

## Seasonal variation of antioxidant enzymatic responses in the desiccation-tolerant bryophyte *Syntrichia ruralis* (Hedw.) Web. & Mohr.

Ruchika<sup>1</sup> – Zsolt CSINTALAN<sup>1†</sup> – Katalin VERES<sup>3</sup> – Evelin Ramóna PÉLI<sup>2</sup>

1: Hungarian University of Agricultural and Life Sciences, Department of Plant Physiology and Plant Ecology, Institute of Crop Production, H-2100, Páter Károly utca 1, Gödöllő, Hungary, e-mail: [ruchika.110291@gmail.com](mailto:ruchika.110291@gmail.com)

2: University of Veterinary Medicine, Department of Botany, H-1078, István utca 1., Budapest, Hungary, e-mail: [pelievelin@gmail.com](mailto:pelievelin@gmail.com)

3: Centre for Ecological Research, Institute of Ecology and Botany, H-2163, Alkomány út 2-4, Vácraátót, Hungary

† Deceased February 19, 2019

**Abstract:** Bryophytes are poikilohydric organisms that can be used as model plants to study desiccation tolerance mechanisms. The main objective of this study was to examine the activities of the antioxidant enzymes ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (POD) in the rehydrated and desiccated states in *Syntrichia ruralis* (Hedw.) Web. & Mohr. from two slopes, one North-east (NE) and one South-west (SW) facing and collected in different seasons. Our results showed seasonal variation in the enzymatic activities of APX, CAT and POD between the slopes in both the rehydrated and desiccated states. The mean value of all the activities of APX, CAT and POD and MDA contents (a measure of lipid peroxidation) tended to be higher in moss cushions collected from the NE compared to the SW facing slopes except in summer season. The mean values of all enzymatic activities were higher in desiccated states as compared with rehydrated states. Protein content has lower values in summer and winter season. Differences in the antioxidant activities of the mosses growing on the two slopes may reflect adaptations to desiccation stress.

**Keywords:** Poikilohydric, catalase, ascorbate peroxidase, guaiacol peroxidase, lipid peroxidation

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### Introduction

The soil surface in arid areas is frequently covered by cryptobiotic crusts (CBCs) comprising communities. These formations were previously often referred to as algal crusts (Komáromi 1979; 1980; 1983), microbiotic soil crusts (Eldridge and Greene 1994), biological soil crusts (Evans and Johansen 1999, Belnap et al. 2001), or cryptogamic crusts (Strandling et al. 2002). Nowadays, however, the “cryptobiotic crust, CBC” has become generally accepted (Pócs 2006. 2008). Arid and semi-arid areas are subjected to frequent drought, and as a result are often rich in desiccation-tolerant species. These

species make a significant contribution to grassland diversity and facilitate fundamental ecosystem functioning such as carbon storage in nutrient-poor environments, increase water retention capacity and interacting with vascular plants by seedling establishment (Lindo and Gonzalez, 2010). Furthermore, bryophytes in grasslands could be used as indicators to track nutrient fluxes, pollutants, or climate change in grasslands (Müller et al. 2012). However, global climate change is affecting CBCs, and therefore their species composition may be affected (Rodriguez-Caballero et al. 2018). Therefore, investigations to study the seasonal

variations on desiccation tolerant species can provide important information related to the aspects of desertification. Some poikilohydric mosses (desiccation tolerants) can survive during dry conditions and can fully recover on rehydration (Alpert and Oliver 2002). In the Hungarian steppe, desiccation tolerant mosses such as *Syntrichia ruralis* (Hedw.) Web. & Mohr. have been reported to be abundant between the scattered tufts of dominant grasses *Festucetum vaginatae danubiale* association (Csintalan et al. 2000). More generally, *S. ruralis* has a worldwide distribution, and is an important component of many biological soil crusts (Belnap et al. 2016).

