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## **Seasonal changes in antioxidant enzymes, polyphenol oxidase enzyme, flavonoids and phenolic content in three leafy liverworts**

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# Seasonal changes in antioxidant enzymes, polyphenol oxidase enzyme, flavonoids and phenolic content in three leafy liverworts

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Seasonal changes in the total phenolic content, flavonoid content, specific activity of the enzyme polyphenol oxidase (PPO) and that of the antioxidative enzymes-catalase (CAT) and peroxidases (POX) were analyzed in three leafy liverworts, namely *Solenostoma crenulata*, *Chiloscyphus gollani* and *Fossombronia himalayensis*. In the rainy season (July–September), the plants were in their young juvenile stage and the levels of activity of antioxidative enzymes catalase (CAT) and peroxidases (POX) were observed to be the lowest. The enzymes showed maximum activity during the months January–March which constitute a dormant season for bryophytes. Activity of POX is found to be higher than that of CAT in all the three seasons. Highest content of flavonoids was observed towards the end of the growing season (January–March). Total phenolic content was found to be highest in winter season (October–December). PPO showed highest activity in the rainy season (July–September) and lowest during winter season. Activity of enzyme PPO and that of phenolic content showed inverse relationship.

Bryophytes constitute a group of small plants which form essential components of terrestrial ecosystems. They can successfully colonize and survive in extreme environmental conditions. The plants synthesize secondary metabolites as a response to biotic or abiotic stresses. Since the bryophytes lack lignins, resins and silica on the epidermis unlike vascular plants and also bear no spines, thorns and trichomes on the leaves for protection, they might have developed biochemical capability in devising active molecular mechanism to make them unpalatable and provide protection against grazing as part of their survival strategy.

Several natural processes like photosynthesis, respiration and stress responses generate reactive oxygen species (ROS) as a byproduct. These ROS can lead to the disruption of the normal physiological and cellular functions (Asada 1994) and also damage biomolecules of plasma membranes and cell walls, thus affecting directly the cell survival (Schutzen-dubel and Polle 2002). Plants which can respond and adapt to drought stress are equipped with complex and highly efficient antioxidative defense systems composed of protective non-enzymatic as well as enzymatic mechanisms that efficiently scavenge ROS and prevent damaging effects of free radicals (Breusegem et al. 2001). Plants produce antioxidant enzymes, such as catalase and peroxidase to get rid of these reactive oxygen species.

Bryophytes produce a number of secondary metabolites and also possess strong antioxidative machinery which helps them to cope with biotic and abiotic stresses (Xie and Lou 2009, Dey and De 2012). The antioxidant defenses appear to provide crucial protection against oxidative damage in cellular membranes and organelles in plants grown under unfavorable conditions. Antioxidant enzymes protect cells against oxidative stress and are associated with stress tolerance and longevity. ROS react with cellular constituents such as protein and lipids, leading to damage and, thus affecting directly the cell survival.

Hirata et al. (2000) studied enzyme peroxidase in the thallose liverwort *Marchantia polymorpha* and characterized it as a glycoprotein which is dissimilar to any known tracheophyte peroxidase. Paciolla and Tommasi (2003) studied the antioxidant systems in a moss, *Brachythecium velutinum* and a liverwort, *Marchantia polymorpha*. Both taxa were revealed to use enzyme ascorbate peroxidase in the removal of hydrogen peroxide. Montenegro et al. (2009) reported the presence of various phenolic compounds such as caffeic, gallic, vanillic, chlorogenic, p-coumaric, 3-4 hydroxybenzoic and salicylic acid in the moss *Sphagnum magellanicum* by Reverse phase high pressure liquid chromatography.

In India, Krishnan and Murugan (2013a, 2013b) determined the presence of phenols, flavonoids, saponins, tannins and glycosides in *Marchantia polymorpha* and also indicated positive correlation between antimetastatic effects and the flavonoids present in *M. linearis*. Dey et al. (2013) studied the effect of altitude and tissue type on the antioxidant potential of vegetative and reproductive tissues of *Pellia endiviifolia*. Krishnan et al. (2014) studied the

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effect of light intensity, cations, organic carbon source and the size of inocula on the productivity of flavonoids. Kadam (2015) reported direct relationship between the content of polyphenol and the growth.

Earlier reports on seasonal variations in the storage compounds and enzymatic activities in West Himalayan liverworts (Kapila et al. 2014, Kapila and Thakur 2016, Thakur and Kapila 2016) indicate that the changes in temperature and relative humidity due to the seasonal changes in climatic conditions have a pronounced effect on the biochemical constituents.

