



Effect of ABA treatment on activities of antioxidative enzymes in selected bryophyte species

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ABSTRACT: The effect of the signal molecule and stress phytohormone abscisic acid (ABA) on activities of antioxidant enzymes was tested in three bryophyte species, viz., the liverwort *Marchantia polymorpha* and the phylogenetically unrelated mosses *Physcomitrella patens* and *Atrichum undulatum*. Production of reactive oxygen species increases in plants exposed to both abiotic and biotic stress. Antioxidant enzymes are very effective and usually represent the plant's first line of defence against the cytotoxic effects of these reactive oxygen species. The activities of enzymes of the antioxidative system (POX, CAT, SOD) in the tested bryophyte species are shown to be increased by treatment with lower concentrations of exogenous ABA. Higher concentrations of exogenous ABA did not significantly influence activities of the tested antioxidative enzymes. The obtained results point to possible involvement of ABA as a signal molecule in the first line of defence against stress in all three bryophyte species.

KEYWORDS: mosses, liverworts, abscisic acid, peroxidase, superoxide dismutase, catalase

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INTRODUCTION

Bryophytes were among the first land plants and had to cope with various stresses once they abandoned their aquatic environment. However, they remain less studied than the sister lineage of vascular plants. A complete understanding of how plants interact with the environment is lacking, and this is especially true for the less complex plants (i.e., bryophytes). In addition, bryophytes include many different groups exhibiting various strategies of survival in harsh environments. One of the main barriers to the advancement of this area of plant biology has been the paucity of simple and appropriate experimental models that would enable the researcher to dissect biochemically and genetically the response of less complex plants to environmental stress (OLIVER *et al.* 2000). Lately, more bryophytes have come to be regarded

as model systems for use as powerful experimental tools to elucidate complex biological processes in plants. This has been due to the discovery and development of homologous recombination technologies in the moss *Physcomitrella patens* (Hedw.) Brach & Schimp. However, rather few bryophytes are used to advance our understanding of how plants respond to and survive stressful environments. The response of plants (including bryophytes) to stress includes processes such as signal transduction, transcript regulation, ionic and osmotic homeostasis, biosynthesis of defence proteins and the action of phytohormones (plant growth regulators). One of the plant growth regulators, abscisic acid (ABA), is a unique molecule found in organisms across kingdoms from bacteria to animals, suggesting its ubiquitous and versatile role in physiological functions of various organisms (SABOVLJEVIĆ *et al.* 2014). Abscisic acid is

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known to be involved in increase of stress tolerance in bryophytes (TAKEZAWA *et al.* 2011). Its participation in stress tolerance has been documented among different lineages of bryophytes (VUJIČIĆ *et al.* 2016).

A common consequence of most abiotic and biotic stresses is an increased production of reactive oxygen species (ROS) (POLLE & RENNENBERG 1993). Reactive oxygen species like the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^{\cdot}) are toxic byproducts of processes such a photosynthetic or respiratory electron transport. Plants protect cellular and sub-cellular systems from the cytotoxic effects of these ROS with antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (POX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and catalase (CAT), as well as non-enzymatic substances like glutathione, ascorbic acid, α -tocopherol and carotenoids (ELSTNER 1986; BOWLER *et al.* 1992; MENCONI *et al.* 1995; ALSCHER *et al.* 1997).

Since ABA can be responsible for the ability to tolerate some forms of stress in bryophytes (WANG *et al.* 2008), the physiological and biochemical responses of three different bryophyte species to ABA were studied in the present investigation. It was expected that increase in the presence of ABA in the medium will secondarily enhance the stress defence of the selected bryophytes species.

For this study, three species of bryophytes were chosen: one thallose liverwort [*Marchantia polymorpha* L. (Marchantiales)] and two phylogenetically unrelated mosses [*Physcomitrella patens* (Funariales) and *Atrichum undulatum* (Hedw.) P. Beauv. (Polytrichales)].

MATERIAL AND METHODS

Plant material. The plant material used in these experiments was grown in *in vitro* species-optimised cultures (SABOVLJEVIĆ *et al.* 2006; VUJIČIĆ *et al.* 2010). Cultures of *A. undulatum* and *M. polymorpha* were grown on half-strength MS (MURASHIGE & SKOOG 1962) medium enriched with sucrose (15.0 g l^{-1}), while those of *P. patens* were grown on BCD (SABOVLJEVIĆ *et al.* 2009) medium with sucrose (15.0 g l^{-1}). The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 25 min. In order to study the effects of ABA on morphogenesis (VUJIČIĆ *et al.* 2016) and antioxidative enzyme activities, different concentrations (0, 0.03, 0.1, 1, 3 and $10 \mu\text{M}$) of this plant hormone were added to all basal media. Cultures were grown at $25 \pm 2^\circ\text{C}$ under short-day conditions (8-h photoperiod) at $47 \mu\text{mol m}^{-2} \text{ s}^{-1}$ PFD (provided by cool-white fluorescent tubes). They were grown under these conditions for 4 weeks, after which the plant material was frozen at -70°C .

