influences of cutting on proliferation of protonemata

We cut sterile protonemata into 2-mm long fragments with a sterilized scalpel in an aseptic Petri dish, and then transferred the cut fragments into a flask with 45 ml basic MS solid medium (autoclaved at 121 °C for 20 min), and conducted the same treatment but with protonemata without cutting. We put the flasks into a sterile culture room at 25 °C, 12 hours light (ca. 200 μmol m^{-2} s^{-1})/12 hours dark with a relative humidity of 60%. We recorded dry mass monthly after drying the protonemata at 40 °C for three hours. In each treatment, we initially inoculated six flasks of protonemata. After a one-month culture, we randomly selected three of the six flasks and weighed protonemata they contained after drying. Protonemata from the remaining three flasks were weighed after a two-month culture.

In order to get the initial dry mass of the treatment, we picked out three pieces of protonemal colony of almost the same size as the uncut treatment, got rid of the agar in the colony, dried and weighed them; we used this in the treatments as the initial dry mass.

Influences of phytohormones on protonemal induction from leaves and stem fragments of gametophores

In order to compare the influences of different combinations of phytohormones on the protonemal induction of *R. giganteum*, we cut leaves from the gametophores (which were produced from the third-generation protonemata of *R. giganteum*) on a clean plate and cultured them on MS media with the adaxial surface of the leaves down (two pieces of leaves per flask). The design of the media with different phytohormones is given in Table 1. We conducted the same treatment on MS medium without phytohormones as the control. Treatments were conducted in triplicate. We calculated protonemal induction rates of the different treatments by checking if the leaves produced protonemata after a one-month culture.

In order to compare the differences between stems and leaves of *R. giganteum* in their capability of protonemal induction in the media with 2,4-D at different concentrations, we selected ~1-cm high gametophores of *R. giganteum*, cut the stems and leaves into 2-mm fragments with a sterile scalpel on a clean plate, then transferred the fragments into a four flasks, each containing 45 ml MS solid medium with 0 mg l^{-1}, 0.5 mg l^{-1}, 1.0 mg l^{-1}, and 2.0 mg l^{-1} of 2,4-D, respectively. Each flask contained about 50 gametophytic fragments. We calculated the induction rate for each treatment after a three-week culture. Each treatment (e.g. each concentration of 2,4-D) was conducted with three duplications.

Statistical analyses

We report the results as means ± SEs. Differences among treatments were tested with one-way ANOVA. Statistical analyses were carried out using SPSS ver. 15.0.

Table 1. Media used in the experiments.

Treatment	MS medium + hormone (mg l^{-1})	Treatment	MS medium + hormone (mg l^{-1})
Control	MS medium	9	MS + 2,4-D (1.0)
1	MS + 2,4-D (1.0) + 6-BA (0.1)	10	MS + 2,4-D (2.0)
2	MS + 2,4-D (1.0) + KT (0.1)	11	MS + NAA (1.0)
3	MS + 2,4-D (2.0) + 6-BA (0.1)	12	MS + NAA (2.0)
4	MS + 2,4-D (2.0) + KT (0.1)	13	MS + 6-BA (1.0)
5	MS + NAA (1.0) + 6-BA (0.1)	14	MS + 6-BA (2.0)
6	MS + NAA (1.0) + KT (0.1)	15	MS + KT (1.0)
7	MS + NAA (2.0) + 6-BA (0.1)	16	MS + KT (2.0)
8	MS + NAA (2.0) + KT (0.1)		

Results

Appropriate sterilization scheme of explants

The responses of the explants to disinfection with 0.1% $HgCl_2$ for different times were quite different. Because there were no sporophytes of *R. giganteum* available in our collection, stem fragments and shoots were adopted as explants in the culture. Sterilizing with 0.1% $HgCl_2$ for eight minutes was most suitable for the present culture (Table 2).

Culture medium selection

The dry mass of *R. giganteum* protonemata cultured on MS medium for two months was the highest of the three treatments (Table 3). However, after a further one-month culture, the protonemata on modified Knop medium weighed more than the others.

After a three-month culture, the protonemata of *R. giganteum* on modified Knop medium were able to extend on the surface of the medium, but rarely formed gametophores; on modified White medium, the protonemata were not only able to grow, but also to produce numerous gametophores; on MS medium, the protonemata were able to produce abundant branches, which led to a high density of the colony and a limited extension (Fig. 1). Compared with those on the other two media, the protonemata on MS medium aged early during the three-month culture.