Drought and heat are two of the main stresses that limit the survival of moss biocrusts in arid areas (Chongfeng et al. 2017). These stresses increase reactive oxygen species (ROS) production inside the plant cell (Cruz de Carvalho et al. 2012). ROS include superoxide radicals ( $O_2^{\cdot-}$ ), hydroxyl radicals ( $OH\cdot$ ), hydrogen peroxide ( $H_2O_2$ ), and singlet oxygen ( $^1O_2$ ). They can oxidize cellular components such as lipids (membranes), proteins (enzymes), DNA and RNA, which can eventually lead to cell death (Leprince et al. 2000; Mittler 2002; Cruz de Carvalho 2008). Plants, including mosses, have an antioxidant system for the detoxification of excess ROS using enzymatic and non-enzymatic antioxidants. Enzymes that remove ROS include catalase (CAT), superoxide dismutase (SOD) and guaiacol peroxidase (POD) while non-enzymatic antioxidants include glutathione (GSH), vitamin C, and carotenoids. Furthermore, some enzymes regenerate oxidized ROS scavenging molecules such as glutathione reductase (GR) (Zhang et al. 2017). Stress has often been shown to increase the activity of these enzymes, for example SOD activity and catalase in drought tolerant *Syntrichia ruralis* reached maximum levels after slow drying for 5h (Dhindsa and Mattowe 1981) and

SOD activity was greatly increased during desiccation in the moss *Atrichum androgynum* (Mayaba and Beckett 2003). Similarly, Onele et al. (2018) showed that slow drying induced POD in *Dicranum scoparium*.

Stress tolerance varies greatly between bryophytes, but species growing on open exposed dry habitats, rock surfaces can tolerate fast, prolonged desiccation and frequent dry and wet cycles (Bewley, 1972; Proctor et al. 2007a). The desiccation tolerant moss *S. ruralis* has been used as an experimental model plant to understand how plants respond to environmental stress (Oliver et al. 2000b; Dinakar et al. 2012). It has both constitutive and inducible mechanisms that can reduce and repair cellular damage and enable it to regain its normal metabolism within minutes on rehydration (Péli et al. 2005). Studies have been carried out at the molecular level on *S. ruralis* (Scott and Oliver 1994; Wood and Oliver, 1999; Zeng et al. 2002; Oliver et al. 2004) and also at the physiological level (Tuba et al. 1996; Csintalan et al. 1999, 2000; Kalapos and Mázsa 2001; Proctor et al. 2007; Barón et al. 2009). However, further research is needed to clarify and understand the metabolism at the enzymatic level and to study the mechanism behind the species distribution in open grasslands. Therefore, activities of some antioxidant enzymes were examined in this study and could be helpful in finding the role of these enzymes in bryophytes. The main objective of this study was to observe the seasonal variation in the antioxidant enzymes CAT, APX, POD along with protein and MDA contents during rehydration and desiccation in *S. ruralis* to understand the response of these antioxidant enzymes to stress. We hypothesized that there can be seasonal variation in antioxidant enzymatic activities under different degrees of environmental stresses such as drought, heat, and variation in extreme temperatures.

## Materials and Methods

### *Plant Material*

*Syntrichia ruralis* (Hedw.) Web. & Mohr (synonym: *Tortula ruralis*) (Pottiaceae) is also known as sandhill screw moss. This species grows as extensive mats on open exposed areas of sandy dunes in semi-arid grassland and plays an important role in CSCs by binding sand particles. Moss cushions of *S. ruralis* were collected in an air-dried state from semi-arid sandy grassland near Bócsa-Bugac in the Kiskunság region (central Hungary 46° 53' 29" N, 19° 26' 35.6" E) in late winter (March 2018), spring (May 2018), summer (July 2018) and autumn (October 2018). Samples were selected from two different slopes, a north-east (NE) and a south-west (SW). Average annual temperature and average annual precipitation along with monthly changes in meteorological parameters such as photosynthetically active radiation, temperature, precipitation, and relative humidity were also recorded during investigated year (2018) at the sample site. These climatic conditions were presented in Ruchika et al. 2020.

### *Experimental set-up*

Air-dried field samples were transported back to the laboratory in paper envelopes and kept at room temperature for two days in opened paper envelopes. Later, samples were cleaned by removing sand particles. For the rehydration treatment, five replicates of moss cushions were transferred on wet filter paper trays placed in transparent plastic boxes partially filled with water. They were sprayed with distilled water, and maintained constantly hydrated for 72 h. For the desiccation treatment, samples were slowly desiccated by placing them in petri dishes for 48 h. Samples (0.3 g) were divided in two different treatments: rehydrated (Rehy) and desiccated (Desic) to determine the activities of antioxidant enzymes and the protein con-

tent. 0.2 g was used to determine lipid peroxidation products (MDA content). A similar experiment set-up was followed in each season. Rehydrated and desiccated shoot tips of *S. ruralis* are shown in Figure 1 (A and B, respectively).

