Botanica SERBICA 41 (1): (2017) 17-24

Original Scientific Paper

Protective effects of polyamines against UV-A and UV-B illumination in *Physcia semipinnata* thalli

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ABSTRACT: The damage to DNA induced by UV-A and UV-B and protective effects of the polyamines putrescine (put), spermidine (spd) and spermine (spm) were investigated on the lichen *Physcia semipinnata* in the present study. Our results suggest that significant alterations of the photosynthetic quantum yield ratio occurred in response to increased UV-A and UV-B exposure time. The photosynthetic quantum yield ratio gradually decreased in *P. semipinnata* following exposure to UV-A and UV-B. *Physcia semipinnata* thalli which were treated with a polyamine in a concentration of 1 mM were not affected by UV-A exposure for 72 h. In the case of UV-B treatment, the protective polyamine dosage was 0.25 mM. We also used the random amplified polymorphic DNA (RAPD) technique to detect DNA damage. The main changes observed in the RAPD profiles, which were obtained using 12 RAPD primers, were the appearance or disappearance of different bands and variation of their intensities. The use of at least three different primers allowed detection of specific band patterns in both UV-A- and UV-B-exposed samples treated with polyamines as compared to untreated ones.

Keywords: RAPD-PCR, putrescine, spermidine, spermine, lipid peroxidation

Received: 11 February 2016 Revision accepted: 23 May 2016

UDC: [547.415:613.648.2]:582.29
DOI:

INTRODUCTION

Depletion of the stratospheric ozone allows more solar ultraviolet (UV) radiation to reach the Earth's lower atmosphere and surface. The UV wavelengths most affected by ozone (O_3) reductions are in the UV-B band (280–315 nm), with some effect also in the UV-A band (315–400 nm) (Madronich *et al.* 1998). The discovery of severe depletion of the stratospheric ozone layer has attracted increased attention to the potential effects of enhanced UV-B radiation on all living organisms (McKenzie *et al.* 2003). Of the three usual wavebands of UV light, UV-A may be as harmful as UV-B (Holzinger & Lutz 2006), and it stimulates changes in the metabolism of non-vascular plants (Kováčik *et al.* 2010), including lichens (Unal *et al.* 2008; Unal *et al.* 2009). Environmental stress, irrespective of its nature, enhances the formation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide. Increased input of UV light has also been shown to stimulate increase in ROS (Kováčik & Klejdus 2008; Wang *et al.* 2010). Ultraviolet radiation affects plant structure and metabolism, especially photosystem II (PS II), in two ways. One way is by causing direct damage to key components such as the D1 protein of photosystem II, so that the photosynthetic apparatus undergoes oxidative degradation, while the other is by inhibiting the activation of Rubisco (Aro *et al.* 1993). Many organisms display adaptations to avoid or minimise UV-induced damage. For example, photosynthetic organisms have mechanisms to efficiently repair damage to DNA and the photosynthetic machinery, and they exhibit a variety of

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responses to UV stress, including the formation of UV-protective pigments such as scytonemins and carotenoids (Castenholz & Garcia-Pichel 2000).

As polycationic molecules containing several groups of amines, polyamines (PAs) are widely present in the cells of most living species, ranging from bacteria to animals, and can be found at concentrations on the mM order of magnitude in the cell. These molecules are indispensable cellular components implicated in many physiological functions, such as DNA replication and repair, transcription, protein synthesis, post-translational protein modifications and the stabilisation of membrane structures (Tabor & Tabor 1985; Balasundaram 1991). In addition, polyamines may be required for normal cell growth, cellular proliferation and protection of DNA from damage in many eukaryotic cells (Snyder & Sunkara 1990). Polyamines have been reported to interact with the phosphate groups of DNA, with anionic components of phospholipids and with cell wall components such as pectic polysaccharides (Galston & Sawhney 1990; Bouchereau *et al*. 1999). Furthermore, PAs protect DNA from damage by neutralising charge and conformational changes of DNA (Kakkar & Sawhney 2000). However, the protective role of PAs still remains unclear.

Over the past decade, the use of molecular techniques has provided novel insight on the diversity of genotoxicological responses underlying cellular function in different organisms. The one that we used in this study was RAPD-PCR. To date, RAPD markers have been used for species identification, analysis of population structure, analysis of the genetic impact of environmental stress and analysis of genetic diversity.

