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The effect of aluminium-stress and exogenous spermidine on chlorophyll degradation, glutathione reductase activity and the photosystem II D1 protein gene (*psbA*) transcript level in lichen *Xanthoria parietina*

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ABSTRACT

In this study, the effects of short-term aluminium toxicity and the application of spermidine on the lichen *Xanthoria parietina* were investigated at the physiological and transcriptional levels. Our results suggest that aluminium stress leads to physiological processes in a dose-dependent manner through differences in lipid peroxidation rate, chlorophyll content and glutathione reductase (EC 1.6.4.2) activity in aluminium and spermidine treated samples. The expression of the photosystem II D1 protein (*psbA*) gene was quantified using semi-quantitative RT-PCR. Increased glutathione reductase activity and *psbA* mRNA transcript levels were observed in the *X. parietina* thalli that were treated with spermidine before aluminium-stress. The results showed that the application of spermidine could mitigate aluminium-induced lipid peroxidation and chlorophyll degradation on lichen *X. parietina* thalli through an increase in *psbA* transcript levels and activity of glutathione reductase (GR) enzymes.

Introduction

Lichens can accumulate a large amount of metals because they have large surface areas and do not have cuticles or stoma in the thalli. Particles coming from the air through dry or wet deposition or soluble heavy metals are taken in by lichens *via* passive transport mechanisms, such as ion exchange and ion diffusion, or via active transport mechanisms (Nash III, 1996). Therefore, the accumulation, toxicity, and tolerance of metals in lichen thalli have been widely studied (Chettri et al., 1998; Garty et al., 1985; Sanita di Toppi et al., 2008; Unal et al., 2010; Pirintsos et al., 2004). Chlorophyll degradation and altered assimilation pigments of lichens in the field and in the laboratory were previously correlated with the presence of metals (Chettri et al., 1998). Currently, there are many studies on the different mechanisms of metal detoxification, including the production of lichen acids, phytochelatins, non-thiol compounds and antioxidant enzymes. Sanita di Toppi et al. (2008) showed that soluble antioxidants, such as glutathione and ascorbate, and antioxidant enzymes, such as superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), and peroxidases (POD), are involved during the entire process of metal detoxification and participate in metal-chelation (“first line”) and antioxidant protection (“second line”).

Polyamines, including putrescine (Put), spermidine (Spd), and spermine (Spm) are a class of biogenic amines with multiple *in vivo* effects on the cellular processes in most organisms (Bouchereau et al., 1999). Polyamines are important in protecting plants against abiotic stresses, such as potassium deficiency, osmotic shock, drought, metal stress and UV irradiation, because these compounds have roles in osmotic adjustment, maintenance of membrane stability and free-radical scavenging (Duan et al., 2009; Unal et al., 2008). In addition, an induction of polyamine biosynthesis has been shown to be a signal of stress tolerance in several systems. The data on polyamines in lichens indicate a correlation between polyamines and metal accumulation (Pirintsos et al., 2004), effects on nitrogen stress (Pirintsos et al., 2009) and a protective role against UV-A irradiation (Unal et al., 2008). Despite the large number of publications describing the effects of metal tolerance and toxicity on lichen physiology, the protective effects of polyamines under metal stress are unexplored.

The main target of photodamage is photosystem II (PSII), which is multisubunit membrane protein complex that catalyses the light-induced splitting of water. Photodamage of PSII is mainly due to damage to the D1 protein, which forms a heterodimer with

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the D2 protein in the reaction centre of PSII, and the subsequent rapid degradation of the D1 protein (Prasil et al., 1992). The D1 protein of thylakoid membranes has been shown to be a sensitive protein to various environmental stress conditions (Giardi et al., 1997). Photosynthetic organisms often suffer from an imbalance of synthesis/degradation of the D1 protein when exposed to stress.

The D1 protein is encoded by the *psbA* gene, which plays a critical role in replacement of injured D1 protein. Repair of photodamaged PSII requires several steps including the degradation of the damaged D1 protein, the *de novo* synthesis of D1 protein, and the incorporation of the newly synthesised D1 into PSII. Previous studies have reported that salt stress (Allakhverdiev et al., 2002), metal stress (Qian et al., 2009) and oxidative stress (Nishiyama et al., 2004) prevents the repair of photodamaged PSII by inhibiting the transcription and translation of the *psbA* genes. Wang et al. (2011) showed that drought stress remarkably inhibited the transcription of the *psbA* gene in PSII in wheat.

