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In vitro study on the reproductive behavior of the endemic and threatened Indian liverwort: *Cryptomitrium himalayense* Kashyap (Aytoniaceae)

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Abstract – Axenic cultures of the thalloid liverwort *Cryptomitrium himalayense* Kashyap were established from spores and propagated *in vitro* under a variety of culture media and controlled environments to determine the optimum conditions for the onset of sexual phase. Enhanced vegetative growth in form of overlapping rosette-forming thalli occurs on half strength Knop's medium as well as in Hoagland medium under continuous illumination of 2.93 W/m^2 at 21ºC from the production of innovations from the basal parts of the dorsal surfaces of the thalli. Thalli became shorter and produced tuberous swelling following nutrient depletion and drying out of the medium. Sex organs were produced only on Hoagland medium supplemented with 1% sucrose under a long day regime with colder nights (16 hours light at 21ºC and of 8 hours darkness at 15ºC). Thalli acclimatized and transferred on soil also produced sex organs under similar conditions of photoperiod and temperature.

***Cryptomitrium himalayense* / photoperiod / reproduction / sucrose / temperature**

INTRODUCTION

In recent years, establishment of axenic culture and *in vitro* propagation of bryophytes have received attention not only for morphogenetic, genetics, physiological and metabolic studies (Ono *et al.*, 1988; Cove *et al.*, 2006; Pressel & Duckett, 2009) but also for bioprospecting investigations (Ohta *et al.*, 1990; Sauerwein & Becker, 1990; Morais *et al.*, 1991; Tazaki *et al.*, 1995; Chiou *et al.*, 2001; Hohe & Reski, 2005; Decker & Reski, 2008; Sabovljevic *et al.*, 2009). In addition a number of culture protocols have been developed for the *ex situ* conservation of bryophytes (Basile & Basile, 1988; Kowalczyk *et al.*, 1997; Sastad *et al.*, 1998; Ramsay & Burch, 2001; Hohe *et al.*, 2002; Duckett *et al.*, 2004; Buczkowska *et al.*, 2006; Gonzalez *et al.*, 2006; Rowntree, 2006; Cvetic *et al.*, 2007; Chen *et al.*, 2009; Liang *et al.*, 2010; Rowntree *et al.*, 2011).

In the present contribution, we describe growth responses and reproduction in an endemic and threatened Indian liverwort *Cryptomitrium himalayense* Kashyap (Aytoniaceae, Marchantiopsida) on different culture media and controlled physical conditions towards the optimization of *ex situ* conservation strategies (Awasthi *et al.*, 2010a). *Cryptomitrium himalayense* is the only Indian species of *Cryptomitrium*. This species is endemic to India and restricted to the western Himalaya and Sikkim (Udar & Srivastava, 1983; Singh, 1997). Pant (1983) listed this species under threatened bryophytes and discussed the effects of urbanization resulting in disappearance of several its localities in the Himalaya.

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MATERIALS AND METHODS

Spores were procured from mature sporophytes of *C. himalayense* (India, Western Himalaya, Uttarakhand, Nainital, Dhobighat, *ca* 2120 m, on rock, 6 Nov. 2008, *A. K. Asthana & V. Sahu, 249110* (LWG)), surface sterilized with 1% sodium hypochlorite solution for 8-10 minutes. Cultured in half strength Knop's macronutrients medium (Knop, 1865) under axenic conditions they produced well differentiated thalli. These were sub-cultured in half strength Knop's macronutrients as well as in Hoagland medium (Hoagland & Arnon, 1950), MS (Murashige & Skoog, 1962), half strength Knop's macronutrients + Nitsch trace elements (Kaul *et al*., 1962), Gamborg B-5 (Gamborg *et al*., 1968) medium without and with 1% sucrose. The pH of the media was adjusted at 5.8 and all the media were solidified with 0.8% agar (bacto-grade). The controlled conditions of light and temperature are given in Table 1.

