THE PHYSIOLOGICAL AND DNA DAMAGE RESPONSE OF IN THE LICHEN *HYPOGYMNIA PHYSODES* TO UV AND HEAVY METAL STRESS

HAMUTOĞLU, R.[1] – DERICI, M. K.[2] – ARAS, E. S.[3] – ASLAN, A.[4,5] – CANSARAN-DUMAN, D.[6*]

[1]*Histology and Embryology Department, Faculty of Medicine, Cumhuriyet University, Sivas, Turkey*

[2]*Medical Pharmacology Department, Faculty of Medicine, Kırıkkale University, Kırıkkale, Turkey*

[3]*Biology Department, Faculty of Science, Ankara University, Ankara, Turkey*

[4]*Department of Biology, Faculty of Arts and Science, Kyrgyz-Turkish Manas University, Bishkek, Kryrgyzstan*

[5]*Department of Pharmacology, Faculty of Pharmacy, Yüzüncü Yıl University, Van, Turkey*

[6]*Biotechnology Institute, Ankara University, Ankara, Turkey*

**Corresponding author*
e-mail: dcansaran@yahoo.com; phone: +90-312-596-1032/2563

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Abstract. This work aims to determine the response of *Hypogymnia physodes* (L.) Nyl. (hooded tube lichen) collected in an unpolluted site (Yenice Forest in Karabük, Turkey) to stress conditions. In the present study, the effect of exposure to different heavy metals (Cd^{+2}, Pb^{+2}, and Cr^{+6}) for different durations and UV radiations dosages on lichen was examined at the physiological and molecular levels. The effects of stress conditions were determined in the case of different parameters concerning heavy metal, protein, chlorophyll, and carotenoid contents and changes in the DNA profiles. According to the results obtained that exposure to heavy metals and UV radiations leads to a physiological response in a concentration and dose-dependent manner through differences in chlorophyll, protein content in heavy metals and UV treated lichen specimen. Furthermore, changes in RAPD assay and DNA methylation analysis showed that homologous nucleotide sequences in the genome from untreated and stress conditions treated lichen specimen showed different band patterns and methylation under heavy metals and UV stress. The results determined that lichen specimen suggest as a possible bioindicator able to measure the biological effects of heavy metal pollution and damage to UV radiation.
Keywords: *lichen, UV radiations, metal uptake, chlorophyll content, DNA alteration*

Introduction

Due to population and rapid economic growth and urbanization, air pollution creates a major threat both to the environment and living organisms in the world. Environmental Protection Agency (EPA) has reported that some pollutants are poisonous, and inhaling them, in particular, can rapidly increase problems in human health (Cheloni et al., 2014; Mateos et al., 2018). Environmental pollution caused by heavy metals is one of the most serious problems at a global extent (Pescott et al., 2015; Ares et al., 2017). Among all heavy metals, cadmium (Cd^{+2}), chromium (Cr^{+3} and Cr^{+6}) and lead (Pb^{+2}), in particular, cause an ever-increasing international concern. The excessive Cr^{+6}, Cd^{+2} and Pb^{+2} concentrations in contaminated areas can give rise to a

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variety of problems, which is metal toxicity to mainly humans and animals (Frohnmeyer and Staiger, 2003). Another main problem in ecotoxicology is the damage of the stratospheric ozone layer due to anthropogenic activities, which results in increased UV radiation on the surface of the Earth and causes damage to the biological system (Singh et al., 2012). Pollutants and UV radiation interrupt metabolic activities at the cellular level and damage biological molecules such as proteins and nucleic acids (Pourrut et al., 2011).

In contrast to organic pollutants, heavy metals cannot be degraded through any known biological processes (Brown et al., 2005), and thus, there remains an urgent need for remediation of contaminated areas by environmentally friendly technology. An appropriate and cost-effective method to remove pollutants and UV radiation from the environment is needed. Biological methods, instead of physical and chemical methods, enable to direct assessment of environmental stressors. Thus, information obtained from biological data can be allowed to determinate the environmental impact of global problems on biological organisms. The solution to many global problems, such as bioremediation of toxic chemicals in the environment and decreasing the effect of UV radiation will require further research on cellular and molecular biology with biological organisms (Bah et al., 2011). For instance, *Typha angustifolia* shows protein changes when exposed to Cr^{+6}, Cd^{+2} and Pb^{+2} heavy metals. Results of the study suggest that abiotic stress, such as heavy metal, temperature, drought and salt stress, induces changes in protein expression level in plants (Cho and Seo, 2005; Pandey et al., 2005). The liverwort (*Lunularia cruciate*) has been used to evaluate the effect of heavy metals on the cellular levels, including changes in the mRNA level (Basile et al., 2005). Lichen species have used to determinate the response of physiological and molecular mechanisms in stress conditions in recent years (Aras et al., 2010; Cansaran-Duman, 2011; Matos et al., 2015). Due to lichen species lack protective cuticle and roots, they can adsorb all metals directly from contaminated areas and ability to absorb in high quantity heavy metals in contaminated areas are usually used as model organisms for various purposes by the environmental area.

Damage to DNA may generate mutations, altered bases and strand breaks (Shugart and Theodorakis, 1994), and afterward, to carcinogenesis, teratogenesis and health disorders (Kurelec, 1993). Some researchers have reported that positively charged metal ions can react with negatively charged oxygen atoms located in both chains of the DNA in phosphate groups (Anastassopoulou, 2003). The DNA damage generated by environmental stress sources has been shown with various studies conducted in our laboratory (Cansaran-Duman et al., 2011; Cansaran and Aras, 2014; Vardar et al., 2014).

In recent years, some studies have shown that low UVB fluence rate is the cause of cellular changes in higher plants (Brown et al., 2005; Brown and Jenkins, 2008). It has been demonstrated that low UVB fluence rate responses involve specific receptors and appear to be photoregulatory. The changes caused by UV radiation on plant development, morphology and physiology have been observed in several studies (Mackerness, 2000; Frohnmeyer and Staiger, 2003; Jenkins, 2009; Hideg et al., 2013). Generally, energy-rich UV radiation gives rise to the formation of free radicals that damage DNA, proteins, and the damage of photosynthetic pigments (Hideg et al., 2013).

This study aimed to understand, predict and expand the knowledge of the possible biological response of *Hypogymnia physodes* lichen specimen to different types and

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exposure periods of both UV and heavy metal solutions on cellular organization and physiological responses by examining bioaccumulation performance. Firstly, we evaluated heavy metal contents, chlorophyll (chl-a, chl-b, total chl, and carotenoids) and protein content in response to different heavy metal accumulation and UV radiations exposure to lichen specimen. Secondly, RAPD and MSAP-AFLP techniques were examined in *H. physodes* for possible changes in the DNA exposed to different heavy metal solutions and UV radiations. Although the capacity of heavy metal accumulation on lichen specimen has been extensively studied, only a few studies to date have shown that lichen specimen exposes to UV radiations at molecular and biochemical levels and heavy metal accumulation. This is the first manuscript examining methyl profiles in lichen specimen. Thus, this study will provide a better understanding of the molecular mechanisms of cellular protection against different stress factors on lichen species.

Materials and methods

Lichen sample and stress treatment

All procedures were carried out at Ankara University Department of Biology Molecular Systematic Laboratory and Ankara University Biotechnology Institute Central Laboratory, Ankara, Turkey. *Hypogymnia physodes* lichen specimen was selected as suitable bioindicators due to it has a large thallus surface. The lichen specimen was obtained from the unpolluted area of Yenice Forest, Karabük, Turkey in 2011 (41°10'N, 32°23'E). The sample collected from Yenice Forest was firstly washed and stored at appropriate conditions (4 °C) for a few days and sprayed distilled water. Then, the lichen specimen was sprayed constantly with distilled water in the laboratory. The sample was kept at room temperature for 2-3 h. During the treatments, thallus was placed in a climate chamber at 15 °C, exposure a photosynthetic photon flux density (PPFD) of 75 μmol m^2 s^{-1} and a 16 h photoperiod. All analytical procedures were carried out at least three replicates (n = 3).