The protonemal colonies cultured on the three media varied greatly after the first one-month culture. Within the cultural time, the growth of the protonemata on MS medium decelerated but accelerated on modified Knop medium (Table 4).

Integrating protonemal colony diameter and mass increment, we found that modified Knop medium was suitable for the growth of the protonemata for about two months without producing gametophores, while modified White medium was good for the induction of the gametophores, and MS medium was appropriate for branch formation of protonemata, which makes it increase the dry mass fast in a short period.

Table 2. Influence of disinfection time on explants of *Rhodobryum giganteum.*

Sterilization time	Explant response	
	After a 10-day culture	After a 35-day culture
5 min	All of ten explants turned brown and were contaminated	–
8 min	All of ten explants turned brown and eight of them were contaminated	One uncontaminated explant produced protonemata and the other produced a little gametophore
10 min	All of ten explants turned brown and five fragments were contaminated	One brown explant produced protonemata

Table 3. Dry mass change ± SE of *Rhodobryum giganteum* protonemata in three media after different culture times. Means with the same letters are not significantly different at the 0.05 level (LSD test).

Media	Protonemal dry mass (mg)		
	Initial	After a two-month culture	After a three-month culture
Modified Knop medium	0.53 ± 0.03	7.53 ± 0.09^b	20.43 ± 0.22^a
Modified White medium	0.53 ± 0.03	7.83 ± 0.03^b	16.87 ± 0.09^a
MS medium	0.53 ± 0.03	11.37 ± 0.20^a	17.90 ± 0.06^b
		F_8 = 104.54	F_8 = 179.66
		MSE = 13.63, $p < 0.001$	MSE = 10.10, $p < 0.001$

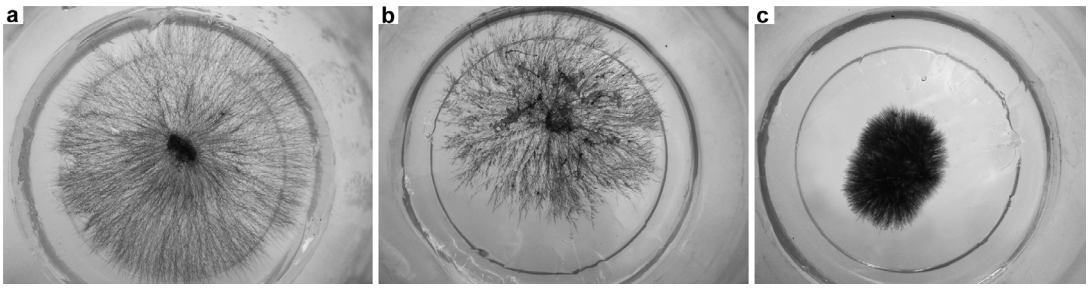

Fig. 1. Protonemal colonies of *Rhodobryum giganteum* on (**a**) Knop, (**b**) modified White, and (**c**) MS media after a 3-month culture.

Influences of cutting of the protonemata on its proliferation

The cutting of the protonemata increased their dry mass through proliferating protonemata *in vitro*. The protonemata, if cut before culture, were able to increase their proliferation rate, their dry mass being 2.17 times higher than that of the control. After a two-month culture, such proliferation rate of the protonemata decreased (Table 5).

Effect of 2,4-D in inducing leaves and stem fragments to form protonemata

2,4-D alone was able to induce the leaves to produce protonemata effectively. KT had a low induction effect, which is similar to that of the control. In addition, no callus from *R. giganteum* leaves was induced in any of the treatments (Table 6).

Protonemata of *R. giganteum* were more easily induced from the stems than from the leaves. All the stems on the media with 2,4-D at 0.5 mg l^{-1} or 0 mg l^{-1} were able to produce

Table 4. Protonemal colony diameters (cm) ± SE of *Rhodobryum giganteum* on three media after different culture times.

Media	Diameter of protonemal colony after		
	one-month culture	two-month culture	three-month culture
Modified Knop medium	2.09 ± 0.11^a	3.45 ± 0.03^a	5.61 ± 0.12^a
Modified White medium	1.80 ± 0.06^b	2.99 ± 0.03^b	4.67 ± 0.10^b
MS medium	1.13 ± 0.06^c	2.11 ± 0.08^c	2.43 ± 0.14^c
	$F_{17} = 3.97$	$F_{17} = 8.78$	$F_8 = 18.97$
	MSE = 0.72, $p = 0.09$	MSE = 1.43, $p = 0.03$	MSE = 7.97, $p = 0.005$

Table 5. The influence of cutting *Rhodobryum giganteum* protonemata on their protonemal dry mass (mg) ± SE.

