

***In vitro* conservation strategy for endemic and endangered Himalayan liverwort *Stephensoniella brevipedunculata* Kashyap (Marchantiophyta)**

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Abstract

During the present study an effort has been made to propagate (*in-vitro*) the endangered and endemic Himalayan liverwort *Stephensoniella brevipedunculata* Kashyap using different culture media under controlled Laboratory conditions. Axenic cultures of the taxon have been established using tubers as explants. Seven combinations of media with Full Strength Knop's macronutrients; Half-strength Knop's macronutrients; Half-strength Knop's macronutrients + Vitamins; Half-strength Knop's macronutrients + 0.2 mg L⁻¹ IBA + 0.1 mg L⁻¹ BAP; Half-strength Knop's macronutrients + 0.1 mg L⁻¹ Kinetin + 0.1 mg L⁻¹ 2,4D; Half-strength Knop's macronutrients + 0.1 mg L⁻¹ IBA + 0.2 mg L⁻¹ BAP and Hoagland no. 2 basal salt mixture were used for culture. The best growth was observed in the Hoagland no. 2 basal salt mixture medium, in which dichotomously branched young thalli were successfully formed. Subsequently healthy population of culture grown plants has been raised on soil in pots for the first time.

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1. Introduction

Stephensoniella brevipedunculata Kashyap was instituted by Kashyap nearly a Century ago as a monotypic and endemic species from the western Himalayas in India, however in a recent phylogenetic study of the complex thalloid liverworts by Villarreal *et al.* (2015) and Long *et al.* (2016), the genus *Stephensoniella* Kashyap has been transferred under genus *Exormotheca* Mitten in the family Exormothecaceae. But the morphological data are so strong and valid to maintain the genus *Stephensoniella* as such and is being treated as it is. Since its inception it was known only from western and North western Himalaya (Himachal Pradesh, Jammu and Kashmir and Uttarakhand) in some restricted pockets only (Kashyap, 1914; Mehra and Mehra, 1939; Udar *et al.*, 1983, Pant *et al.*, 1994; Singh, 1997; Sharma *et al.*, 2011; Awasthi and Pande, 2015). This species has been placed under endangered category due to rapid decrease in its population and non availability of this species at its known localities (Singh, 1999, 2008). It has been listed among globally endangered liverworts in the world list of bryophytes by IUCN (Bryophyte Specialist Group) 2000 (Tan *et al.*, 2000). The possible factors which inhibit its luxuriant growth may be attributed to its restricted distribution, non synchronized development/maturity of sex organs and to some extent limited spore germination due to vegetative propagation by means of tubers. Reproduction

through tubers in nature imposes serious limitations on its dispersal and expanse to wider area. There are several factors which are responsible for endangered status of taxon like over collection, pollution due to urbanization of the hills, tourist movement and also some natural disturbances like landslides, grazing etc. Various workers from time to time carried out detailed study on endangered bryophyte taxa (Mehra and Kachroo, 1952; Udar *et al.*, 1983; Awasthi *et al.*, 2010, 2013). The spore germination study on this plant has been earlier done by Mehra and Kachroo (1952). Tubers are perennating organs and show more compact arrangement of scales and rhizoids (Udar *et al.*, 1983). During the present study an effort has been made to propagate the plants *in vitro* using different culture media under controlled Laboratory conditions. For the study, semidried plants of *S. brevipedunculata* were recently collected from Nainital (Uttarakhand). Axenic cultures of the taxon have been established using tubers as explants. Seven combinations of culture media were used for culture. After one month dichotomously branched thalli were developed in Hoagland medium. Subsequently these were transferred to pots and in 60 days a well grown population of plants has attained for the first time.

2. Materials and Methods

Specimens of *Stephensoniella brevipedunculata* were

Table 1: Observations on the growth of explants of *Stephensoniella brevipedunculata* Kashyap in different culture media.