Two different stress treatments (exposure to heavy metals and UV radiations) were applied in the study. A schematic representation of stress treatments in lichen specimen was shown in *Figure 1*. *H. physodes* was subjected to Pb^{+2}, Cd^{+2} and Cr^{+6} (30, 60 and 120 mg/L) heavy metal stress for different time periods (0.5, 1, 2, 6, 18, 24, 48 and 72 h). In brief, the stock solutions of Cd^{+2}, Pb^{+2} and Cr^{+6} (200 mg/L) were prepared by dissolving a weighed quantity in deionized water. In this study, the required concentrations were prepared from the stock solution with dilution. Lichen thallus (10 g weight) was immersed for an incubation period of 0.5, 1, 2, 6, 18, 24, 48 and 72 h. The lichen specimen was studied with three unexposed samples. The procedure followed for UV exposure; thallus sample placed in a petri dish was exposed to UV radiations at different doses of 4, 8, 12, 20 and 40 j/cm^2 (352 nm, 50 Hz, 0.60 Amps) by using dose-meter at 25 °C. Irradiation chamber BS-03 (Dr. Gröbel UV-Electronic GmbH) and dosemeter were used to lichen for determining to expose to UV radiations (UVA, UVB, UVC, daylight, UVA + UVB, UVA + day light) ratios. The control (non-stressed) group was analyzed with molecular markers and was indicated as a 'control sample'. Negative control was also used to determine the presence of any contamination.

Determination of heavy metals concentration

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The determination of heavy metal contents was performed from the previous study of Cansaran-Duman (2011). Lichen sample exposed to Cd^{+2} and Pb^{+2} heavy metals was collected at different time intervals. The sample was dried at 90 °C for 9 h and mineralization using HNO_3 and H_2O_2 (2:1, v/v) under ultrasonication. The set of standard calibration curves with good linear regression and better relative standard deviations were achieved for Cd^{+2} and Pb^{+2} metals in *H. physodes*. Cd^{+2} and Pb^{+2} concentrations of lichen sample were analyzed using Flame Atomic Absorption Spectroscopy (FAAS; Instrument PM Avarta, GBC Scientific Equipment, Australia). Standard reference material, CRM 482 Lichen (*P. furfuracea*), was used in the study to determine whether within range of the recommended values. The measured recovery % ± SD for Cd^{+2} and Pb^{+2} in the added CRM 482 reference material were 98 ± 7, 99 ± 6, 98 ± 3, respectively. The recovery was about 100% for Cd^{+2} and Pb^{+2} in the lichen sample, and the results with the CRD 482 material suggested that sample preparation and analyses were accurate. The conventional spectrophotometric method of Cr^{+6} was analyzed via 1.5–diphenylcarbazide measured at 540 nm in acid solution (APHA, 1985).

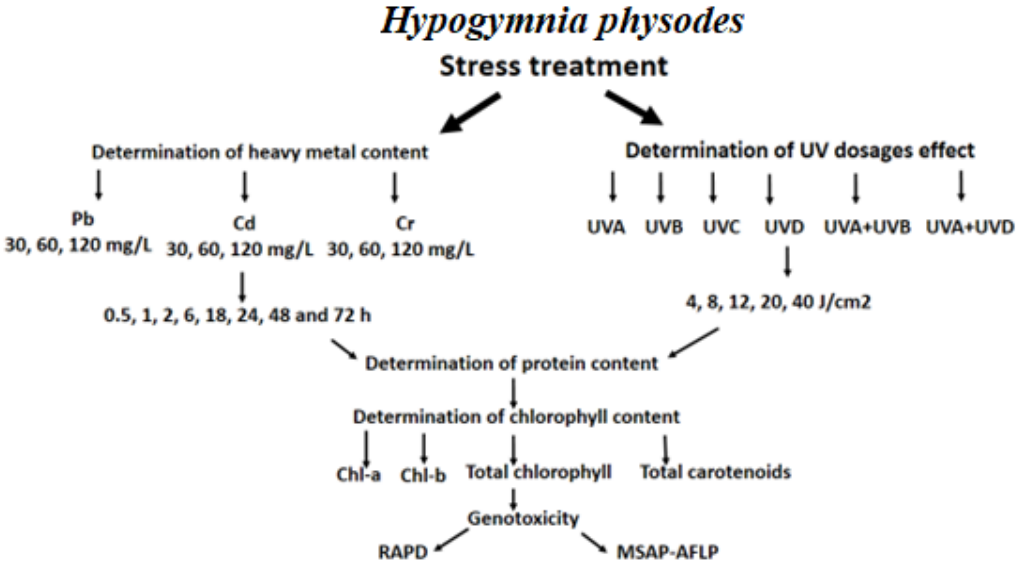

Figure 1. *A schematic representation of the treatments in Hypogymnia physodes lichen specimen. In this study evaluated three different metal stress treatments (Pb^{+2}, Cd^{+2} and Cr^{+6}) for eight different time periods (0.5, 1, 2, 6, 18, 24, 48 and 72 h) and in five different UV doses (4, 8, 12, 20, 40 J/cm^2 for UVA, UVB, UVC, daylight, UVA + UVB, UVA + daylight) in lichen sample. Also, the genotoxic effect of heavy metals and UV radiations was investigated for lichen specimen by RAPD and DNA methylation analysis*

Determination of total soluble protein content

Lichen specimen was homogenized (1:1, w/v) with 0.2 M phosphate buffer (pH 7.0). The obtained homogenate was centrifuged at 27.000 × *g* for 20 min. The supernatant was removed after centrifugation period. The total soluble protein content was determined according to the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) standard. The experiments were repeated three times (n = 3).

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Determination of chlorophyll contents

Unexposed lichen sample was immersed in distilled water for 5 min to obtain photosynthetic activity. Lichen specimen was exposed to Cd^{+2}, Pb^{+2}, and Cr^{+6} heavy metal solution and different UV radiations, and the chlorophyll content were determined by taking samples from this sample exposed to stress at certain time intervals (*Fig. 1*). Contents of chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll a/b, and total carotenoids were spectrophotometrically determined (Metzner et al., 1965). Briefly, a 40-60 mg lichen sample was kept within 3 ml of dimethylsulfoxide (DMSO) for 1 h at 65 °C in the dark. Lichen sample kept in the dark for 1 h was centrifuged at 12000 *g* for 10 min at 20 °C. The supernatant was transferred to a fresh tube, and then, spectrophotometric measurements were taken at wavelengths of 644 (chl-a), 663 (chl-b), 452 (total chl) and 470 nm (Chls) (Ronen and Galun, 1984; Wellburn, 1994). This process was performed triplicate (n = 3). Each analysis was replicated three times.

Genomic DNA extraction and RAPD assay

H. physodes was exposed to Pb^{+2}, Cd^{+2} and Cr^{+6} (30, 60 and 120 mg/L) heavy metal stress for different periods (0.5, 1, 2, 6, 18, 24, 48 and 72 h), UV radiations and DNA of the lichen sample was extracted (*Fig. 1*). DNA extraction was performed according to the protocol defined by Aras and Cansaran (2006). Concentration and purity of the DNA extracted are measured at OD 260 and with a 260 nm/280 nm absorbance ratio by nanodrop (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, USA), respectively.

Primer screening for RAPD analysis was performed using 10 primers. The six of the ten primers amplified clear and reproducible bands in heavy metal stress and UV stress, respectively. PCR components were determined as follows; 200 ng genomic DNA, 2.5 µL 10X reaction buffer, 3.0 mM $MgCl_2$, 20 mM dNTPs, 0.3 mM primer, and 0.5 unit of Taq polymerase (Promega, Madison, USA), and ddH_2O was added to the final volume of 25 µL. Negative controls were used in all PCR reactions. The PCR program operated with the following steps: 95 °C for 30 s for denaturation, 36 °C for 1 min at 35 cycles for annealing step, 72 °C for 2 min for extension and a final extension at 72 °C for 10 min. PCR products were loaded on 1.5% agarose gels and run at 80 V for 4 h.

MSAP-AFLP analysis

In the MSAP-AFLP analysis was used OPC10 primer which is one of the primers giving sharp bands during RAPD analysis. The genomic DNA (200 ng) of the two stress exposed samples were separately digested with *Eco*R I/*Msp* I and *Eco*R I/*Hpa* II restriction enzymes at 37 °C for 2.0 h. Subsequently, the digested aliquot was ligated to specific adopters for examined restriction enzymes because it was applied to avoid reconstruction of restriction sites one for *Eco*R I sticky ends and other for *Msp* I or *Hpa* II sticky ends, at 20 °C for 90 min. The ligated DNA was diluted with Tris-EDTA buffer, and then the diluted products were amplified using different combinations of *Eco*R I and *Msp* I or *Hpa* II primer each with three selective nucleotides at 5′ and 3′, respectively. MSAP-AFLP analyses were conducted following the method described by Mastan et al. (2012). Briefly, selective amplifications were performed at 65 °C temperature for the first cycle, the annealing temperature was successively reduced by 0.7 °C for the other 11 subsequent cycles. The remaining 23 from 45 cycles were run at 56 °C annealing temperature. After the formamide dye added PCR product, the

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electrophoresis assay was run. The gels were stained with silver nitrate and scanned (Bassam et al., 1991). To test the reproducibility of the AFLP-PCR, the experiments were repeated at least twice for each primer, faint bands were not involved in this study.