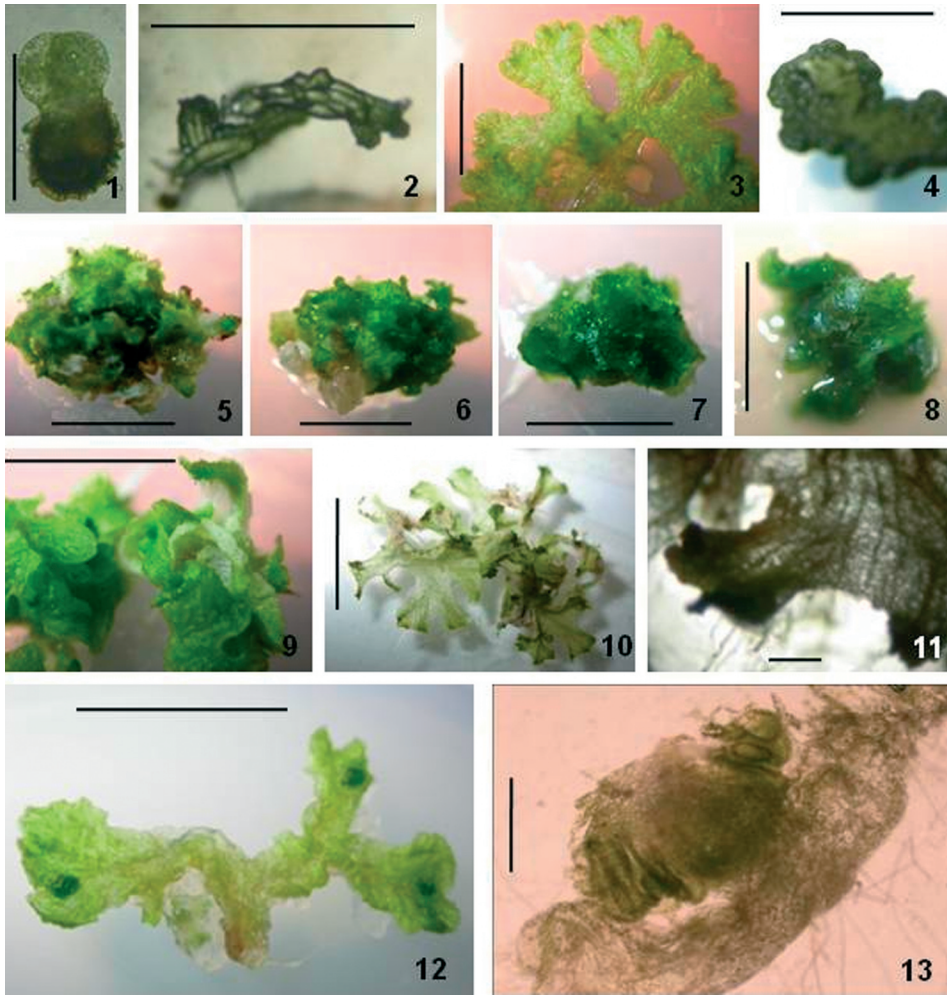
The reproductive behavior of *C. himalayense* was also observed on soil. The mature and well differentiated thalli were acclimatized and transferred to sterilized soil mixed with peat following the protocols in Awasthi *et al.* (2010b) and maintained under controlled physical conditions (Table 1).

Table 1. Controlled conditions of light and temperature provided in the experiments