The aims of this study were as follows: (i) to determine the DNA mutation rate during both UV-B and UV-A radiation stress in *Physcia semipinnata* (J. F. Gmel.) Moberg using the RAPD-PCR technique; (ii) to determine the role of polyamines in DNA damage; (iii) to evaluate MDA (malondialdehyde) formation for the purpose of ascertaining lipid peroxidation; and (iv) to determine photosynthetic efficiency.

MATERIALS AND METHODS

Sample collection and experimental design. *Physcia semipinnata* samples were collected from tree branches in Karagöl, Izmir, Turkey (38°33' N, 27°13' E, 840 m) in September. The lichens were detached from their substrate, transferred to the laboratory and rinsed immediately for 15 s with double-distilled water at room temperature. This procedure was repeated three times to eliminate dust. The samples were then incubated in distilled water for 30 minutes to rehydrate the thalli and maintained in Petri dishes for the duration of the experiments. The experiments were carried out in a closed cabinet to avoid evaporation, the water levels in the Petri dishes being checked constantly to keep them stable.

The work consisted of three steps: 1 - determining the UV-A and UV-B durations for experimentation; 2 - determining the polyamine (spm, spd, put) doses; and 3 - designing an experiment to determine the protective effect of polyamines on DNA.

In the first step, we determined which durations of UV-A and UV-B radiation exposures give the maximum quantum yield. For this purpose, one set of lichen thalli was exposed to UV-A (352 nm) at an intensity of 20 J s^{-1} m^2 and another set to UV-B (314 nm) at an intensity of 12.5 J s^{-1} m^2 for 30 min, 1 h, 2 h, 24 h, 48 h and 72 h. The UV-A lamps (XX-20BLB, *Philips*) and UV-B lamps (FSX24T12-UVB-HO, Philips) used here were fixed 15 cm above the sets of lichen thalli. The control groups were incubated in a growth chamber under light with intensity of 120 µmol m^{-2} s^{-1}. According to temperature measurements, there was no heat effect from the indicated height. Temperature was stabilised at 24°C (the same temperature as at the sample collection site) and checked constantly to avoid heat effects. Stabilisation of the UV lamps' temperature was controlled manually (Garty *et al.* 2004). Approximately 10 thalli were not exposed to UV radiation as a negative control.

In the second step, we determined the most effective doses of polyamines (spm, spd, put). For this purpose, lichen thalli were treated with 0.25, 0.5, 1, 2.5 and 5 mM concentrations of polyamines (spm, spd and put) for 30 min, after which each group was exposed to UV-B for 24 h to determine effective concentrations of polyamines for subsequent experiments. No dose experiment was conducted for UV-A radiation, since Unal *et al*. (2008) reported a polyamine dose of 1 mM to be effective against UV-A radiation in their study.

In the third step, we set out to determine the protective effects of polyamines on DNA. Lichen thalli were treated with spm and spd at the effective concentration (0.25 mM for UV-B, 1 mM for UV-A) and exposed to UV-A and UV-B for 72 and 24 h, respectively. The thalli were kept at -20°C until DNA extraction. All experiments were repeated three times.

Chlorophyll flourescence. Chlorophyll *a* fluorescence of samples was measured for both UV-A- and UV-B-exposed samples with a plant efficiency analyser (Handy PEA, Hansatech) as previously described by Pirintsos *et al.* (2009). Before measurement of chlorophyll fluorescence, thalli were dark-acclimated for 5 min. The Fv/Fm parameter (maximum quantum yield efficiency of PSII) was used as a stress indicator. It was determined experimentally from fluorescence induction curves of 5-s duration recorded at an irradiance of 1800 µmol m^{-2} s^{-1} from light-emitting diodes. All experiments were repeated three times.

Measurements of lipid peroxidation. Formation of malondialdehyde (MDA) was evaluated as an indicator

Table 1. RAPD-PCR primers and sequences.

Primer #	Primer Name	Sequence
1	OPB8	5'-GTCCACACGG-3'
2	OPB10	5'-CTGCTGGGAC-3'
3	OPB17	5'-AGGGACGAG- 3'
4	OPB1	5'-GTTTCGCTCC- 3'
5	OPB11	5'-GTAGACCCGT- 3'
6	OPB12	5'-CCTTGACGCA- 3'
7	OPB7	5'-GGTGACGCAG- 3'

of lipid peroxidation. Determination of MDA with thiobarbituric acid-reactive substances (TBARS) was performed by the method described by Hagege *et al*. (1990). The difference of absorbance between 532 and 600 nm was used to calculate formation of MDA as a by-product of lipid peroxidation. Each treatment consisted of three replicates.