DAYS	CULTURE MEDIA USED						
	½ Knop's	Knop's	½ Knop's + Vitamins	½ Knop's + IBA + BAP	½ Knop's + Kinetin + 2,4D	½ Knop's + IBA + BAP	Hoagland Basic
After 6 days	No development	No development	Tuber developed to thalli	No development	No development	Development just started	Tuber start developing and dichotomy initiated
After 15 days	Tubers give rise to thalli, dichotomy initiated, numerous rhizoids formed	Thalli grow downward, ventral scale formation initiated	Tubers give rise to thallus formation with dichotomy, thalli $\pm 4.35 \times \pm 1 \text{ mm}$, numerous rhizoids formed	2-3 thalli formed from one tuber, thalli $\pm 1.23 \times \pm 0.67 \text{ mm}$, numerous rhizoids	Thalli grow downwards, thalli $\pm 2.41 \times \pm 1.2 \text{ mm}$, numerous rhizoids formed	Dichotomy initiated, thalli $\pm 2.3 \times \pm 1.3 \text{ mm}$, numerous rhizoids formed	Thalli developed, pores seen, thalli $\pm 4.15 \times \pm 1.24 \text{ mm}$, numerous rhizoids formed
After 21 days	Dichotomy initiated, numerous rhizoids formed, thalli $\pm 3.60 \times \pm 1.10 \text{ mm}$	Tuber gives rise to thalli (long thin thalli), thalli $\pm 4.4 \times \pm 3.10 \text{ mm}$, numerous rhizoids formed	Dichotomy initiated, pore formation on thalli appeared, thalli $\pm 4.5 \times \pm 1.40 \text{ mm}$, numerous rhizoids formed	Thalli developed downwards curled, thalli $\pm 2.22 \times \pm 0.99 \text{ mm}$, numerous rhizoids formed	Thalli developed downward highly curled, thalli $\pm 5 \times \pm 1.47 \text{ mm}$, numerous rhizoids formed	Dichotomy initiated, thalli $\pm 4.82 \times \pm 1.52 \text{ mm}$, numerous rhizoids formed	Thalli developed, pores seen, numerous rhizoids formed
After 30 days	Thalli with dichotomous branching, 2-4 thalli formed from tuber, thalli $\pm 4.96 \times \pm 2.09 \text{ mm}$	Thalli with dichotomous branching, incurved thalli with numerous rhizoids	Thalli with dichotomous branching, thalli $\pm 5.22 \times \pm 2.29 \text{ mm}$, numerous rhizoids formed	Incurved thalli, thalli $\pm 3.09 \times \pm 1 \text{ mm}$, numerous rhizoids formed	Thalli developed, thalli $\pm 5.5 \times \pm 1.76 \text{ mm}$, numerous rhizoids formed	Thalli with ventral scales developed, pores clearly seen, thalli $\pm 7.05 \times \pm 1.56 \text{ mm}$, numerous rhizoids formed	Thalli developed with dichotomous branching and ventral scales, pores clearly seen, thalli $\pm 9.24 \times \pm 3.62 \text{ mm}$

collected in semidried condition [India, Western Himalaya, Uttarakhand, Nainital, Bada-patthar, ca. 2100 m on soil covered rock, November 15, 2015, A.K. Asthana, 300616 (LWG)]. Only tubers were viable, therefore the tubers were used as explants. Tubers were washed thoroughly with tap water first and then with double distilled water. They were surface sterilized with 0.01% sodium hypochlorite solution for 15 minutes and finally they were rinsed three times in sterile deionised water. Seven combinations of culture media viz., Full Strength Knop's macronutrients; Half-strength Knop's macronutrients; Half-strength Knop's macronutrients + Vitamins; Half-strength Knop's macronutrients + 0.2 mg L⁻¹ IBA + 0.1 mg L⁻¹ BAP; Half-strength Knop's macronutrients + 0.1 mg L⁻¹ Kinetin + 0.1 mg L⁻¹ 2,4D; Half-strength Knop's macronutrients + 0.1 mg L⁻¹ IBA + 0.2 mg L⁻¹ BAP and Hoagland no. 2 basal salt mixture were prepared. The pH of media was

maintained at 5.8 before autoclaving and media were gelled with Clerigel (0.4%). All the media were autoclaved at 15 psi for 1 hour and poured in petridishes. After pouring 1 to 2 tubers were inoculated in each set of three petridishes. The cultures were maintained under controlled condition of light (2500-3000 lux, continuous light and temperature ($\pm 22^\circ\text{C}$)). The growth of tubers was observed regularly.

