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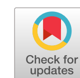
Biochemical characterization of peroxidases from the moss *Dicranum scoparium*



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ABSTRACT

Mosses are a convenient model to study stress responses of plants because of their remarkable stress tolerance. Peroxidase (EC 1.11.1.7) activities were tested in three moss species, namely *Dicranum scoparium*, *Hylocomium splendens* and *Pleurozium schreberi* growing together in the same location in a boreal forest. Peroxidase activity in *D. scoparium* was twice as high as in other mosses. Total peroxidase activity in unstressed *D. scoparium* was constitutively high; furthermore, long-term desiccation caused a significant increase in activity after 48 h of drying. Interestingly, when thalli desiccated for a week were rapidly rehydrated, peroxidase activity initially declined and then increased after 2 h rehydration. Diverse anionic and cationic isoforms were detected by native isoelectric focusing and PAGE of both crude extracts and partially purified peroxidases. The ability of peroxidases from *D. scoparium* to produce superoxide radical ($O_2^{\bullet-}$) was confirmed using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay and in-gel nitroblue tetrazolium chloride (NBT) staining; specific $O_2^{\bullet-}$ producing isoforms were revealed using 2D electrophoresis. Given a quinone and chelated Fe^{3+} *D. scoparium* could produce extracellular hydroxyl radical ($^{\bullet}OH$), and production was increased by desiccation/rehydration stress. The possible roles of peroxidases and quinone reductases in apoplastic $^{\bullet}OH$ production is discussed. Our data demonstrate that *D. scoparium* possesses high constitutive peroxidase activity that can be further increased by desiccation stress. Among the diverse moss peroxidases, some anionic isoforms displayed both pro- and antioxidative activities. These findings suggest that the ability of peroxidases to produce and detoxify reactive oxygen species is an evolutionarily ancient characteristic, important for plant stress tolerance.

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1. Introduction

Bryophytes are non-vascular plants represented today by three phyla, namely liverworts (phylum *Hepatophyta*), mosses (phylum *Bryophyta*), and hornworts (phylum *Anthocerophyta*) (Záveská et al. 2015). Bryophytes are believed to be among the first plants to colonize the land (Ponce de León and Montesano 2013). Mosses are currently represented by approximately 10,000–15,000 species that grow in some of the harshest environments on earth such as dry heaths, rock faces, tree trunks and even deserts (reviewed in Dey and De 2012). They exhibit distinctive adaptations which allow them to occupy this wide range of environments, including the ability to tolerate drying out, i.e., they are desiccation tolerant (Proctor et al. 2007). The dominant phase of the moss life cycle is a haploid gametophyte, meaning that any mutations will immediately be visible in the phenotype (Chobot et al. 2008). Furthermore, they respond to some hormones and environmental stimuli in the same way as vascular plants (Cove et al. 1997). These features, taken together with their lack of waxy cuticle, epidermis and well-developed conduction system make mosses good models to study mechanisms of stress tolerance in plants, particularly desiccation tolerance.

Among the mechanisms of desiccation tolerance, regulating the levels of stress-induced reactive oxygen species (ROS) plays a crucial role. ROS such as the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) are produced in healthy plants, but their production greatly increases when plants become stressed. Once produced, ROS can attack cellular components by causing damage to lipids, proteins, nucleic acids and activate cell death (Demidchik

Abbreviations: ABTS, 2,2′-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid; DMBQ, 2,6-Dimethoxy-1,4-benzoquinone; IEF, isoelectric focusing; MDA, malondialdehyde; NBT, nitroblue tetrazolium chloride; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide..

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2015; Cruz de Carvalho et al. 2017). Multiple antioxidative systems function to keep ROS at a safe level. However, ROS also play positive roles in plants such as signaling, and a diverse range of stress responses (Choudhury et al. 2017). Among these responses, the oxidative burst, involving the rapid production of several types of ROS in the apoplast, occurs in response to abiotic or biotic stresses (Mika et al. 2004). Stress-induced formation of ROS has been shown in bryophytes, for example, in liverworts (Li et al. 2010), hornworts (Chasov et al. 2015), and mosses (Mayaba et al. 2002; Frank et al. 2007; Lehtonen et al. 2009; Cruz de Carvalho et al. 2012). However, the mechanisms of ROS formation in mosses are not yet fully understood.

In higher plants, apoplastic (class III) peroxidases are an important source of ROS for the oxidative burst. These enzymes can both scavenge H_2O_2 during their peroxidative cycle, and also behave as pro-oxidants by producing ROS, including $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$, during their hydroxylic cycle (Mika et al. 2004; Passardi et al. 2004; Almagro et al. 2009). The ability of plant peroxidases to switch from anti- to pro-oxidative regimes is important during stresses such as desiccation. It is likely that in mosses peroxidases are also key enzymes involved in stress reactions and ROS metabolism. Peroxidases in bryophytes are structurally closely related to those found in higher vascular plants, and some of them demonstrate similar responses to biotic and abiotic stresses (Ros et al. 2007). For example, chitosan, a derivative of fungal chitin, can initiate a variety of stress responses in plants that prepare themselves for pathogen attack. In *Arabidopsis*, chitosan triggers an increase in peroxidase-induced ROS production, leading to stomatal closure (Khokon et al. 2010). In the moss *Physcomitrella patens* chitosan induces peroxidase secretion into the culture medium (Rensing et al. 2008), and a rapid burst of ROS formation (Lehtonen et al. 2009). Knock-out peroxidase mutants of *P. patens* were unable to release peroxidase upon chitosan treatment and were more susceptible to fungi (Lehtonen et al. 2009). Similarly, cuticle-free suspension cultures of the liverwort *Marchantia polymorpha* readily release extracellular peroxidases capable of producing H_2O_2 (Ishida et al. 1985, 1987). A variety of stresses, e.g., heavy metals, upregulate Class III peroxidase activity in mosses (Saxena and Saiful-Arfeen 2009; Wu et al. 2009), suggesting that in addition to a general role in scavenging stress-induced ROS peroxidases also play a role in generating ROS. Among mosses, *P. patens* is a 'model moss' whose genome sequence has been determined and 45 putative genes for Class III peroxidase have been revealed (Lehtonen et al. 2009). However, little information is available about the diversity and biochemical characteristics of peroxidases in other moss species.