DNA isolation and RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction). After polyamine treatment and UV-radiation exposures, DNA was isolated from *P. semipinnata* thalli using a modified CTAB method (Doyle & Doyle 1987; Tuney & Sukatar 2010).

The RAPD-PCR was realised using a combination of 12 different RAPD primers (Table 1). Amplification for the PCR was conducted using an ABI Prism 9700 thermal cycler. The PCR took place in a 25-ml volume containing 50 pmol of each primer, 1.25 mM dNTPs, 1 U of Taq polymerase, 25 mM 10 x Taq polymerase buffer and 10 ng/ml of genomic DNA.

Denaturation was conducted at 94°C for 1 min, annealing at 40°C for 30 s and extension at 72°C for 1 min for 40 cycles; this was followed by denaturation at 94°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 45 s, then by final extension at 72°C for 11 min. The presence or absence of PCR bands was confirmed using agarose gel electrophoresis following PCR analysis.

The PCR products were resolved on 1.5% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) and electrophoresed at 80 V for about 1 h using a gel electrophoresis system (Sub-Cell GT Cell, Bio-rad). In all gels, a 100-bp molecular size standard was run in addition to the amplified products to determine the approximate band size. Agarose gels were stained with EtBr and visualised under a UV lamp.

The genetic distance between the treated groups was calculated with the PopGene 32 program (Yeh & Yang 1999). The presence of bands was numbered as '1' and the absence of bands as '0' to build a matrix.

RESULTS

Thalli exposed to UV-A for different durations ranging from 30 min to 72 h, exhibited different responses of PSII activity, an assertion based on permanent reduction of the maximal efficiency of PSII (Fv/Fm) and estimates of the rate of photoinhibition. The Fv/Fm values were significantly lower for 48 and 72 h of UV exposure compared to shorter exposure times, indicating a photodestructive effect of UV-A on photosystem II after prolonged exposure (Table 2).

In the next step, to determine if polyamine treatment would protect the cells from photoinhibition by UV-A exposure, we treated the cells with different doses of spd, spm and put and then exposed them to UV-A for 72 h. Permanent reduction of maximal PSII efficiency was assessed by measuring Fv/Fm, whose values were significantly lower in untreated samples compared to polyamine-treated samples (Fig. 1), pointing to a potential protective effect of polyamines; interestingly, we did not observe any great differences in the responses of thalli to the three polyamines used.

The photodestructive effect of UV-B on chlorophyll fluorescence was apparent after 24 h (Table 2), at which point Fv/Fm decreased relative to shorter incubations and the control treatment. We then incubated *P. semipinnata* thalli with polyamines in different concentrations for 24h (Fig. 2).

The Fv/Fm values for UV-B exposure revealed both toxic effects of polyamines on PSII at high concentrations (> 0.5 mM) and protective effects at lower concentrations (< 0.5 mM) (Fig. 2).

Table 2. Maximal quantum yield results for different UV-A and UV-B durations in the lichen *P. semipinnata.* Values in bold are significantly different from control samples. Significance of differences ($p<0.05$) was checked by one-way analysis of variance (ANOVA). Values are means of three replications ±SD.

	Control	30 min	1 h	2 h	24 h	48 h	72 h
UV-A	0.728±0.06	0.755±0.12	0.741±0.09	0.723±0.12	**0.412±0.05**	**0.229±0.012**	**0.171±0.55**
UV-B	0.682 ± 0.016	0.714 ± 0.014	0.681 ± 0.016	0.701 ± 0.028	**0.456 ± 0.011**	**0.303 ± 0.094**	-

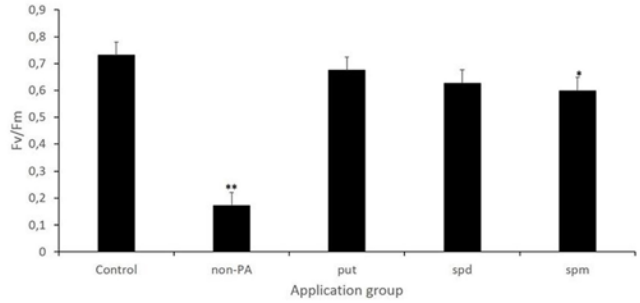

Figure 1. Maximal quantum yield of 1 mM polyamine-treated thalli after 72 h in the lichen *P. semipinnata.*
*Represents a statistically difference of $p < 0.05$ when compared with the control.
** Represents a statistically significant difference of $p < 0.01$.