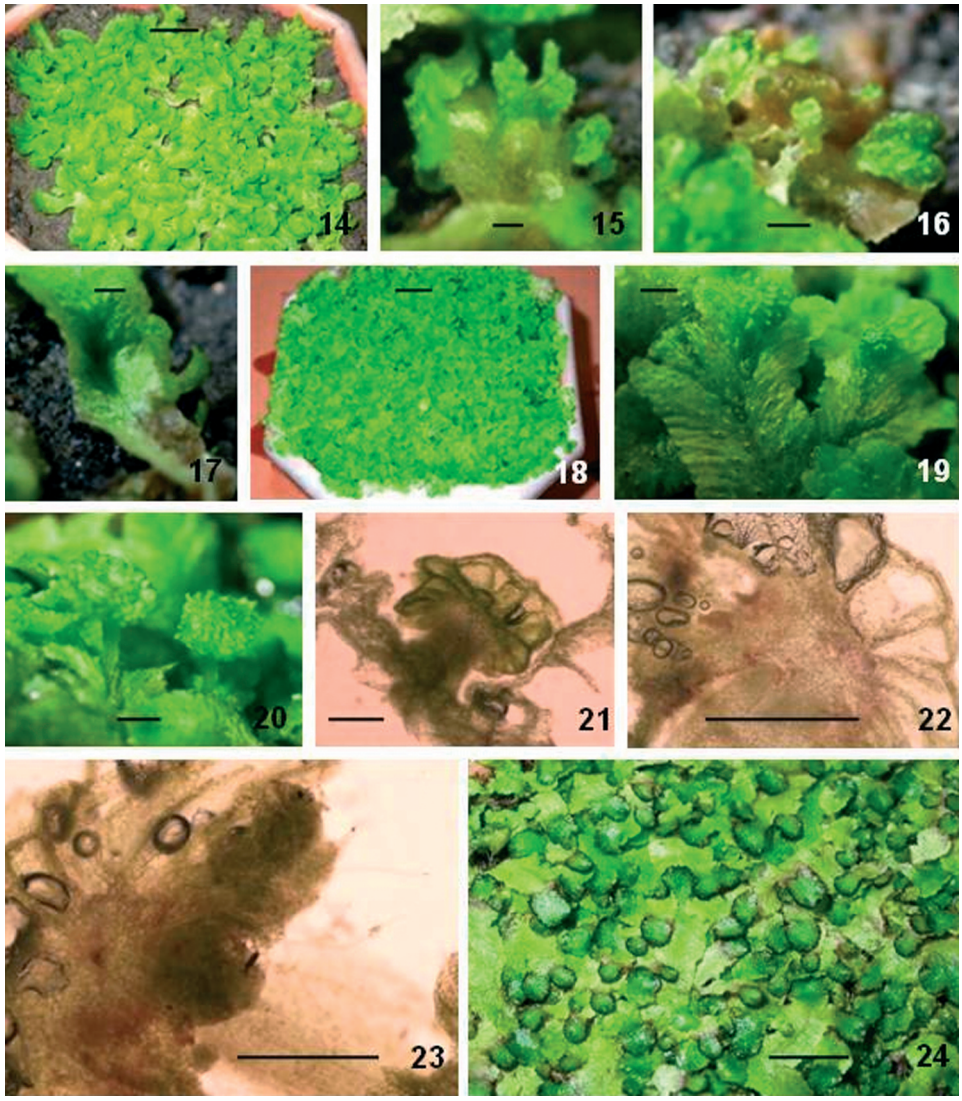
Photoperiod	*Temperature*
Continuous illumination of 2.34-2.93 W/m^2	21 ± 2ºC continuously 15-18ºC continuously
Alternate light (2.93 W/m^2) for 16 hrs and dark period for 8 hrs (Long day regime)	21 ± 2ºC continuously 15-18ºC continuously 21ºC during light and 15ºC during dark 15ºC during light and 21ºC during dark
Alternate light (2.93 W/m^2) for 8 hrs and dark period for 16 hrs (Short day regime)	21 ± 2ºC continuously 15-18ºC continuously 21ºC during light and 15ºC during dark 15ºC during light and 21ºC during dark

RESULTS

Spores of *C. himalayense* germinated readily after 3-4 days of inoculation in half strength Knop's macronutrients medium (Figs 1, 2) and produced well differentiated fan-shaped thalli within 80 days (Fig. 3). Continuous illumination of 2.34-2.93 W/m^2 at 21 ± 2ºC was found to be optimal for the best growth. In these conditions, single innovations, emerging from the dorsal surface of the basal part of each thallus (Fig. 3), developed into rosettes of thalli just above the previous ones. In other media viz. half strength Knop's macronutrients + Nitsch trace elements, MS and Gamborg B-5 with and without sucrose, the spores failed to germinate. Further differentiation of the sporelings into well differentiated thalli was seen only in half strength Knop's macronutrients.