For each treatment, shoot/thallus explants were cultured in 90-mm Petri dishes, each containing 10 explants. The effects of ABA were evaluated in terms of

its influence on the activities of antioxidative enzymes (POX, CAT and SOD).

Tissue extract preparation. Before determination of enzyme activities and protein concentrations, frozen plant material was homogenised in liquid nitrogen. Crude proteins were extracted with a buffer containing 50 mM Tris, 1 mM EDTA, 30% glycerol, 1.5% PVPP, 10 mM DTT and 1 mM PMSF. The homogenates were centrifuged for 5 min at $12000 \times g$ and 4°C (on a Sorvall Heareus Biofuge Stratos centrifuge). The supernatants were filtered prior to protein determination by the BRADFORD (1976) method and stored at -70°C .

Analytical assays. Activity of peroxidase (POD, in U mg^{-1} of soluble protein) was assayed by measuring the increase in absorbance at 430 nm. The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 6.5), $10 \mu\text{l}$ of enzymatic extract, $60 \mu\text{l}$ of 1 M pyrogallol (Sigma, $A_{430} \epsilon = 2.4 \text{ mM}^{-1}\text{cm}^{-1}$) as a hydrogen donor and $30 \mu\text{l}$ of 1 M H_2O_2 . Absorbance was measured using an Agilent 8453 UV-visible spectrophotometer.

Activity of catalase (CAT, in $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}\text{mg}^{-1}$ of soluble protein) was determined spectrophotometrically by measuring the decrease in absorbance at 240 nm (AEBI 1984). The reaction mixture (3 ml) contained 50 mM potassium sodium phosphate buffer (pH 7.0), $20 \mu\text{l}$ of enzymatic extract and 30% H_2O_2 ($A_{240} \epsilon = 0.04 \text{ mM}^{-1}\text{cm}^{-1}$).

Activity of superoxide dismutase (SOD, in units of enzyme activity per mg of soluble protein) was determined according to BEYER & FRIDOVICH (1987). The reaction mixture (3 ml) contained 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 260 mM L-methionine, 1.5 mM nitroblue tetrazolium chloride (NBT), 0.04 mM riboflavin and 0-50 μl of enzymatic extract. The mixture was kept under fluorescent light (Tesla Pančevo, 65 W) for 60 min at 25°C . One unit of SOD was taken to be the amount of the enzyme where the NBT reduction (to blue formazan) ratio was 50%. The NBT reduction ratios were measured with an ELISA microplate reader (adjusted to 540 nm).

Statistical analysis. For every treatment, there were four replications, each representing 10 gametophyte shoots of the same height (1.0 cm). The experiment was repeated twice. Data were analysed using StatGrafics software, version 4.2 (STSC Inc., Rockville, Maryland, USA), followed by analysis of variance (ANOVA). Fisher's least significant difference (LSD) test was used to separate mean values. The term significant was used to indicate differences for which $P \leq 0.05$.

Results were analysed using StatGrafics software, version 4.2 and Origin 8.0. Significance of differences between mean values was tested by bifactorial ANOVA assuming $P < 0.05$, followed by Duncan's multiple comparison tests.

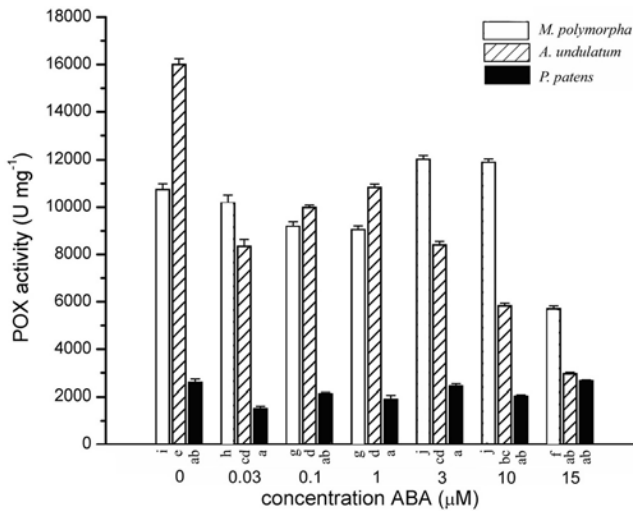


Figure 1. POX enzyme activity of three bryophyte species treated with different ABA concentrations (units are given per mg of protein).