In the present study, we investigated peroxidase activity in the boreal acrocarpous moss *Dicranum scoparium*. The boreal forest ecosystem occupies 11% of the Earth's terrestrial surface (Bonan and Shugart 1989), where *D. scoparium* and the feather mosses *Pleurozium schreberi* and *Hylocomium splendens*, often account for more than 65% of the forest floor (DeLuca et al. 2002). We studied the kinetic properties of peroxidases from *D. scoparium* using various substrates and documented the diversity of peroxidase isoforms using PAGE and isoelectric focusing (IEF). We further studied the effect of drying and wetting cycles on peroxidase activity. We partially purified peroxidases using ion exchange chromatography, determined their cellular localization, and finally, tested the ability of peroxidases to produce superoxide anion and hydroxyl ion radicals. Our overall aim was to increase our understanding of peroxidases in mosses, specifically their roles in desiccation tolerance.

2. Material and methods

2.1. Plant material

Dicranum scoparium Hedw, *Hylocomium splendens* (Hedw.) B.S.G. and *Pleurozium schreberi* (Brid.) Mitt were collected growing together in the Aisha forest, Tatarstan, Russia (55°53′21.3″N 48°38′14.3″E). After cleaning, thalli were allowed to air-dry slowly for 2 d between sheets of paper, and then stored refrigerated at 4 °C in the dark before use. Peroxidase activity remained consistently high in material stored for several months (data not shown).

2.2. Desiccation treatment

The effects of a drying and wetting cycle on peroxidase activity were studied as follows. Each replicate comprised 0.5 g dry mass of 2 cm apical stem segments. Dry thalli were initially fully hydrated by incubation in 25 ml distilled H_2O for 10 min with gentle shaking on the orbital shaker, and then slowly dried by placing them in an exicator over 35% $CaCl_2$ solution (resulting in a relative humidity of 41%) for 0 to 168 h. Samples were taken before rehydration (time 0), immediately after rehydration (time 0.17 h) and at the following time intervals during desiccation: 1, 2, 4, 24, 48, 120, 144 and 168 h. At each time interval the relative water content and peroxidase activity were measured. After dehydration for 168 h, thalli were rapidly rehydrated by placing them in distilled H_2O and incubating for 1 and 2 h. In another set of experiments, air-dry thalli of *D. scoparium* and pre-hydrated thalli (24 h, +5 °C) were incubated in distilled H_2O for 10, 20, 30, 60, 120 min and 24 h. At each time interval material was taken to measure extracellular and intracellular peroxidase activity.

2.3. Assessment of relative water content

Water content in thalli at full turgor was determined by weighing thoroughly blotted material that had been incubated in 25 ml distilled H_2O for 10 min with gentle shaking on the orbital shaker (turgid mass), then subtracting the dry mass, obtained by drying over 35% $CaCl_2$ solution for 0 to 168 h. The relative water content (RWC) after drying was estimated as (fresh mass after drying − dry mass)/(turgid mass − dry mass).

2.4. Determination of redox enzyme activity

Peroxidase (EC 1.11.1.7) activities were typically measured using 1 mM *o*-dianisidine and 1 mM H_2O_2 as substrates in 0.07 M Na-citrate buffer, pH 5.5. Extracellular peroxidase activity was measured in the leachate and intracellular peroxidase activity was measured in supernatants of homogenized thalli after centrifugation at 4300*g*, 5 °C for 20 min. Substrate specificity was determined using the following substrates: 0.5 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) ($\varepsilon_{405} = 36.8\ mM^{-1}\ cm^{-1}$); 0.1 mM *o*-dianisidine ($\varepsilon_{460} = 30.0\ mM^{-1}\ cm^{-1}$); 0.1 mM *p*-coumaric acid ($\varepsilon_{310} = 16.6\ mM^{-1}\ cm^{-1}$), and 0.1 mM caffeic acid ($\varepsilon_{311} = 9.91\ mM^{-1}\ cm^{-1}$). Phenol oxidase (EC 1.14.18.1) activity was determined similarly to the peroxidase, but H_2O_2 was omitted from the mixture. Catalase (EC 1.11.1.6) activity was assayed by a decrease in the H_2O_2 content ($\varepsilon_{240} = 40.0\ mM^{-1}\ cm^{-1}$) in the reaction medium containing 0.05 M Na-citrate buffer, pH 7.0, 40 mM H_2O_2. Enzyme activity was expressed per g of dry mass or protein basis as required. Protein concentration was measured according to Bradford (1976).

2.5. Cell wall protein fractionation in D. scoparium

Redox activity in cytosol and various cell wall fractions in *D. scoparium* was tested as described in Li et al. (2010). Briefly, proteins from the *D. scoparium* thallus were extracted sequentially using Tris–HCl buffer, Sorensen's phosphate buffer, digitonin, and NaCl. The following fractions were obtained: intracellular soluble fraction (C) and fractions of proteins bound with various bonds to the cell wall: hydrogen bonds (B_1), van der Waals bonds and hydrophobic interactions (B_2), and ionic bonds (B_3).

2.6. Oxygen radical measurement

2.6.1. Superoxide anion radical formation

To assay $O_2^{\bullet-}$ formation, extracts were incubated with 0.1 mM NADH and 0.1 mM 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) in 0.08 M Na-citrate buffer, pH 7.8 in the presence and absence of 250 units ml^{-1} of superoxide dismutase (SOD) for 50 min, and conversion of XTT to XTT formazan ($\varepsilon_{470} = 21.6\ mM^{-1}\ cm^{-1}$) measured (Sutherland and Learmonth, 1997). To test for in-gel $O_2^{\bullet-}$ production, gels were stained with nitroblue tetrazolium chloride (NBT) as described in López-Huertas et al. (1999). Superoxide specificity was confirmed by incubating gels in the absence of NADH. Broad range molecular mass markers (Bio-Rad, CA) were run with the gels and stained by Coomassie Brilliant Blue G250.

2.6.2. Hydroxyl radical formation

Hydroxyl radical production was estimated by measuring the oxidation of deoxyribose (Gómez-Toribio et al. 2009; Moyo et al. 2017). Unless indicated otherwise, material was hydrated for 5 d in the dark at +5 °C on non-cellulosic cloth. Three to five replicates each containing an equivalent of 0.2 g dry mass were then shaken in 20 ml of 20 mM phosphate buffer, pH 5 containing 0.5 mM 2,6-dimethoxy-1,4-benzoquinone (DMBQ), 0.1 mM $FeCl_3$, 0.6 mM oxalic acid and 2.8 mM deoxyribose. Samples (990 μl) were taken at the start and after 10, 20, 30, 60 and 120 min, 10 μl 50% H_3PO_4 added to stop reactions and then samples frozen. Later, 50 μl of samples were mixed with 250 μl of 2.5% TCA and 250 μl of 1% thiobarbituric acid in 50 mM NaOH and distilled water to make a volume of 1 ml. The mixture was then heated in boiling water (100 °C) for 10 min and absorption measured at 532 nm. Readings were converted to malondialdehyde (MDA) equivalents ($\varepsilon_{532} = 0.156\ mM^{-1}\ cm^{-1}$, Devasagayam et al. 2003). Solutions lacking *D. scoparium* material but otherwise treated in the same way were used as blanks. Production rates of $^{\bullet}OH$ were very low when either the quinone or $FeCl_3$ were omitted from the incubation solution. To check the specificity of the assay, we confirmed that known $^{\bullet}OH$ scavengers (thiourea and sorbitol) reduced the oxidation of deoxyribose (data not shown).