Figs 1-13. *In vitro* propagation of *Cryptomitrium himalayense* Kashyap. **1.** Germinating spore. **2.** Growing sporeling. **3.** Well differentiated fan shaped broader thalli in creeping rosette with innovations (80 days old) in half strength Knop's macronutrients medium. **4.** Formation of callus after sub-culturing in half strength Knop's macronutrients + 2% sucrose (60 days old). **5.** Shorter abnormal thalli in half strength Knop's macronutrients + Nitsch trace elements medium. **6.** Thalli, after sub-culturing in MS medium. **7.** Callus-like growth from sub-cultured thallus in MS medium + 1% sucrose (50 days old). **8.** Rotted blackish green tissue in Gamborg B-5 medium. **9.** Well differentiated dark green coloured thalli in Hoagland medium. **10, 11.** Thalli with tuberous apical swellings on drying medium. **12, 13.** Fertile thallus with archegonial disc in Hoagland medium with 1% sucrose. Scales: Fig. 1 = 0.1 mm; Figs 2, 11, 13 = 1.0 mm; Figs 3-10, 12 = 1.0 cm.



Figs 14-24. Growth of *in vitro* raised thalli of *Cryptomitrium himalayense* Kashyap after acclimatization and introduction to soil. **14.** *In vitro* raised thalli, after transferring on soil (after 50 days). **15, 16, 17.** Innovations regenerated from margins, old decaying tissue and basal part of thalli respectively. **18.** Formation of thalli in bulk on soil. **19.** Induction of gametangiophores under long day regime with cold temperature on soil. **20.** Well developed archegoniophores. **21.** Section of thallus through female disc. **22, 23.** Archegonia and sporophytes on a female disc. **24.** Population of mature thalli on soil with prominent tuberous apices. Scales: Figs 15-17, 19-23 = 1.0 mm; Figs 14, 18, 24 = 1.0 cm.

When the innovations, apical parts of cultured thalli or intact thalli were sub-cultured in half strength Knop's macronutrients + 2% sucrose, callus formation took place (Fig. 4). Sub-culturing of such secondary inocula in half strength Knop's macronutrients + Nitsch trace elements with or without 1% sucrose resulted into either death of the explants or the development of short abnormal, twisted and narrower thalli (Fig. 5). When intact thalli were transferred from half strength Knop's macronutrients to MS medium or Gamborg B-5 medium, they became generally decolourized. In a few however, the apical parts remained green. Dark green callus-like tissue developed after about a month from these. Subsequently, several new thalli regenerated from the callus but these remained very short even after 50 days and did not differentiate into normal thalli (Fig. 6). The formation of callus-like tissue was even more prominent on media supplemented with 1% sucrose (Fig. 7). Many blackish necrotic regions developed after 20 days. These spread over the entire mass of tissue resulted in rotting and death after about 50 days (Fig. 8). On Hoagland medium, sub-culturing of intact thalli or apical parts of thalli resulted in the formation of darker green thalli in compact clusters (Fig. 9). Thus among all the media, half strength Knop's macronutrients and Hoagland medium were the most suitable for the differentiation and rapid clonal propagation of thalli in continuous illumination of 2.34-2.93 W/m^2 at 21 ± 2ºC. The morphological responses of cultures on different media are summarized in Table 2.

After 150 days, when the cultures were drying out and nutrients were exhausted, the thalli tended to be smaller and produced tuberous swelling at their apices (Figs 10, 11). Thalli became fertile and archegonial discs appeared (Figs 12, 13) only in Hoagland medium supplemented with 1% sucrose and in a long day regime at 21ºC in light and 15ºC in darkness after 70-90 days of sub-culturing. Hoagland medium without sucrose did not induce the gametangial formation.