Water content (WC%) were measured and calculated by using the fresh (FW) and oven-dried (DW) weight of the samples after small intervals of rehydration (2h, 6h, 12h, 24 h, 72 h) and drying out at 80 °C, respectively;  $WC = [(FW - DW) / DW] \times 100$  (Péli et al. 2011). Figure 2 shows changes in water content during rehydration-dehydration cycle.

### *Extraction of plant material and antioxidant enzyme assays*

Moss shoots (0.3 g) from both slopes and in rehydrated and desiccated states were ground to a fine powder in liquid nitrogen and homogenized in 2 mL of potassium phosphate extraction buffer (125 mM, pH =7.8) using a pre-chilled mortar and pestle. The extract was centrifuged at 4 °C for 10 min at  $15000 \times g$  (RCF) in a cooling centrifuge. The supernatant was used to determine the assay of catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (POD) according to (Dazy et al. 2009) with some modifications. The molar extinction coefficient ( $\epsilon$ ) was used to calculate the enzymatic activities and expressed as Units  $mg^{-1}$  protein content.

#### *Ascorbate peroxidase (APX, EC 1.11.1.11)*

The APX reaction mixture consisted of 125 mM potassium phosphate buffer (pH= 7.0), 5 mM Na-ascorbate, 1 mM  $Na_2$ -EDTA, 100 mM  $H_2O_2$  and 0.1 mL plant enzyme extract. The decrease of ascorbate concentrations was measured for 100 sec at 25 °C ( $\epsilon_{290} = 2.8 \text{ mM}^{-1}\text{cm}^{-1}$ ).

#### *Catalase (CAT, EC 1.11.1.6)*

Catalase activity was determined by measuring the decrease in the  $H_2O_2$  concentration. The CAT reaction mixture (1 mL) contained

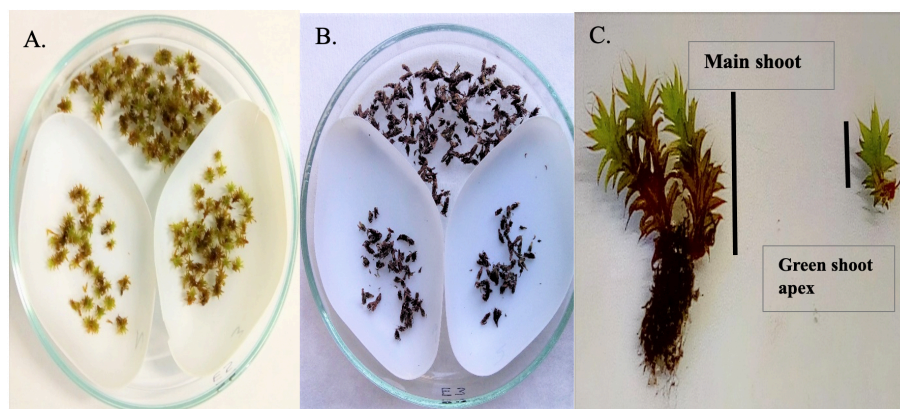


Figure 1. Shoots of the moss *Syntrichia ruralis* in the (A.) rehydrated state and (B.) desiccated state (slow drying) and two parts of the green shoot (C.).

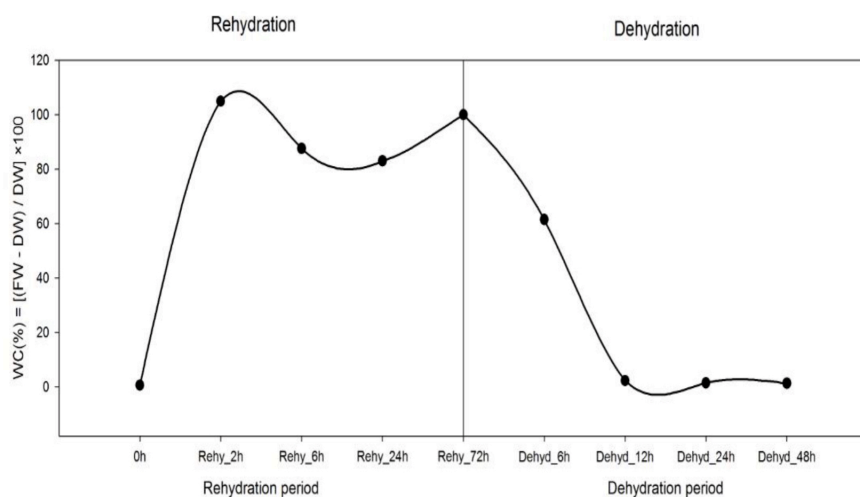


Figure 2. Graphical representation of water content percentage (WC%) during rehydration period (left) and dehydration period (right).