3. Results and Discussion

The efforts have been made to establish the axenic cultures of *Stephensoniella brevipedunculata* using tubers as explants which started to grow after 6 days of inoculation in different media (Table 1). Green coloured small innovations were emerged from single tuber after 6 days of inoculation (Fig. 1-2). After 15 days young thalli were formed (Fig. 2C & D). Subsequently after 21 days thalli developed with well

differentiated dichotomous branching (Fig. 3A, C & D). In 30 days of time tubers have given rise to well developed mature thalli. Plants exhibited best growth in Hoagland's medium and Half-strength Knop's + Vitamins as compared to all other culture media used (Fig. 4C & D). Thus present study revealed that the plant population of *Stephensoniella brevipedunculata* can be successfully grown on Hoagland and Half-strength

Knop's + Vitamins under controlled laboratory conditions. Successfully grown plant population in Hoagland media were transferred to soilrite in pots (Fig. 4I & J) where they grew successfully. Further, these plant populations will be hardened and acclimatized in moss house. After successful acclimatization, efforts can be made for replantation in their natural and native habitat. Thus for conservation and preservation

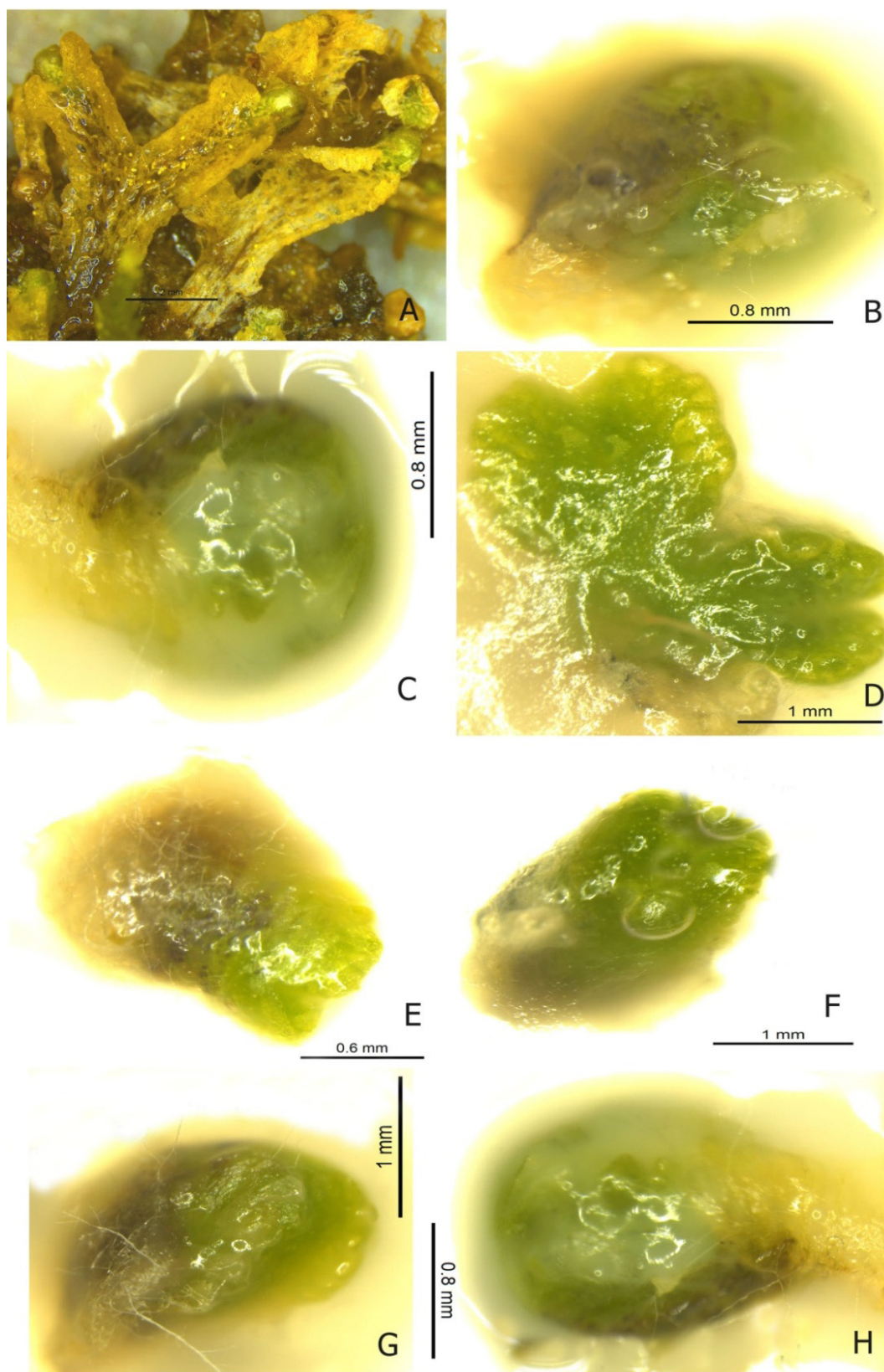


Fig. 1: *Stephensoniella brevipedunculata* Kashyap. *In vitro* propagation of tubers after 6 days (A-H), A: Tubers present at the apex of thalli used as ex plants, B: Tubers in Half-strength Knop's macronutrients medium C: Full Strength Knop's macronutrients medium, D: Hoagland's medium, E: Half-strength Knop's macronutrients medium + Vitamins, F: Half-strength Knop's macronutrients medium + IBA+ BAP, G: Half-strength Knop's macronutrients medium + Kinetin + 2, 4D, H: Half-strength Knop's macronutrients medium+IBA+BAP.