When 120-day-old thalli were transferred to sterilized peat-mixed soil saturated with half strength Knop's macronutrients, uniform populations of well differentiated, broader and vigorously growing thalli was developed in pot within 50-60 days (Fig. 14) under constant low temperature of 21ºC and high humidity (90-100%) and alternate light of 2.93 W/m^2 for 16 hours and dark period of 8 hours. In these conditions thalli remained vigorous and broader, while at moderate temperature 25ºC and low humidity (40-60%) thalli tended to be shorter and the basal parts began to decay. Tuberous apical swellings were also apparent at the apices of thalli. Lower temperatures (21 ± 2ºC) and higher humidity (90-100%) resulted in emergence of several innovations from apical as well as lateral margins (Fig. 15) and from the decaying basal regions (Figs 16, 17) which gave rise the population in bulk (Fig. 18).

When the thalli acclimatized on soil were kept under long day regime with colder night (receiving 2.34-2.93 W/m^2 for 16 hours at 21ºC and dark period of 8 hours at 15ºC), after 20-25 days well developed umbrella-shaped archegoniophores were produced(Figs 19, 20, 21) and subsequently archegonia (Fig. 22) and sporophytes (Fig. 23). Each lobe of the thallus produced a single archegonial disc. Thus the number of archegonial discs and subsequent sporophytes depended on the extent of thallus branching. In continuous light, in short day regimes or in a continuous cold temperature of 15ºC with any photoperiod no sex organ developed, instead the thalli developed prominent tuberous apices (Fig. 24) after 100 days. In such case, the remaining part of thalli gradually decayed and new thalli regenerated from the persistent tuberous apices. The reproductive events after transferring and acclimatization on soil under different conditions of photoperiod and temperature are summarized in Table 3.

Table 2. Morphological response of *Cryptomitrium himalayense* Kashyap in different growing media (90 days old) under continuous illumination (2.34-2.93 W/m^2) at 21 ± 2ºC temperature. Each result is the mean ± standard error from 10 sampling unit. Each sampling unit contained 3-15 thalli

Growth media	*Morphological response of culture*			
	Average length of thalli (in mm)	*Average breadth of thallus lobe (in mm)*	*Remarks*	*% number of thalli giving respective response*
In wild	14.08 ± 0.60	3.96 ± 0.09	Very delicate, closely creeping, once or twice forked thalli	–
1/2 K	20.27 ± 0.61	4.00 ± 0.04	Well differentiated fan shaped thalli in creeping rosette along with several innovations	100 ± 00
1/2 K + N	9.69 ± 0.40	2.34 ± 0.13	Shorter abnormal thalli having sicky appearance (Yellowish, twisted and narrower thalli) or death of thalli	76.93 ± 3.48
1/2 K + N + 1S	7.66 ± 0.18	2.39 ± 0.10	Shorter abnormal thalli having sicky appearance (Yellowish, twisted and narrower thalli) or death of thalli	72.39 ± 3.80
1/2 K + 2S	–	–	Callus formation occurred	67.45 ± 4.68
MS	6.30 ± 0.21	1.81 ± 0.17	Several innovations or miniature of thalli regenerated from dark green callus like mass of tissue	100 ± 00
MS + 1S	–	–	Rottened dark blackish green coloured callus like pulpy mass of twisted, deformed thalli	88.94 ± 3.43
B-5	–	–	Rottened blackish green mass of tissue	100 ± 00
H	18.09 ± 0.48	3.90 ± 0.08	Well differentiated dark green coloured thalli	100 ± 00
H + 1S	23.48 ± 0.39	5.18 ± 0.18	Well differentiated dark green coloured thalli (Sporophyte produced by 75.05 ± 3.48 % of thalli in long day condition)	100 ± 00
On soil after acclimatization at 21± 2ºC	28.40 ± 0.83	5.34 ± 0.13	Well differentiated, two or three times dichotomously branched thalli	88.70 ± 1.29
On soil after acclimatization at 25 ± 2ºC	12.27 ± 0.59	3.94 ± 0.19	Shorter thalli	81.34 ± 1.47