125 mM potassium phosphate buffer (pH=7.0), 100 mM H<sub>2</sub>O<sub>2</sub>, and 0.1 mL plant enzyme extract. The decrease in the H<sub>2</sub>O<sub>2</sub> concentration in a reaction mixture was measured for 340 sec at 25 °C ( $\epsilon_{240} = 36.6 \text{ mM}^{-1}\text{cm}^{-1}$ ).

#### *Guaiacol peroxidase (POD, EC 1.11.1.7)*

The POD reaction mixture (1 mL) contained 125 mM potassium phosphate buffer (pH=7.0), 34 mM guaiacol, 100 mM H<sub>2</sub>O<sub>2</sub>, and 0.1 mL plant enzyme extract. The increase in tetra guaiacol concentration in a reaction

mixture was measured for 150 sec, 25 °C ( $\epsilon_{470} = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$ ).

#### *Protein determination*

The concentration of protein was determined according to the (Bradford,1976) with some modification. Coomassie blue dye-binding assay was used for the quantification of soluble protein content. Bovine serum albumin (BSA) was used for the preparation of the standard curve. Enzyme extracts of samples from both slopes were measured spectrophotometrically at 595 nm. Protein content were

calculated later using the standard curve and expressed in mg.

#### *Lipid peroxidation*

Lipid peroxidation was measured as the MDA content determined by the thiobarbituric acid (TBA) reaction according to Heath and Packer (1968) with some modifications. Moss shoots (0.2 g) were homogenized in 2 mL of 0.1% TCA extraction buffer under cold conditions. The suspension was centrifuged at  $15000 \times g$  (RCF) for 10 min at 4 °C and supernatant was collected. Samples consisted of 200  $\mu\text{L}$  of the supernatant, to which 1800  $\mu\text{L}$  of TCA (20%) containing TBA (0.5%) buffer was added. The assay mixture was heated at 95 °C for 30 min. The content was cooled to stop the reaction for 5-10 min on ice and re-centrifuged at  $10000 \times g$  (RCF) for 10 min at 4 °C. Each sample from the slopes comprised of five replicates. MDA concentration (mM) was calculated as  $(A_{532} - A_{600} / 155)$  expressed as  $\text{nmol g}^{-1}$  dry weight.

#### *Statistical analysis*

All the variables were tested for normality and equal variance using the Shapiro-Wilk test and Levene's test, respectively. ANOVA post-hoc (Tukey's test) was performed on the experimental data comparing the different antioxidant enzymatic activity between the north-east and south-west slopes with respect to different seasons in rehydrated and dehydrated states. Differences are significant at a level ( $p \leq 0.05$ ). Statistical analyses were performed using the statistical software R programming language version 3.5.3 for Windows (R development Core Team, Auckland, New Zealand).

## **Results**

#### *Activity of APX, CAT, POD in mosses collected from the NE and SW slopes in the rehydrated and desiccated states*

Antioxidant enzymatic activity results were represented in two different states, i.e., rehydrated (rehy) and desiccated (desic) between north-east (NE) and south-west (SW) slopes with respect to different period of collection (Figure 3). The activities of APX, CAT and POD observed to be higher in material from the NE while compared to the SW facing slopes in all seasons except opposite trend was seen in summer season. All the activities tended to be higher in desiccated states than in rehydrated material for both slopes. All the activities were followed similar trend upon rehydration and desiccation, these activities increased first from spring to summer season and then declined in autumn season. Again, it was increased in the colder winter season. In both rehydrated and desiccated states, all activities were higher in summer and winter season and lower in spring and autumn. APX (Figure A-B) and POD (Figure E-F) activities were showed variations throughout the year in both rehydrated and desiccated states between both NE and SW slopes. CAT activities did not vary much throughout the year in both rehydrated and desiccated states for the material from the NE and SW facing slopes (Figure C-D).