Statistical analysis

The results of heavy metal content, ratio of UV radiation and total soluble protein content of the lichen specimen were evaluated by multifactor analysis of variance (univariate ANOVA) or Student's t-test followed by posthoc Tukey test as appropriate (SPSS for Windows version 11.0) to display the effects of both stress sources on the exposed lichen.

Chlorophyll a, b, total chlorophyll and carotenoids were calculated by the following equations by Shakoor et al. (2014). The experiments were repeated three times (n = 3) and then evaluated with multifactor analysis of variance (ANOVA).

Estimation of profiling scoring and data analyses for RAPD assay

The RAPD analysis of the results was evaluated by considering the number of bands, which appear in the control (non-stressed) sample. Polymorphism calculated in RAPD profiles included the disappearance and appearance bands when compared with the non-stressed sample (Atienzar et al., 1999; Liu et al., 2005). Each polymorphic DNA band on the gel was treated as an individual locus and scored for their presence (1) or absence (0). Genomic template stability (GTS%) was calculated as followed by Atienzar et al. (1999). Changes in RAPD patterns were evaluated as decreases in GTS, compare with the number of RAPD profiles exposed to a different stress condition, and profiles obtained from the control samples.

Estimation of profiling scoring and data analyses for methylation analyses

MSAP data analysis was scored reproducible bands. All the amplified bands obtained from the MSAP-AFLP analysis were divided into four methylation types based on the presence or absence of groups as indicated by Li et al. (2009). According to this classification, the bands indicating each methylation type (I, II, III, and IV) were counted and placed in their location in *Table 1*.

The percentage of methylation was evaluated as the number of methylated bands × 100 by dividing the total number of bands. The percentage of methylation polymorphism was calculated using the formula (=number of polymorphic methylated bands×100/number of methylated bands).

Table 1. *Methylation types created by the cutting ability of HPAII and MspI restriction enzymes*

Methylation type	Methylation pattern	HpaII	MspI
Type I	CCGG C<u>C</u>GG GGCC GGCC	Active	Active
Type II	<u>C</u>CGG GGCC	Active	Inactive
Type III	C<u>C</u>GG GGCC	Inactive	Active
Type IV	<u>C</u>CGG GGCC	Inactive	Inactive

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Results

We analyzed *H. physodes* lichen specimen for three different metal stress treatments were applied (Pb^{+2}, Cd^{+2} and Cr^{+6}) in three different doses (30, 60 and 120 mg/L) for eight different periods (0.5, 1, 2, 6, 18, 24, 48 and 72 h). The same concerns exposure to UV radiations on lichen specimen examined six combinations of UV radiations (UVA, UVB, UVC, daylight, UVA + UVB, UVA + daylight) with five different doses of 4, 8, 12, 20 and 40 j/cm^2. Subsequently, in the analyzed sample, heavy metal, total soluble protein, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids contents were measured (*Fig. 1*). The changes of DNA profile exposed to different heavy metals and UV radiations determined RAPD and DNA methylation analysis assays.

Determination of heavy metal contents in lichen specimen

The 30 mg/L Cd^{+2} metal absorption by *H. physodes* was very fast and optimum equilibrium was reached at 30 min ($P < 0.05$) (*Fig. 2; Table 2*). *H. physodes* was found to have an optimum absorption rate of 70.5% after 30 mg/L Pb^{+2} applications in 72 h ($P < 0.05$). It was noticed that 60 mg/L Cr^{+6} exposure significantly reduced (79.9%) heavy metal content in *H. physodes* (47.8 mg/L) in 72 h when compared to unexposed sample (59.8 mg/L) ($P < 0.05$). The highest absorption efficiency (94.2%) was obtained at 120 mg/L Cr^{+6} for 72 h in *H. physodes* lichen specimen ($P < 0.05$).

The highest absorption efficiency was achieved as about 92.2%, 95.1% and 95.5% using 30, 60 and 120 mg/L Cr^{+6} for 18 h, respectively. According to the results of 30, 60 and 120 mg/L Pb^{+2} application in lichen specimen, the absorption capacity percentage decreased from 43.4% to 34.5% as *H. physodes* was applied to 30 mg/L Pb^{+2} for 24 h. 60 mg/L Pb^{+2} heavy metal absorption decreased from 58.1% for 24 h in *H. physodes*. At 120 mg/L Pb^{+2}, the absorption efficiency was found to be low in *H. physodes* (59.2%) for 24 h ($P < 0.05$).

The optimum absorption capacity against 30, 60 and 120 mg/L Cd^{+2} heavy metal stress was found as 43.8% for 30 min, 63.3% for 12 h and 57.5% for 6 h in *H. physodes*, respectively ($P < 0.05$).

Table 2. *30 mg/L Cd^{+2} content of Hypogymnia physodes lichen specimen with ANOVA (Same letters in a column indicate the absence of significant differences at $P < 0.05$ by ANOVA)*

Samples	N	Mean	Std. deviation	Std. error	95% confidence interval for mean		Min	Max
					Lower bound	Upper bound		
Control	3	29.7000-	.10000	.05774	29.4516	29.9484	29.60	29.80
30 min	3	12.9000	.20000	.11547	12.4032	13.3968	12.70	13.10
1 h	3	11.6000	.10000	.05774	11.3516	11.8484	11.50	11.70
2 h	3	12.1000	.10000	.05774	11.8516	12.3484	12.00	12.20
6 h	3	11.8000	.30000	.17321	11.0548	12.5452	11.50	12.10
18 h	3	11.2000	.20000	.11547	10.7032	11.6968	11.00	11.40
24 h	3	10.9000	.20000	.11547	10.4032	11.3968	10.70	11.10
48 h	3	10.9000	.40000	.23094	9.9063	11.8937	10.50	11.30
72 h	3	10.9000	.30000	.17321	10.1548	11.6452	10.60	11.20
Total	27	13.5556	5.85526	1.12685	11.2393	15.8718	10.50	29.80
	Sum of	**df**	**Mean**	**F**	**Sig.**			

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	squares		**square**		
Between groups	890.407	8	111.301	2044.301	.000
Within groups	.980	18	.054		
Total	891.387	26			

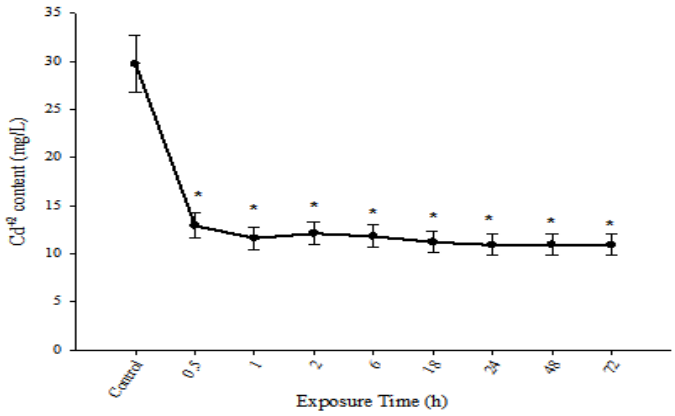

Figure 2. *The time-concentration curve of Cd^{+2} content of the stock solution that exposed to Hypogymnia physodes. (* = statistically different from control (P < 0.05) Cd^{+2} removal by H. physodes at initial Cd^{+2} concentration for 72 h*

Determination of protein content in lichen specimen

Effect of heavy metals on protein content

Protein content decreased with the increase in the concentration of heavy metals in *H. physodes* at the examined time range (30 min–72 h). Maximum protein content in *H. physodes* was increased for 30 mg/L Cr^{+6} and 30 mg/L Cd^{+2} until 30 min; however, there was a significant decrease in protein content afterward (from 60 min to 72 h) ($P < 0.05$). Protein content of lichen specimen was also significantly increased at both heavy metal stress levels (60 mg/L Cr^{+6} and Cd^{+2} heavy metals) for 30 min ($P < 0.05$). Protein content of *H. physodes* decreased all concentrations of Pb^{+2} stress when compared with the unexposed sample. The results of this study showed that the protein content of lichen specimen significantly decreased ($P < 0.05$) by 120 mg/L concentration of Cr^{+6} (*Table 3*) and Cd^{+2} at 1 h after treatment (0.023 mg/L, respectively). However, the protein content of *H. physodes* significantly decreased by 120 mg/L Pb^{+2} exposure for 1 h when compared with the unexposed lichen sample (20.73 and 19.23, 19.9 and 18.32 mg/L, respectively) ($P < 0.05$). Protein content significantly decreased after from 2 to 72 h exposure to 120 mg/L Pb^{+2}.