Abbreviations: 1/2 K: Half strength Knop's macronutrients; 1/2 K + N: Half strength Knop's macronutrients + Nitsch trace elements with 10 ppm ferric citrate; 1/2 K + N + 1S: Half strength Knop's macronutrients + Nitsch trace elements with 10 ppm ferric citrate + 1% sucrose; 1/2 K + 2S: half strength Knop's macronutrients + 2% sucrose; MS: Murashige and Skoog medium; MS + 1S: Murashige and Skoog medium supplemented with 1% Sucrose; B-5: Gamborg B-5 medium; H: Hoagland medium; H + 1S: Hoagland medium supplemented with 1% Sucrose.

Table 3. Reproductive efforts made by *Cryptomitrium himalayense* Kashyap after transferring and acclimatization on soil under different conditions of photoperiod and temperature. Each result is the mean ± standard error of percentage number of thalli giving respective response from 10 sampling units. Each sampling unit contained 15-60 thalli

Temperature	*Photoperiod conditions*		
	Continuous light (2.34-2.93 W/m^2)	*Alternate light (2.93 W/m^2) for 16 hrs with dark period of 8 hrs (long day)*	*Alternate light (2.93 W/m^2) for 8 hrs with dark period of 16 hrs (short day)*
21 ± 2ºC Continuously	Innovations (95.52 ± 0.93)	Innovations (82.45 ± 1.16)	Innovations (70.52 ± 1.19)
15-18ºC continuously	Tuberous apices (81.55 ± 0.75)	Tuberous apices (97.18 ± 0.77)	Tuberous apices (78.04 ± 1.39)
21ºC during light and 15ºC during dark	–	Gametangia and subsequent sporophytes (82.88 ± 2.32)	Tuberous apices (68.14 ± 1.26) Innovations (30.15 ± 0.69)
15ºC during light and 21ºC during dark	–	Tuberous apices (70.69 ±0.62) Innovations (27.15 ± 0.87)	Innovations (54.07 ± 1.32) Tuberous apices (43.03 ± 0.92)

DISCUSSION

This paper demonstrates that the endangered liverwort *C. himalayense* readily grew *in vitro* on half strength Knop's macronutrients medium and then transferred to soil. Gametangia are readily produced on Hoagland medium supplemented with 1% sucrose, while in media viz., half strength Knop's macronutrients + Nitsch trace elements, MS and Gamborg B-5 with and without sucrose, the spores failed to germinate and often resulted into callus formation (when explants are apical part of thalli or intact thallus).

The formation of dark green, callus-like tissue from entire surface of explants (apical part of thalli or intact thalli) in MS and Gamborg B-5 media may be due to excessively high dose of nitrogen in ammonium as well as nitrate and relatively less calcium in these media in comparison to half strength Knop's macronutrients. This may be due to which cell wall formation not keeping pace with rapid cell division. Kumra (1984) also observed such callus formation, initiated from lower epidermis in *Asterella wallichiana* (Lehm. *et* Lindenb.) Pandé, K.P.Srivast. *et* Sultan Khan *ex* Grolle grown in MS medium. Gamborg B-5 medium produced dark blackish green coloured callus-like pulpy mass of twisted and deformed thalli perhaps as a result of calcium deficiency. Addition of 2% sucrose into Knop's macronutrients also resulted in callus formation as has been noted previously in the thalli of other liverworts (Kaul *et al*., 1962; Mehra & Pental, 1976). Healthy growth and normal differentiation of thalli in half strength Knop's macronutrients indicates that calcium and low level of nitrogen are optimal for the growth of this species.