#### *Variation in protein determination (protein content) between the slopes (NE and SW) in seasons in the rehydrated and desiccated states*

Protein content were represented in two different states, i.e., rehydrated (rehy) and desiccated (desic) between north-east (NE) and south-west (SW) slopes with respect to different period of collection (Figure 4A-D). On rehydration, the protein content was observed increased and desiccation resulted in a decrease level of the protein synthesis in all seasons in both NE and SW slopes. Overall, in spring and autumn season, protein content was found increased whereas in summer and winter season it become decreased. Based on slope-wise, protein content was not sig-

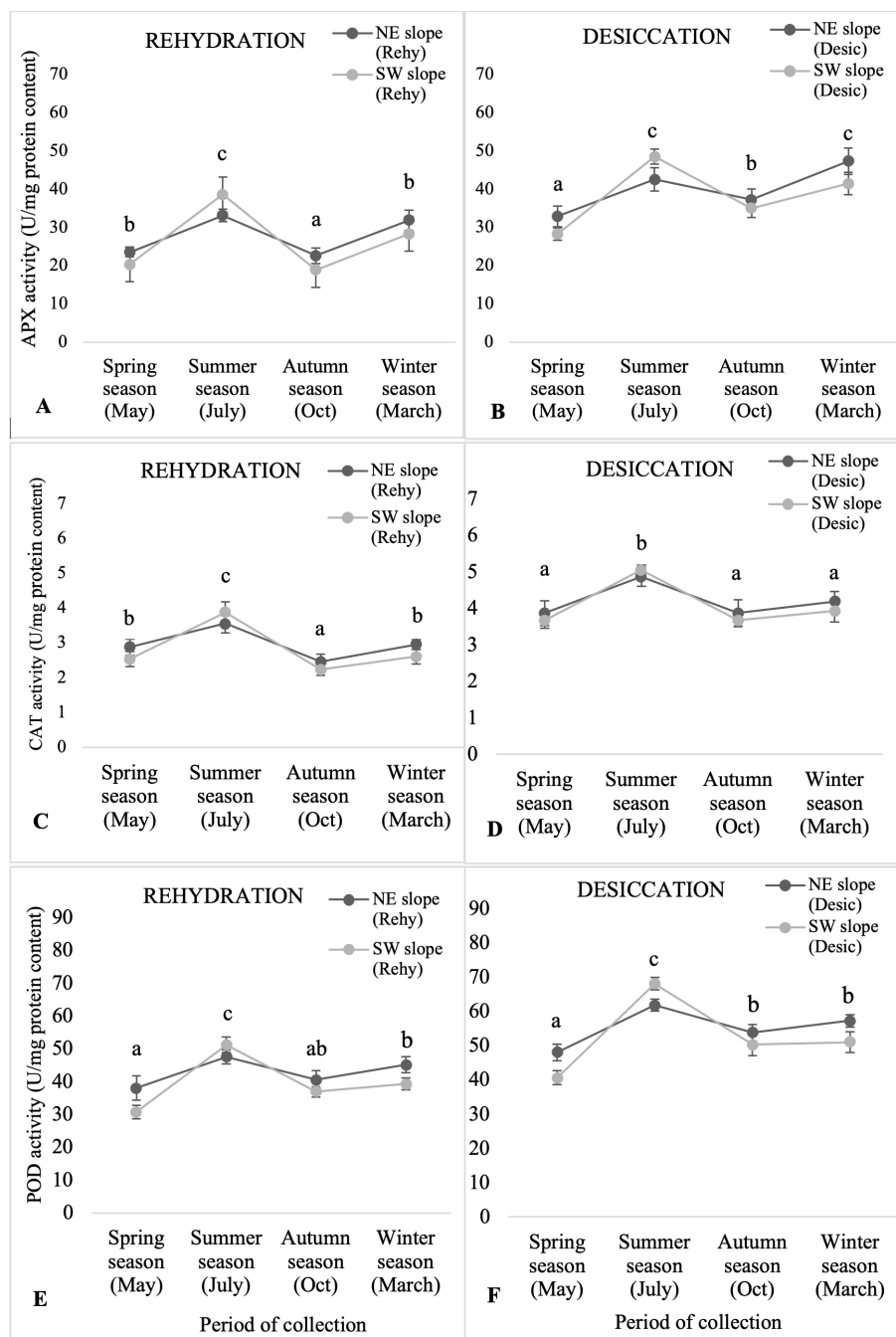


Figure 3. Effect of activity of antioxidant enzymes in *S. ruralis*: (A-B) APX ; (C-D) CAT; (E-F) POD in rehydrated (Rehy) and desiccated (Desic) states between north-east (NE) and south-west (SW) slopes with respect to different period of collection (Spring, Summer, Autumn, Winter season). The mean values ( $n = 5$ )  $\pm$  SD with different alphabetical letters is significantly different at  $p \leq 0.05$  using ANOVA post-hoc (Tukey's test).

nificantly different in rehydrated states ( $p \geq 0.05$ ) while significant different in desiccated states. Based on season-wise, protein content was significantly different ( $p \leq 0.05$ ).

