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ACCUMULATION OF CR^{6+}, PB^{2+} AND CD^{2+} AND ULTRAVIOLET RADIATION ALTER METHYLATION AND GENOMIC DNA STATUS IN *RAMALINA FARINACEAE*

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ABSTRACT. In this study was aimed to determine the genotoxic effect of *Ramalina farinacea* lichen species against stress sources at the molecular level. After applying three different heavy metals (Pb^{2+}, Cd^{2+}, and Cr^{6+}) to the *R. farinacea*, the extent to which the lichen sample absorbed these metals was determined by Flame Atomic Absorption Spectroscopy. RAPD and MSAP-AFLP assays were also used to determine the status of DNA damage. The heavy metal analysis showed that *R. farinacea* had high levels of Pb^{2+}, Cd^{2+}, and Cr^{6+} content. According to the results obtained from molecular analyses, band changes were observed against seven primers heavy metal stresses and three primers against UV stress. An increase in Genomic Template Stability (GTS) was determined during the time in *R. farinacea* treated with all heavy metal concentrations. The effect of UV radiations in *R. farinacea* revealed the highest polymorphism and the lowest GTS rate depending on the dose. Among all methylation combinations, Type II was found to show altered in *R. farinacea* in response to Pb^{2+}, Cd^{2+}, and Cr^{6+} contents and UV radiations. *R. farinacea* can be used at the molecular level as a biomarker of suitable genotoxic effect. This is the first study to reveal DNA damage against stress sources using a sample of *R. farinacea* lichen species.

Keyword and phrases. Ramalina farinacea, heavy metals, ultraviolet radiation, genotoxicity

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1. INTRODUCTION

The developments in the field of industry led to a serious increase in environmental pollution by releasing most of the heavy metals and various harmful pollutants [1,2]. The increase in the amount of heavy metals in the environment together with many other contaminants effects the living cells by disrupting the balance between reactive oxygen species (ROS) & antioxidant systems, and in this case, it causes diseases that negatively effects life [2]. In particular, soil, water and air analyzes, which are the physical elements of the natural environment, cannot provide sufficient data to reveal heavy metal genotoxicity. Lichens are important biological organisms that do not have organs such as root, stem, leaves and cuticles. Thanks to these properties, lichens have the ability to absorb elements such as heavy metals emitted from pollutant sources [3]. In recent studies, lichen species have been identified as suitable organisms that can be used to reflect atmospheric rates by measuring pollutants such as metals, elements and radionuclides accumulated in their thalli [4,5,6]. Particularly, epiphytic lichens are considered to be one of the important bioindicators of air quality because they easily obtain water and essential nutrients from the air [7-10].

It is known that lichens show different reactions to the accumulation of heavy metals. Some types of lichen can even tolerate high doses of some heavy metals such as Cu [11], Fe [12], Pb [12], Cd [13] and Mn [13]. With the accumulation of metals in lichen samples at a level that cannot be tolerated, problems such as decreased photosynthesis rate, decreased nitrogen fixation, thylakoid and plasma damage, chlorophyll deterioration can be observed. Environmental pollution of heavy metals has increasingly become of serious concern around the world [14-16]. Among all heavy metals, cadmium (Cd^{2+}), chromium (Cr^{3+} and Cr^{6+}) and lead (Pb^{2+}), in particular, continue to be a world concern [16]. The increased concentrations of Cr^{6+}, Cd^{2+}, and Pb^{2+} in polluted areas may pose a variety of problems and hazards [17]. In the study conducted by Liu et al. [18], *Orzya sativa* was treated with Cd and DNA damage was evaluated. According to the Random Amplified Polymorphic DNA (RAPD) analysis results, they stated that cadmium pollution has serious effects on DNA level. In the study, the researchers found that some bands disappeared and changes occurred in some bands compared with DNA of the control sample (unpolluted sample).

Ultraviolet (UV) radiation has vital importance as it has negative effects on humans, animals and plants. Plants have responded to the stress by repairing and enhancing the affected efficient systems and by incorporating the UV protective substances that prevent damage. While the substances used by different groups of

organisms are chemically various, the screening of UV radiation is used universally [19,20]. Numerous colourless lichen compounds absorb UV-B strongly, and some, such as parietin also assimilate photosynthetic active radiation (PAR) [21]. Studies have shown that lichens may generally vary in their responses to UV exposure by increasing the production and accumulation of secondary metabolites that block UV penetration [20,22]. Hall et al. [23] have found that secondary metabolites such as phenolic compounds accumulate at a high level in the outer layers of the medulla as a result of the short and long-term effects of UV-B radiation in some lichen species, and this accumulation reduced the transmission of UV radiation to thalli, and aromatic components in lichens played a protective role against UV radiation.