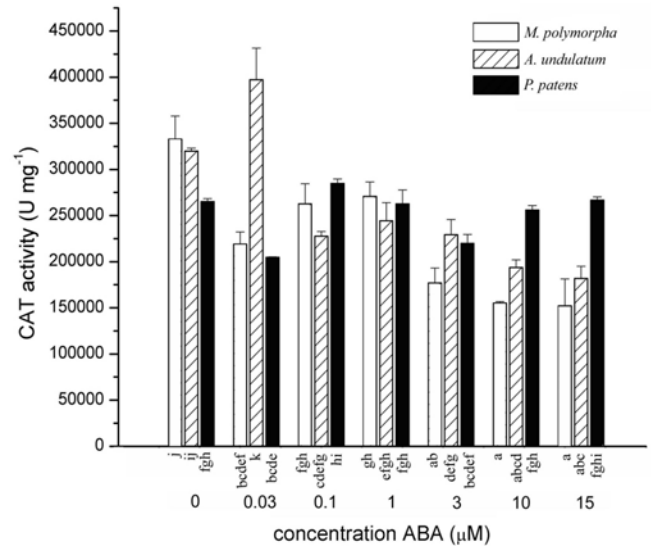


Figure 2. CAT enzyme activity of three bryophyte species treated with different ABA concentrations (units are given per mg of protein).

RESULTS AND DISCUSSION

The putative involvement of ABA levels in the development of plants as a signal of environmental changes is a result of changes in both their hormonal status and their response to activated oxygen species. Abscisic acid is known to trigger the production of H₂O₂, which activates a ROS signaling process that can be viewed as a node in a network linking various stresses. These status characteristics and responses can differ drastically between terrestrial plants like *P. patens* and *A. undulatum* on the one hand and semi-aquatic species adapted to flooded conditions like *M. polymorpha* on the other, as has been demonstrated before for various vascular plants, but not for bryophytes (TAKAHASHI & KAUFMAN 1992).

The activities of antioxidative enzymes responded to exogenous ABA in all three of the tested bryophyte species. The general pattern of the response of POX, CAT and SOD activities in the tested species can be summarised as follows: when exogenous ABA was applied in a lower concentration, all of the group of oxidative stress enzyme activities increased, while at a higher ABA concentration the enzyme activities showed no clear response related to ABA concentration in comparison with the control plants (Figs. 1-3). Generally, by converting O^{2•-} into less toxic H₂O₂, SOD constitutes the first link in the defence of plants against stress. Then POX and CAT take part in it. In the examined species, the activity of SOD was the lowest, while POX and CAT activities were significantly higher than in the exogenous ABA-free control. The lowest POX activity was recorded in *P. patens* (Fig. 1). This is in accordance with ecology of the studied species, namely a quick life span under environmental conditions optimal for growth and survival through dormant spores in unfavourable seasons. In *A. undulatum*, POX activity

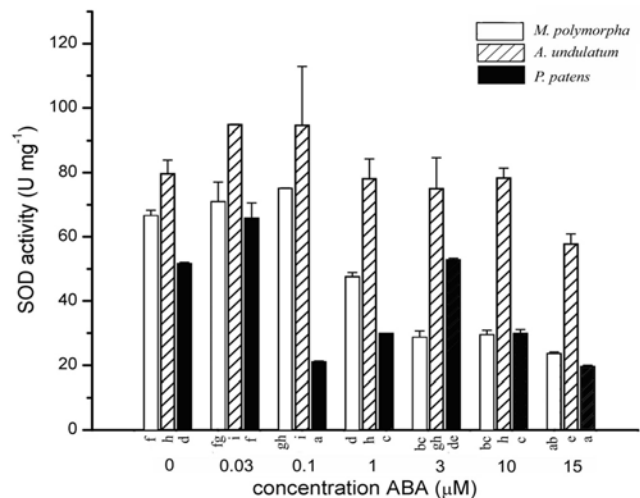


Figure 3. SOD enzyme activity of three bryophyte species treated with different ABA concentrations (units are given per mg of protein).

was significantly higher in the control plants than in those treated with ABA. This species can survive long periods of drought and has a much wider ecological valence of resistance to various environmental stresses compared to *P. patens*. The higher activity of POX can therefore be considered an adaptation to fairly often repeated stages of rehydration/dehydration.

The most important function of ABA is in water stress signaling and regulation of water loss. Bryophytes are highly dependent on water in the process of development, i.e., they have a rather narrow ecovalence in relation to the presence of water. In nature, the highest concentration of ABA is present in bryophytes that are adapted to arid conditions and the lowest concentration in aquatic species,

indicating that ABA may play a role in drought tolerance (HARTUNG *et al.* 1994). In the case of *Funaria hygrometrica* Hedw., ABA makes the protonema resistant to drought, and it induces drought tolerance within the Marchantiales. It has been shown that gradual drying of the moss *F. hygrometrica* leads to an increase in ABA content up to 10 nmol/g of dry weight (WERNER *et al.* 1991).