Variation in lipid peroxidation (MDA content) between the slopes (NE and SW) in seasons in the rehydrated and desiccated states MDA content differed significantly between

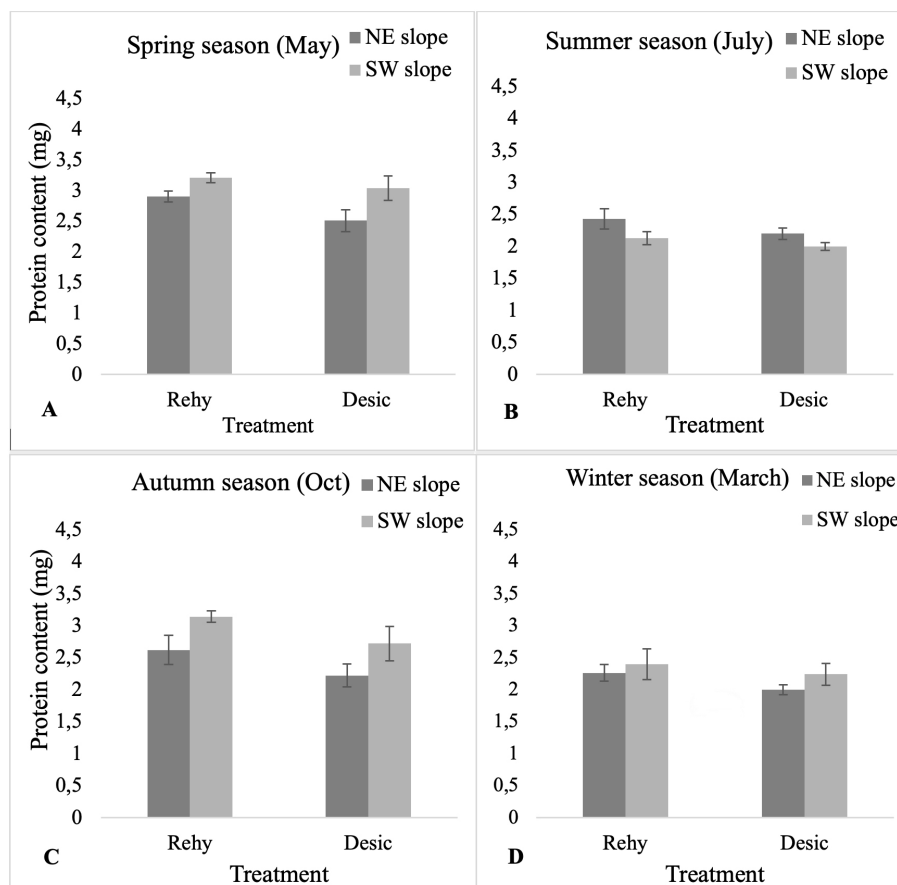


Figure 4. Protein content in *S. ruralis* (A-D) in rehydrated (Rehy) and desiccated (desic) states between north-east (NE) and south-west (SW) slope with respect to different period of collection (Spring, Summer, Autumn, Winter season). The mean values ( $n = 5$ )  $\pm$  SD are represented using ANOVA.

each season in rehydrated states and desiccated states. It was not significantly different between the slopes ( $p \geq 0.05$ ) although there was significant difference between seasons ( $p \leq 0.05$ ). The concentration of the oxidized lipid MDA tended to be higher in desiccated material than rehydrated material (Figure 5A-B). In all seasons, MDA content was found higher in NE slope except summer season as compared to SW slope.