Here we account for the seasonal variation in total phenolic content and flavonoids, and the specific activity of the enzyme polyphenol oxidase (PPO) and that of antioxidant enzymes catalase (CAT) and peroxidase (POX) in three leafy liverworts, namely *Solenostoma crenulata*, *Chiloscyphus gollani* and *Fossombronia himalayensis* during three bryological seasons.

## Material and methods

### Collection and identification of plant material

Study on seasonal variation has been conducted by collecting plant material in three different seasons relevant to the life histories of bryophytes i.e. rainy season (July–September), winter season (October–December) and at the end of the growing season (January–March). The information on range of temperature and total precipitation during three seasons was collected from the Meteorological Centres of respective cities and are given in Table 1. All the samples of leafy liverworts namely *Solenostoma crenulata* growing on wet soil near stream, in shady habitat at Mandi (altitude 1044 m), *Chiloscyphus gollani* growing on wet soil at Shimla (altitude 2200 m) and *Fossombronia himalayensis* growing in shaded habitat on wet soil at Kasauli (altitude 1927 m) were collected in three different seasons from the same site. The plant species were carefully selected and cleaned of soil and other contaminants.

### Preparation of extracts

Extracts for the estimation of enzymatic activity was prepared by homogenizing 500 mg of frozen plants in 0.1 M phosphate buffer (pH 7.0). Then the homogenate was filtered through four layers of muslin cloth. The filtrate was centrifuged at 10 000 rpm for 20 min at 4°C. The supernatant was used as a source of enzymes. The method given by Cakmak

and Marschner (1992) was followed to determine the specific activity of enzyme catalase. The method of Eglely et al. (1983) was followed to determine the activity of enzyme peroxidase. The activity of enzyme polyphenol oxidase (PPO) was determined by the method of Van Lelyveld and Pretorius (1973).

Total phenolic content (TPC) was estimated by the method of Swain and Hillis (1959) with gallic acid as standard. The TPC was calculated as mg gallic acid equivalents per gram of fresh sample ( $\text{mg g}^{-1}$ ).

Extract for the estimation of flavonoid was prepared in 80% acetone. Total flavonoid content was estimated by the method of Luximon-Ramma et al. (2002) using rutin as a standard. Calculation of total flavonoids was done as equivalent of per mg rutin (RE) per gram of fresh sample ( $\text{mg g}^{-1}$ ).

### Statistical analysis

Analyses were performed with three replicates for each species for three seasons at each of the three sites; therefore the non-parametric test i.e. the Kruskal–Wallis test was applied using IBM SPSS 20 software. For comparing the groups further, Mann–Whitney test was applied as we were comparing two groups out of the three groups at a time. Differences at  $p \leq 0.05$  were considered statistically significant.

## Results

The results obtained from the present study are given graphically in Fig. 1–5. Activities of enzymes catalase and peroxidase (Fig. 1, 2) showed significant ( $p \leq 0.05$ ) seasonal variation in the three periods of collection using Kruskal–Wallis test (Table 2, 3). Post hoc comparisons using the Mann–Whitney test reveal that the activities of both peroxidase and catalase differ significantly ( $p \leq 0.05$ ) among all three seasons. Among the three species of leafy liverworts, *Fossombronia himalayensis* showed highest activity of both the antioxidant enzymes catalase and peroxidase in all the seasons.

In all the three species, peroxidase showed highest activity at the end of growing season i.e. January–March ( $0.078 \pm 0.002 \text{ Kat s}^{-1} \text{ mg}^{-1} \text{ protein}$  in *Chiloscyphus gollani*,  $0.118 \pm 0.001 \text{ Kat s}^{-1} \text{ mg}^{-1} \text{ protein}$  in *Solenostoma crenulata* and  $0.167 \pm 0.003 \text{ Kat s}^{-1} \text{ mg}^{-1} \text{ protein}$  in *F. himalayensis*), whereas the lowest activity was observed in the rainy season i.e. July–September ( $0.018 \pm 0.001 \text{ Kat s}^{-1} \text{ mg}^{-1} \text{ protein}$  in

Table 1. The range of temperature and total precipitation during the three seasons.