2.7. Electrophoretic protein separation

PAGE was conducted in either gradient (3–12%) or 10% gels in a native Laemmli (1970) system using a MiniPROTEAN Tetra Cell (BioRad, USA) with no SDS and mercaptoethanol, and without heating samples. Peroxidase activity was visualized using 5 mM *o*-dianisidine in 70 mM Na citrate buffer, pH 5.5 with addition of 1 mM H_2O_2 producing brown bands. Isoelectric focusing (IEF) was conducted using a 5% gel with the addition of Ampholine of pH 3.5–10.0 (LKB, Sweden) and Ampholyte of pH 5–8.2 (Bio-Rad, USA) using the IEF system (HiiuKalur, Estonia). A set of IEF M1A standards (3.6–9.3) (Sigma, USA) was used for determination of isoelectric points of the proteins.

2.8. Ion-exchange chromatography

Extracts were prepared by grinding material in 50 mM phosphate buffer pH 7.0, and then proteins precipitated by $(NH_4)_2SO_4$ (30–80%), centrifuged at 23,500*g* for 20 min, and the resulting pellet re-suspended in phosphate buffer. After dialysis proteins were partially purified using the anion exchange column Hi-Trap Q FF (GE Healthcare, Sweden) equilibrated with 25 mM Tris–HCl buffer, pH 7.5 (chromatography system ÄKTA™ start). Proteins were eluted with a linear gradient of NaCl (0–1 M) at a flow rate of 1 ml min^{-1}. Peroxidase activity was determined in each fraction.

2.9. Statistics

Experiments were performed in three to six biological and three to nine analytical replicates. Data were processed using the Microsoft Excel software. Representative results of each experiment series are presented, unless indicated otherwise. Tables and figures present mean values and their standard deviations. Statistical analysis was performed using the Student *t*-tests at $P \le 0.05$ (*), $P \le 0.01$ (**), $P \le 0.001$ (***).

3. Results

3.1. Redox enzyme activities and peroxidase isoforms in three moss species

Peroxidase activity in *D. scoparium* was about twice as high as that in *H. splendens* and *P. schreberi* (Fig. 1A). Gradient PAGE (3–12%) of crude extracts showed that *D. scoparium* has two main peroxidase isoforms with molecular masses of 197 and 263 kDa. In *H. splendens* five major isoforms were visualized with masses of 58, 122, 358, 708, 848 kDa, while *P. schreberi* had four isoforms with masses of 53, 704, 848, 1352 kDa. In *D. scoparium*, when substrates were supplied at 1 mM, peroxidases displayed the highest activity, exceeding catalase activity by two orders of magnitude and phenol oxidase activity by three orders of magnitude (Table 1). The affinities of peroxidase for substrates (K_m) increased in the order: *p*-coumaric acid < caffeic acid < *o*-dianisidine < ABTS, while the maximum velocity (V_{max}) increased in the order: ABTS < *p*-coumaric acid < caffeic acid < *o*-dianisidine (Table 2).

3.2. Activities of intra- and extracellular peroxidases in D. scoparium during long-term desiccation and rehydration

The RWC in the thallus of *D. scoparium* gradually declined during desiccation over a 35% $CaCl_2$ solution, reaching 25% after 168 h (Fig. 2A). During desiccation, peroxidase activity gradually increased, reaching almost double the initial activity after a week (Fig. 2B). Although rapid rehydration initially reduced rates, activity quickly recovered, and after 2 h rates were similar to those present at the end of long-term desiccation (Fig. 2B).

3.3. Intra- and extracellular peroxidase activities and hydroxyl radical formation during rehydration of air-dry D. scoparium

Extracellular peroxidase activity in the solution in which pre-hydrated mosses were incubated was low and did not change with time (Fig. 2C). Intracellular peroxidase activity in crude extracts of the same samples also showed no significant changes (data not shown). Peroxidase activity in the rehydration solution of air-dry samples of *D. scoparium* was up to six times higher than the activity in the incubation solutions of pre-hydrated mosses (Fig. 2D). Activity remained stable during 2 h of rehydration and declined after 24 h (1440 min) of incubation. The activity of intracellular peroxidases after hydration of air-dry *D. scoparium* did not change with time (Fig. 2E). Given a quinone and chelated Fe^{3+}, the thalli of *D. scoparium* could readily produce extracellular $^{\bullet}OH$ (Fig. 2F). Rates of $^{\bullet}OH$ production in air-dry mosses after rehydration were c. two times higher than those in pre-hydrated thalli in the same time intervals (Fig. 2G). In dry and hydrated material rates of extracellular $^{\bullet}OH$ production greatly declined after 1 h.

3.4. Purification of peroxidases from D. scoparium by anion-exchange chromatography

Both supernatant of crude extract (S) and proteins precipitated with ammonium sulfate (ASP) displayed thirteen peroxidase isoforms with pIs of 4.0, 4.1, 4.2, 4.3, 4.5, 4.6, 4.8, 5.1, 8.8, 9.1, 9.3, 9.5, 9.8 after separation by IEF; most isoforms were anionic (Fig. 3B). Precipitated proteins purified by anion-exchange chromatography displayed a single peak of maximum peroxidase activity that eluted at c. 0.3 M NaCl (Fig. 3A). IEF of proteins from this peak fraction revealed several anionic isoforms (Fig. 3B); flow-through fractions ($\sum$ № 1–8) contained mostly cationic isoforms. Isoforms from peak fractions were further investigated by 2D