## Discussion

In this present study, the activities of the antioxidative enzymes APX, CAT and POD were compared between the mosses grow-

ing on NE and SW slopes of semi-arid sandy grassland collected at different period of the year. Our results showed that mosses growing on the NE slope have higher enzymatic activities in both the rehydrated and desiccated states as compared to the SW slope except in summer season. It seems likely that the differences in the enzyme activities in the mosses growing on the two slopes are a consequence of the more stressful conditions on the NE facing slopes. Conditions on the SW slope are more optimal (e.g., favourable light conditions, better availability of water) for moss growth. This is suggested by a recent study on the photosynthetic efficiencies of mosses sampled from the two slopes

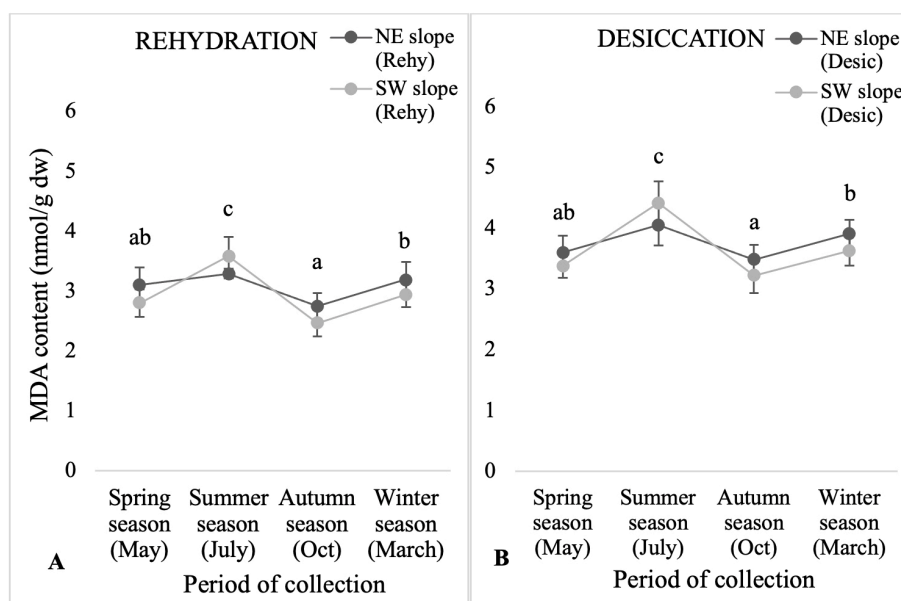


Figure 5. MDA content in *S. ruralis* in rehydrated (Rehy) and desiccated (desic) states between north-east (NE) and south-west (SW) slope with respect to different period of collection (Spring, Summer, Autumn, Winter season). The mean values ( $n = 5$ )  $\pm$  SD with different alphabetical letters is significantly different at  $p \leq 0.05$  using ANOVA post-hoc (Tukey's test).

(Ruchika et al. 2020). Similarly, higher activities of antioxidative enzymes suggests that mosses growing on the NE slope might be experiencing greater stress. In summer and winter season, qN and NPQ values were reported higher that may indicate the stressful environmental conditions (high light exposure and temperature variations) in these two seasons. In this study, it also showed higher activities in summer and winter season.

In plants, the production of antioxidant enzymes is one of the strategies to defend themselves from ROS injury during desiccation (Seel et al. 1991; Oliver and Bewley 1997). These mosses were collected from exposed areas in semi-arid sandy grassland that showed increased antioxidant enzymatic activities. However, similar results were also reported in the moss *S. caninervis* Mitt. collected from exposed areas that showed the highest antioxidant enzyme activity (Yin and Zang 2016).

Ascorbate peroxidase (APX) enzyme plays

a key role in eliminating  $H_2O_2$  and therefore it is an important component of the antioxidant system (Najami et al. 2008). APX activity was lower following rehydration (Figure 3A), presumably because of the reduction in oxidative stress. Bansal and Srivastava (2017) also reported a reduction in APX activities during rehydration in the moss *Brachythecium procumbens*. Catalase enzyme (CAT) is an important antioxidant enzyme that breaks down  $H_2O_2$  to form water and oxygen (Zhang et al. 2017). In rehydrated mosses, CAT activities were observed similar in NE and SW slope (Figure 3C). Results are generally consistent with studies on other mosses that have found that CAT activity does not vary greatly during wetting /drying cycles, suggesting that CAT is probably a largely constitutive defence against oxidative stress (Mayaba and Beckett 2003). Guaiacol peroxidase (POD) activity, which will also remove  $H_2O_2$ , increased during slow desiccation in all moss



samples as compared to rehydrated states (Figure 3F). Similar increases in POD activity have been observed during desiccation in *Brachythecium velutinum* (Paciolla and Tommasi 2003), *B. procumbens* (Bansal and Srivastava 2017), *Octoblepharum albidum* (Lubaina et al. 2013) and *Dicranum scoparium* (Onele et al. 2018).