Period of collection	Rainy season (July–September)	Winter season (October–December)	End of the growing season (January–March)
Kasauli (altitude 1927 m)			
Mean air temp. (°C)	29.27	19.34	15.67
Total precipitation (mm.)	195–70	12–24	43–53
Shimla (altitude 2200 m)			
Mean air temp. (°C)	20.6–19.4	17.2–10.6	8.3–13.9
Total precipitation (mm.)	424–160	33–28	60–61
Mandi (altitude 1044 m)			
Mean air temp. (°C)	25.5–25.3	23.1–17.4	16.8–21
Total precipitation (mm.)	240–130	25–10	30–22

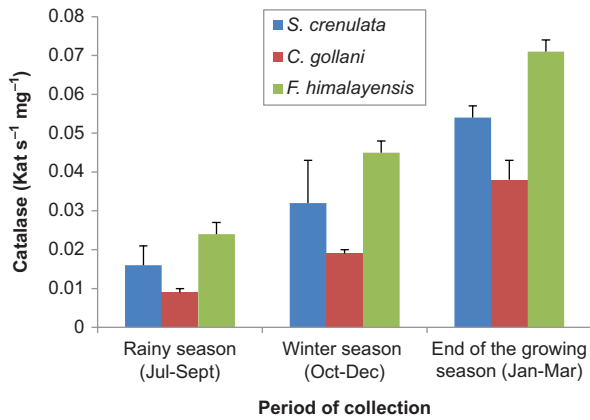


Figure 1. Activity of catalase in three leafy liverworts in three periods of collection. All observations within species differ significantly among seasons ( $p \leq 0.05$ ).

*C. gollani*,  $0.033 \pm 0.001$  Kat s<sup>-1</sup> mg<sup>-1</sup> protein in *S. crenulata* and  $0.083 \pm 0.003$  Kat s<sup>-1</sup> mg<sup>-1</sup> protein in *F. himalayensis*).

The enzyme catalase showed lower activity than the enzyme peroxidase but both the enzymes showed same seasonal pattern. *C. gollani* showed lowest activity of the enzyme catalase compared to *S. crenulata* and *F. himalayensis* in all the three seasons of collection.

The total phenolic content was found the highest during October–December in all the studied taxa ( $21.58 \pm 0.18$  mg g<sup>-1</sup> fw in *C. gollani*,  $20.5 \pm 0.07$  mg g<sup>-1</sup> fw in *F. himalayensis*,  $23.72 \pm 0.08$  mg g<sup>-1</sup> fw in *S. crenulata*) and the lowest in the rainy season ( $8.82 \pm 0.14$  mg g<sup>-1</sup> fw in *C. gollani*,  $9.85 \pm 0.37$  mg g<sup>-1</sup> fw in *F. himalayensis*,  $10.36 \pm 0.08$  mg g<sup>-1</sup> fw in *S. crenulata*, Fig. 3). Kruskal–Wallis test (Table 4) used for total phenolic content revealed that all the three leafy liverworts showed significant ( $p \leq 0.05$ ) seasonal variations in all the three seasons of collection i.e. July–September ( $9.85 \pm 0.37$  mg g<sup>-1</sup> fw in *F. himalayensis*,  $8.82 \pm 0.14$  mg g<sup>-1</sup> fw in *C. gollani*,  $10.36 \pm 0.08$  mg g<sup>-1</sup> fw in *S. crenulata*), October–December ( $20.50 \pm 0.07$  mg g<sup>-1</sup> fw in *F. himalayensis*,  $21.58 \pm 0.18$  mg g<sup>-1</sup> fw in *C. gollani*,  $23.72 \pm 0.08$  mg g<sup>-1</sup> fw in *S. crenulata*) and January–March ( $18.49 \pm 0.70$  mg g<sup>-1</sup> fw in *F. himalayensis*,  $19.36 \pm 0.21$  mg g<sup>-1</sup> fw in *C. gollani*,  $21.83 \pm 0.21$  mg g<sup>-1</sup> fw in *S. crenulata*).

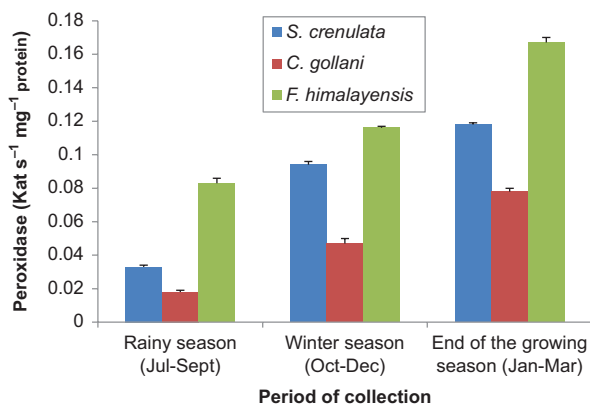


Figure 2. Activity of peroxidase in three leafy liverworts in three periods of collection. All observations within species differ significantly among seasons ( $p \leq 0.05$ ).