Rapid advances in molecular biology in recent years have provided new methods to detect DNA damage [23-25]. With the help of these sensitive molecular markers used, any genotoxic damage occurring in the biological organism can be easily detected [24-26]. The contamination of soil with heavy metals has a genotoxic effect on plants that have to grow in these areas, causing changes in the DNA profile, such as mutation [27]. Recently, RAPD and Amplified Fragment Length Polymorphism (AFLP) techniques have been successfully utilized to identify temporary DNA changes caused by heavy metal stress [6,16,28-32]. Current molecular genetics and genomic studies have provided insight into the importance of cytosine methylation, which plays a significant role in gene regulation [33]. Methylation creates differences in plants by causing DNA polymorphisms or epigenetic variations. The plant genome can respond to environmental and genetic stresses that result dynamically in both genetic and epigenetic methylation polymorphisms. Stress-triggered genotypes can contribute significantly to phenotypic innovation and the development of biological organisms [33].

Another method that makes it possible to indicate the effect of environmental pollutants on DNA size is comet assay. Single-cell gel electrophoresis (Comet) assay is a sensitive and simple tool capable of specifying DNA damage in the cells of biological organisms [34-36]. Moreover, RNA sequence and quantitative Real-Time-PCR techniques have been also used to determine the genetic damage that occurred in the samples collected from some polluted areas. In recent years, the genotoxic effect of pollutants in biological organisms other than lichen species has been identified with these two techniques [35]. However, the fact that lichens are a biological organism consisting of algae and fungi makes it difficult to apply these techniques based on RNA in the lichens. In this situation, the RAPD technique is still the best option for determining the molecular size genotoxic effect of pollutants using lichens, which is the best bio-indicator organism. However, in

order to make our results more detailed and reliable, a study was also carried out with the MSAP-AFLP technique [34-36].

The main objective of the present work was to elucidate whether the genotoxic effect of lichens, one of the best biomonitor organisms against pollutants, by using two different molecular techniques. *R. farinaceae* lichen species was exposed for heavy metals (Pb^{2+}, Cd^{2+}, and Cr^{6+}) and UV radiations (UVA, UVB, UVC, daylight, UVA+UVB, UVA+day light) for evaluating the impact of pollution. In this study, RAPD and MSAP-AFLP assays were used in *R. farinacea* for possible changes in DNA status after the exposure to different stress factors. Thus, this study will provide a more comprehensive understanding of the molecular mechanisms of cellular protection against different stress factors on *R. farinacea* lichen specimen. According to our knowledge, this study could be the first to evaluate the genotoxic effects on DNA of *R. farinacea*.

2. MATERIALS AND METHODS

2.1. Lichen samples and stress treatment

Ramalina farinacea was collected from Yenice Forest in Karabük, Turkey (41°10'N, 32°23'E). Three heavy metals and different UV radiation stress were applied to lichen samples at different time intervals in the study.

Thallus sample was placed in a petri dish in laboratory condition. It was exposed to different doses of UV radiation using a dose-meter (352 nm, 50Hz, 0.60 Amps) at 25 °C. The different levels of UV radiations were obtained with the UV irradiation chamber BS-03 (Dr. Gröbel UV-Electronic GmbH) and a dosimeter to determine the exposure of different UV radiation rates.

2.2. Determination on heavy metal content

Lichen sample exposed to Cd^{2+}, Cr^{6+} and Pb^{2+} heavy metals were analyzed by Flame Atomic Absorption Spectroscopy (FAAS; Instrument PM Avarta, GBC Scientific Equipment, Australia). The heavy metal content was determined according to Hamutoğlu et al. [16] studies.

2.3. Genomic DNA extraction and RAPD assay

DNA extraction was carried out under the protocol developed by Aras and Cansaran [37]. DNA purity was measured using nanodrop (NanoDrop ND-1000

Spectrophotometer, Thermo Scientific, Wilmington, USA). 10 primers were used in RAPD analysis and all primers showed amplified clear & reproducible bands, seven with metal and UV stress, respectively. PCR components were determined according to the protocol of Hamutoğlu et al. [16]. PCR products were loaded and visualized on agarose gel stained with ethidium bromide. The samples unexposed stress sources were used as control samples Negative control was also used to verify that the presence of any contaminating nucleic acid has been introduced into the master mix during the process.