Effect of UV radiations on protein content

The protein content of *H. physodes* significantly decreased at 40 j/cm^2 among the other four UV radiations. Exposure to 40 j/cm^2 UVA + UVB radiations, protein content significantly decreased by 28% in *H. physodes* ($P < 0.05$), similarly after the 4, 8, 12 and 20 j/cm^2 UV radiations exposure decreased protein content of *H. physodes*. The lowest UVB radiation (4 j/cm^2) exposure was recorded as 0.0470 mg/L protein content and the highest UV radiations exposure (40 j/cm^2) was measured as 0.0157 mg/L protein content in *H. physodes* when compared to unexposed sample (0.0505 mg/L) ($P < 0.05$).

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Determination of chlorophyll content

Effect of heavy metals on chlorophyll-a content

The results of the study showed that the chl-a content was significantly decreased by 30, 60 and 120 mg/L concentration of Cr^{+6}, Pb^{+2} and Cd^{+2} after 2 h exposure (15.42 and 14.46, 14.55 and 11.49 mg g^{-1}, respectively) ($P < 0.05$). 120 mg/L Cr^{+6} exposure for 2 h significantly decreased the chl-a content of *H. physodes* by 18% (0.404 mg g^{-1}) ($P < 0.05$).

Table 3. *Protein content of exposing to 120 mg/L Cr^{+6} of Hypogymnia physodes lichen specimen with ANOVA (Same letters in a column indicate the absence of significant differences at P < 0.05 by ANOVA)*

Samples	N	Mean	Std. deviation	Std. error	95% confidence interval for mean		Min	Max
					Lower bound	Upper bound		
Control	3	.0176289	.00003006	.00001736	.0175542	.0177035	.01760	.01766
30 min	3	.017689	.00002009	.00001160	.0178190	.0179188	.01785	.01789
1 h	3	.0231703	.00002001	.00001155	.0231206	.0232200	.02315	.02319
2 h	3	.0145604	.00006000	.00003464	.0144114	.0147095	.01450	.01462
6 h	3	.0101416	.00004010	.00002315	.0100420	.0102413	.01010	.01018
18 h	3	.0132290	.00003005	.00001735	.0131543	.0133036	.01320	.01326
24 h	3	.0116093	.00001008	.00000582	.0115842	.0116343	.01160	.01162
48 h	3	.0097306	.00003002	.00001733	.0096560	.0098051	.00970	.00976
72 h	3	.0124985	.00007005	.00004044	.0123245	.0126725	.01243	.01257
Total	27	.0144930	.00418853	.00080608	.0128361	.0161500	.00970	.02319

	Sum of squares	df	Mean square	F	Sig.
Between groups	.000	8	.000	37374.901	.000
Within groups	.000	18	.000		
Total	.000	26			

Effect of UV radiations on chlorophyll-a content

In UV radiations exposure, chl-a content of the unexposed sample of *H. physodes* was determined as 2.300 mg g^{-1}. In terms of the applied stress level among all UV radiations types (UVA, UVB, UVC, daylight, UVA + daylight, UVA + UVB), the highest increase was observed at 40 j/cm^2 ($p<0.05$). After the implementation of 40 j/cm^2 UVA + UVB radiations level, the highest chl-a content increase (10.760 mg g^{-1}) was observed about 5 fold when compared to the unexposed sample (2.300 mg g^{-1}) ($p<0.05$). In contrast, exposure to UVA radiation alone at a dose of 40 j/cm^2 increased the amount of the lowest chl-a (2.742 mg g^{-1}) ($p<0.05$).

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Effect of heavy metals on chlorophyll-b content

This study was determined the changes in chlorophyll-b content after exposure to heavy metals and UV radiations compared to the unexposed sample. The results of the study showed that chl-b content of *H. physodes* unexposed sample (1.518 mg g^{-1}) significantly decreased with 30, 60 and 120 mg/L concentration of Cr^{+6}, Pb^{+2} and Cd^{+2} after 48 h treatment ($P < 0.05$). After 30, 60 and 120 mg/L Pb^{+2} treatments, the highest decrease rate at all concentrations when compared to the unexposed specimen was determined as 0.013, 0.209 and 0.057 mg g^{-1}, respectively ($p<0.05$).

Effect of UV radiations on chlorophyll-b content

H. physodes lichen specimen was observed 1.629, 2.873, 2.659, 2.229, 2.033 and 2.853 mg g^{-1} chl-b content exposed to 8 j/cm^2 UVA, UVB, UVC, daylight, UVA + UVB, UVA + day light when compared to the unexposed sample (1.518 mg g^{-1}), respectively.

Effect of heavy metals on total chlorophyll content

Total chlorophyll content in the unexposed sample in *H. physodes* was observed as 3.818 mg g^{-1}. The optimum result of total chlorophyll content in *H. physodes* was obtained at three heavy metal exposures (Cr^{+6}, Pb^{+2}, and Cd^{+2}) and concentrations (30, 60 and 120 mg/L) after a 60 min treatment. The highest decrease rates of Cr^{+6} heavy metal among all concentrations were observed as 0.857, 1.130 and 0.583 mg g^{-1} in *H. physodes* lichen specimen, respectively ($P < 0.05$).

Effect of UV radiations on total chlorophyll content

Total chlorophyll content of *H. physodes* was observed as 4.341 and 5.394 mg g^{-1} expose to 8 j/cm^2 UVA + day light radiations. The highest content of total chlorophyll was determined as 14.923, 12.989, 12.159 and 16.010 mg g^{-1} after exposure to 20 j/cm^2 UVB, UVC, UVA + daylight, and UVA + UVB radiations, respectively ($p<0.05$). Especially, after the 20 j/cm^2 UVA + UVB radiations treatment, the maximum decrease of total chlorophyll content (16.010 mg g^{-1}) was observed in *H. physodes*, when compared to the unexposed sample (3.818 mg g^{-1}) ($P < 0.05$).

Effect of heavy metals on the ratio of chlorophyll a/b

Variations in the ratio of chlorophyll a/b were caused by increasing lichen metal content. The ratio of chlorophyll a/b in *H. physodes* exposed to 30, 60 and 120 ppm Cr^{+6} heavy metal stress showed a significant decrease after 30 min of all three heavy metal treatments ($p<0.05$). Exposed to 30 ppm Cd^{+2} and Pb^{+2} heavy metal stress in *H. physodes* lichen specimen, the treatments showed a marked increase in the ratio of chlorophyll a/b (86.02 mg g^{-1}) as a result of 18 and 24 h, respectively ($p<0.05$). After treatments, the ratio is gradually decreasing towards the lowest level.

Effect of UV radiations on the ratio of chlorophyll a/b

An increase in the ratio of chlorophyll a/b was observed after 4 j/cm^2 treatment in *H. physodes* lichen specimen exposed to UVB radiation.

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Determination of total carotenoid content

Effect of heavy metals on total carotenoid content

The content of total carotenoid in the control sample of *H. physodes* was observed as 0.092 mg g^{-1}. The optimum time of after all heavy metal exposure was determined at 18 h. All examined heavy metal concentrations (30, 60 and 120 mg/L) significantly decreased total carotenoid contents in *H. physodes* when compared to unexposed sample ($P < 0.05$). The maximum change in total carotenoid content was observed as 0.056 mg g^{-1} after 18 h 120 mg/L Cd^{+2} treatment in *H. physodes* ($P < 0.05$).