Items	Protonemal dry mass		
	Initial	After a one-month culture	After a two-month culture
Without cutting treatment (Control)	1.97 ± 0.03	5.57 ± 0.12^a	14.40 ± 0.15^a
Cutting treatment	1.97 ± 0.03	12.07 ± 0.18^b	20.63 ± 1.14^b
		$F_5 = 927.44$,	$F_5 = 29.34$,
		MSE = 63.38, $p < 0.001$	MSE = 58.28, $p = 0.01$

protonemata. For the leaves, MS medium with 2,4-D at 0.5 mg l^{-1} led to a higher protonemal induction rate, with a rate of 82.40% (Table 7). The induction rate of the protonemata on MS medium with 1.0 mg l^{-1} 2,4-D is similar to that without 2,4-D. However, the medium with 2,4-D at a concentration above 1.0 mg l^{-1} could restrain the production of the protonemata.

Discussion

The tissue cultures of bryophytes usually start from sporophytes; the initiation from spores via surface sterilization appears to be generally highly successful (Sokal *et al*. 1997, Sabovljevic *et al*. 2003). However, for some species such as those of *Rhodobryum*, spores are not regularly available, and also some species do not produce sporophytes regularly. In these cases gametophores are adopted as explants, but the success rate of procedures is highly variable and contamination is rather common (Rowntree 2006). Rowntree *et al*. (2005) reported that protonemata were sensitive to several compounds, but Gonzalez *et al*. (2006) successfully cultured protonemal explants of *Splachnum ampullaceum in vitro*. Asexual propagules were also used as explants, although they were mostly vulnerable to disinfectants. Rhizoidal tubers and bulbils in axils of moss taxa and multicellular gemmae in liverworts are relatively tolerant to disinfectants (Ono 1973, Duckett *et al*. 2004). Though difficult, protonemata and gametophores have been induced successfully from gametophyte explants, as Sabovljevic *et al*. (2003) induced shoots of *Eurhynchium praelongum* with 7% and 9% NaClO effectively; Maeda (1979) obtained axenic protonemata of *Plagiomnium vesicatum* from leaves and stem fragments. Duckett *et al*. (2004) believed that spores, gemmae and fragments of gametophytes germinated to protonemata first, but in our experiment, from shoot fragments of *R. giganteum* we obtained both newborn protonemata and gametophores.

The three types of media showed different effects on the protonemal growth and differentiation. Modified Knop medium contains high

Table 6. Influences of 2,4-D, KT, and 6-BA on protonemal induction from gametophyte leaves of *Rhodobryum giganteum.*

Hormone (mg l^{-1})	Protonemal induction rates (%)	Hormone (mg l^{-1})	Protonemal induction rates (%)
MS (control)	33	MS + 2,4-D (1.0)	100
MS + 2,4-D (1.0) + 6-BA (0.1)	0	MS + 2,4-D (2.0)	67
MS + 2,4-D (1.0) + KT (0.1)	33	MS + NAA (1.0)	0
MS + 2,4-D (2.0) + 6-BA (0.1)	0	MS + NAA (2.0)	0
MS + 2,4-D (2.0) + KT (0.1)	0	MS + 6-BA (1.0)	0
MS + NAA (1.0) + 6-BA (0.1)	0	MS + 6-BA (2.0)	0
MS + NAA (1.0) + KT (0.1)	0	MS + KT (1.0)	33
MS + NAA (2.0) + 6-BA (0.1)	0	MS + KT (2.0)	0
MS + NAA (2.0) + KT (0.1)	33		

Table 7. Influences of 2,4-D on protonemal induction of *Rhodobryum giganteum* from gametophyte leaves and stems.

2,4-D concentrations (mg l^{-1})	Protonemal induction rates (%)	
	leaves	stems
0	64.1 ± 4.1^{b}	100.0 ± 0.0^{a}
0.5	82.4 ± 3.2^{a}	100.0 ± 0.0^{a}
1.0	67.2 ± 2.3^{b}	91.3 ± 1.6^{b}
2.0	19.7 ± 0.3^{c}	53.2 ± 2.1^{c}
	$F_{11} = 66.3$, MSE = 0.2, $p < 0.001$	$F_{11} = 113.9$, MSE = 0.2, $p < 0.001$