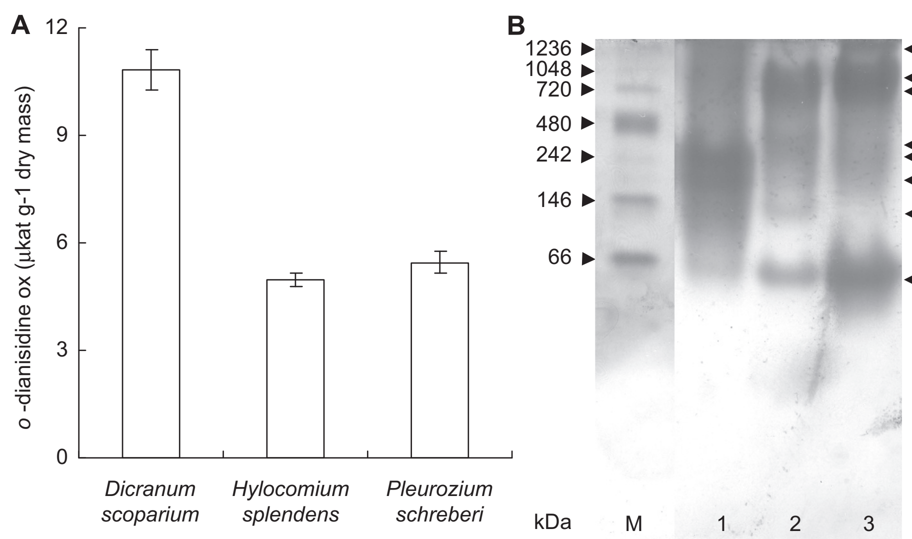

Fig. 1. Activity of peroxidase in crude extracts from three species of moss (A). Peroxidase activity was measured with 50 µl of enzyme extract (supernatant) from each species, 1 mM *o*-dianisidine, 70 mM Na-citrate buffer, pH 5.5 and 1 mM H_2O_2. (B) Isoforms of peroxidases from gradient electrophoresis (3–12%). M – Molecular mass markers. 1, *Dicranum scoparium*; 2, *Hylocomium splendens*; 3, *Pleurozium schreberi*. Peroxidase activity was visualized by staining with 5 mM *o*-dianisidine and 1 mM H_2O_2 in 70 mM Na-citrate buffer, pH 5.5. Markers were stained with Coumassie Brilliant Blue R250. Peroxidase isoforms from all gels are marked with arrow heads on the right-hand side.

Table 1
The activity of redox enzymes in crude extracts of *Dicranum scoparium.*

Enzymes	Substrates (1 mM)	Activity (nkat g^{-1} dry mass)
Peroxidase	*o*-Dianisidine	9846 ± 876
Catalase	H_2O_2	94 ± 18
Phenol oxidase	*o*-Dianisidine	2 ± 0

gel electrophoresis, which involved IEF (Fig. 3C) followed by PAGE separation (Fig. 3D). Each of the three major peroxidases (with pIs of 4.6, 4.8, 5.1) comprised two isoforms with molecular masses of 197 and 263 kDa, while the peroxidase with a pI 4.0 was represented by a single isoform with a molecular mass of 263 kDa (Fig. 3D).

3.5. Cellular location of peroxidases

Fractionation of *D. scoparium* was performed to distinguish the intracellular soluble proteins (fraction C) from the proteins bound to the cell wall with hydrogen bonds (fraction B_1), van der Waals bonds and hydrophobic interactions (fraction B_2), and ionic bonds (fraction B_3). Activity in fraction C was 8-fold higher than that in the other fractions (Fig. 4A), but a significant proportion of peroxidases occurred in the cell wall. IEF separation of proteins from all fractions revealed 8 major isoforms with pIs of 4.0, 4.3, 4.5, 4.6, 4.8, 5.1, 9.5, 9.8 (Fig. 4B).

3.6. Superoxide formation

The XTT reduction assay indicated that fraction C could produce $O_2^{\bullet-}$ when supplied with NADH (Fig. 5A); however, SOD only inhibited the rate of reduction by 50%, indicating that some XTT reduction occurred by reactions other than those mediated by $O_2^{\bullet-}$. Electrophoretic studies strongly suggested that the major source of $O_2^{\bullet-}$ production in all fractions was peroxidase. Running the various cellular fractions on a 10% PAGE showed that peroxidase isoforms visualized from fractions C, B_1, B_2 and B_3 (Fig. 5B) corresponded to bands that produced $O_2^{\bullet-}$ in the presence of NBT and NADH (Fig. 5C). In-gel staining with NBT in the absence of NADH (Fig. 5D) did not reveal $O_2^{\bullet-}$ production (Fig. 5C). However, a relatively light band from fraction C that demonstrated $O_2^{\bullet-}$ producing activity did not correspond to any bands with peroxidase activity (Fig. 5B). In-gel staining of proteins from fraction B_1 subjected to 2D gel electrophoresis with IEF (Fig. 5E) followed by PAGE (Fig. 5F) also visualized spots corresponding both to peroxidase activity (Fig. 5F) and $O_2^{\bullet-}$ production (Fig. 5G). Comparable results were obtained for other cell wall fractions (data not shown).

Table 2
Michaelis constant (K_m) and maximum rate (V_{max}) for the oxidation of different substrates by peroxidases in crude extracts of *Dicranum scoparium.*

Substrates	K_m (µM)	V_{max} (µM min^{-1})
ABTS	99	0.1
o-Dianisidine	246	877.2
p-Coumaric acid	643	72.7
Caffeic acid	365	552.5

4. Discussion

One of the key components of plant stress tolerance is the ability to maintain cellular redox status and control ROS levels. As discussed in the Introduction, the haploid, non-vascular bryophytes are good models to study mechanisms of stress tolerance in plants, specifically desiccation tolerance. This is because, first, bryophytes are highly stress tolerant. Desiccation tolerance is a common phenotype in bryophytes and more than 200 mosses have been experimentally verified to be desiccation tolerant (Wood 2007). Secondly, moss genes display high homology with genes in vascular plants. For example, approximately 66% of genes identified from expressed sequence tag analyses of gene expression identified in gametophytes of *P. patens* have homologs in the *Arabidopsis* genome (Alam et al. 2012). Analysis of the genome of the moss *P. patens* shows that it possesses large multigenic families involved in stress tolerance. These gene families probably initially facilitated the evolutionary movement of the bryophytes to land and subsequently were recruited for stress tolerance during the evolution of vascular plants (Rensing et al. 2008). Therefore, it is not surprising that redox enzymes such as peroxidases are well represented in the bryophyte genome. Results of the present study provide good evidence that class III peroxidases play important roles in the desiccation tolerance of the moss *D. scoparium*, with activity almost doubling during slow drying for a week (Fig. 2B). As in other organisms, in *D. scoparium* peroxidases