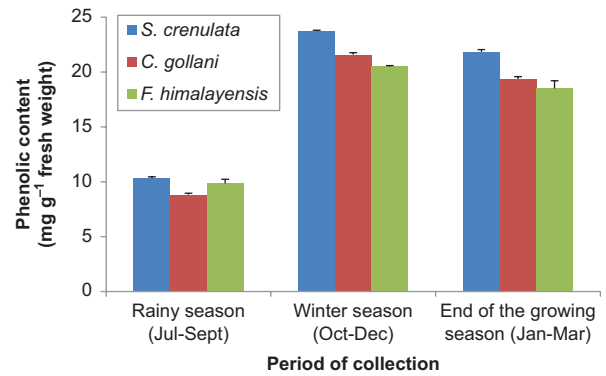


Figure 3. Total phenolic content in three leafy liverworts in three periods of collection. All observations within species differ significantly among seasons ( $p \leq 0.05$ ).

Post hoc comparisons using the Mann–Whitney test reveal that the total phenolic content differ significantly ( $p \leq 0.05$ ) among all three seasons.

Total flavonoid content analysed in all the three seasons of bryological growth indicated significant ( $p \leq 0.05$ ) seasonal changes using Kruskal–Wallis test (Table 5). Post hoc comparisons using the Mann–Whitney test reveal that the total flavonoid content differ significantly ( $p \leq 0.05$ ) among all three seasons. The flavonoid content in all the three leafy liverworts (Fig. 4) was found to be maximal at the end of growing season i.e. January–March ( $16.68 \pm 0.08$  mg g<sup>-1</sup> fw in *F. himalayensis*,  $18.07 \pm 0.38$  mg g<sup>-1</sup> fw in *C. gollani*,  $20.77 \pm 0.11$  mg g<sup>-1</sup> fw in *S. crenulata*) and minimal in the rainy season i.e. July–September ( $5.98 \pm 0.30$  mg g<sup>-1</sup> fw in *C. gollani*,  $7.82 \pm 0.10$  mg g<sup>-1</sup> fw in *S. crenulata*,  $8.39 \pm 0.37$  mg g<sup>-1</sup> fw in *F. himalayensis*). Low seasonal variation in the content of flavonoids was observed in the winter season i.e. October–September and at the end of the growing season i.e. January–March.

All the three leafy liverworts showed significant ( $p \leq 0.05$ ) seasonal variation for the activity of PPO in all the three periods of collection using Kruskal–Wallis test (Table 6). The specific activity of PPO was at the peak (Fig. 5) in the

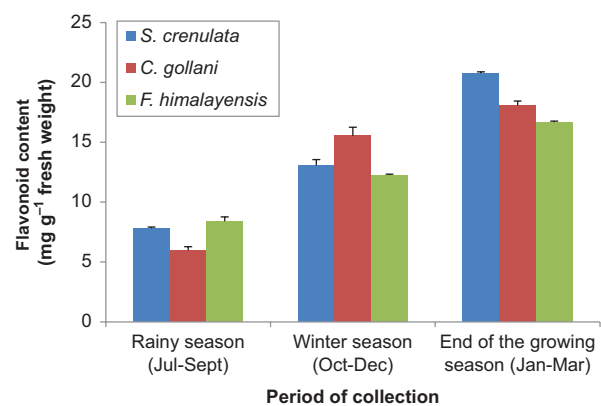


Figure 4. Total flavonoid content in three leafy liverworts in three periods of collection. All observations within species differ significantly among seasons ( $p \leq 0.05$ ).

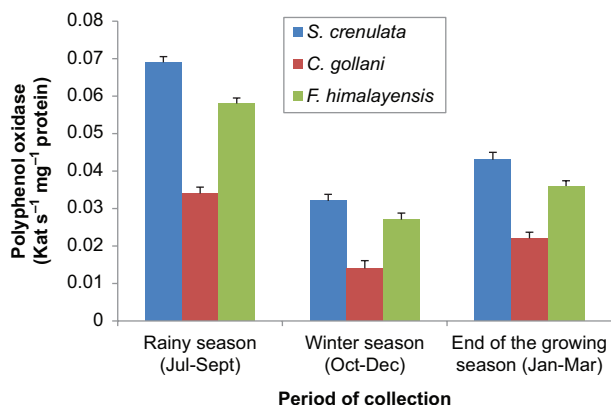


Figure 5. Activity of polyphenol oxidase in three leafy liverworts in three periods of collection. All observations within species differ significantly among seasons ( $p \leq 0.05$ ).

rainy season and found minimum in the winter season i.e. October–December and then again showed an increase during January–March i.e. at the end of growing season. Post hoc comparisons using the Mann–Whitney test reveal

that the activity of polyphenol oxidase differ significantly ( $p \leq 0.05$ ) among all three seasons.