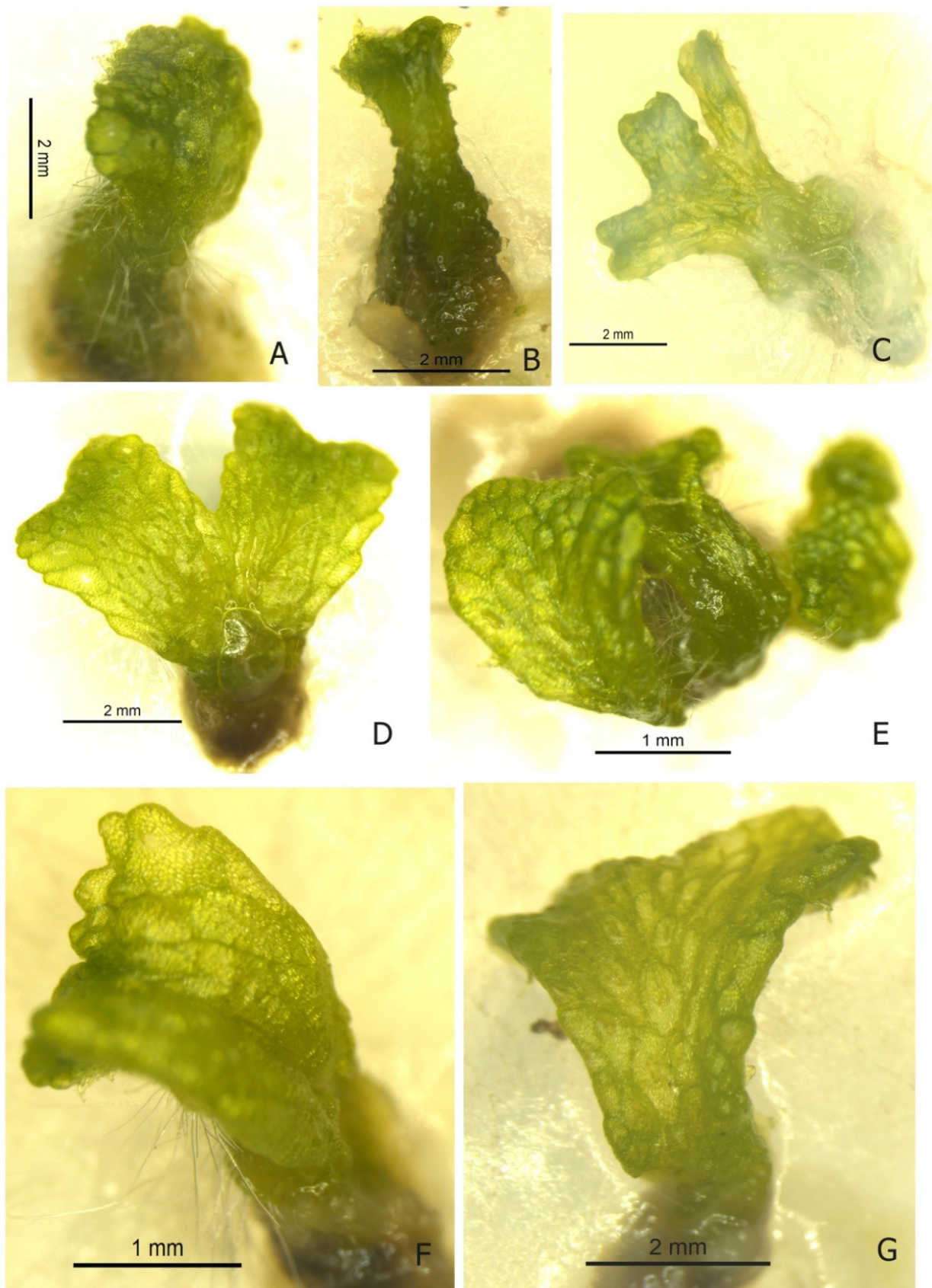


Fig. 2: *Stephensoniella brevipedunculata* Kashyap. *In vitro* propagation of tubers after 15 days (A-G), A: Growing thallus in Half-strength Knop's macronutrients medium, B: Full Strength Knop's macronutrients medium, C: Dichotomy in thalli in Hoagland's medium, D: Dichotomy in thalli in Half-strength Knop's macronutrients medium + Vitamins, E: Half-strength Knop's macronutrients medium + IBA+ BAP, F: Half-strength Knop's macronutrients medium + Kinetin + 2,4D, G: Half-strength Knop's macronutrients medium + IBA+ BAP.