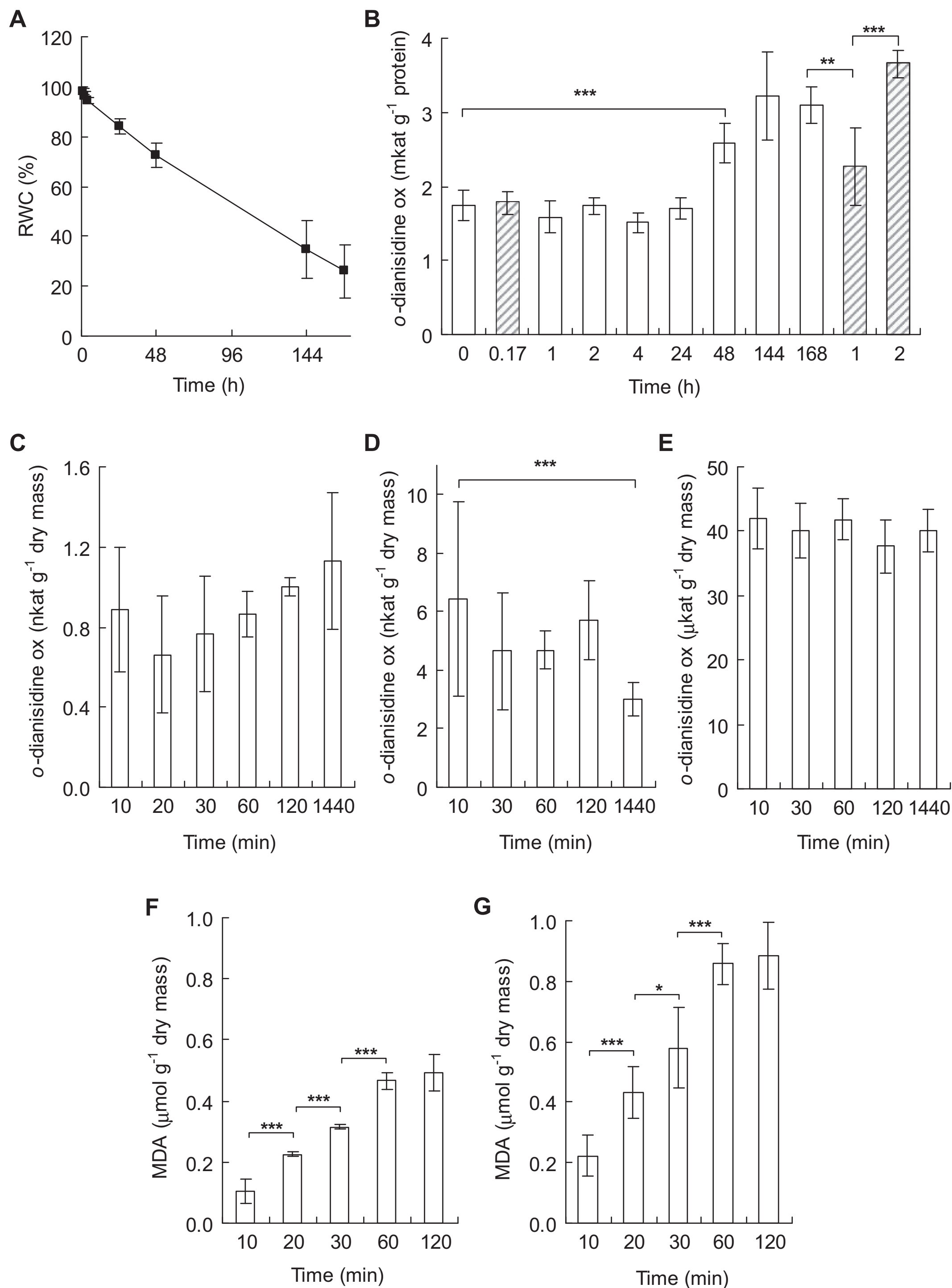

Fig. 2. (A) RWC in the thallus of *D. scoparium* during long-term desiccation over saturated solution of 35% $CaCl_2$. (B) Activities of intra- and extracellular peroxidases in *D. scoparium* during long-term desiccation and rehydration. Time zero corresponds to dry thalli, 1/6 (shaded) corresponds to material rapidly rehydrated for 10 min, and times up to 168 h correspond to the drying time. After 168 h, the bars labeled 1 and 2 (shaded bars) correspond to material rehydrated for 1 and 2 h. (C) Peroxidase activity in the leachate of mosses hydrated for 24 h and then incubated in distilled H_2O. (D) Peroxidase activity in the leachate of air-dry *D. scoparium* incubated in distilled H_2O. (E) Activity of intracellular peroxidases during hydration of air-dry *D. scoparium*. (F) Production of $^{\bullet}OH$ in pre-hydrated thalli of *D. scoparium*. (G) Production of $^{\bullet}OH$ during the rehydration of air-dry mosses. (A–G) difference is significant at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), $n = 6$. (D, E, G) $n = 9$.

occur as multiple anionic and cationic isoforms (Figs. 3B). In addition to their well-known roles in detoxifying H_2O_2, peroxidases from *D. scoparium* can function as pro-oxidants and produce $O_2^{\bullet-}$. Other redox processes such as $^{\bullet}OH$ production also occur in the apoplast of *D. scoparium*, but the precise role of peroxidases in these reactions remains uncertain. However, the presence of peroxidases with both anti- and pro-oxidant properties that play important roles in stress tolerance appears to be an ancestral characteristic of plants.

4.1. Importance of moss peroxidases in the boreal forest ecosystem

Dicranum scoparium, together with other acrocarpous mosses such as *Polytrichum* and *Sphagnum*, and the pleurocarpous "feather mosses"