The data on photoperiod and gametangial induction in liverworts are somewhat contradictory. *Marchantia polymorpha* L. has been described both as a long day plant (Miller & Coldiace, 1969; Courtoy, 1972) and also as day neutral (Benson-Evans, 1964). Among other liverworts, long day plants are *Preissia quadrata* (Scop.) Nees, *Conocephalum conicum* (L.) Dumort. (Benson-Evans, 1961), short day plants *Riccia glauca* L. (Benson-Evans, 1961), *Asterella tenella* (L.) P.Beauv. (Bostic, 1981), *Marchantia papillata* Raddi subsp. *grossibarba* (Steph.) Bischl. (Awasthi *et al*., 2011) and day neutral plants *Riccia crystallina* L. (Chopra & Sood, 1973a), *Targionia hypophylla* L. (Bapna *et al*., 1984).

Temperature also induces a variety of response in bryophytes. In some bryophytes gametangial induction depends on low temperature comparable with vernalization. Some taxa like *Lunularia cruciata* (L.) Dumort., *Aneura mirabilis* (Malmb.) Wickett & Goffinet, *Riccia crystallina* L. and *Leptobryum pyriforme* (Hedw.) Wils. required either low temperature alone or first low then higher temperature (Bopp, 1983). *Philonotis turneriana* (Schwaegr.) Mitt. and *Funaria hygrometrica* Hedw. required a critical temperature of 18ºC for gametangial induction (Kumra & Chopra, 1983; Nath *et al.*, 2009). Bostic (1981) and Hohe *et al.* (2002) suggested that both temperature and day length affect sexual reproduction.

In *Cryptomitrium himalayense*, alone low temperature in continuous light did not induce gametangial formation. Both low temperature (21ºC during light and 15ºC during dark) and alternate light and dark periods of 16 hours and 8 hours respectively were required for gametangial induction. This was true both in culture and on soil and fits closely with climatic conditions when gametangia are produced in nature. Thus an interdependence of temperature and photoperiod coordinate gametangial production (Hohe *et al*., 2002).

Nutrient supply has been shown to have a major role in gametangial induction in many liverworts. Selkirk (1979) showed that limited nitrates led to gamete production in several species of the liverwort *Riccia*. In *Cephalozia media* Lindb., exogenous inorganic nitrogen did not significantly affect sexual or asexual reproduction while organic nitrogen in form of certain amino acids or kinetin overdo photoperiodic control of the reproductive response (Lockwood, 1975). A relatively low N: high C ratio in *Marchantia polymorpha* stimulated production of sexual branches (Wann, 1925). On the other hand in *Fossombronia porphyrorhiza* (Nees) Prosk., nitrogen as nitrate caused more gametangial production than when it was supplied as ammonium (Chin *et al*., 1987 as in *Fossombronia brasiliensis* Steph.). An exogenous supply of sucrose has also been shown to be an essential requirement for the development of sex organs in several bryophyte taxa *in vitro* viz., *Riccia crystallina* L. (Chopra & Sood, 1973a; 1973b; Chopra & Bhatla, 1983), *Athalamia pusilla* (Steph.) Kashyap (Mehra & Pental, 1976), *Targionia hypophylla* L. (Bapna *et al*., 1984), *Riccia discolor* Lehm. *et* Lindenb. (Sarala, 1992), *Riccia frostii* Austin (Chopra & Vashistha, 1993). Bapna *et al.* (1984) presumed that a hypothetical chemical substance named "Bryonin" was synthesized from sucrose like precursors under low temperature and dark condition in *T. hypophylla*. On agar medium, *C. himalayense* produced sex organs only in Hoagland medium supplemented with 1% sucrose in alternate light / dark conditions. Hoagland medium and peat mixed soil contain optimum nitrogen as ammonium. This suggests that both ammonium and sugars together with temperature and photoperiod have key roles in gametangium induction in *C. himalayense*.

Under *in vitro* conditions *C. himalayense* reproduces asexually by means of innovations from the basal part of thalli when the physical conditions are favorable for vegetative growth and nutrients are available. At the onset of

dryness and exhaustion of nutrients, thalli become smaller and produce tuberous swelling at their apices. Similarly, under continuous low temperature, thalli produce persistent tuberous apices. These tuberous apices are a diagnostic feature of this species that occurs at the high altitude region of Himalaya, where cold temperature is prevailing throughout the year.

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