In vitro propagation of the endemic and threatened Indian liverwort: *Cryptomitrium himalayense* Kash.

Cryptomitrium himalayense Kash., a thalloid liverwort belonging to the family Rebouliaceae of the order Marchantiales, was first instituted by Kashyap[1] on the basis of specimens collected from western Himalaya (Mussoorie, Shimla) at an altitude of ca. 6000–7000 ft. *Cryptomitrium* is among the 55 monotypic liverwort genera in India[2]. Besides being monotypic, this species is endemic to India, distributed only in the western Himalaya[3,4] and Sikkim (eastern Himalaya)[5]. The species has a delicate thallus growing in moist places under the dense shade of trees or in moist hollows in the hilly regions of Himalaya, which are also good tourist spots, attracting a large number of visitors throughout the year. Thus this taxon is exposed to considerable stress due to various biotic interferences, inevitably disturbing their habitat and threatening their very survival. Pant[5] enlisted this species under threatened bryophytes and discussed the effects of urbanization resulting in disappearance of plant from its localities in the Himalaya. Though this species has not been sighted in the recent past, it was seen at Dhobighat locality (alt. ca. 6956 ft) in Nainital in November 2008. The plants with their sporophytes were collected and deposited in the Bryophyte Herbarium of National Botanical Research Institute, Lucknow (LWG-249110).

C. himalayense being an endemic and threatened species, an attempt was made for *in vitro* propagation through spore culture for *ex situ* conservation and maintenance of its *in vitro* germplasm so that bioprospection of the species may be carried out. The findings of Sauerwein and Becker that *in vitro* cultures of a liverwort *Fossombronia pusilla* produce the same terpenoid constituents as produced by plants growing in nature are encouraging in this regard[6].

In India, culture studies on bryophytes, particularly on Marchantiales, have been confined to *in vitro* spore germination[7–9], the effects of abiotic factors on thallus growth[10,11], regeneration[12] and reproductive biology[13]. But so far no work has been carried out on *in vitro* propagation of such endemic and threatened bryophytes, very restrictively distributed in small populations, with the objective of *ex situ* conservation and utilization of valuable biodiversity without overharvesting from its natural habitat.

Spores procured from the surface sterilized mature capsule of *C. himalayense* with 1% sodium hypochlorite solution for 8–10 min were cultured in Knop's macronutrients medium[14] at full, half and one fourth strength under aseptic conditions. Addition of Nitsch trace elements along with 10 ppm ferric citrate[15] and 1% sucrose to the half strength Knop's macronutrients was also tried. Other commonly used plant tissue culture media, viz. MS[16] and Gamborg B-5[17] were also used. The pH of the media was adjusted at 5.8 and all the media were gelled with 0.8% agar (bactograde). Controlled and aseptic conditions were maintained throughout the experiment. Spores of *C. himalayense* germinated readily after 3–4 days of culture (Figure 1 *a*)