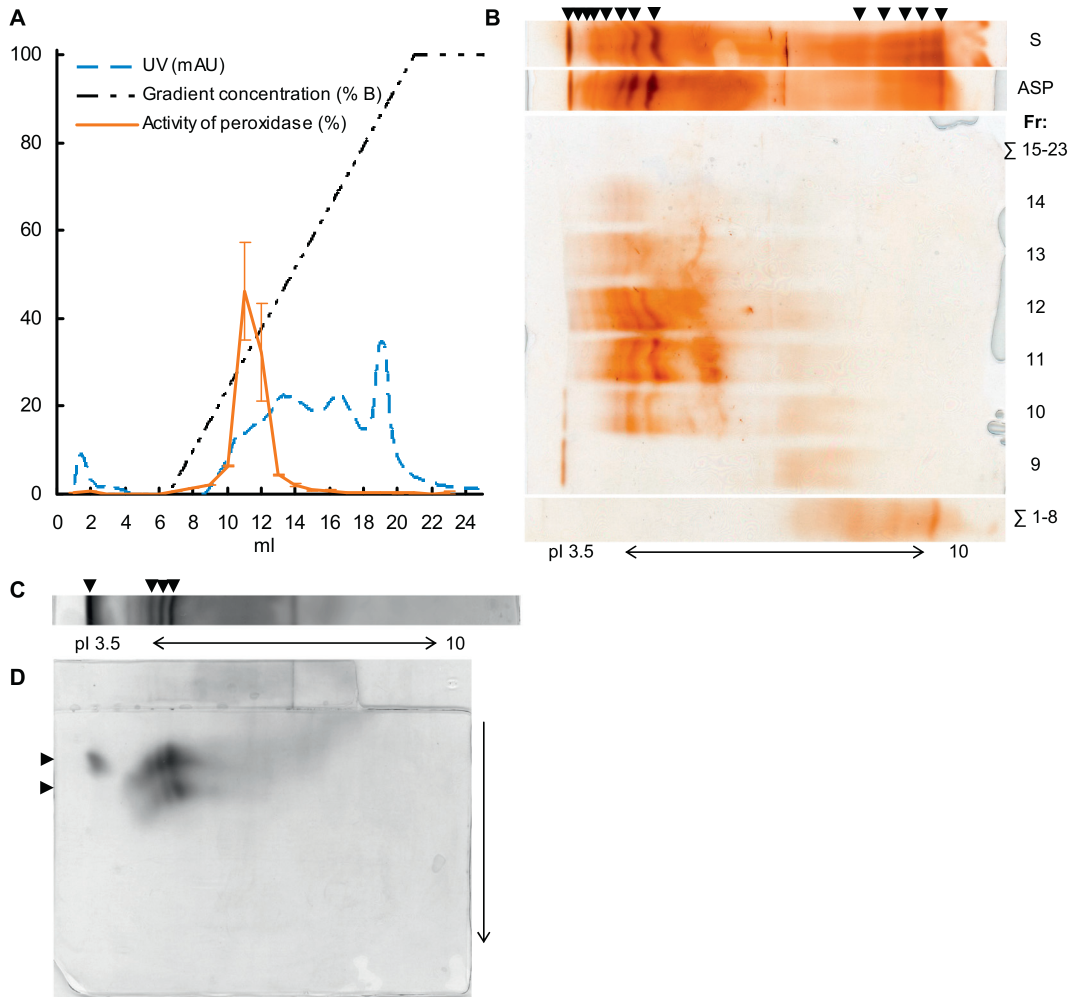

Fig. 3. (A) Purification of a crude extract from *D. scoparium* by anion exchange chromatography (Hi-Trap Q FF). Column was equilibrated with 25 mM Tris–HCl buffer, pH 7.5 and proteins eluted with NaCl in a linear concentration gradient (0–1 M). (B) IEF of peroxidase isoforms in a pH gradient of 3.5–10. In all gels peroxidases were visualized with 5 mM *o*-dianisidine and 1 mM H_2O_2 in Na-citrate buffer, pH 5.5. S, supernatant of crude extract; ASP, proteins after ammonium sulfate precipitation; numbered fractions, fractions with proteins eluted from an anion-exchange column. (C) IEF of the peroxidase isoforms in the peak fractions after anion-exchange chromatography and (D) subsequent 2D-electrophoretic separation of proteins.

of *H. splendens* and *P. schreberi* are extremely important components of the boreal forest ecosystem. These mosses account for 30–95% of the average cover of the boreal forest floor and yield a net primary productivity (NPP) of 200 to >400 kg ha^{-1} yr^{-1}, and comprise a total biomass of between 0.1–2.0 Mg C ha^{-1} as live moss and 2.0–4.0 Mg C ha^{-1} as dead moss (Vogel and Gower 1998; Turetsky 2003). Furthermore, the moss-cyanobacteria associations of the feather mosses are the major gateway of entry of N into boreal systems (Rousk et al. 2013), although *D. scoparium* apparently does not normally possess these associations (Bay et al. 2013). No attempt has been made to systematically survey

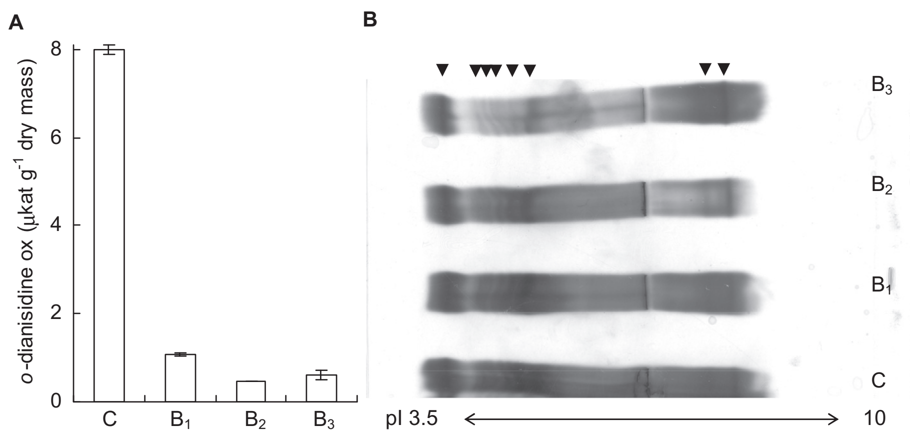

Fig. 4. (A) Cellular location of peroxidases from *D. scoparium*. Activity of peroxidase in the intracellular fraction (C) and bound to the cell wall by hydrogen bonds (B_1), van der Waals bonds and hydrophobic interactions (B_2) and ionic bonds (B_3). (B) IEF of peroxidase isoforms from fractions C, B_1, B_2, B_3. Peroxidase isoforms are indicated with arrow heads. Peroxidases were visualized as in Fig. 3.