2.4. MSAP-AFLP analysis

The genomic DNA (200 ng) of samples exposed to the heavy metal and UV stress was cut separately with *Eco*R I/*Msp* I and *Eco*R I/*Hpa* II restriction enzymes at 37 °C for 2 h (Table 1). MSAP-AFLP analysis was performed according to the protocol of Hamutoğlu et al. [16]. AFLP-PCR experiments were repeated at least twice for each primer and faint bands were not recorded in this study.

TABLE 1. Types of methylation produced by the cleavage of HPAII and MspI restriction enzymes.

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

2.5. Statistical analysis

The results of data analysis were done with the multifactor analysis of variance (univariate ANOVA). The experiments were independently repeated three times. (n=3).

2.5.1. Estimation of profiling scoring and data analyses for RAPD assay

The rate of polymorphism was calculated by the disappearance or appearance of bands formed when the sample was exposed to stress [16,29,38].

2.5.2. Estimation of profiling scoring and data analyses for methylation analyses

All the amplified bands that are identified with the MSAP-AFLP analysis were classified into four categories based on the presence or absence of each amplicon as indicated by Li et al. [39].

3. RESULTS

3.1. The determination of heavy metal contents

The highest absorption ratio was determined 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 *R. farinacea* lichen specimen was applied to 30 mg/L Pb^{2+} for 24 h. 60 mg/L Pb^{2+} heavy metal absorption decreased from 58.1 % to 35.1 % for 24h in *R. farinacea*. At 120 mg/L Pb^{2+}, the absorption efficiency was found to be lower than 30 and 60 mg/L Pb^{2+} in *R. farinacea* (82.5 %) for 24 h ($p<0.05$) (Table 2). The highest removal efficiency (21 % for 2 h, 73.3 % for 24 h and 83.3 % for 24 h) was determined 30, 60 and 120 mg / L Cd^{2+} in *R. farinacea* lichen species ($p<0.05$).

TABLE 2. The measurements of heavy metal contents with AAS after exposure of the *R. farinacea* lichen sample to 120 mg / L Pb^{2+} (ANOVA analysis was performed and the same letters in a column indicate no significant differences with ANOVA test at $p < 0.05$).

Samples	***N***	***Mean***	***Std. Deviation***	***Std. Error***	***95% Confidence Interval for Mean***		***Min***	***Max***
					Lower Bound	***Upper Bound***		
Control	3	119.9(a)	.20	.11	119.4	120.3	119.7	120.1
30 min	3	96.7(b)	.20	.11	96.2	97.1	96.5	96.9
1 h	3	88.2(c)	.20	.11	87.7	88.6	88.0	88.4
2 h	3	79.6(d)	.10	.05	79.3	79.8	79.5	79.7
6 h	3	72.2(e)	.10	.05	71.9	72.4	72.1	72.3
18 h	3	71.8(f)	.40	.23	70.8	72.7	71.4	72.2
24 h	3	70.9(g)	.10	.05	70.6	71.1	70.8	71.0
48 h	3	70.9(h)	.20	.11	70.4	71.3	70.7	71.1
72 h	3	70.9(i)	.10	.05	70.6	71.1	70.8	71.0
Total	27	82.3	16.16	3.11	75.9	88.7	70.7	120.1
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**			
Between Groups	6796.0	8	849.5	21237.5	.000			
Within Groups	.70	18	.04					
Total	6796.7	26						

3.2. Determination of heavy metals and UV radiations on RAPD profiles in *R. farinacea*

R. farinacea control sample obtained a total of 42 and 15 bands in heavy metal and

UV stress respectively by using RAPD analyses (Table 3). Total number of bands were more in *R. farinacea* variety treated with Cr^{6+} (42 in control, 30 mg/L Cr^{6+}: 132; 60 mg/L Cr^{6+}: 139; 120 mg/L Cr^{6+}: 192 band) when compared to *R. farinacea* treated with Pb^{2+} (28 in control, 30 mg/L Pb^{2+}: 85; 60 mg/L Pb^{2+}: 69; 120 mg/L Pb^{2+}: 83 band) and Cd^{2+} (42 in control, 30 mg/L Cd^{2+}: 122; 60 mg/L Cd^{2+}: 113; 120 mg/L Cd^{2+}: 91 band) (Table 3-5). After 120 mg/L Cr^{6+} treatment, 11 extra bands appeared and 20 extra bands disappeared at 30 min in *R. farinacea* variety, and this species showed the highest levels of change in bands treated with Cr^{6+} (Figure 1) (Table 3). The lowest number of band changes (7 bands) was detected at 60 mg/L Pb^{2+} after 1 h treatment in *R. farinacea* lichen specimen (Figure 2) (Table 4).

