MATERIAL AND METHODS

Selection of Materials

To study the effect of various physical and chemical factors on protonemal differentiation and bud formation in mosses following two species were selected after screening of a number of mosses for their growth under ordinary cultural conditions. These species were collected from northern western Himalayas, India.

1. Hydrogonium arcuatum (Griff.) Wijk. & Marg.
2. Anoectangium clarum Mitt.

Composition and Preparation of Medium

Aseptic cultures of these species were raised from spores on basal medium (NB) containing Knop’s major salts (diluted by one half), Nitsch’s trace elements, Ferric citrate (Nitsch’s & Nitsch’s, 1956) and Sucrose. The quantity of various constituents in one liter of basal medium was as follows:

- **Macro elements**
  - Ca(NO$_3$)$_2$.4H$_2$O 500 mg
  - MgSO$_4$.7H$_2$O 125 mg
  - KNO$_3$ 125 mg
  - KH$_2$PO$_4$ 125 mg

- **Nitsch’s trace elements**
  - MnSO$_4$.4H$_2$O 3.0 mg
  - H$_3$BO$_3$ 0.5 mg
  - ZnSO$_4$.7H$_2$O 0.5 mg
  - CuSO$_4$.5H$_2$O 0.025 mg
  - Na$_2$MoO$_4$.4H$_2$O 0.025 mg
- **Iron source**
  Ferric citrate  
  10 mg

- **Carbon source**
  Sucrose  
  10 gm

Above mentioned chemicals of analytical grade were used for preparation of medium with double - distilled water and was gelled with agar (8 gm/l) of bacteriological grade. Before sterilizing medium in an autoclave, pH of medium was adjusted to 5.8 using 0.1N - hydrochloric acid or sodium hydroxide. In each culture tube of 15 x 2.5 cm of dimension, about 20 ml of the medium was poured and culture tubes were plugged with non-absorbent cotton wrapped in cheese cloth. The tubes were then autoclaved at 15 psi for 20 minutes. After autoclaving these culture tubes were kept in a slanted position to obtain slants of the basal medium.

**Establishment of cultures**

Aseptic cultures were initiated from spores. For this gametophores with healthy sporophytes were selected and sporophytes were detached from them. Capsules were thoroughly washed in running water for 2-3 hours and then surface-sterilized with saturated chlorine water followed by 2-3 washings in sterile water. Capsules were punctured with the help of sterile needles and spores were transferred aseptically to the semi-solid Nitsch’s basal medium. After spore germination, one of the cultures was selected and its protonemata subcultured for further experimentation. A small amount of bud free protonema was used as inoculum in each tube and care was taken to keep equal amount of inoculum in each tube.

**Cultural conditions**

Cultures were kept in culture room under controlled conditions of light and temperature (25±2°C). Continuous illumination of 3,500 lux was provided by combination of fluorescent tubes. Variation in light intensities were provided by varying the number of fluorescent tubes.
Effects of following chemicals, individually, on protonemal growth and bud formation, were studied by adding them into the basal medium.

- **Auxins**
  - Indole-3-acetic acid (IAA)
  - 2,4-Dichlorophenoxyacetic acid (2,4-D)
  - α- Naphthaleneacetic acid (NAA)
  - β- Naphthoxyzacetic acid (NOA)

- **Cytokinins**
  - 6-Benzylaminopurine (BAP)
  - 6-Furfurylaminoopurine (KINETIN)
  - 2-Isopentenyl adenine (2 iP )

- **Gibberellic acid**
- **Cadmium acetate**
- **Cadmium nitrate**
- **Cadmium sulphate**
- **Lead acetate**
- **Lead nitrate**

For each experiment ten replicates were maintained along with a control. The experiments were run for 60 days. Observations were made periodically under a stereoscopic binocular microscope. For detailed observation and photography temporary glycerine (1%) mounts of germinating spores, protonemata, buds and gametophores were prepared.
Screening of antimicrobial activity

Following moss species were selected for antimicrobial activity:

1. *Entodon myurus* (Hook.) Hamp.
2. *Hydrogonium javanicum* (Doz. & Molk.) Hilp.

Microorganisms used

1. *Bacillus macerans* Schardinger
5. *Aspergillus niger* Van Tieghem.

Preparation of plant extracts

Fresh moss plants were collected from some selected localities of Uttaranchal and Himachal Pradesh. Samples were carefully washed under running tap water and finally with sterile distilled water. They were then dried on blotting papers at room temperature for a week. Samples were crushed in pestle and mortar and homogenized to fine powder with electric grinder. Finally, they were stored in air tight bottles for further experimentation.

Three different solvents namely distilled water, methanol and petroleum ether were used for extraction from the fine powder as described by Quiroga *et al.* (2001) with some modifications. For making aqueous extract, 10 gm of powdered plant material were taken and added to their respective solvents viz. methanol, petroleum ether and distilled water (w/v, 10 gm/100ml) in a 250 ml conical flask, plugged with cotton and then kept on a rotary shaker at high speed for 24 h. After shaking, it was filtered through Whatman filter paper number 1 and the supernatant was collected and dried in oven at 45°C. Extracts were then re-dissolved in their respective solvents to obtain final concentration of 12.5, 25, 50 and 100µg/ml for each plant and stored at 4°C in air tight bottles for their antimicrobial assay.
Antibacterial assay

For assaying antibacterial activity, the agar well diffusion method of Perez et al. (1990) and Singh and Sangwan (2011) were used with minor modifications. Cultures of the bacteria and fungi were obtained from NCIM, Pune (India).

Bacteria were subcultured in nutrient agar slants. The bacterial culture was inoculated on LB broth separately and kept at 37°C overnight. This LB broth mixed with bacterial culture was used for antibacterial assay of various plant extracts. Petri plates were poured with nutrient agar medium. After solidification, LB broth mixed with bacterial culture was spreaded over it and then punched with a 6 mm diameter cork borer to prepare wells and filled with about 50µl of the relevant plant extract of desired concentration. Simultaneously, Streptomycin and Ketoconazole was used as positive control for bacteria and fungi, respectively. Similarly, a negative control was also tested using the different solvents. The test was carried out in triplicates. The petriplates were incubated at 35°C for 24 hours. Zone of inhibition was then measured. MIC of various plant extracts was determined. It is the least concentration of each extract showing a clear zone of inhibition. The inhibitory activities of plant extract were measured in terms of percentage relative inhibition zone diameter (RIZD) by applying the expression:

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\% \text{ RIZD} = \frac{\text{IZD Sample} - \text{IZD Negative Control}}{\text{IZD Antibiotic Standard}} \times 100
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Where, RIZD is the relative inhibition zone diameter (mm) and IZD is the inhibition zone diameter. The resulting IZD of the samples was either higher than or equal to the IZD of the blanks. The test was considered negative when the IZD of the samples was equal to the IZD of the blank.

Similarly, the antifungal activity was also assayed using agar well diffusion method. The fungal culture was subcultured in PDA medium. PDA slants were prepared by dissolving solid PDA media in distilled water. Test tubes were then inoculated with fungal cultures. From these slants fungal inoculums was inoculated separately on PDA medium and test tubes were kept at 27°C undisturbed. These cultures were used for antifungal assay.