## Discussion

Our study showed that the activity of antioxidant enzymes peroxidase and catalase in all the three leafy liverworts increased significantly during January–March when the conditions are not favourable for the bryophytic growth and this period is considered dormant for the growth of these plants. This could be related to the protective mechanism of these plants against the stressful conditions towards the end of the growing season. Plants induce catalase and peroxidase to scavenge reactive oxygen species (ROS) and provide protection against inactivation of cell functions (Singh et al. 2006). The activity of peroxidase increases in overwintering organs and plays protective roles in enhancing their tolerance against unfavorable conditions (Citadin et al. 2002, Ghorbanli et al. 2012). Catalase – an oxidoreductase enzyme decomposes  $H_2O_2$  to water and molecular oxygen and is one of the key enzymes involved in removal of toxic peroxides

Table 2. Kruskal–Wallis analysis values for the enzyme catalase.

Seasons	n	Mean rank	$\chi^2$	df	p
Rainy season (July–September)	9	6.56	16.745	2	0.000
Winter season (October–December)	9	13.61			
End of the growing season (January–March)	9	21.83			

Table 3. Kruskal–Wallis analysis values for the enzyme peroxidase.

Seasons	n	Mean rank	$\chi^2$	df	p
Rainy season (July–September)	9	6.89	12.633	2	0.002
Winter season (October–December)	9	15.06			
End of the growing season (January–March)	9	20.06			

Table 4. Kruskal–Wallis analysis values for total phenolic content.

Seasons	n	Mean rank	$\chi^2$	df	p
Rainy season (July–September)	9	5.00	19.574	2	0.000
Winter season (October–December)	9	21.28			
End of the growing season (January–March)	9	15.72			

Table 5. Kruskal–Wallis analysis values for total flavonoid content.

Seasons	n	Mean rank	$\chi^2$	df	p
Rainy season (July–September)	9	5.00	22.592	2	0.000
Winter season (October–December)	9	14.22			
End of the growing season (January–March)	9	22.78			

Table 6. Kruskal–Wallis analysis values for the enzyme polyphenol oxidase.

Seasons	n	Mean rank	$\chi^2$	df	p
Rainy season (July–September)	9	20.83	12.945	2	0.002
Winter season (October–December)	9	7.39			
End of the growing season (January–March)	9	13.78			



present in intracellular compartments, thereby enhancing the freezing tolerance by reducing the hydrogen peroxide concentration (Baek et al. 2000). Low temperature and water stress lead to overproduction of reactive oxygen species which cause oxidative damage to cell (Sattler et al. 2007). During oxidative stress, hydrogen peroxide – a toxic intermediate is produced which is decomposed by peroxidases (Reddy et al. 2005). Increased activity of peroxidase and catalase has been suggested as an adaptive mechanism to provide protection against oxidative damage.

Haribal and Renwick (2001) reported the seasonal variation in flavonoid content of *Alliaria petiolata* to be dependent on light content or day length. Lee et al. (2003) observed a positive correlation between the content of flavonoid aglycones in samples of *Angelica keiskei* and the duration of exposure to sunshine. Presently, higher content of flavonoids observed during the months of January–March (sunnier as compared to the other two seasons) also indicates that low temperature and high light conditions lead to accumulation of flavonoids. Due to the phenolic hydroxyl groups present in flavonoids they are capable of effectively scavenging the ROS (reactive oxygen species) and are potent antioxidants (Cao et al. 1997).

Total phenolic content was observed to be highest in the winter season i.e. October–December. They have a vital role in absorbing and neutralizing free radicals and decomposing peroxides (Shah et al. 2010).

In the present study, a significant variation in the activity of enzyme Polyphenol oxidase was also observed. Highest activity of enzyme PPO was observed in rainy season when the TPC was lowest. PPO activity is negatively related with total phenolic compounds. This may be due to the formation of insoluble cell wall bound polyphenols from soluble phenolics (Malmir 2014).

It is concluded that there exist wide seasonal variations in total phenolic content, flavonoids, antioxidant enzymes and polyphenol oxidase enzyme in all the three presently studied species of leafy liverworts which might be due to fluctuations in temperature, precipitation, conditions of their habitat, the duration and intensity of sunshine as well as the photoperiods. The present work can be useful in finding the appropriate growth conditions for the harvesting of particular desired compounds.

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