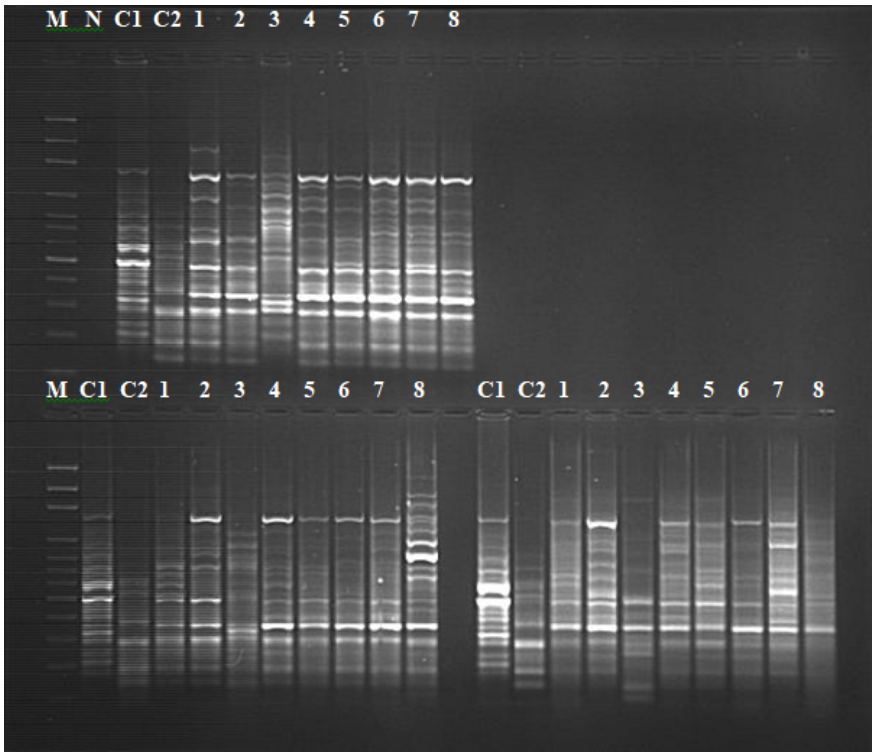

FIGURE 1. DNA band profile of 30 mg/L (top-left), 60 mg/L (lower-left) and 120 mg/L (lower-right) concentration of Cr^{6+} in *Ramalina farinacea* using RAPD with primer OPC7 primers. (M: Marker, C1-C2: Control, N: Negative control, 1: 30 min, 2: 1 h, 3: 2 h, 4: 6 h, 5: 18 h, 6: 24 h, 7: 48 h, 8: 72 h).

TABLE 3. Varying band number using OPC 02, OPC 04 and OPC 07 primers as a result of treating 30, 60 and 120 mg/L Cr^{6+} heavy metal stress in *Ramalina farinacea* lichen specimen.

30 mg/L Cr^{6+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 02	**14**	3	4	1	7	4	5	2	5	3	3	3	4	3	5	1	3
OPC 04	**14**	2	4	2	2	1	2	2	2	1	0	0	2	1	3	1	4
OPC 07	**14**	2	6	3	5	1	6	3	3	2	6	3	2	1	1	2	2
	42	7	14	6	14	6	13	7	10	6	9	6	8	5	9	4	9
	a+b	**21**		**20**		**18**		**17**		**15**		**14**		**14**		**13**	
60 mg/L Cr^{6+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 02	**14**	2	3	1	3	3	4	2	3	1	4	1	0	2	7	2	5
OPC 04	**14**	1	4	2	1	3	2	2	5	2	5	1	4	3	2	1	1
OPC 07	**14**	4	6	6	4	2	4	3	1	1	4	4	3	0	5	4	6
	42	7	13	9	8	8	10	7	9	4	13	6	7	5	14	7	12
	a+b	**20**		**17**		**18**		**16**		**17**		**13**		**19**		**19**	
120 mg/L Cr^{6+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 02	**14**	5	6	4	7	4	6	5	3	4	4	2	4	2	3	5	0
OPC 04	**14**	4	6	5	1	3	2	2	6	3	3	1	1	2	5	1	6
OPC 07	**14**	2	8	4	8	5	8	4	3	2	6	5	7	2	5	1	7
	42	11	20	13	16	12	16	11	12	9	13	8	12	6	13	7	13
	a+b	**31**		**29**		**28**		**23**		**22**		**20**		**19**		**20**	