Aluminium (Al) toxicity can cause excessive reactive oxygen species (ROS) production, including the superoxide radical ($O_2^{\cdot-}$), the hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2). ROS that results from photosynthesis and oxidative metabolism, such as superoxide anions and hydrogen peroxide, can be involved in a number of stress responses (Foyer et al., 1994). It has been suggested that Al induces oxidative stress because this ion is involved in various processes, including enzyme activities related to ROS (Cakmak and Horst, 1991), and is associated with peroxidative damage of membrane lipids due to the stress-related increase in the production of highly toxic oxygen free radicals. Many studies have also shown that Al inhibits CO_2 assimilation and photosynthesis in many plant species. However, the effect of exogenous Spd on *psbA* transcript levels under aluminium stress has not been studied in lichens.

The aims of this study are as follows: (i) to determine the physiological response during Al stress in *Xanthoria parietina* by analysis of the chlorophyll content, the rate of chlorophyll degradation, lipid peroxidation and GR activity, (ii) to determine the effects of exogenous Spd on the transcription levels of the *psbA* gene, chlorophyll degradation and GR activity under Al stress, and (iii) to determine the effects of different concentrations of Al on the transcription levels of the *psbA* gene by semi-quantitative RT-PCR .

Results

The results of this study showed that the chlorophyll a content was significantly ($p < 0.001$) decreased by 0.5 and 1.0 mM concentration of aluminium 24 and 48 h after treatment (15.42 and 14.46, 14.55 and 11.49, respectively, as seen in Table A.1). However, the chlorophyll a content was no change significantly by exogenously adding polyamines for 24 and 48 h (20.73 and 19.23, 19.9 and 18.32, respectively, as seen in Table A.1).

The content of chlorophyll b increased significantly with increased aluminium concentration (Table A.1). The chlorophyll a/b ratio was significantly decreased after 24 and 48 h exposure to 0.5 and 1.0 mM aluminium. A positive effect of spermidine (no decrease in the chlorophyll a/b ratio) was observed in *X. parietina* after 24 and 48 h treatment with the high concentration of aluminium.

Fig. A.1 and A.2 show the malondialdehyde (MDA) content of thalli after exposure to aluminium for 24 and 48 h. Aluminium exposure significantly increased the MDA content of thalli at 0.5 and 1.0 mM for 48 h (19.5 and 30.1 nmol/g fresh wt., respectively), but not at 0.25 mM ($p < 0.01$). The increase in MDA level, as an index of lipid peroxidation, was in a concentration- and time-dependent manner. MDA production levels between Al-treated samples and exogenously Spd treated samples were also shown to be significantly different ($p < 0.01$). The 0.5- and 1.0 mM-Al

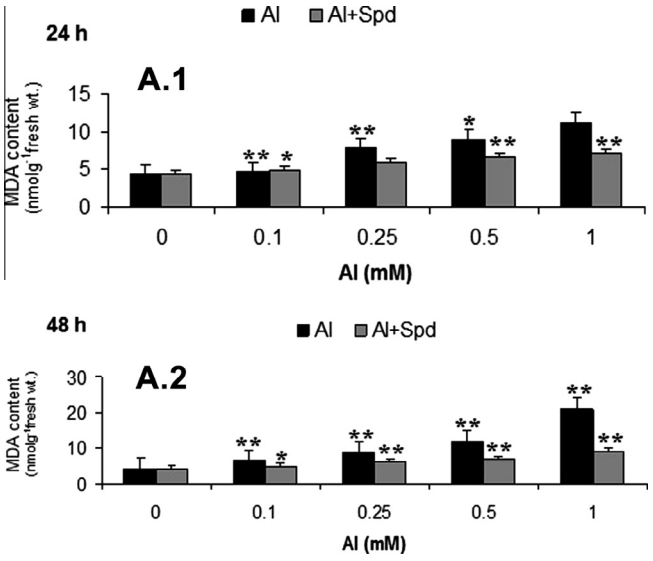

Fig. A.1 and A.2. The effects of exogenous spermidine on the MDA content of *X. parietina* thallus under different concentrations of aluminium. *Represents a statistically significant difference of $p < 0.05$ when compared with the control, **represents a statistically significant difference of $p < 0.01$.