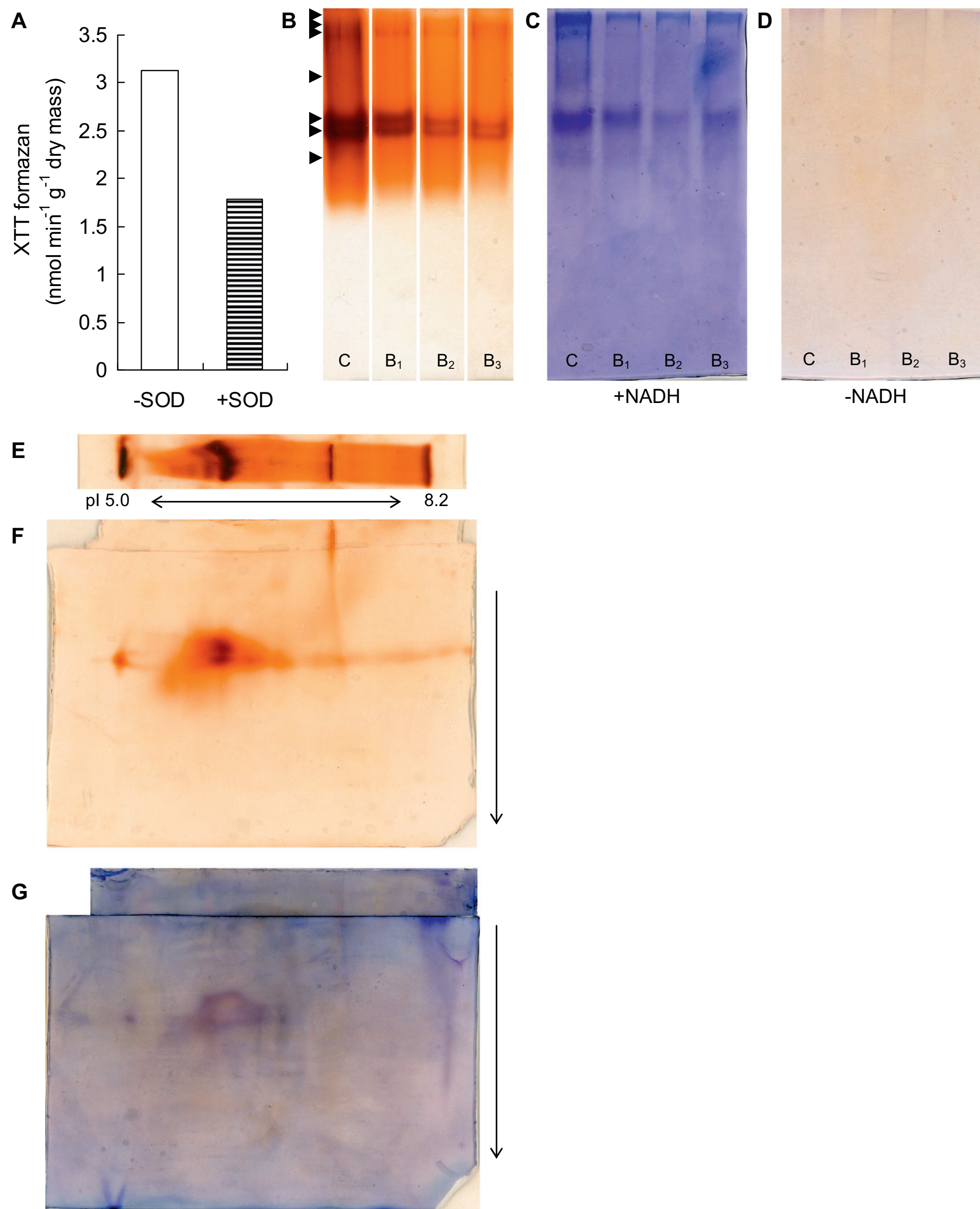

Fig. 5. (A) Rate of $O_2^{•-}$ production in fraction 'C' of *D. scoparium* assayed with XTT and 0.1 mM NADH as a reductant in the presence and absence of 250 U ml^{-1} SOD in Na-citrate buffer, pH 7.8. (B, C, D) 10% PAGE of fractions C, B_1, B_2, and B_3. Peroxidase isoforms are marked with arrow heads. (B) Peroxidase activity, visualized as in Fig. 3; (C) NADH dependent $O_2^{•-}$ production, visualized with NBT; (D) as for (C) but omitting NADH from the staining solution. (E, F, G) 2D-electrophoretic separation of proteins from fraction B1. Peroxidase visualization after IEF (E) followed by 10% PAGE (F). Visualization of $O_2^{•-}$ producing activity after IEF followed by 10% PAGE (G).

peroxidase activity in boreal mosses, so in initial experiments we measured activity in *D. scoparium* and two feather mosses. All species displayed high peroxidase activity, and a high diversity of peroxidase isoforms (Fig. 1). As activity was highest in *D. scoparium*, this species was selected for further study. Among redox enzymes measured, peroxidases displayed the highest activity (Table 1) with *o*-dianisidine being the most readily metabolized substrate (Table 2). Interestingly, cellular fractionation indicated that in *D. scoparium* a significant proportion of peroxidase activity is cell wall bound (Fig. 4), and can be leached (Fig. 2C), particularly when dry moss is rehydrated (Fig. 2D). It has been shown that peroxidases leached from plants into the soil may oxidize soil phenolics, liberating carbohydrates and proteins that are otherwise locked in soil humus and thus inaccessible to soil hydrolases and microbes (Tian and Shi 2014). Given to recent advances in molecular biology diagnostics that facilitate recognition of the specific organisms contributing to enzymes present in soils (e.g., Kellner and Vandenbol 2010), further work is needed to document the presence of moss peroxidases in boreal soils. Given the large biomass and high peroxidase activity of mosses in boreal forests, leaching of these enzymes into soils may significantly affect carbon turnover in these systems.

4.2. Role of moss peroxidases in desiccation tolerance in D. scoparium

Results presented here show that activity of class III peroxidases tends to increase during slow drying in *D. scoparium*, particularly when the water content drops below c. 80%, corresponding to 48 h of

drying under the conditions used here (Fig. 2A,B). Maintenance or increase of peroxidase activity during water stress is important to prevent ROS accumulation and oxidative damage (Wang et al. 2003). There are few studies on the effects of desiccation on class III peroxidases in mosses, although Seel et al. (1992) found that relatively fast desiccation had little effect on activity in either a tolerant (*Tortula* (*Syntrichia*) *ruraliformis*) or a sensitive (*Dicranella palustris*) species. Similarly, no changes in peroxidase transcripts were reported in a comprehensive study on changes in the transcriptome of a desiccation tolerant moss during a dry and wetting cycle (Gao et al. 2015, 2017). However, these authors desiccated mosses extremely quickly, with thalli reaching a RWC of 2% after 4 h of desiccation. Such rapid drying probably never happens in boreal forests, where drying rates of "cushion" mosses are much slower (Zotz et al. 2000). Results from the present study suggest that peroxidase activity may significantly increase when mosses are desiccated at ecologically realistic rates. Rehydration of desiccated *D. scoparium* reduced peroxidase activity after 1 h (Fig. 2B), probably because of damage to peroxidases caused by the burst of ROS production that occurs when desiccation tolerant tissues are re-wetted (Mayaba et al. 2002; Oliver et al. 2004; Cruz de Carvalho et al. 2012). However, activity rapidly recovered, and 2 h after rehydration, rates were similar to those present at the end of long-term desiccation (Fig. 2B). This is consistent with the report of Oliver et al. (2004) of an increase in transcripts involved in protection from oxidative stress during the rehydration of a desiccation tolerant moss. Taken together, the increase in activity during slow drying, and the rapid recovery of activity following rehydration suggest that peroxidases play an important role in desiccation tolerance of mosses by reducing stress-induced ROS.