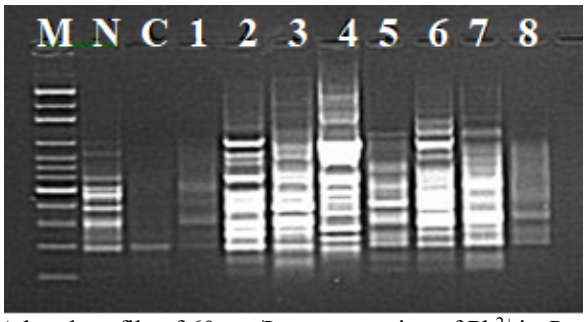

FIGURE 2. DNA band profile of 60 mg/L concentration of Pb^{2+} in *Ramalina farinacea* using RAPD with primer OPC10 primers. (M: Marker, C: Control, N: Negative control, 1: 30 min, 2: 1 h, 3: 2 h, 4: 6 h, 5: 18 h, 6: 24 h, 7: 48 h, 8: 72 h).

TABLE 4. Varying band number using OPC 04 and OPC 10 primers as a result of treating 30, 60 and 120 mg/L Pb^{2+} heavy metal stress in *Ramalina farinacea* lichen specimen.

30 mg/L Pb^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 04	**14**	2	5	2	4	1	2	1	4	3	2	2	5	2	2	1	4
OPC 10	**14**	3	2	2	3	2	5	3	2	2	1	1	0	0	3	1	3
	28	5	7	4	7	3	7	4	6	5	3	3	5	2	5	2	7
	a+b	**12**		**11**		**10**		**10**		**8**		**8**		**7**		**9**	
60 mg/L Pb^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 04	**14**	3	5	1	6	5	3	1	4	4	2	2	4	4	1	3	2
OPC 10	**14**	1	1	2	2	2	0	2	2	0	1	0	2	1	1	0	2
	28	4	6	3	8	7	3	3	6	4	3	3	2	6	5	3	4
	a+b	**10**		**11**		**10**		**9**		**7**		**8**		**7**		**7**	
120 mg/L Pb^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 04	**14**	4	5	3	2	2	5	3	1	2	3	0	4	2	3	1	1
OPC 10	**14**	2	4	4	3	4	1	2	5	0	4	3	1	1	2	2	4
	28	6	9	7	5	6	6	5	6	2	7	3	5	3	5	3	5
	a+b	**15**		**12**		**12**		**11**		**9**		**8**		**8**		**8**	

In terms of Cd^{2+} stress in *R. farinacea*, the highest GTS value was observed expose to 30 mg/L Cd^{+2} heavy metal stress at 48 h and 72 h (69.0 %), 60 mg/L Cd^{2+} at 48 h (73.8 %) and 120 mg/L Cd^{2+} at 48 h (83.3 %) treatments. The lowest GTS values (54.7, 59.5 and 59.5 %) were obtained at 30 min treatments in all Cd^{2+} heavy metal stress concentrations, respectively (Table 6). As regards Cr^{6+} stress in *R. farinacea*, the highest GTS value was seen expose to 30 mg/L Cr^{6+} heavy metal stress at 72 h (69.0 %), 60 mg/L Cr^{6+} stress at 18, 48 and 72 h (54.7 %) and 120 mg/L Cr^{+6} stress at 48 h (54.7 %). The lowest GTS value (50.0, 38.0 and 26.1 %) was obtained at 30 min treatments in all Cr^{6+} concentrations (Table 6).

TABLE 5. Varying band number using OPC 01, OPC 04 and OPC 08 primers as a result of treating 30, 60 and 120 mg/L Cd^{2+} heavy metal stress in *Ramalina farinacea* lichen specimen.

30 mg/L Cd^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	**14**	1	6	1	5	3	2	5	3	0	4	2	4	3	3	3	2
OPC 04	**14**	2	3	1	3	0	5	1	1	2	1	2	0	2	1	0	2
OPC 08	**14**	2	5	4	3	3	2	1	5	4	4	3	3	1	3	2	4
	42	5	14	6	11	6	9	7	9	6	9	7	7	6	7	5	8
	a+b	**19**		**17**		**15**		**16**		**15**		**14**		**13**		**13**	
60 mg/L Cd^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	**14**	3	5	2	4	3	2	1	5	2	5	1	3	1	4	0	3
OPC 04	**14**	2	3	1	2	2	3	1	1	0	3	0	1	2	1	0	1
OPC 08	**14**	2	2	4	3	6	1	1	5	0	4	2	5	1	2	3	5
	42	7	10	7	9	11	6	3	11	2	12	3	9	4	7	3	9
	a+b	**17**		**16**		**17**		**14**		**14**		**12**		**11**		**12**	
120 mg/L Cd^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	**14**	4	2	3	5	2	2	0	5	3	2	1	2	1	1	2	2
OPC 04	**14**	3	1	2	2	3	1	2	2	0	1	0	0	1	1	0	0
OPC 08	**14**	2	5	1	2	0	7	2	1	0	3	1	4	1	2	0	4
	42	9	8	6	9	5	10	4	8	3	6	2	6	3	4	2	6
	a+b	**17**		**15**		**15**		**12**		**9**		**8**		**7**		**8**	