Table A.1
Chlorophyll a (Chla), chlorophyll b (Chlb), and chlorophyll a/b (Chla/b) of the lichen *Xanthoria parietina* with control and aluminium supplement of different concentration and exogenous polyamine application groups.

	n	Chla (mg/g dw)		Chlb (mg/g dw)		Chla/b	
		24 h *X* ± SD	48 h *X* ± SD	24 h *X* ± SD	48 h *X* ± SD	24 h *X* ± SD	48 h *X* ± SD
Control	3	22.78 ± 0.03	22.19 ± 0.12	6.68 ± 0.28	6.17 ± 0.24	3.41 ± 0.08	3.59 ± 0.12
0.1 mM $AlCl_3$	3	22.85 ± 0.19	21.91 ± 0.14	6.96 ± 0.05	6.83 ± 0.04	3.28 ± 0.2	3.21 ± 0.04
0.25 mM $AlCl_3$	3	19.41 ± 0.12	18.62 ± 0.09	6.58 ± 0.08	6.98 ± 0.013	2.95 ± 0.06	2.66 ± 0.08
0.5 mM $AlCl_3$	3	15.42 ± 0.09	14.55 ± 0.18	7.77 ± 0.23	8.18 ± 0.07	1.98 ± 0.05	1.78 ± 0.08
1 mM $AlCl_3$	3	14.46 ± 0.14	11.49 ± 0.03	9.25 ± 0.26	8.74 ± 0.22	1.56 ± 0.11	1.33 ± 0.07
0.1 mM $AlCl_3$ + 1 mM Spd	3	25.30 ± 0.08	24.21 ± 0.16	6.47 ± 0.013	6.74 ± 0.11	3.91 ± 0.12	3.6 ± 0.11
0.25 mM $AlCl_3$ + 1 mM Spd	3	22.37 ± 0.06	21.68 ± 0.03	6.82 ± 0.07	7.19 ± 0.08	3.28 ± 0.08	3.013 ± 0.083
0.5 mM $AlCl_3$ + 1 mM Spd	3	20.73 ± 0.12	19.9 ± 0.012	7.52 ± 0.26	7.94 ± 0.08	2.76 ± 0.015	2.48 ± 0.03
1 mM $AlCl_3$ + 1 mM Spd	3	19.23 ± 0.05	18.32 ± 0.09	8.14 ± 0.18	8.53 ± 0.23	2.36 ± 0.075	2.17 ± 0.07
ANOVA							
F statistic		0.88	1.18	4.12	92746.11	90769.25	20396496.74
p		0.5537	0.3643	0.0060	<0.0001	<0.0001	<0.0001