Lipid peroxidation. Levels of MDA production in polyamine- treated samples that were previously exposed to UV-A and UV-B for 72 and 24 h (Fig. 3a, b), respectively, also showed significant differences ($p < 0.05$). The exogenously spd-treated sample had a lower concentration of MDA than in the spm- and put-treated samples following UV-A and UV-B exposure for 72 and 24 h (Fig. 4a, b), respectively. The MDA concentrations in spm-, spd- and put-treated samples subjected to UV-A treatment were 1.33, 0.87 and 1.6 nmol g^{-1} of fresh weight, respectively. In the case of samples subjected to UV-B treatment, the corresponding concentrations were 1.52, 0.95 and 1.82 nmol g^{-1} of fresh weight, respectively. In the experimental group of UV-B, different polyamine concentrations had an effect on the rate of lipid peroxidation. The content of MDA also increased after treatment with polyamines in concentrations of 5, 2.5, 1 and 0.5mM for 24 h (Fig. 5).

RAPD-PCR. To determine the protective effects of polyamines on DNA under UV-A and UV-B radiation, we used 12 different RAPD primers in our study and found different banding patterns for both UV-A- and UV-B-exposed samples treated with polyamines. The primers OPB6, OPB10, OPB11 and OPB12 were most successful in the UV-B experiment (Fig. 6), while the most successful ones in the UV-A experiment were OPB6, OPB10 and OPB12 (Fig. 7).

The RAPD-PCR band distance between UV-A-exposed and negative control samples was calculated as 57%, while the genetic distance between negative control and polyamine-treated UV-A-exposed samples ranged from 12 to 14 to 20% (spm, spd, put, respectively).

DISCUSSION

Polyamines are important for protecting plants against various abiotic stresses, owing to their roles in osmotic adjustment, maintenance of membrane stability and free-radical scavenging (Duan *et al.* 2009). In addition, 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 radiation (Unal *et al.* 2008). In the present study, our results demonstrated different responses of the lichen *P. semipinnata* when exposed to UV radiation at high (i.e., toxic) and low (i.e., protective) concentrations of polyamines. The results of determining the photosynthetic quantum yield and MDA levels (Tables 4, 5, 6) indicate that increasing doses of polyamines have toxic effects on the lichen *P. semipinnata* under stress induced by UV-B radiation. Kramer *et al.* (1991) indicated in their study that UV-B radiation induces intracellular polyamine biosynthesis. If this similarly occurred during our experimental incubations, it is likely that the UV-B-induced polyamine biosynthesis, combined with the exogenously added polyamines, contributed to the toxic effects observed in the cell. Although specific concentrations of biosynthesised polyamines were not measured, the response across increasing concentrations of exogenous polyamines suggests that the switch from protection to toxicity occurs at doses in excess of naturally produced concentrations. Galston & Sawhney (1990) and Bouchereau *et al.* (1999) explained how polyamine

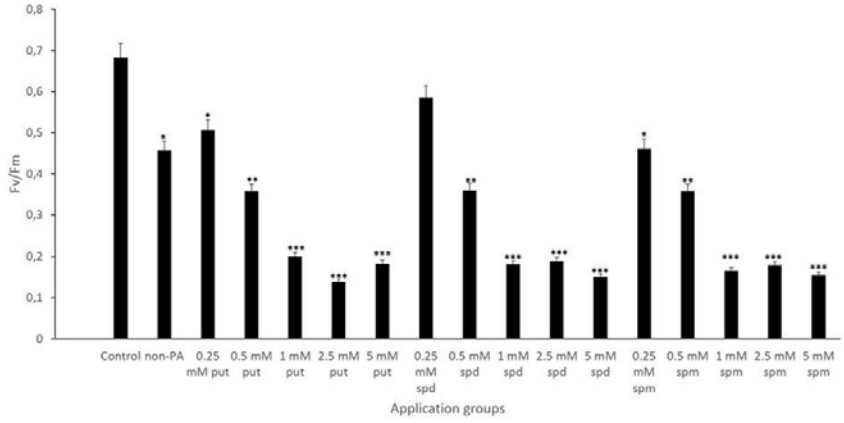

Figure 2. Maximal quantum yield of 1 mM polyamine-treated thalli in different concentrations after 24-h UV-B exposure in the lichen *P. semipinnata.*
* Represents a significant difference of $p < 0.05$ when compared with the control.
** Represents a statistically significant difference of $p < 0.01$.
*** Represents a statistically significant difference of $p < 0.001$

