SUMMARY

Bryophytes are the most primitive among land plants and all aspects of their life cycle are quite interesting among land plants. Earlier studies concentrated more on floristics, morphology and phylogeny of this group, whereas other aspects such as physiology, biochemistry and morphogenesis received little or no attention as bryophytes were thought to be of little economic importance.

Bryophytes provide suitable system to study morphogenesis in plants because of their small size, high regeneration potential, short life cycle, simplicity of organization, their definite responses to plant hormones, plasticity in their differentiation capacity and well defined haploid and diploid phases in the life cycle.

Recent studies have shown that bryophytes can play a significant role in biomonitoring as many bryophytes are sensitive to pollution and show visible and specific symptoms of injury even in the presence of minute quantities of pollutants. Due to these features bryophytes can serve as good indicator of degree of pollution and also of the nature of pollutants. Some bryophytes are known to concentrate in their body large amount of heavy metals. These species act as sinks, thus preventing recycling in the ecosystem for different periods of time (see Tyler, 1990, Gerdol et al., 2000; Aceto et al., 2003; Rühling and Tyler, 2004; Schintu et al., 2005, Chakrabortty & Paratkar, 2006; Cao et al., 2008 ; Basile et al.,2009; Vats et al., 2010 )

In recent years, the antimicrobial activity of bryophytes has also drawn the attention of several researchers. Although the information about the occurrence of antimicrobial substances in mosses are scanty. These plants have great potential as remarkable reservoir of various biological active compounds. Realization of importance of bryophytes as a potential source of large number of biological active compounds of commercial value has led to research on developmental aspects of this group (see Asakawa, 2007; Dulger et al., 2005; Sabovljevic et al., 2006; Sabovljevic & Sabovljevic, 2008; Veljic et al., 2010)

In the present study, effects of various physical factors and chemical factors including growth regulators and some heavy metals on various developmental phases of two mosses: Anoectangium clarum and Hydrogonium arcuatum have been studied. Attempt has also been made to study antimicrobial activity of Entodon myurus, Hydrogonium javanicum and Physcomitrium coorgense.
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- Naphthoxyacetic acid (NOA)

- **Cytokinins**
  - 6-Benzylaminopurine (BAP)
  - 6-Furfurylaminopurine (KINETIN)
  - Isopentenyladenine (2 iP)

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

For each experiment ten replicates were maintained alongwith 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 northern western Himalayas, India. 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 aqueous, 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.
Results

Spores of Hydrogonium javanicum germinated in 24 hours after planting on NB medium, whereas in Anoectangium clarum spore germination was observed after 3 days under ordinary cultural conditions. In both the species, both unipolar and bipolar germination was observed. Spores showed considerable increase in diameter. This was followed by greening of plastids and rupture of exine. Intine emerged in the form of germ papilla and transverse division and branching at the base formed chloronema. Caulonema differentiated after 7 and 14 days in Hydrogonium javanicum and Anoectangium clarum, respectively. In both the species protonema exhibited heterotrichous habit. In Hydrogonium both aerial and prostrate systems were equally developed. In Hydrogonium, protonema produced buds after 35 days under ordinary cultural conditions, whereas under similar conditions in Anoectangium, protonema remained bud-free.

Among the physical factors, effect of light intensity and hydration was studied on protonemal growth and bud formation. In both species, protonemal growth increased with increase in light intensity up to 3,500 lux. In Hydrogonium javanicum maximum number of buds were observed at 3,500 light intensity. In Anoectangium, variation in light intensity was ineffective in inducing buds. In Hydrogonium, buds formed normal leafy shoot.

In Hydrogonium as well as in Anoectangium maximum protonemal growth was recorded on media containing 0.8% agar, whereas higher levels of agar adversely affected the growth. In Anoectangium variation in hydration of the medium failed to induce buds.

Among chemical factors, effect of sucrose, auxins (2,4-D, IAA, NAA, NOA), cytokinins (BAP,2iP, Kinetin) and gibberellic acid (GA3) were studied on protonemal growth and bud formation. To study the effect of heavy metals, salts of heavy metals Cadmium (cadmium acetate, cadmium nitrate, cadmium sulphate) and Lead (lead acetate, lead nitrate) were incorporated individually to NB medium in the concentration range $10^{-8}$ to $10^{-4}$M.