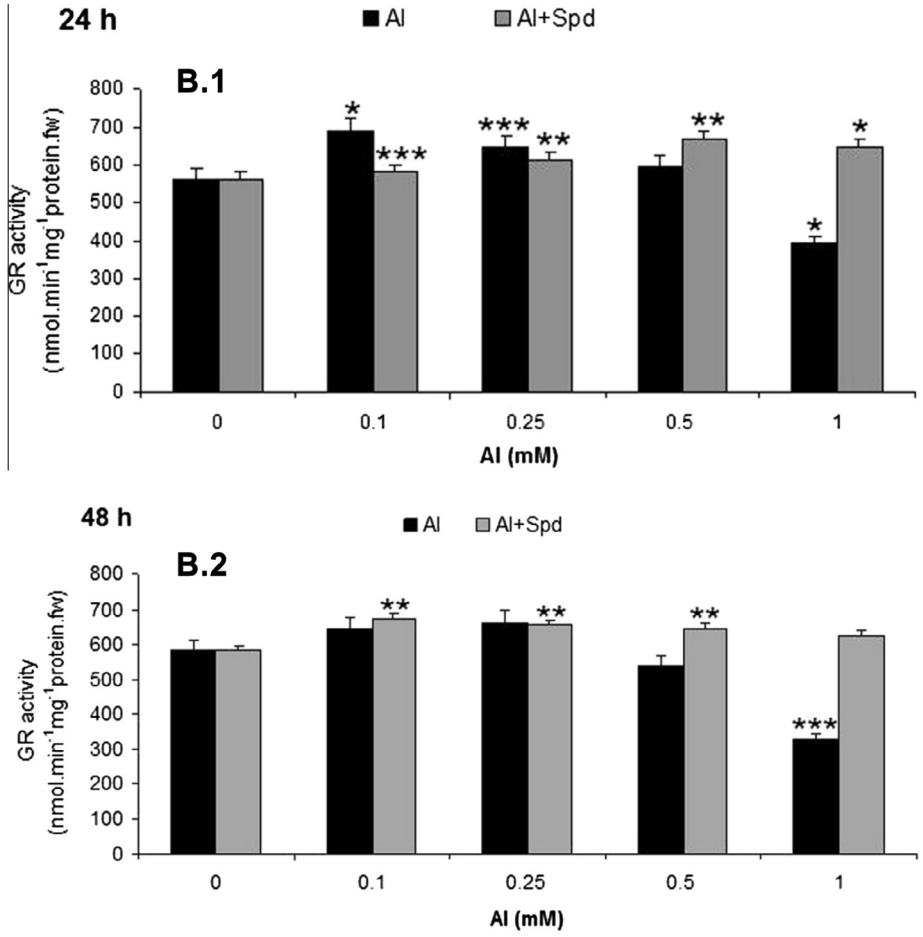

Fig. B.1 and B.2. The effects of spermidine on GR activity of *X. parietina* thalli under different concentrations of aluminium. *Represents a statistically significant difference of $p < 0.05$ when compared with the control, **represents a statistically significant difference of $p < 0.01$, ***represents a statistically significant difference of $p < 0.001$.

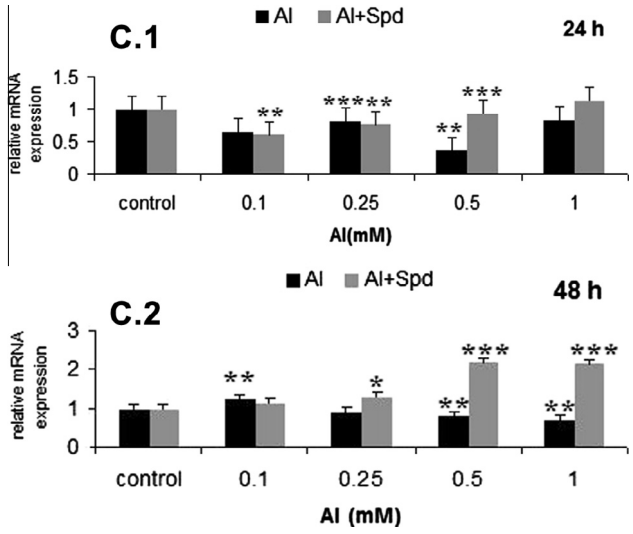

Fig. C.1 and C.2. The effects of exogenous spermidine on the relative expression of *psbA* in *X. parietina* under different concentrations of aluminium. *Represents a statistically significant difference of $p < 0.05$ when compared to the control, **represents a statistically significant difference of $p < 0.01$, ***represents a statistically significant difference of $p < 0.001$.

samples treated exogenously with Spd had a lower content of MDA (6.8 and 8.21 nmol/g fresh wt., respectively) than the other groups for 48 h (Fig. A.2).

GR activity increased in samples treated with 0.1, 0.25 and 0.5 mM Al for 24 h (Fig. B.1 and B.2). In the *X. parietina* thalli of lichens treated with 1.0 mM Al, the GR activity decreased (327.57 nmol min^{-1} mg^{-1} protein) by 55% after 48 h. Exogenous Spd application significantly improved the GR activity in all of the Al treatment groups for 24 and 48 h ($p < 0.05$, $p < 0.01$ and $p < 0.001$). Additionally, samples treated with exogenous Spd had higher GR activity than the control and Al-treated samples at 24 and 48 h (Fig. B.1 and B.2). In the *X. parietina* thalli samples treated with Spd and Al, GR activity increased by 15.46%, 16.34%, 11.00% and 7.39% after 48 h (672.83, 655.14, 646.3 and 625.4 nmol min^{-1} - mg^{-1} protein, respectively).