Effect of UV radiations on total carotenoid content

Total carotenoid content of lichen specimen was founded a decrease expose to all UV radiations when compared to the control sample. The maximum change in total carotenoid contents was determined after 12 j/cm^2 expose to UV radiations ($P < 0.05$). The highest decrease after 12 j/cm^2 treatment was observed after UVA + UVB radiations exposure (0.070 mg g^{-1}) in *H. physodes* ($P < 0.05$).

Determination of heavy metals and UV radiations on RAPD profiles in Hypogymnia physodes

A representative example of the results obtained by RAPD analysis is shown in *Figure 3*. The concentrations measured for the DNA samples were approximately in the range of 1285–2012 ng/µl for all heavy metals and 964–2077 ng/µl for all UV radiations exposures at 260 nm/280 nm ratios between 1.64–1.97 and 1.54–1.99, respectively.

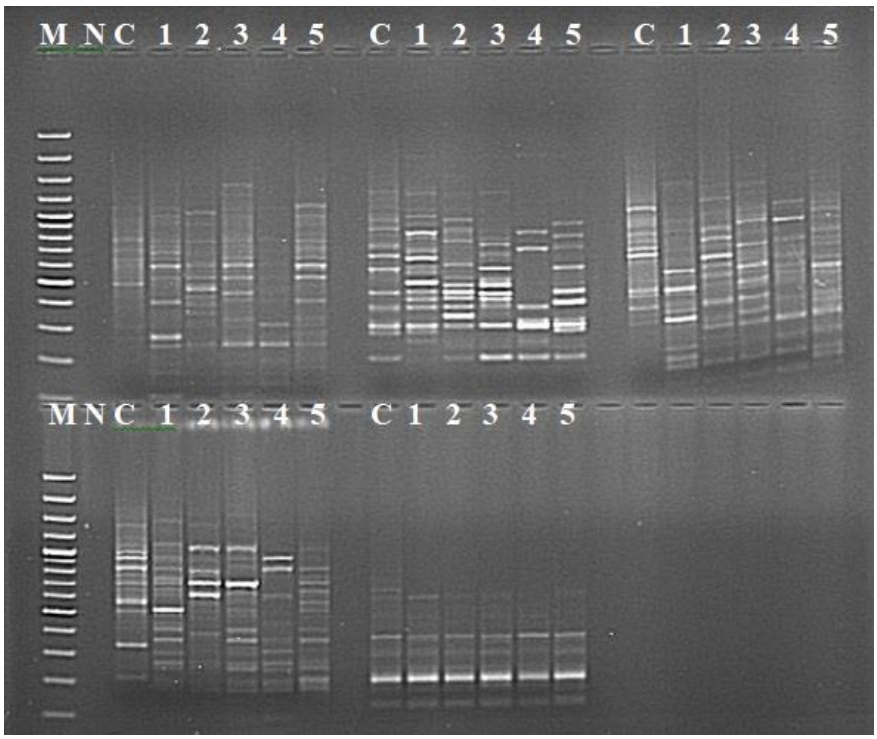

Figure 3. *The results of RAPD-PCR treating at UVB radiation in Hypogymnia physodes (top-left OPC02, top-center OPC04; top-right TubeA05, lower-left OPC07, lower-center OPC10*

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primers). (M: Marker; N: Negative control; C: Control sample (non-stressed sample); 1: 4 j/cm^2; 2: 8 j/cm^2; 3: 12 j/cm^2; 4: 20 j/cm^2; 5: 40 j/cm^2)

In RAPD analyses, some of the primers displayed significant differences in band patterns formed by loss of normal bands and appearance of new bands in the treated heavy metals and UV radiation exposure in comparison to the untreated sample profiles. The highest number of appearance and disappearance of new bands was observed at 30 mg/L Cd^{+2} concentrations with ten primers. Most of the new band appearances/disappearances were shown in Cd^{+2} contaminations (33 bands) observed in 30 mg/L Cd^{+2} contaminations. The lowest band appearances/disappearances were determined in Cd^{+2} (8 bands) and Pb^{+2} (8 bands) exposure to 30 min. The highest band appearances/disappearances in different UV radiation exposures were shown at 40 j/cm^2 exposure (42 bands).

As an analysis of the samples applied Cd^{+2} heavy metal in *H. physodes*, the highest genomic template stability (GTS%) value occurred after exposure to Cd^{+2} heavy metal stress at 72 h in all concentrations (30, 60 and 120 mg/L). 30, 60 and 120 mg/L Cd^{+2} treatment resulted in the highest GTS value of 91.1%, 85.5% and 86.6%, respectively. The lowest GTS value occurred after exposure to Cd^{+2} at 2 h in all concentrations. The lowest GTS value (63.3%, 64.4% and 70.0%) was obtained in the 30, 60 and 120 mg/L Cd^{+2} heavy metal treatment, respectively. According to this, the highest and lowest band variations were observed at 30, 60 and 120 mg/L Cd^{+2} concentrations at 2 h and 72 h, respectively. In terms of Cr^{+6} stress in *H. physodes* lichen specimen, the highest and the lowest GTS values were detected at 48 h of 30 mg/L (88.5%) and 72 h of 60 mg/L (90.6%) and 72 h of 120 mg/L (90.6%), respectively. The lowest GTS value was obtained at 30 min and 18 h in the 30 mg/L Cr^{+6} (69.7%), 2 h in the 60 mg/L Cr^{+6} (70.8%) and 1 h in 120 mg/L Cr^{+6} (78.1%). In terms of Pb^{+2} stresses in *H. physodes*, the highest GTS value was observed after exposure to Pb^{+2} at 72 h in all concentrations. 30, 60 and 120 mg/L Pb^{+2} treatments resulted in the highest GTS values of 93.0%, 93.0% and 91.3%, respectively. The lowest GTS values were obtained at 30 min, 2 h and 6 h in 30 mg/L (82.6%), 30 min at 60 mg/L (78.2%) and 30 min at 120 mg/L (80.8%) Pb^{+2} treatments.

In terms of UVA radiation stress in *H. physodes* lichen specimen, the highest GTS value (87.8%) was observed after exposure to 4 j/cm^2 UVA radiation. The lowest GTS value (76.5%) was obtained at 40 j/cm^2 UVA radiation. As regards UVA and daylight stress in *H. physodes*, the highest GTS value (88.6%) was observed after exposure to 4 j/cm^2 UVA and daylight radiations. The lowest GTS value (80.86%) was obtained at 12 j/cm^2 UVA and daylight radiations. Despite UVC stress in *H. physodes*, the highest GTS value (92.1%) was observed exposed to 4 j/cm^2 UVC radiation and the lowest GTS value (76.5%) was determined at 40 j/cm^2 UVC radiation. In terms of daylight stress in *H. physodes*, the highest GTS value (89.67%) was realized exposed to 8 j/cm^2 daylight radiation but the lowest GTS value (84.78%) was obtained at 40 j/cm^2 daylight radiation.

When all UV radiations and UVB exposure were compared, maximum changes of UV exposure were determined in UVB radiation in *H. physodes*. The significant changes were determined, such as appearances of some new bands or the disappearance of bands when compared to the control. A maximum of 28 bands disappeared among the exposed *H. physodes* with 40 j/cm^2, while in 20 j/cm^2, a maximum of 13 new bands appeared in *H. physodes* exposed to UVB radiation (*Table 4*). Also, the results showing the most variation among the different heavy metal (Cd^{+2}, Cr^{+6}, Pb^{+2}) and time arrival

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(0.5, 1, 2, 6, 18, 24, 48 and 72 h) combinations applied to the *H. physodes* lichen species are given in *Table 5*. *H. physodes* lichen specimen for three different UV treatments were applied (UVA, UVB, UVC, UVD, UVA + UVB, UVA + UVD) in five different doses (4, 8, 12, 20 and 40 j/cm^2). The results showing the most variation among the UV radiations and doses combinations applied to the *H. physodes* lichen species are given in *Table 6*. The highest GTS value (88.4%) was observed exposed to 4 j/cm^2 UVB radiation (*Table 7*). The lowest GTS value (73.18%) was obtained at 40 j/cm^2 UVB radiation (*Table 7*). Regarding UVA and UVB stress in *H. physodes*, the highest GTS value (84.3%) was observed exposed to 4 j/cm^2 UVA and UVB radiations. The lowest GTS value (63.4%) was obtained at 40 j/cm^2 UVA and UVB radiations.