During dehydration, plants deal with the water-deficit condition which causes lower water potential and declines the primary metabolism in bryophytes (Dinaker et al. 2002). In the desiccated state, accumulation of ROS increases the damage to proteins and lipids in the chloroplast also in mitochondria, peroxisomes, and plasma membrane (Scheibe and Beck 2011). However, there is a down-regulation of the synthesis of proteins during drying conditions (Cruz de Carvalho et al. 2014). Similarly, in this present study, results observed lower protein values during desiccation (Figure 4 A-D) which may indicate the damage of proteins. Higher values in antioxidant enzymatic activities might be indicated higher water deficit condition and imbalance of ROS production in NE slope. In the previous report, protein synthesis induced during rehydration (Oliver et al. 2004). Similarly, it may indicate the higher protein content values in the rehydrated state in both NE and SW slopes.

Lipid peroxidation (MDA content) is used as to indicate the degree of oxidative damage in plants (Liu et al. 2013). Increased stress is probably the reason for the higher MDA levels in mosses growing on the NE slope compared with those growing on the SW slope. Similar results were observed that MDA content increased during desiccation while comparing with rehydration (Figure 5). The lower level of lipid peroxidation in moss shoots suggests that this moss might be better protected from oxidative damage during rehydration. However, in contrast, Zhang et al. (2017) reported that in species *Bryum argenteum* Hedw. and *Bar-*

*bula fallax*, Hedw. MDA content increased first within 24 h and then declined at 48 h and 72 h later stages of desiccation stress. It seems likely that measuring MDA alone may give a rather poor indicator of oxidative stress in tissues and as suggested by De Dios Alché (2019), other molecules such as 4-hydroxy-nonenal (HNE) may be a more sensitive indicator of oxidative stress. Future studies on desiccation-induced changes in lipids in mosses should probably use indicator molecules other than MDA.

In the present study, the activities for all enzymes (APX, CAT, POD) tended to be lower in the rehydrated compared to the desiccated state. Previous studies in *S. ruralis* (Oliver 1991; Oliver and Bewley 1997; Oliver et al. 1998), and *A. viticulosus* and *R. lanuginosum* (Proctor and Smirnov 2000) indicated the importance of constitutive protection with an induced repair mechanism upon rehydration. It appears that the H<sub>2</sub>O<sub>2</sub> scavenging antioxidant enzymes form part of the inducible mechanism. During rehydration, processes such as photosynthesis, respiration and protein synthesis return to normal and suggesting recovery from stress (Oliver 1991; Cruz de Carvalho et al. 2011, 2014).

Although more frequent sampling occasions would have been desirable, our results also suggested that the antioxidant enzymatic activities might be affected by increasing temperatures from spring to summer season (April to July 2018) and by declining temperatures from autumn to winter season (October to late March 2018). In summer and winter season, enzymatic activities differed greatly between collections and treatments, which indicated that anti-oxidative systems may be performed an important role in balancing the production of free radicals and adjust level of protective enzymes to provide protection in extreme environment. In the present study, collections were made on representative days of each of the four seasons, in an attempt to obtain an overview of how the activities of

the enzymes vary throughout the year. In this present study, we investigated the activities of the antioxidant enzymes APX, CAT, POD with protein determination and MDA content in the desiccation-tolerant moss *S. ruralis*, comparing material collected from the NE and SW slopes in different seasons. Our results showed significant seasonal variations in antioxidant enzymatic activities in the rehydrated and desiccated states for the slopes. In general, higher activities of the antioxidant enzymatic activities were found in mosses collected from the NE slope. In both states, the highest activities occurred in mosses collected during summer and winter season and the lowest activities were found during the spring and autumn season. Besides seasonal differences in the activities of the antioxidant enzymes, the small spatial-scale exposures i.e., the NE and SW slope orientation also can modify the expression of these enzymes. The role of some antioxidant enzyme in desiccation tolerance may be different, basically depending

on the actual metabolic balance of mosses. Their activity is influenced not only by water conditions but also by other environmental factors (e.g., exposures, light, and soil conditions) which need to be further investigated in the future.

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