4.3. Diversity of D. scoparium peroxidases

All three moss species tested here displayed a diversity of peroxidase isoforms (Fig. 1B). This is consistent with data from the analysis of the genome sequence of the model moss *P. patens* that revealed 45 putative genes for class III peroxidases (Lehtonen et al. 2009). Partially purified peroxidases from *D. scoparium* by ion-exchange chromatography displayed a single peak of activity bound to the column and some activity in flow-through fractions (Fig. 3A). More detailed analysis by 2D gel electrophoresis showed the presence of a range of anionic and cationic isoforms with major isoforms having molecular masses of 197 and 263 kDa (Fig. 3B–D). These proteins are probably oligomers based on the typical mass of monomeric class III peroxidase being c. 40 kDa (Hirata et al. 2000). At present we do not know if this diversity of peroxidases in *D. scoparium* is due to gene diversity and the synthesis of various gene products or results from post-translational modifications, for example, glycosylation, of existing peroxidase proteins. Interestingly, electrophoretic analysis of peroxidases in cytosol and different cell wall fractions demonstrated that the isoforms present in these cellular locations possess similar pI and molecular masses (Figs. 4 and 5). In future it will be important to determine whether individual class III peroxidases in moss have specific functions, for example by testing how their expression changes during growth, development and following stress.

4.4. Anti- versus pro-oxidative functions of D. scoparium peroxidases

Metabolism of H_2O_2 and typical peroxidase substrates confirms that peroxidases in *D. scoparium* can display typical antioxidative functions (Table 1). However, it is well recognized that higher plant peroxidases, including bryophyte peroxidases, have complex redox cycles and can switch to a pro-oxidative regime (Mika et al. 2004; Chasov and Minibayeva 2009; Minibayeva et al. 2009; Chasov et al. 2015). Strong inhibition of XTT reduction by SOD in crude moss extract suggests the synthesis of $O_2^{\bullet-}$ (Fig. 5A). We tested the possible involvement of peroxidases in $O_2^{\bullet-}$ synthesis by in-gel NBT staining. Results showed that most of the peroxidase isoforms in the intracellular and cell wall fractions can produce $O_2^{\bullet-}$ in the presence of NADH (Fig. 5). Among the possible benefits for pro-oxidative function of peroxidases resulting in ROS production in *D. scoparium* could be defense against pathogens. For example, exposure of *P. patens* tissues to the fungal elicitor chitosan leads to rapid production of ROS, and increased activity of several redox enzymes including the peroxidase *PpPrx34* (Lehtonen et al. 2009, 2012). While gels indicate that peroxidases are the most important enzymes in $O_2^{\bullet-}$ production in *D. scoparium*, some $O_2^{\bullet-}$ producing activity in *D. scoparium* could derive from enzymes other than peroxidases (Fig. 5B), possibly NAD(P)H oxidases. In mosses, ROS production could play an important role in desiccation tolerance, as the severe mechanical and physiological damage that accompanies drying and subsequent rehydration will make the moss vulnerable to pathogen attack. Therefore, ROS production could trigger the hypersensitive response, initiate signaling, reinforce cell walls, and be directly toxic to invading pathogens (Ponce de León and Montesano 2017).

4.5. Role of peroxidases in hydroxyl radical production

Results show that given a quinone and chelated ferric ions, *D. scoparium* can produce $^{\bullet}OH$ (Fig. 2F,G). Rates of production appear linear for 1 h, then rapidly decline between 1 and 2 h. There is no obvious reason for the decline in the rate of $^{\bullet}OH$ production, but possibly the assay reagents, particularly the quinone, were toxic to the moss. Peroxidases have often been proposed to play a key role in $^{\bullet}OH$ production (Richards et al. 2015). In the apoplast of higher plants, the compound III of peroxidase can reduce H_2O_2 and $O_2^{\bullet-}$ to $^{\bullet}OH$, and although this relies on Fe, the mechanistic basis is not yet fully resolved (Richards et al. 2015). However, given the many reports of quinones in bryophytes (e.g. Rycroft and Cole 2001), here we determined $^{\bullet}OH$ production using a method involving supplying quinone and chelated Fe. This method was originally developed for free-living fungi (Gómez-Toribio et al., 2009), where it has been suggested that the Fenton reaction produces $^{\bullet}OH$ and Fe^{3+} from a reaction between H_2O_2 with Fe^{2+}. Peroxidases are important in assisting the oxidation of hydroquinones to quinones which produces H_2O_2, and in the same reaction Fe^{2+} from the reduction of Fe^{3+}. Continuous $^{\bullet}OH$ production occurs by "redox cycling" as the hydroquinones are regenerated by extracellular quinone reductases (Arantes and Goodell 2014). In preliminary studies we found that quinone reductase activity can be readily demonstrated in *D. scoparium* (data not shown), but more work is needed to elucidate the mechanism of $^{\bullet}OH$ production in this moss. Possibly greater rates of radical formation in material rehydrated after desiccation (Fig. 2G) compared to the rates in hydrated material (Fig. 2F) occur because the Fenton reaction is stimulated by H_2O_2 produced by the oxidative burst that is produced during the rehydration of desiccated mosses (Mayaba et al. 2002). If, as seems likely, peroxidases are a major source of ROS, then this would explain why rehydration stimulates both peroxidase release and $^{\bullet}OH$ formation. Hydroxyl radicals may play many roles in plants, including stress signaling, growth control and in the regulation of cell death (for review see Richards et al. 2015). This report appears to be the first to demonstrate extracellular $^{\bullet}OH$ production by bryophytes.

5. Conclusions

The main conclusion of the work presented here is that in the widespread boreal moss *D. scoparium* peroxidases play key roles in desiccation tolerance. The enzyme has constitutively high activity, and activity approximately doubles during slow drying. Furthermore, *D. scoparium* peroxidases can work in both anti- and pro-oxidative modes, suggesting that functional duality of this enzyme is an ancestral characteristic of plants. As for most plants, *D. scoparium* possesses a great diversity of peroxidase isoforms. In future work, we plan to determine the sequences of the main isoforms, allowing us to probe the specific roles of the different isoforms by testing how their expression varies during development or following stress. This will also enable us to make comparisons with the peroxidases of other mosses and vascular

plants, which will increase our understanding of the roles of these enzymes in plant stress tolerance in general.

Authors' contributions

A.O. performed most experiments and contributed to the writing of the manuscript. A.C. designed the study and analyzed kinetic properties of the redox enzymes in three moss species. L.V. performed protein purification. T.T. measured hydroxyl radical production. R.P.B. and F.M. designed the study, discussed the results and wrote the final draft of the manuscript.

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