In terms of Pb^{2+} stress in *R. farinacea*, the highest GTS values were seen expose to 30 mg/L Pb^{2+} heavy metal stress at 48 h (75.0 %), 60 mg/L Cr^{6+} stress at 18, 48 and 72 h (75.0 %) and 120 mg/L Cr^{6+} stress at 24, 48 and 72 h (71.4 %). The lowest GTS values were obtained at 30 min treatments in 30 and 120 mg/L Pb^{2+} and 1 h treatments in 60 mg/L Pb^{2+} heavy metal stress (Table 6).

Varying band-number using primers as a result of UVA, UVB and UVC radiation samples in *R. farinacea* was also shown in Table 7. As regards UVA stress in *R. farinacea* lichen species, the highest GTS value (96.6 %) was observed exposed to 4 j/cm^2 UVA radiation (Table 8). UVC treatments in *R. farinacea*, the highest GTS value (88.6 %) was determined at 4 j/cm^2 UVC radiation. UVB treatments in *R. farinacea*, the highest GTS value (66.6 %) was revealed at 8 j/cm^2 UVB radiation. The lowest GTS value (43.3 %) was observed at 40 j/cm^2 UVB radiation (Figure 3) (Table 8).

TABLE 6. The rates of GTS values using Cd^{2+}, Cr^{6+}, and Pb^{2+} heavy metal stress in *Ramalina farinacea* lichen specimen.

Samples		Rates of GTS (%)	Samples		Rates of GTS (%)	Samples		Rates of GTS (%)
30 ppm Cd^{2+}	30 min	54.76	60 ppm Cd^{2+}	30 min	59.52	120 ppm Cd^{2+}	30 min	59.52
	1 h	59.52		1 h	61.90		1 h	64.28
	2 h	64.28		2 h	61.90		2 h	64.28
	6 h	61.9		6 h	66.66		6 h	71.42
	18 h	64.28		18 h	66.66		18 h	78.57
	24 h	66.66		24 h	71.42		24 h	80.95
	48 h	69.04		48 h	73.80		48 h	83.33
	72 h	69.04		72 h	71.42		72 h	80.95
Samples		**Rates of GTS (%)**	**Samples**		**Rates of GTS (%)**	**Samples**		**Rates of GTS (%)**
30 ppm Cr^{6+}	30 min	50.0	60 ppm Cr^{6+}	30 min	38.09	120 ppm Cr^{6+}	30 min	26.19
	1 h	52.38		1 h	42.85		1 h	30.95
	2 h	57.14		2 h	45.23		2 h	33.33
	6 h	59.52		6 h	50.0		6 h	45.23
	18 h	64.28		18 h	54.76		18 h	47.61
	24 h	66.66		24 h	50.0		24 h	52.38
	48 h	66.66		48 h	54.76		48 h	54.76
	72 h	69.04		72 h	54.76		72 h	52.38
Samples		**Rates of GTS (%)**	**Samples**		**Rates of GTS (%)**	**Samples**		**Rates of GTS (%)**
30 ppm Pb^{2+}	30 min	57.14	60 ppm Pb^{2+}	30 min	64.28	120 ppm Pb^{2+}	30 min	46.42
	1 h	60.71		1 h	60.71		1 h	57.14
	2 h	64.28		2 h	64.28		2 h	57.14
	6 h	64.28		6 h	67.85		6 h	60.71
	18 h	71.42		18 h	75.0		18 h	67.85
	24 h	71.42		24 h	71.42		24 h	71.42
	48 h	75.0		48 h	75.0		48 h	71.42
	72 h	67.85		72 h	75.0		72 h	71.42