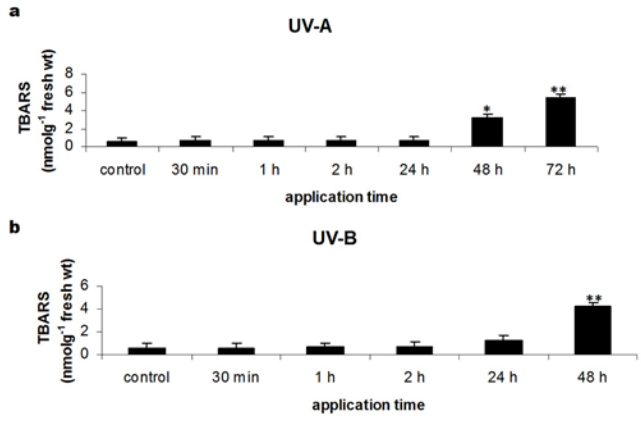

Figure 3. Effects of UV-A (a) and UV-B (b) on MDA content of *P. semipinnata* thallus.
* Represents a statistically significant difference of $p < 0.05$ when compared with the control.
** Represents a statistically significant difference of $p < 0.01$.

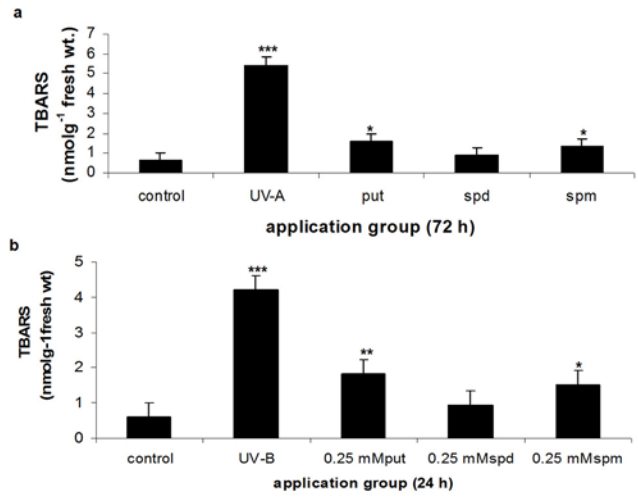

Figure 4. Effects of polyamine on MDA content during UV-A (a) and UV-B (b) stress of *P. semipinnata* thallus.
* Represents a statistically significant difference of $p < 0.05$ when compared with the control.
** Represents a statistically significant difference of $p < 0.01$.
*** Represents a statistically significant difference of $p < 0.001$ when compared with the control.

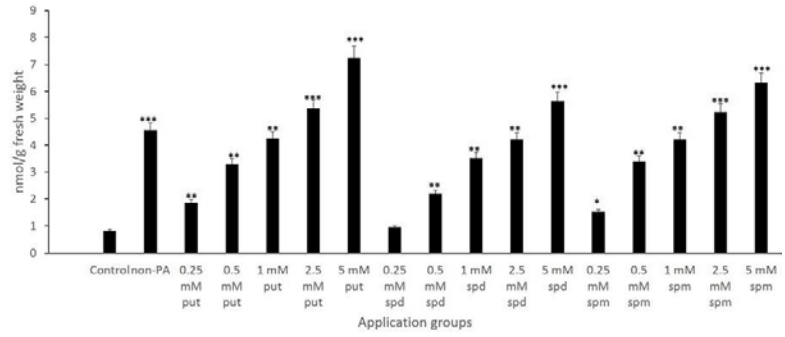

Figure 5. Malondialdehyde content (nmol g^{-1} fresh weight) of *P. semipinnata* exposed to UV-B light (incubated with different concentrations of polyamines and H_2O for 24 h).
* Represents a statistically significant difference of $p < 0.05$ when compared with the control.
** Represents a statistically significant difference of $p < 0.01$.
***Represents a statistically significant difference of $p < 0.001$.

catabolism starts after the polyamine concentration increases in the cell. Polyamine oxidase, which plays a major part in polyamine degradation, causes the release of compounds like gamma-aminobutyric acid, 3- acetamidopropanal, hydrogen peroxide and ammonia following the degradation process. The increase in these compounds has previously been shown to enhance cellular stress, thereby causing cell death. In our assumption, synthesis of the enzyme polyamine oxidase was induced at higher polyamine concentrations, making it likely that intracellular concentrations of hydrogen peroxide (and other compounds) increased, which caused oxidative stress and produced an overall decrease of PSII efficiency. Subsequent experiments were conducted using a lower dose (0.25 mM) of exogenous polyamines to ensure protective rather than toxic effects.