Table 4. *Varying band-number using OPC01, OPC02, OPC04, OPC07, OPC10 and TubeA05 primers as a result of UVB radiation samples in Hypogymnia physodes*

Primers	**C**	**4 j UVB**		**8 j UVB**		**12 j UVB**		**20 j UVB**		**40 j UVB**	
		a	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	23	1	3	1	1	0	3	2	4	2	4
OPC 02	23	2	0	3	2	4	2	3	3	1	3
OPC 04	23	0	0	0	2	0	3	4	3	3	7
OPC 07	23	1	4	2	3	1	3	1	7	0	4
OPC 10	23	2	0	1	2	2	2	1	2	2	6
Tube A05	23	0	3	0	1	2	4	2	0	1	4
138		6	10	7	11	9	17	13	19	9	28
a + b		16		18		26		32		37	

a + b = The total number of band alternations, C = control sample = non-stressed sample

Table 5. *H. physodes lichen specimen for three different metal stress treatments were applied (Pb^{+2}, Cd^{+2}, and Cr^{+6}) in three different doses (30, 60 and 120 mg/L) for eight different periods (0.5, 1, 2, 6, 18, 24, 48 and 72 h). The results showing the most variation among the different heavy metal (Cd^{+2}, Cr^{+6}, Pb^{+2}) and time arrival (0.5, 1, 2, 6, 18, 24, 48 and 72 h) combinations applied to the H. physodes lichen species are given*

Primers	**30 ppm Cd^{+2}**		**60 ppm Cd^{+2}**		**120 ppm Cd^{+2}**		**30 ppm Cr^{+6}**		**60 ppm Cr^{+6}**		**120 ppm Cr^{+6}**		**30 ppm Pb^{+2}**		**60 ppm Pb^{+2}**		**120 ppm Pb^{+2}**		**T**
	TB	**a+b (2 h)**	**TB**	**a+b (2 h)**	**TB**	**a+b (2 h)**	**TB**	**a+b (6 h)**	**TB**	**a+b (2 h)**	**TB**	**a+b (1 h)**	**TB**	**a+b (30 min)**	**TB**	**a+b (30 min)**	**TB**	**a+b (30 min)**	
OPC 01 OPC 02 OPC 04 OPC 07 OPC 10 TubeA05	90	33	90	32	90	27	96	30	96	28	96	21	115	20	115	25	115	22	RAPD
Methylation types (MT)	**TB**	**a+b MTII**	**TB**	**a+b MTII**	**TB**	**a+b MTI**	**TB**	**a+b**	**TB**	**a+b MTII**	**TB**	**a+b**	**TB**	**a+b**	**TB**	**a+b MTII**	**TB**	**a+b**	
Type I Type II Type III Type IV	413	213	413	314	413	351	357	81	357	87	357	98	456	127	456	149	456	181	MSAP-AFLP

a + b = The total number of band alternations, TB = Total band (non-stressed sample = control sample), T = Technic, MT = Methylation types

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Methylation DNA and polymorphism in examined lichen specimen to different levels of stress condition

Band alterations in heavy metals and UV radiations exposure were compared with respect to the untreated control samples. 413 to 691 bands were produced in the untreated sample, and 217 bands with an average of 11 per primer were obtained in the MSAP-AFLP analysis. The total number of band alterations (a + b) was 117 bands for the Cd^{+2} heavy metal and lichen specimen stressed with UVB (*Fig. 4*).

Table 6. *H. physodes lichen specimen for three different UV treatments were applied (UVA, UVB, UVC, UVD, UVA + UVB, UVA + UVD) in five different doses (4, 8, 12, 20 and 40 j/cm^2). The results showing the most variation among the UV radiation and dose combinations applied to the H. physodes lichen species are given*

Primers	**UVA**		**UVB**		**UVC**		**UVD**		**UVA+UVB**		**UVA+UVD**		**T**
	TB	**a+b (40 J)**	**TB**	**a+b (40 J)**	**TB**	**a+b (40 J)**	**TB**	**a+b (40 J)**	**TB**	**a+b (40 J)**	**TB**	**a+b (12 J)**	
OPC 01 OPC 02 OPC 04 OPC 07 OPC 10 Tube A05	115	27	138	37	115	27	115	28	115	42	115	22	RAPD
Methylation types	**TB**	**a+b MTII**	**TB**	**a+b MTII**	**TB**	**a+b MTI**	**TB**	**a+b**	**TB**	**a+b MTII**	**TB**	**a+b**	
Type I Type II Type III Type IV	691	117	691	451	691	141	691	92	691	381	691	204	MSAP-AFLP

a + b = The total number of band alternations, TB = Total band (non-stressed sample = control sample), T = Technic, MT = Methylation types

Table 7. *The rates of GTS values using UVB radiation in Hypogymnia physodes lichen specimen*

Samples-UVB	**Rates of GTS (%)**
4 j/cm^2	88.40
8 j/cm^2	86.95
12 j/cm^2	81.15
20 j/cm^2	76.81
40 j/cm^2	73.18

The highest rate of methylation was obtained with Type-2 primer (28.9%) and Type-3 (59.3%) primers in the Cd^{+2} heavy metal (*Table 5*) and *H. physodes* lichen specimen stressed with UVB radiation (*Table 6*). The highest rate of changes was observed in the *H. physodes* exposed to heavy metal stress for 6 h and UVB radiation stress for 12 and

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24 h. When the results of the MSAP - AFLP analysis were evaluated based on the methylation types in the heavy metal stressed samples, the maximum level of methylation (33.3%) was observed in Type II, and the lowest level of methylation (63.3%) was seen in Type III. Type II methylation was not observed for the first 6 h and then, occurred at a rate of 34.3% at 12 h and 24 h. The rate of Type II methylation (34.3%) remained the same until the end of 12 h and completely disappeared at 24 h in Cr^{+6} heavy metal exposure in *H. physodes*.

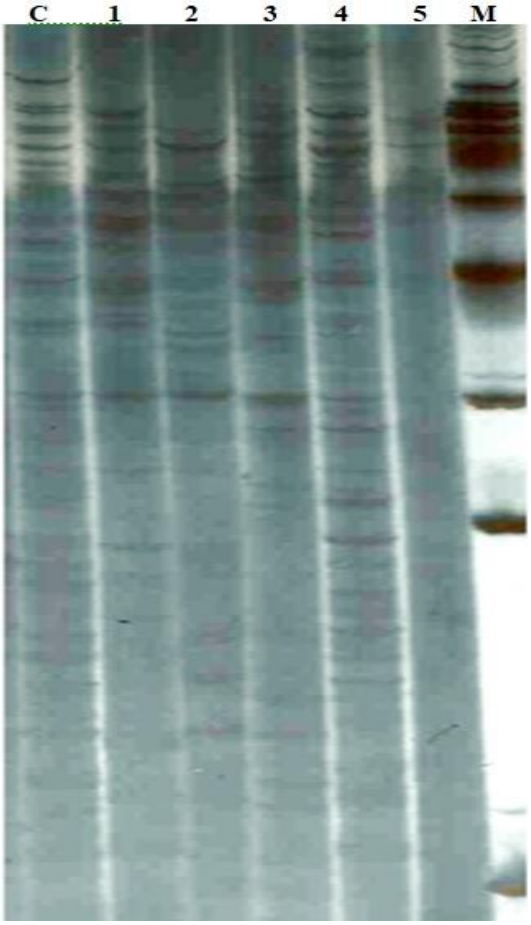

Figure 4. *AFLP profiles generated by Type II metilation from Hypogymnia physodes exposed to UVB radiation. (C: Control sample (non-stressed sample); 1: 4 j/cm^2; 2: 8 j/cm^2; 3: 12 j/cm^2; 4: 20 j/cm^2; 5: 40 j/cm^2; M: Marker)*

Discussion

In our study were applied increasing Cr^{+6}, Pb^{+2} and Cd^{+2} heavy metal concentrations and UV radiations in different doses in *H. physodes* lichen specimen. Heavy metal accumulation capacities, total protein content and chlorophyll parameters constituted against stress were determined in lichen specimen. In addition, the genotoxic effect generated by heavy metals and UV radiations was investigated with RAPD and MSAP - AFLP techniques at a molecular level (*Fig. 1*). This is the first evaluation of the changes in DNA methylation and polymorphism in methylated DNA in lichen specimen under heavy metals and UV radiations stress.