3.3. Methylation DNA and polymorphism in examined lichen species to different levels of stress condition

Band changes after exposure to heavy metals and UV radiation were compared with untreated control samples. 413 to 691 bands were obtained in the untreated sample and a total of 217 bands were determined with an average of 11 per primer in the MSAP-AFLP analysis. The total number of band changes was 117 bands for the heavy metal and lichen species exposed to UVB stress, respectively. The highest methylation was observed for the heavy metal stressed *R. farinacea* at 6 h (91.2 %) and for the UV radiations stressed *R. farinacea* at 30 min and 1 h (89.7 %). The lowest levels of methylation polymorphism were detected at 1 h in the heavy metal stressed *R. farinacea* (30 %) and at 12 and 24h in the UV radiations

TABLE 7. Determination of the genotoxic effect after UVA, UVB and UVC radiation treatment in *Ramalina farinacea* using OPC01, OPC02, OPC04, OPC07, OPC10, OPA16 and TubeA05 primers.

UVA											
		4 j		**8 j**		**12j**		**20 j**		**40 j**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC01	**15**	1	2	2	4	1	3	2	3	2	1
OPC02	**15**	1	1	1	1	2	1	1	0	0	3
OPC04	**15**	0	0	2	0	1	1	0	1	0	2
OPC07	**15**	1	1	3	3	1	5	2	4	3	7
OPC10	**15**	1	1	1	1	2	3	0	2	2	2
TubeA05	**15**	1	3	1	0	0	4	3	6	4	5
	90	5	8	10	9	7	17	8	16	11	20
	a+b	**13**		**19**		**24**		**24**		**31**	
UVB											
		4 j		**8 j**		**12 j**		**20 j**		**40 j**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	**15**	2	3	1	3	2	3	3	6	1	4
OPC 02	**15**	1	4	3	5	3	2	3	5	5	6
OPC 04	**15**	2	3	1	3	1	5	7	2	3	7
OPC 07	**15**	1	4	2	3	2	4	4	5	7	4
OPC10	**15**	2	5	3	2	1	2	1	5	2	8
Tube A05	**15**	1	3	0	4	2	3	2	2	2	5
	90	9	22	10	20	11	19	20	28	17	34
	a+b	**31**		**30**		**39**		**48**		**51**	
UVC											
		4 j		**8 j**		**12 j**		**20 j**		**40 j**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC01	**15**	1	2	2	1	1	5	3	5	4	4
OPC02	**15**	1	3	1	3	1	2	1	4	4	3
OPC04	**15**	1	0	1	1	1	2	2	4	2	3
OPA16	**15**	1	1	2	3	1	3	3	1	4	2
TubeA05	**15**	0	0	1	2	2	4	5	6	3	4
	90	4	6	7	10	6	16	14	20	17	16
	a+b	**10**		**17**		**22**		**34**		**33**	

stressed *R. farinacea* (40 %). MSAP - AFLP analysis results were evaluated according to methylation types in samples exposed to heavy metal stress. In this context, the maximum methylation level (33.3%) was observed in Type II and the lowest methylation level (63.3%) was revealed in Type III status. After the first 6 h of heavy metal exposure, there was no change in the type II methylation level, but then there was a change in methylation rate of 34.3% in 12 and 24 h. The highest methylation rate (79.6%) was determined in *R. farinacea* lichen species exposed to 12 j/cm^2 UVB stress. The lowest methylation rate (39.6%) was observed after UV exposure at 12 j/cm^2. As shown in Figure 4, the methylation rate decreased at 12 j/cm^2 and started to increase again in other UV radiations (20 and 40 j/cm^2) applied after this joule value. The differences were observed in methylation status in lichen samples not exposed to UV stress and *R. farinacea* exposed to 12 j/cm^2 UV stress.

The methylation status of 34.3% was determined in *R. farinacea* exposed to 4 j/cm^2. According to the MSAP-AFLP analysis data obtained from UVB stress, Type II methylation level reached the maximum level (71.2%) at the 6th h. While the minimum rate of Type IV methylation (33.6%) was observed in 24 h, the maximum level of Type III methylation (77.2%) was also detected.