Malondialdehyde is a lipid peroxidation product, and MDA content can therefore 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 present in cellular membranes. Changes in lipid and protein structures result in the loss of membrane integrity and selective permeability. In all of the UV-A and UV-B exposure groups, UV induced MDA accumulation in the present work (Fig. 1a, b). However, many plant physiologists have also suggested that polyamines act as scavengers of active oxygen species and stabilise membranes by reducing lipid peroxidation under different conditions of environmental stress (Galston & Sawhney 1990; Borrell *et al.* 1997; Bouchereau *et al.* 1999). Groppa *et al.* (2007) reported that exogenously applied spm reduced the rate of lipid peroxidation in wheat leaves under Cd and Cu stress. In the present study, results of determining lipid peroxidation were in agreement with those obtained by Borrell *et al.* (1997). When samples were exposed to polyamines before UV-A

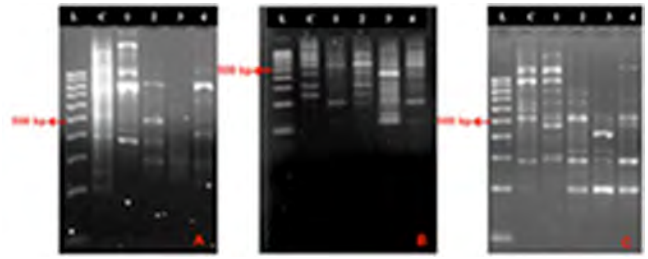

Figure 6. RAPD-PCR band profiles with different primers (A: OPB6, B: OPB10, C: OPB12) of UV-A-exposed *P. semipinnata* thalli. C indicates control thallus unexposed to UV-A radiation, while 1 indicates a thallus exposed to UV-A, but not treated with polyamines. Samples numbered 2, 3 and 4 represent 0.25 mM spm-, spd- and put-treated and then UV-A-treated samples, respectively.

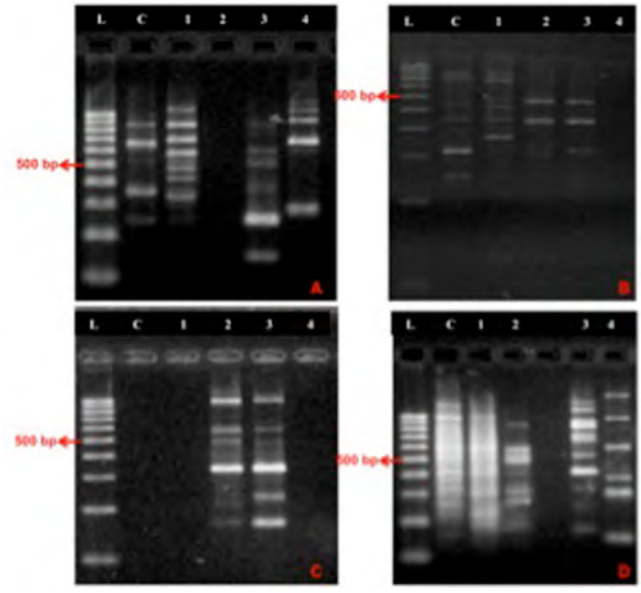

Figure 7. RAPD-PCR band profiles with different primers (A: OPB6, B: OPB10, C: OPB11, D: OPB12) of UV-B-exposed *P. semipinnata* thalli. C indicates control thallus unexposed to UV-B radiation, while 1 indicates a thallus exposed to UV-B, but not treated with polyamines. Samples numbered 2, 3 and 4 represent 0.25 mM spm-, spd- and put-treated and then UV-B-treated samples, respectively.

and UV-B treatment for 72 and 24 h, respectively, MDA content was found to be lower than in the control group and other groups (Fig. 2a, b).