The photosystem II D1 protein gene (*psbA*) was up-regulated after treatment with different concentrations of Al (Fig. C.1 and C.2). The *psbA* gene transcription levels after treatment with 0.1, 0.25, 0.5 and 1.0 mM concentration of aluminium for 24 h are shown in Fig. C.1. When compared with the control, the *psbA* mRNA level was decreased by 0.35-, 0.17-, 0.45- and 0.15-fold. Treatment with 0.1 mM aluminium for 48 h resulted in higher levels of *psbA* gene expression (1.25-fold). However, treatment, with other concentrations of aluminium (0.25, 0.5 and 1.0 mM) for 48 h resulted in decreases by 0.92-, 0.82- and 0.72-fold in the *psbA* mRNA levels relative to control group (Fig. C.2). The application of Spd did not show a positive effect on the *psbA* mRNA transcript level, except in the group treated with 1.0 mM Al for 24 h. However, treatment with 0.1, 0.25, 0.5 and 1 mM Al for 48 h resulted in increases of 1.15-, 1.32-, 2.19- and 2.15-fold, respectively, in the *psbA* mRNA level relative to the control and non-Spd added samples.

Discussion

The effects of aluminium and exogenous spermidine on chlorophyll degradation

Aluminium toxicity has been extensively described in physiological, cellular, and molecular biological aspects and has been shown to cause reactive oxygen species production and inhibit photosynthesis (Kochian, 1995; Ohki, 1986). Chlorophyll is sensitive to stress-initiated oxidative processes, such as photo-oxidation (Chettri et al., 1998). Chlorophyll b is formed from chlorophyll a by the oxidation of the methyl group on ring II of the aldehyde (Chettri et al., 1998), and the ratio of chlorophyll a/b is more sensitive to modification than chlorophyll a + b. In green plants, antenna size is determined by the amount of the light-harvesting chlorophyll a/b protein complex that is associated with the photosystems (Tanaka and Tanaka, 2006) and according to recent relative works antenna size is determined also by polyamines and plastidal transglutaminases (Ioannidis et al., 2012). Conversion of chlorophyll a to chlorophyll b not only impacts the chlorophyll a/b ratio but is also the first step of chlorophyll degradation. In this work, we found that the application of exogenous Spd prevented chlorophyll degradation in the aluminium-stressed *X. parietina* thallus, compared to non-polyamine treated samples (as seen in Table A.1). Besford et al. (1993) also showed that treatment Spd or Spm prevents loss of chlorophyll, indicating preservation of the thylakoid membranes at the site of the chlorophyll-protein complex in osmotically shocked oat leaves. In this study, although the content of Chla was strongly influenced by high aluminium levels, exogenous spermidine treatment could be prevents loss of chlorophyll under aluminium stress.

Effects of aluminium and spermidine on lipid peroxidation

Metal toxicity can induce lipid peroxidation in photosynthetic organisms. This may be due to the production of ROS that distort the membrane architecture, as reported earlier in various higher plants (Shah et al., 2001). Thus, lipid peroxidation may seriously influence the membrane integrity. MDA is a kind of lipid peroxidation product; therefore, MDA content can be an indicator of membrane injury. Turton (1997) suggested that the presence of MDA in biological systems can be related to the peroxidation of unsaturated fatty acids constituting cellular membranes. The consequences of the changes in lipid and protein structure are the loss of membrane integrity and selective permeability. In the present work, all of the tested concentrations of aluminium were verified to induce MDA accumulation. MDA contents were moderately increased by aluminium, as observed in our previous report on chromium (Unal et al., 2010). However, many plant physiologists have suggested that polyamines could act as acid neutralizing compounds and scavengers of active oxygen species that stabilise membranes by reducing lipid peroxidation under different environmental stress conditions (Borrell et al., 1997; Bouchereau et al., 1999). Borrell et al. (1997) determined that the MDA levels were reduced by incubation with Spd and Spm in osmotically stressed oat leaf tissue for 24 and 48 h. Groppa et al. (2007) reported that exogenously Spm reduced the rate of lipid peroxidation under Cd and Cu stress in wheat leaves. In this study, although aluminium toxicity caused an increasing MDA content, the addition of exogenous Spd prevented higher lipid peroxidation (Fig. A.1 and A.2).