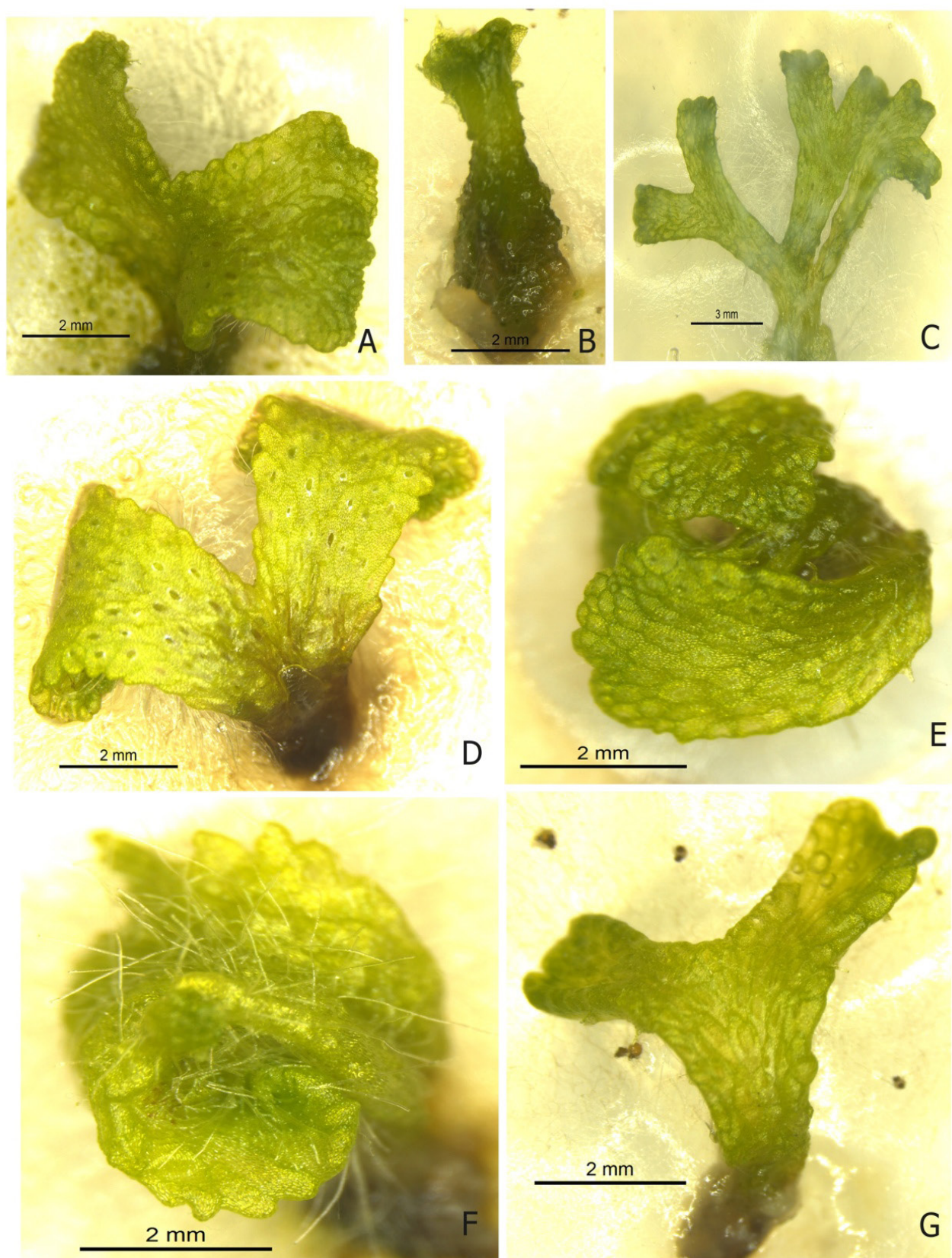


Fig. 3: *Stephensoniella brevipedunculata* Kashyap. *In vitro* propagation after 21 days (A-G), A: Development of thallus in Half-strength Knop's macronutrients medium, B: Full Strength Knop's macronutrients medium, C: Hoagland's medium, D: Half-strength Knop's macronutrients medium + Vitamins, E: Half-strength Knop's macronutrients medium + IBA+ BAP, F: Half-strength Knop's macronutrients medium + Kinetin + 2,4D, G: Half-strength Knop's macronutrients medium+ IBA+ BAP.