Gill et al. (2015) have shown that the toxic effects of Cr^{+6} heavy metal are observed in four different cultivars of *Brassica napus* L. The study determined plant growth and

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biomass ratio changed to exposure to Cr^{+6} concentrations. After Cr^{+6} application, the effect of Malondialdehyde (MDA) and Reactive oxygen species (ROS) increased, which could be an indication of cell damage in the plant. According to the results of our study also found that *H. physodes* lichen specimen was more accumulate in Cd^{+2} and Cr^{+6} heavy metals when compared to Pb^{+2} ($P < 0.05$). In our study, the damage caused by heavy metal application to lichen specimen may be due to the effects of ROS and MDA.

In another study, Fernandez et al. (1992) have indicated that fly ash, tending to be close to the source, is normally associated with the coarse fraction. This study has reported more storage Cr capacity of the samples collected around 5 km from the iron-steel factory. They have also stated that particularly *H. physodes* and *Evernia prunastri* have similar Cr accumulation ratio. Aslan et al. (2006) have reported that *H. physodes* shows high levels of Ca, Ti, Fe and Ba heavy metal accumulation collected from Ordu, province of Turkey. In our study results revealed that *H. physodes* lichen specimen showed to accumulate all heavy metals at a significant level ($P < 0.05$). Koroleva and Revunkov (2017) investigated to create a database of trace elements concentrations in the sample of the epiphytic lichen *Hypogymnia physodes* and to identify the spatial patterns of iron, manganese, nickel, cadmium, silver, lead, strontium, rubidium, and calcium in the Kaliningrad region. They stated that the lichen specimen accumulates microelements more intensively in the west of the Kaliningrad region than in its continental part, which is also due not only to a higher level of urbanization of the territory but also to the region's climatic features. Zulaini et al. (2019) investigated the accumulation of heavy metals on two types of epiphytic lichens, *Parmotrema tinctorum* and *Usnea diffracta*. They stated that *P. tinctorum* can be positively compared to *U. diffracta* for identifying the levels of heavy metals, due to the higher capability to accumulate heavy metals without affecting the internal structure. These lichen species positively responded to the heavy metal accumulation levels. Branquinho et al. (1997) have shown that they were able to determine and quantify the cellular location of Cu in lichens. They have been expected to regulate the extracellular uptake, time or concentration from conventional kinetic studies with other organisms and heavy metals. *Usnea* sp. were most sensitive to Cu uptake compare with other lichen species, since physiological changes occurred for lower supplied Cu concentrations than *R. fastigiata*. It seems that lichen specimen can accumulate much more metal elements than it needs and that accumulation is capable of high tolerance in our study. Thanks to these properties, lichens are defined as the best monitor-indicator organisms that can be used to display atmospheric heavy metal pollution (Abas et al., 2019; Benitez et al., 2019; Ramic et al., 2019). Li et al. (2009) have demonstrated comparative effects of Cd^{+2} and Pb^{+2} on biochemical response and DNA damage in the earthworm *Eisenia fetida* (Annelida, Oligochaeta). The evaluation of DNA damage in earthworms used the comet assay. As a result, it was the aim of the study to determine more detailed information on the effects of heavy metals on earthworms' organisms. When the results of our study and that of Liu et al.'s are compared, both studies clearly show that both of the examined biological organisms were affected by all heavy metal treatments. This study determined that selecting lichen specimen was important for accumulating heavy metals. As a result of this study, *H. physodes* lichen specimen could serve as biomarkers for environmental pollution. It can be used to a novel organism as an environmentally friendly and cost-effective technology for remediation of polluted sites.

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Doğan and Saygıdeğer (2009) have investigated some morphological and physiological changes in *Ceratophyllum demersum* L. at different concentrations of Cd^{+2} (0, 0.01, 0.1 and 1 ml/L) effect in 96 h. They have stated that total soluble sugar and protein content are reduced by the application of Cd^{+2}. Although the amount of protein showed a decrease when compared to the controls, there was no significant correlation between protein content and heavy metals. The reason for the decrease in protein content in plants is often caused by the inhibition of protein synthesis or proteolysis triggered by ROS produced by oxidative stress. Duman et al. (2010) have investigated the biological response against Cr^{+6} heavy metal in *Ceratophyllum demersum* L. Cr accumulation, plant growth, lipid peroxidation, ion escape, photosynthetic pigmentation, protein and proline content have been investigated depending on concentration changes. They have stated a statistically significant difference between 1 mM Cr^{+6} treatment and 5-10 mM Cr^{+6} treatments. In our study, the decrease in total protein in lichen specimen was observed when compared with 60 and 120 mg/L Pb^{+2}, Cr^{+6} and Cd^{+2} heavy metals ($p<0.05$). Particularly, Cd^{+2} and Cr^{+6} heavy metals constitute a high level of damage to protein content when compared with other examined parameters in *H. physodes* ($p<0.05$). In brief, the amount of protein in lichen specimen decreased at a statistically significant level after all heavy metals application ($P < 0.05$). Our study results revealed that the respond of heavy metal stress was similar to the protein content of other study results. We evaluated the change in the protein content of *H. physodes* lichen sample exposure to UV stress. Our study results also demonstrated that total protein content in *H. physodes* lichen specimen applied with UV radiations showed an opposite relation with all samples. Reduction in total protein content was observed depending on the application dose of UVA, UVB, UVC, daylight, UVA + daylight, and UVA + UVB radiations when compared to the unexposed sample. A decrease in protein content of lichen specimen was determined up to 12 j UV dose, but further UV doses were not detected changes of protein content in lichen specimen. For this reason, lichen specimen may have a certain level of tolerance. It is very important to evaluate the protein relationship with the response given to stress conditions. Protein molecules play a significant role in the determination of mainly heavy metal stress and UV radiations exposure. If further protein-related works like proteome studies it may be used in a biomarker for environmental pollution.

A study conducted has shown the physiological change generated at a cellular extent as a result of the exposure of *Cladonia arbuscular* and *Peltigera rufescens* lichen species to mercury (Hg) (Pisani et al., 2011). The results of this study have put forward that photosynthetic pigments are sensitive to $HgCl_2$ in both species. Chl-a, -b, and carotenoids content significantly decreased in *C. arbuscula* subsp. *mitis* but only Chl-a and carotenoids significantly decreased in *P. rufescens* (Pisani et al., 2011). Chettri et al. (1998) were hypothesized that Cu was responsible for the reduced chlorophyll content of lichens growing in mining areas in which Cu, Zn, and Pb were present in the soil. Therefore, they were examined the effect of Cu, Zn, and Pb, individually and in combination, on the respective thallus metal content of the lichens *C. convoluta* and *C. rangiformis*, and the subsequent effect on chlorophyll content. They found that increasing lichen Cu content [up to 1600 μg g^{-1} dry weight (DW)] had no effect on the total chlorophyll content of *C. rangiformis*, whereas Cu concentrations exceeding 175 μg g^{-1} DW caused a decrease in total chlorophyll content in *C. convoluta*, which was 40% at 1560 μg Cu g^{-1} DW. As a result, the Cu effects on chlorophyll were reduced in the presence of Pb and Zn in both lichens, but to a lesser extent in *C. rangiformis*.

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Metal cations appeared to be ionically bound within the cell wall in an exchangeable form with binding affinities of Pb > Cu > Zn. Similarly, Karakoti et al. (2014) found that Pb, Cu, Fe, and Cr have little effect on chlorophyll degradation in lichen *Pyxine cocoes*. Monnet et al. (2001) have observed an increase of chl-a content after applied with 10 mg/L heavy metal stress for 8 days, but then the content of chl-a decreased in ryegrass (*Lolium perenne*). Casano et al. (2015) reported that the proportion of cell walls is important for the capacity to immobilize extracellular Pb^{+2} from the photobiont layer. Therefore, the external cell wall could help to decrease the deleterious effects of Pb on chlorophyll content in a short time (24 h) exposure. Gurbanov and Unal (2018) revealed that Pb^{+2} exposure diminished the total nucleic acid quantity, which could be an important parameter for the elucidation of heavy metal tolerance mechanisms in lichens. Similarly, we observed that the contents of chl-a, chl-b and total chl in *H. physodes* were highest decreased after applied with 30 mg/L Cr^{+6}, Pb^{+2} and Cd^{+2} heavy metal concentration ($P < 0.05$). The contents of the same parameters in *H. physodes* were also significantly decreased after applied with all other 60 and 120 mg/L Pb^{+2} and Cd^{+2} concentrations ($P < 0.05$). Spectrophotometric measures were conducted to determine the carotenoid amount in *H. physodes* applied different heavy metal concentrations and UV radiations. There was a significant decrease in carotenoid amounts after exposed to 8 j/cm^2 all UV radiations in *H. physodes* ($P < 0.05$). In this study, we found that the application of heavy metals and UV exposure prevents loss of chlorophyll and carotenoid. UVA and UVB radiations together have more harmful effects, although *H. pyhsodes* may have shown quite a tolerance. Similarly, *H. pyhsodes* have been deposited at higher rate heavy metals.