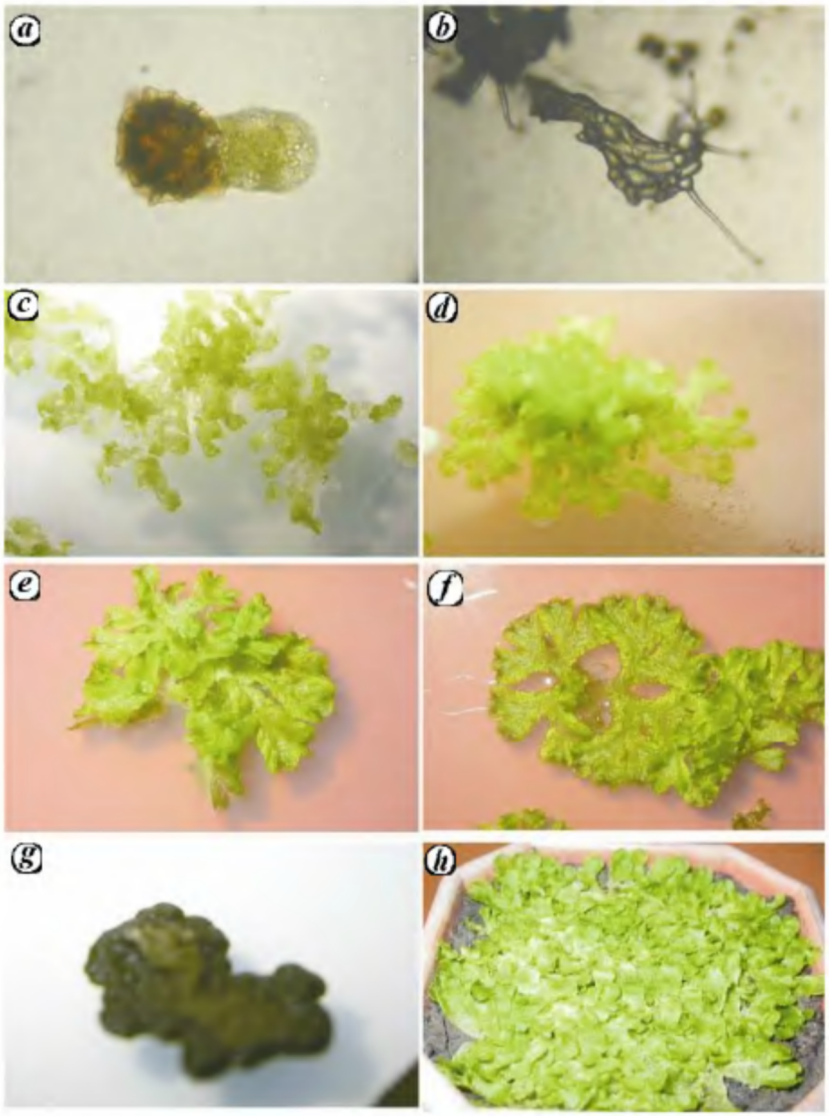

Figure 1. ***a***, Germinating spores (3, 4 days). ***b***, Sporeling stage (15 days). ***c***, ***d***, Young thalli after 30 and 50 days respectively. ***e***, ***f***, Well-differentiated thalli after 70 and 90 days respectively. ***g***, Callus formation. ***h***, Plants transferred to soil in a pot.

only in Knop's macronutrient medium. Germination of spores *in vitro* was apparently similar to that described by Kachroo[18]. In other media, viz. half strength Knop's macronutrients + Nitsch trace elements, MS and B-5 with and without sucrose, spores failed to germinate. Further differentiation of the sporeling (Figure 1 *b*) into well-differentiated thalli was seen only in half strength Knop's macronutrients. Other media used did not favour the growth and differentiation of sporelings even when they were used as secondary inocula. When sporelings or very delicate young thalli were transferred into media supplemented with 1% sucrose, callus formation took place (Figure 1 *g*). Subculturing of the growing young thalli (Figure 1 *c* and *d*) into freshly prepared half strength Knop's macronutrients and providing continuous light of 4000–5500 lux and low temperature of 20°C resulted in rapid growth of thalli and its differentiation (Figure 1 *e* and *f*). Temperature above 30°C proved lethal even for a short time. In about 3 months, a good population of *C. himalayense* was raised from cultured spores that were maintained continuously.

Preference for a dilute culture solution has been noted in other bryophytes[19]. On account of very delicate thallus, the culture of *C. himalayense* shows strong resemblance to *Anthoceros bharadwajii* Udar *et* Asthana[20]; for both the species half strength Knop's macronutrients medium favours growth, while addition of trace elements, sucrose and other media inhibited the growth probably on account of having high osmotic pressure. Induction of callus took place when the young thalli or sporelings were inoculated into nutrient medium supplemented with 1% sucrose. Higher concentration of sucrose (1–4%) has been reported to induce callus formation in the thalli of many liverworts[21]. The development of rhizoids, scales and well-differentiated thalli on medium devoid of any external support of growth regulators suggests that *C. himalayense* has natural endogenous auxin and cytokinin. *In vitro* grown plants have been successfully transferred to soil in pots (Figure 1 *h*) for hardening and multiplication, and a good number of plants under controlled conditions have been obtained. It has paved the way for the use of these plants for bioprospection studies and further they may be planted in their natural habitat for restoration of such an important taxon.

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