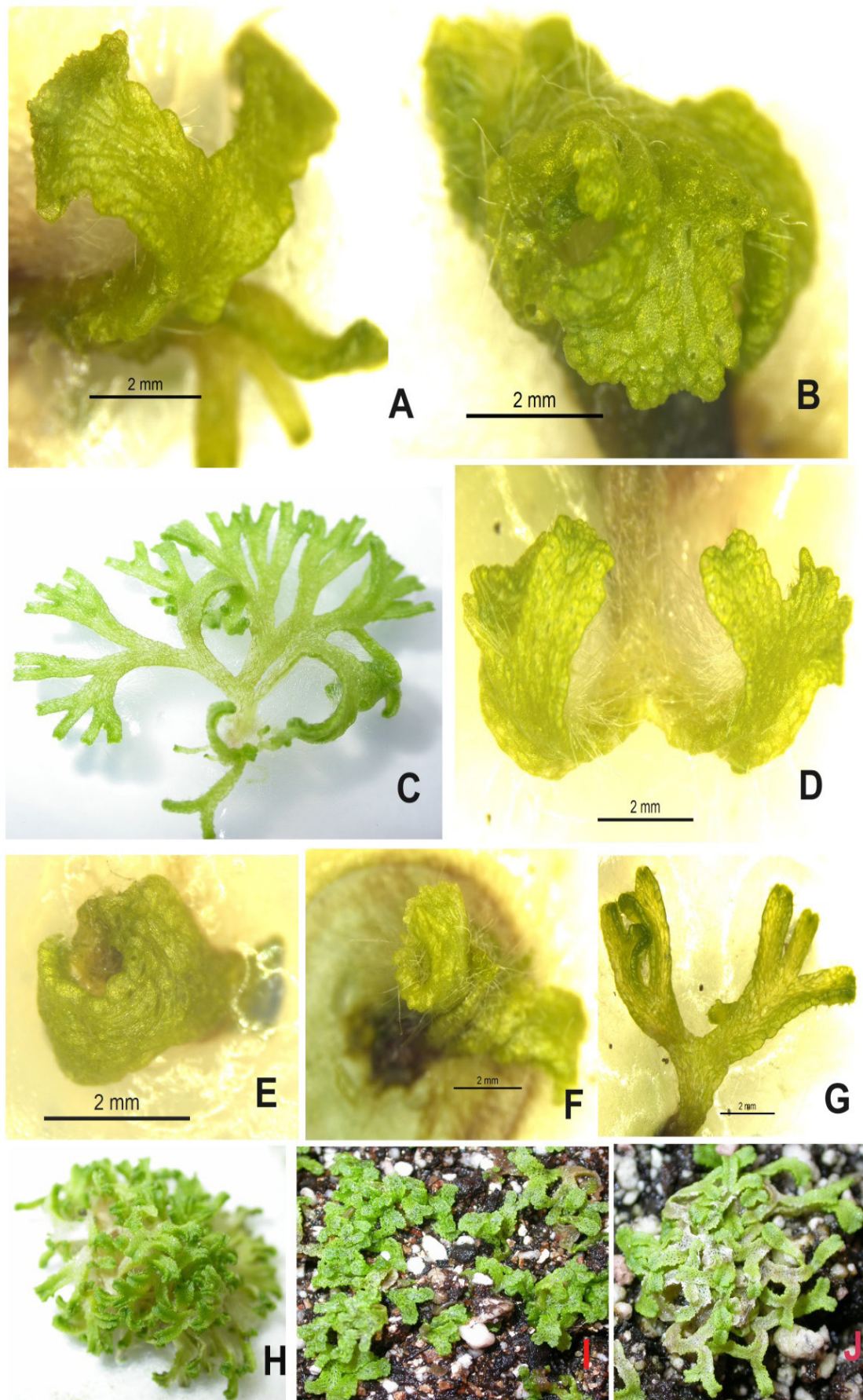


Fig. 4: *Stephensiella brevipedunculata* Kashyap. *In vitro* propagation after 30 days (A-G) A: Thalli in Half-strength Knop's macronutrients medium, B: Full Strength Knop's macronutrients medium, C: Well grown, dichotomously branched thalli in Hoagland's medium, D: Half-strength Knop's macronutrients medium + Vitamins, E: Half-strength Knop's macronutrients medium + IBA+ BAP, F: Half-strength Knop's macronutrients medium + Kinetin + 2,4D G: IBA+ BAP, H: Growth of a population of plants after 2 months, I,J: Plants on soilrite for hardening.

of living germplasm of this important and endemic species present study can play a major role.

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