Glutathione reductase activity and spermidine application

Although the protective role of polyamines under different environmental conditions had been widely discussed, the precise mode of action largely remains a matter of speculation. Polyamines can indirectly reduce ROS formation by inhibiting NADPH oxidase (EC 1.6.3.1) activity, and the effect order in this process is Spm, Spd, Put (Shen et al., 2000). Among the literature regarding the protective role of polyamines, more reports have focused on Spm (Groppa et al., 2001). Shu et al. (2011) showed that exogenous Spm significantly increased the activities of glutathione reductase (GR). Verma and Mishra (2005) also reported that the effect of Put on antioxidant enzymes activity could be APX > GR > CAT > SOD > -POD. Similarly, we observed that the activities of GR in the aluminium-stressed thalli were enhanced by Spd application, which was consistent with Spd reducing the MDA content and chlorophyll degradation in thalli of *X. parietina*.

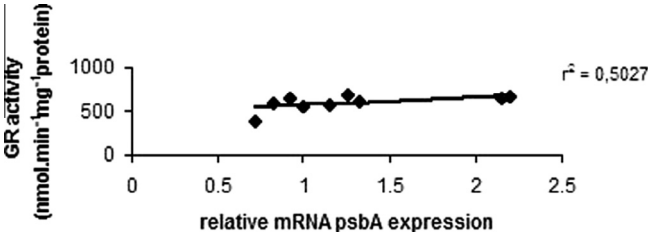

Fig. D.1. The relationship between GR activity and the relative mRNA *psbA* level of the thalli.

PSII repair capacity and spermidine application

Repair of photodamaged PSII requires several steps including the degradation of the damaged D1 protein, the synthesis *de novo* of D1 protein, and the incorporation of the newly synthesised D1 into PSII. Previously, studies have reported that salt stress (Allakhverdiev and Murata, 2004) and oxidative stress (Nishiyama et al., 2004) prevent the repair of photodamaged PSII by inhibiting the transcription and translation of *psbA* genes. Qian et al. (2009) demonstrated that copper and cadmium inhibit the expression of *psbA* and *rbc*L at the transcriptional level in *Chlorella vulgaris*. In this study, the expression of the *psbA* gene in the photobiont of *X. parietina* was analysed by semi-quantitative RT-PCR using GAPDH as the internal control. Our data show that Al application reduced the mRNA level of the *psbA* gene (Fig. C.1 and C.2). The reduction of *psbA* mRNA transcripts may decrease the activity of PSII and electron transfer rates, which was observed by a decrease in chlorophyll content (Fig. D.1, Table A.1).

Several reports have shown that the exogenous application of polyamines, especially Spd, which is a known stimulator of PSII activity (Ioannidis and Kotzabasis, 2007), alleviates the decrease in protein transcripts of PSII major proteins and retards the loss of D1 and D2 under drought and osmotic stress (Duan et al., 2006). Hamdani et al. (2011) suggested that polyamines can maintain the stability of D1 and D2 proteins of PSII and retard their apparent degradation under stress conditions. The *psbA* gene is responsible for the reproduction of new D1 protein and plays an important role in D1 protein turnover during stress resistance (Mulo et al., 2009). In the present study, we have found that the expression of *psbA* is clearly decreased under Al treatment but was increased by Spd application (Fig. C.1 and C.2).

Conclusions

Our results suggest that exogenous Spd could significantly mitigate effects of aluminium-stress in *X. parietina* thalli through an increase in GR, *psbA* transcription and decreased chlorophyll degradation. Future work is necessary to determine on how spermidine-related regulatory mechanisms interact with D1 protein and GR activity in response to abiotic stress.

Experimental

Lichen material

Epiphytic green algal (*Trebouxia*) lichen species foliose *X. parietina* (L.) Th. Fr., were collected from *Pinus* sp. in Gulumbe/Bilecik, Turkey (N 40° 11.526′, E 029° 57.962′) and used for laboratory experiments. Samples were transferred to the laboratory in plastic bags, cleaned of impurities, and washed three times for 5 s with distilled water to remove dust from the surface.

Aluminium treatment

The thallus of *X. parietina* (approx. 10 g) was incubated for 30 min in 50 ml of $AlCl_3$ solution (0.1, 0.25, 0.5, and 1.0 mM) and compared with control samples soaked in deionised water. After Al treatments samples were rinsed three times for 5 s in deionised water and kept the laboratory for 24 and 48 h in Petri dishes.