concentrations of major elements (N, P, K, Ca, and Mg), NO_3 was added as the nitrogen source, and no organic matter was added; MS medium included some organic matter, and NO_3^- and NH_4^+ were avalable in equal quantities; modified White medium has relatively low concentrations of major elements and the sole nitrogen source was NO_3^-. In modified Knop medium, nitrogen — mainly available as NO_3^- — and trace amounts of NH_4^+ (in tartrate ammonia) are also present. According to Hohe and Reski (2002), tartrate ammonia may keep moss protonemata in the state of chloronemata for a long time, meanwhile reducing caulonemata and bud differentiation. Takio *et al*. (1986) reported that with equally large amounts of NO_3^- and NH_4^+ in MS medium, protonemata consumed ammonia nitrogen first, which reduced the pH of medium — after months of culture until pH < 4.0 — and, as a result, the protonemata growth became inhibited, and such inhibition further held back the nitrate uptake and finally the cultures reached senescence. That can explain why, in our experiment, the mass of the protonemata of *R. giganteum* in MS medium increased faster and the protonemata aged earlier. In modified White medium, nitrate nitrogen alone enables growth of the protonemata on an even keel accompanied by the growth of some gametophores, which is consistent with the research results reported by Gonzalez *et al*. (2006).

The protonemal growth and differentiation into gametophores *in vitro* depend not only on internal factors transported from cell to cell, but also on interactions with the substrate (Schween *et al*. 2003). Schween *et al*. (2003) showed that most of the hormones were extra-cellular, and about 65%–85% of the cytokinins were excreted into the medium; the differentiation processes in mosses are clearly dependent on the amount of auxin and cytokinin (Bopp & Atzorm 1992). In the present experiment, the protonemata of *R. giganteum* cultured on MS medium produced a large number of new branches covering a limited area within a short period, which resulted in cytokinin and auxin accumulation at higher concentrations in the limited area, and the feedback effect inhibiting the protonemal growth. By contrast, the protonemata on the modified Knop and modified White media grew well with relatively low density and gradual extension growth, which is similar to the reports on the tissue cultures of bryophytes (Reski & Abel 1985, Bopp & Jacob 1986, Schulz *et al*. 2000).

To obtain young distal cells of linear protonemata, Ono *et al*. (1987) used the method proposed by Grimsley *et al*. (1977) of blending the developed protonemata in a homogenizer and then allowing them to branch. The cutting treatment of the protonemata used in our experiments doubled the biomass in a short period, but transferring was necessary. According to Duckett *et al*. (2004), cellophane is a feasible material to prevent the protonemata from growing into the medium.

There are numerous reports about the influences of cytokinin and auxin on moss species. 2,4-D and 6-BA are usually effective for callus inducement in *Physcomitrella patens* (Chen *et al*. 2006), *Cratoneuron filicinum* (Gao *et al*. 2003), *Atrichum undulatum* (Gang *et al*. 2003), and *Polytrichum commune* (Ono *et al*. 1988), but not in our study. Pan *et al*. (2005) reported that the gametophyte of *Physcomitrella patens* was able to produce protonemata in the media with 2,4-D, but no buds were formed. The experiment of *Pogonatum urnigerum* indicated that the auxin IAA favored budding and the cytokinin 6-BA restrained gametophore growth; both hormones could accelerate protonemal aging in a long culture period (Cvetic *et al*. 2007). Our results coincide with the previous papers, but with all the cytokinin and auxin used, no callus was induced from leaves or from stem fragments.

Conclusion

The gametophores of *Rhodobryum* coltured in the wild with stems full of rhizoids and large upper leaves, which form comal rosettes, are difficult to sterilize and nobody has reported successful sterilization. Our study showed it was possible to establish a culture of *R. giganteum in vitro* by sterilizing new shoots with 0.1% $HgCl_2$ for 8 min. To get a large amount of *R. giganteum*, we first induced the protonemata from the explants, and then produced gametophores from the protonemata. According to this study, there are two ways to increase the protonemal prolif-

eration: one is cutting the old protonemata into pieces and culturing on MS medium until their branches form for a short period and then inoculating onto modified Knop medium to extend the protonemal length; the other way is cutting the plants into fragments, inducing into protonemata on MS medium + 0.5 mg l^{-1} 2,4-D, then transferring onto modified Knop medium after a one-month culture. Compared with the other media, modified White medium is likely to produce gametophores earlier from the protonemata, but with a low yield. The protonemata on modified Knop medium may overgrow the flask after a three-month culture; after that time the protonemata start to differentiate to form gametophores as the concentration of cytokinin secreted from the protonemata reaches the response level, and such a differentiation may last a long time.

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