At the highest concentration of ABA applied (15 µM), POX and SOD activities decreased in all three studied species (Figs. 1-3). It can be asserted that there are no statistically significant changes in the SOD activity of *A. undulatum*. At low concentrations of exogenously added ABA, a slight increase of SOD activity occurred in comparison with the control plants.

The highest CAT activity was recorded in the control group of the liverwort *M. polymorpha* (Fig. 2). Activity of CAT in treated liverwort plants decreased when exogenous ABA was applied. In the other two studied species, no obvious pattern of CAT activity related to ABA concentrations was observed.

However, of all the tested enzyme activities, that of CAT showed the highest values (in U mg⁻¹ of soluble protein). These results suggest that enzymes of oxidative stress (namely POX, SOD and CAT) in bryophytes possibly act as the first level of the anti-oxidative defence system. Non-enzymatic constituents (such as phenols, which bryophytes are rich in) could be the second level of the antioxidative defence system. This may explain why the activities of oxidative stress enzymes decreased at higher concentrations of exogenous ABA. The biological system's ability to readily detoxify reactive intermediates or repair the resulting damage to cell function varies from species to species. In contrast to the case of vascular plants, the response of bryophytes to stress conditions can be regarded as a cell response, not a response of tissues or organs.

Our results indicate that liverworts (in this case *M. polymorpha*) also react to exogenous ABA, although it is commonly accepted that in liverworts lunularic acid plays the part of ABA in other plants. Abscisic acid has been reported to have a photosystem-protective role in some mosses. Thus, BECKETT *et al.* (2000) found that ABA pretreatment of *A. undulatum* speeds up recovery of photosystem II (PSII) during rehydration following desiccation and increases non-photochemical quenching. Similarly, ABA-hardening of plants of *Sphagnum angustifolium* (C.E.O. Jensen ex Russow) C.E.O. Jensen upon rehydration after intensive water stress (drying) improved recovery of PSII activity and provided effective protection of PSII activity against light stress (MARSCHALL & BORBELY 2011). However, MAYABA *et al.* (2001) reported that ABA does not affect starch metabolism or protect chlorophyll from breakdown during desiccation. In our case, the tested moss and liverwort species reacted to ABA. It can be inferred from the obtained results that ABA is part of the first level of the anti-oxidative defence system. With increase in the concentration of exogenous ABA, the

index of multiplication, content of chlorophyll and that of carotenoids decreased in all three of the tested bryophyte species, while their biomass varied slightly (VUJIČIĆ *et al.* 2016).

Further study of non-enzymatic components involved in the defence of bryophytes against stress is needed because such components, in addition to antioxidant enzymes, play a key role in elimination of ROS.

CONCLUSION

The activities of enzymes of the antioxidative system (POX, CAT, SOD) in the tested bryophyte species are shown to be increased by treatment with lower concentrations of exogenous ABA. Higher concentrations of exogenous ABA did not significantly influence activities of the tested antioxidative enzymes. The obtained results point to possible involvement of ABA as a signal molecule in the first line of defence against stress in all three bryophyte species.

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REZIME

Aktivnost enzima antioksidativnog stresa tokom tretmana apscisinskom kiselinom kod odabranih vrsta briofita

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Efekat signalnog molekula i hormona stresa, apscisinske kiseline, testiran je na aktivnost antioksidativnih enzima kod tri vrste briofita: jetrenjače *Marchantia polymorpha*, i filogenetski nesrodnim mahovinama *Physcomitrella patens* i *Atrichum undulatum*. Kod biljaka izloženim i biotičkom i abiotičkom stresu povećava se produkcija reaktivnih kiseoničnih vrsta. Negativni citotoksični efekat reaktivnih kiseoničnih vrsta biva ublažen delovanjem enzima antioksidativne zaštite, pre svega peroksidaza, superoksid dismutaza, katalaza. Kod tri ispitivane briofite aktivnost navedenih enzima bila je povećana u uslovima niskih koncentracija egzogeno dodate apscisinske kiseline. Povećanje koncentracije egzogeno dodate apscisinske kiseline nije bitno uticalo na aktivnost ispitivanih enzima antioksidativne zaštite. Dobijeni rezultati ukazuju na mogućnost učešća apscisinske kiseline kao signalnog molekula u prvim linijama odbrane od stresa kod sve tri ispitivane vrste.

KLJUČNE REČI: mahovine, jetrenjače, apscisinska kiselina, peroksidaze, superoksid dismutaze, katalaze