Spermidine treatment

To examine the role spermidine, we combined the Al treatment with an exogenously supply of 1.0 mM Spd-hydrochloride salt.

Chlorophyll content analysis

Chlorophyll analysis was determined using the method described by Wellburn (1994). Approximately 20 mg of thalli were extracted in the dark for 1 h at 65 °C in 3.0 ml dimethyl sulfoxide (DMSO) in the presence of polyvinylpyrrolidone to minimise chlorophyll degradation. The absorbance of the extracts reflection of turbidity was checked at 750 nm with UV–visible spectrophotometer (Perkin Elmer) to be checked that it was always less than 0.01. For the chlorophyll analysis, measurements were taken at the wavelengths 665.1 and 649.1 nm. Each treatment comprised of three replicates.

Analysis of glutathione reductase Activity

Glutathione reductase activity was analysed according to the protocol of the Glutathione Reductase Enzyme Immunoassay kit (Lot # fr19.30442). Each treatment comprised three replicates.

Lipid peroxidation

Formation of malondialdehyde (MDA) was evaluated as an indicator of lipid peroxidation. The determination of MDA was performed by the thiobarbituric acid reactive substances method (Heath and Packer, 1968). The absorbance differences between 532 and 600 nm was used to calculate MDA formation as a by-product of lipid peroxidation. Each treatment comprised three replicates.

Isolation of RNA and RT-PCR analysis

For RNA extractions, 100 mg of lichen thallus was ground in liquid nitrogen with a mortar and pestle to a fine powder. Then, 1.0 ml of TRIZOL reagent was added for homogenisation. 0.2 ml of chloroform per 1.0 ml of TRIZOL Reagent was added. The samples were vortexed vigorously for 15 s and incubated at room temperature for 2–3 min. The samples were centrifuged at 12,000*g* for 15 min at 4 °C. Samples were incubated at 15–30 °C for 10 min and centrifuged at 12,000*g* for 10 min at 4 °C. The RNA pellet was washed once with 75% ethanol by adding at least 1.0 ml of 75% ethanol per 1.0 ml of TRIZOL reagent used for the initial homogenisation. The samples were mixed by vortexing and were centrifuged at 7500*g* for 5 min at 8 °C. The washing procedure was repeated once, and any leftover ethanol was removed. The RNA pellet was air-dried for 5–10 min. RNA samples have an A_{260}/A_{280} ratio <1.8.

Reverse Transcriptase-PCR was performed using the Intron he High Capacity cDNA Reverse Transcription Kit (Invitrogen, Cat No.: 4398814).

Semi-quantitative RT-PCR

To determine the usefulness of the total RNA isolates, a routine PCR protocol was also applied to cDNAs. PCR reactions were carried out using GAPDH primers. In addition, the following primer sequences were used for *psbA*: sense (CACTAATCCGTGAAACTACT) and antisense (TAATCGTCCAAAGTAACCGTG). GAPDH primers were used as an internal control. The basic protocol for PCR was carried out using 1 μl of cDNA, 2.5 μl of 10X PCR buffer minus Mg^{++}, 0.65 μl of 50 mM $MgCl_2$, 0.5 μl of 10 mM dNTP mix, 0.6 μl of BSA, 0.5 μl of each 10 μM primer and 0.4 μl of recombinant *Taq* DNA polymerase (Fermentas). The thermal cycle was started at 95 °C for 5 min to denature double-strand DNA. PCR cycle numbers were chosen to ensure that the amplification of PCR products was in the exponential range; 34 cycles of denaturation at 95 °C for 1 min, annealing at 47 °C for 1.15 min, and elongation at 72 °C for 1.15 min. Amplification ended at 72 °C for 10 min, and the products were stored at −20 °C. Each Primer set was amplified using an optimised number of PCR cycles to ensure the linearity requirement for semi-quantitative RT-PCR analysis. Aliquots of PCR products (≈10 μl) were analysed by electrophoresis using a 2% agarose gels containing 0.5 μg ml^{-1} of ethidium bromide.

Statistical analyses

The statistical analyses were performed using SPSS for Windows, including one-way analysis of variance (ANOVA) and Tukey's pairwise comparisons.

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