The RAPD-PCR method has been successfully used to detect DNA damage induced by heavy metals, radiation (gamma, X-ray, UV) and pesticides (Atienzar *et al.* 1999; Kuroda *et al.* 1999). Atienzar *et al.* (1999) and Atienzar *et al.* (2000) showed that changes of band patterns observed in DNA fingerprint analyses reflect DNA alterations ranging from single base changes to complex chromosomal rearrangements. Similarly, UV-B and UV-A exposure of *P. semipinnata* in the present study caused almost complete loss of different DNA fragments, suggesting severe inhibition of DNA amplification in RAPD (Figs. 3, 4). The variation of band intensities and disappearance of bands might be linked with the presence of DNA photoproducts produced by UV radiation. We also observed DNA structural changes such as deletions, insertions or breaks after UV radiation, which we therefore postulate to act as a genotoxic agent.

In the present study, the genomic template stability decreased after UV-A exposure of *P. semipinnata* thalli. In the RAPD assay, the genetic distance between the control group and the group composed of samples exposed to UV-A for 72 h was found to be 57%. The genetic distances of samples treated with spm, spd, and put and exposed to UV-A radiation were found to be 12, 14 and 20%, respectively. Similarly, the genetic distance between UV-B-exposed samples and the negative control was calculated as 60%, while it varied between 12 and 25% in the groups that were polyamine-treated and then exposed to UV. Our results showed three kinds of polyamines, put, spm and spd, to be capable of protecting cells of *P. semipinnata*, although the degree of protection was different among different polyamines and between UV-A and UV-B radiation treatments. To judge from the RAPD-PCR results, UV-B is potentially more harmful to DNA than UV-A. Furthermore, the polyamine putrescine has greater ability to protect against UV-B radiation compared to spm and spd.

CONCLUSIONS

Our results suggest that exogenous polyamine treatment can significantly mitigate UV-induced oxidative damage in *P. semipinnata* thalli through a decrease of lipid peroxidation and can also protect the photosynthetic quantum yield. Moreover, it is evident that UV-A and UV-B treatment affects the template stability of DNA. This effect may be due to structural damage to DNA caused by oxidative stress. Polyamines, especially put and spd, play a part in protecting DNA against base alternations during exposure to UV illumination. These compounds are produced naturally by plants and are likely to be important in protecting them against exposure to environmental UV radiation. It is evident that sensitivity of the RAPD assay depends on the mutation levels caused by stress, and that different stressors elicit distinct responses with respect to mutation. Results of the present study suggest that sensitive approaches like RAPD-PCR can be used to further investigate both the protective and the toxic roles of biophysical and biochemical interactions in experimental and potentially occurring natural systems.

Acknowledgements — This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) (2209-BIDEB-2007). We thank our colleague Dr. Katherine Hubbard for her invaluable suggestions and for language editing.

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Botanica SERBICA

REZIME

Zaštitna uloga poliamina protiv UV-A i UV-B zračenja u talusu *Physcia semipinnata*

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U ovom radu su istraživani efekti oštećenja DNK prouzrokovanih UV-A i UV-B zracima, kao i zaštitna uloga poliamina putrescina (put), spermidina (spd) i spermina (spm) na lišaj *Physcia semipinnata*. Naši rezultati pokazuju da je došlo do značajne promene odnosa fotosintetskog kvantnog prinosa kao odgovor na povećano vreme izlaganja UV-A i UV-B zračenju. Naime, nakon izlaganja UV-A i UV-B odnos fotosintetskog kvantnog prinosa kod *P. semipinnata* postepeno opada. Talus *Physcia semipinnata* tretiran sa 1 mM poliamina nije pogođen izlaganjem UV-A u trajanju od 72 h. Kod tretmana UV-B, zaštitna doza poliamina je bila 0.25 mM. Za utvrđivanje oštećenja DNK u ovoj studiji korišćena je i RAPD tehnika. Glavne promene uočene u RAPD profilima, za koje je korišćeno 12 prajmera, sastojale su se u pojavi ili odsustvu različitih traka i u varijacijama u njihovom intenzitetu. Korišćenjem najmanje tri različita prajmera mogu se uočiti specifični obrasci u uzorcima izloženim UV-A i UV-B i tretiranim poliaminima, u odnosu na one koji nisu.

Ključne reči: RAPD-PCR, putrescin, spermidin, spermin, lipidna peroksidaza