In Hydrogonium, 1.0% sucrose supported maximum protonemal growth and buds developed at all levels but their number was maximum at 1.0% sucrose. In Anoectangium, protonema remained bud-free.
Of the auxins tested protonemal growth was stimulated in *Hydrogonium* at lower levels of 2,4-D and NAA. Maximum response in terms of area of protonemal patch was elicited by 2,4-D at $10^{-7}$M. Bud initiation was promoted by all the tested auxins at lower levels and in terms of bud number 2,4-D at $10^{-8}$M elicited maximum response. It was followed by NOA, IAA and NAA. In *Anoectangium*, of the four auxins tested, protonemal growth promoted at lower concentrations of 2,4-D, NAA and NOA whereas IAA proved inhibitory at all levels. In terms of bud number, 2,4-D at $10^{-7}$M proved effective followed by NAA, NOA and IAA. Thinner and longer shoots were produced at lower levels of all the tested auxins, whereas higher concentrations resulted in the development of comparatively thicker and shorter shoots.

In *Hydrogonium*, of the three cytokinins tested, protonemal growth was stimulated slightly at lower levels of all cytokinins whereas higher levels proved inhibitory. In terms of bud number all cytokinins elicited maximum response at $10^{-5}$M and 2-iP proved most effective followed by BAP and Kinetin. In *Anoectangium*, all the tested cytokinins have inhibitory effect on protonemal growth except at $10^{-8}$M kinetin. In terms of bud number, 2iP was most effective at $10^{-4}$M whereas BAP and Kinetin were almost equally effective. All the three cytokinins induced moruloid buds at their higher levels, whereas lower concentrations supported development of normal gametophores.

In *Hydrogonium*, growth of protonema was enhanced slightly at lowest concentrations of GA$_3$ ($10^{-8}$M). Higher concentrations proved inhibitory for protonemal growth and inhibition increased with increase in its concentration. Bud formation was highest at lowest concentration ($10^{-8}$M) of gibberellic acid. Bud number decreased with increase in concentration from $10^{-7}$ to $10^{-4}$ M. In *Anoectangium*, Protonemal growth was promoted at lower concentrations ($10^{-8}$ and $10^{-7}$M) of gibberellic acid and maximum protonemal growth was observed at $10^{-7}$M. GA$_3$ was effective in inducing buds on the protonema. Maximum number of buds were produced at $10^{-7}$M. At all the concentrations of gibberellic acid buds developed into leafy gametophores. The gametophores produced on media containing lower concentration of gibberellic acid were long, thin and possessed distantly placed leaves, whereas gametophores developing on higher concentration had comparatively thicker with shorter axes.

Cadmium salts (cadmium acetate, cadmium sulphate, cadmium nitrate) and Lead salts(lead acetate, lead nitrate) caused retardation of protonemal growth as well as bud
formation in both the species. Higher concentrations of heavy metals adversely affected the morphology of protonema. Swelling of tip cells, rounding of intercalary cells, irregular divisions and branching of protonema were frequent.

The antimicrobial activity of bryophytes depends upon the types of solvents used for extraction in Entodon myurus, Physcomitrium coorgense, Hydrogonium javanicum against four bacterial and one fungal strain. Methanolic extract was most effective in controlling growth of microorganisms whereas other two (aqueous and petroleum ether) extracts were either ineffective or less effective in inhibiting growth of these microorganisms. P. coorgense was found to be most effective as it inhibited growth of all microorganisms at one or the other concentration. On the other hand H. javanicum exhibited its effect against three microbial strains at higher concentrations only. E. myurus showed considerable antimicrobial activity against P. putida only. The aqueous or petroleum ether extract of most of the bryophytes did not show any visible antimicrobial activity. The antimicrobial studies of the selected mosses revealed that certain mosses possess significant antimicrobial property and can be used for therapeutic purposes.