Chettri et al. (1998) have indicated that the ratio of chlorophyll a/b is more sensitive to changes in the content of the lichen. They determined a marked decrease in the ratio of chlorophyll a/b, from 3.0 to 0.4 for *C. convoluta* and from 3.2 to 0.8 for *C. rangiformis*, occurred when the thallus Cu content exceeded 175 and 200 mg g^{-1} DW, respectively. Similarly, we found that there is a significant decrease with increasing treatments the ratio of chlorophyll a/b content in *H. physodes*. Larsson et al (2009) reported that the ratio of chlorophyll a/b ratio increased with increasing UVB radiation in *Xantoria aureola* lichen species at 21 °C degrees. We also found that after 4 j/cm^2 treatments, the ratio of chlorophyll a/b increase with increasing UVB radiation.

Heavy metal and UV stress toxicity have been extensively described in physiological, cellular and molecular biological aspects in the literature. The development of several PCR based techniques provides many advantages in the analysis of genetic toxicology. PCR based technique is simple, fast and capable of detecting point mutations and also a temporary alteration of DNA. Many studies have displayed that molecular technique may potentially form to detect DNA damage and mutational events in cells of different organisms. Genotoxic pollutants in the environment continue to grow rapidly, thereby stressing the need for rapid monitoring of genotoxicity in lichen species. Lichens can be used for this purpose due to the best biomonitor organism. The RAPD band profile generated after exposure to Cr^{+6}, Cd^{+2} and Pb^{+2} heavy metals in the lichen specimen was determined with the RAPD profile of the controls, differences of band number were detected. Cansaran-Duman et al. (2011) have determined the genotoxic contamination in the *Evernia prunastri* lichen species with the RAPD analyses. Körpe-Aksoy and Aras (2010) have studied eggplant seeds in a controlled setting applied to Cu^{+2} metal stress in different concentrations. The results showed that the changes in the GTS rates of the seeds that developed in different Cu^{+2}

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concentrations could be determined by generating RAPD profiles. This study significantly determined the maximum change of band intensities. The highest number of bands that appeared and disappeared was observed in the *H. physodes* lichen sample for UVB exposure. Especially, different changes in GTS rates (%) were shown in lichen specimen. Monitoring of various environmental stresses induced genotoxic effect directly on genomic DNA by RAPD analysis is mainly associated with unexposed sample and lichen treated with different stresses conditions. The changes in physiological and biochemical parameters such as shoot–root length, photosynthetic pigment contents, and antioxidative enzyme level support genomic template stability (Atienzar et al., 1999).

DNA methylation has been shown as a significant indicator of epigenetic and mutagenic effects and it causes differential gene expression, cell differentiation, chromatin inactivation and carcinogenesis in eukaryotic organisms (Xu et al., 2000; Mastan et al., 2012). The epigenetic organization of plant development can be used in production as much as an adaptation to stress since gene expression can be affected by the structure of the chromatin related to the epigenetic organization (Mirouze and Paszkowski, 2011). The level of the presence of methylcytosine can be determined with multiple approaches in many terms since there is a very appropriate technique to determine the methylation rate in the genome (Rein et al., 1997). MSAP-AFLP is a very potent technique to study the genome methylation status. The use of MSAP-AFLP assay has been useful in the detection of the tolerance level toward different environmental stresses. Therefore, biochemical and physiological assays are not enough to obtain deeper insight into the lichen stress response to biotic or abiotic stresses. In the present study, assessment of changes in DNA methylation by MSAP-AFLP in lichen specimen subjected to heavy metals and UV exposures. *H. physodes* thallus 69% methylation was determined in heavy metal stress. Similarly, 58% polymorphism was showed in methylated DNA from *H. physodes* on both stresses. According to the MSAP analysis, compared control with all stress treated; lichen specimen showed different patterns of methylation under heavy metals and UV radiations stress. In comparison to salt-untreated plants, Lu et al. (2007) have detected an increase of total methylated bands in plants exposed to 50-200 mM salt. In our study, as a result of the MSAP - AFLP analyses conducted in lichen specimen exposed to different heavy metals (Cr^{+6}, Pb^{+2}, and Cd^{+2}) and UV radiations (UVA, UVB, UVC, daylight, UVA + UVB, and UVA + daylight) stresses, serious changes of methylation were observed (*Fig. 4*).

Several studies published for determining the genotoxicity by DNA molecular markers of heavy metals exposure in cryptogams and algae so far. Sorrentino et al. (2017) were investigated by ISSR molecular markers in moss *Sphagnum palustre* for determining metal-induced genotoxicity. As a result of their study, both Cd and Pb salts showed a genotoxic effect with a dose-dependent trend. At concentration > 10^{-5} Cd also induced a general toxic effect in *S. palustre*, leading to chlorophyll degradation and moss death. The 12 primers used for the analysis provided a total of 169 reproducible bands, ten of which gave polymorphisms (appearance/disappearance of bands), indicating a clear genotoxic effect induced by the metals (Sorrentino et al., 2017). In our study, some primers showed significant differences in band patterns formed by loss of normal bands and appearance of new bands in the treated heavy metals and UV radiation exposure in comparison to the untreated sample profiles. We determined a total of 138 bands using six primers to evaluate UVB radiation samples in *H. physodes*.

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Both *Sphagnum palustre* moss and *H. physodes* lichen species could be used as bioindicator organisms for genotoxicity.

The study evaluated the genotoxicity of MnONP in *Physcomitrella patens* a model plant system utilized for epigenetic alterations (Ghosh et al., 2018). DNA methylation pattern at the level of single cells was examined by the methylation-sensitive comet assay with HpaII and MspI. MnONP incited DNA hypomethylation in *P. patens* gametophores exposed with 20 μg/mL MnONP concentration. In this study, the highest rate of methylation was obtained in *H. physodes* lichen specimen stressed with UV exposure with Type-2 primer (28.9%).

Compared with the RAPD and MSAP - AFLP analyses, the rate of change in the methylation model in lichen sample applied with heavy metal stress showed more diversity than sample applied with different UV radiations. While sample applied with heavy metal stress had a higher GTS rate in RAPD, GTS rate was determined to decrease based on the increasing UV radiations. The effect of UV stress on DNA stability was found to be higher than heavy metal stress in lichen specimen. The stability of the methylation model applied with heavy metal stress was thought to be less than UV stress in terms of epigenetics.

Conclusion

In the present study, we examined physiological and DNA damage indicator capacity of lichen specimen that has different features than other organisms. Certain organisms, such as lichens, may have properties such as being more tolerant to some pollutants than other organisms in contaminated areas or being able to accumulate the pollutant factor. These properties of organisms are used to provide qualitative information about the level of pollution in their environment. Therefore, it should be possible to make a correlation between observing and measuring the changes in the organism and the sources of the pollutant, pollution and the intensity of the pollution. This study was shown that the use of lichen specimen could eliminate the contaminants that cause environmental pollution. Detailed presentation of possible changes generated in the DNA structure and the presentation of contamination at a molecular level were ensured with our study enriched with molecular techniques. Therefore, the effect of UV stress on lichen DNA stability was thought to be greater than genetically heavy metals. The results indicated that *H. physodes* has great potential to be used in phytoremediation exercises. In addition to the properties of this lichen, the literature data has proved their antibacterial and antioxidant activity as well as cancer cell cytotoxicity and inhibitory effects on enzymes (Song et al., 2014; Mitrovic et al., 2015; Studzinska-Sroka and Zarabska-Bozjewicz, 2019). Finally, it was possible to observe the strong mechanisms of *H. physodes* lichen specimen against stressors such as heavy metals and UV radiation, allowing it to persist under highly stressful conditions. In future studies, the investigation of the biosorption capacity to accumulate different heavy metal tolerant lichen species in laboratory work still need further research. Determination of the biological response of lichens against toxic chemicals could be useful for the treatment of wastewater with lichens. These and similar studies will contribute to the identification of DNA markers.

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