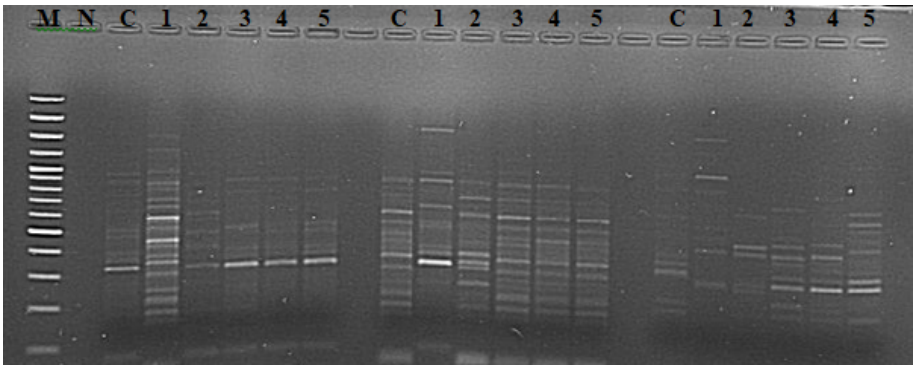

FIGURE 3. The results of RAPD-PCR treating at UVB radiation in *Ramalina farinacea* lichen specimen (left OPC01, center OPC02; right OPC04 primers). (M: Marker, N: Negative control; C: Control, 1: 4 j/cm^2, 2: 8 j/cm^2, 3: 12 j/cm^2, 4: 20 j/cm^2, 5: 40 j/cm^2).

TABLE 8. The rates of GTS values using UV radiations in *R. farinacea* lichen specimen.

	Samples	Rates of GTS (%)		Samples	Rates of GTS (%)		Samples	Rates of GTS (%)
UVA	4 j/cm^2	96.66	UVB	4 j/cm^2	65.55	UVC	4 j/cm^2	88.69
	8 j/cm^2	80.0		8 j/cm^2	66.66		8 j/cm^2	80.86
	12 j/cm^2	75.75		12 j/cm^2	56.66		12 j/cm^2	77.39
	20 j/cm^2	73.33		20 j/cm^2	46.66		20 j/cm^2	67.82
	40 j/cm^2	62.42		40 j/cm^2	43.33		40 j/cm^2	62.60
	Samples	**Rates of GTS (%)**		**Samples**	**Rates of GTS (%)**		**Samples**	**Rates of GTS (%)**
UVD	4 j/cm^2	81.33	UVA+UVB	4 j/cm^2	66.66	UVA+UVD	4 j/cm^2	84.76
	8 j/cm^2	80.0		8 j/cm^2	53.33		8 j/cm^2	79.04
	12 j/cm^2	70.66		12 j/cm^2	53.33		12 j/cm^2	72.38
	20 j/cm^2	60.0		20 j/cm^2	33.33		20 j/cm^2	68.57
	40 j/cm^2	54.66		40 j/cm^2	26.66		40 j/cm^2	65.71

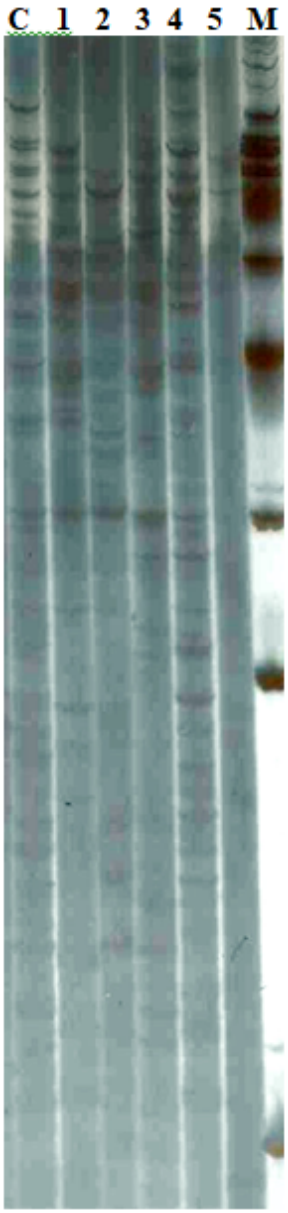

FIGURE 4. AFLP profiles resulting from Type II methylation in *Ramalina farinacea* lichen species exposed to UVB radiation. (M: Marker, C: Control, 1: 4 j/cm^2, 2: 8 j/cm^2, 3: 12 j/cm^2, 4: 20 j/cm^2, 5: 40 j/cm^2).

4. DISCUSSION

Environmental stressors such as heavy metal pollution can induce mutations and toxic effects, leading to the disruption of DNA integrity [40]. Therefore, the determination of DNA damage in terrestrial organisms living in polluted areas is a considerable exponent in ecotoxicology studies [6,41]. As a result of the comparing of band differences of RAPD and Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) analysis, it was determined that the rate of change in the methylation model of the samples exposed to heavy metal stress varies more than the samples exposed to UV radiation. It was observed in the present study that the samples with heavy metal stress had a high GTS rate in the RAPD-PCR results, but the GTS rate decreased due to increasing radiation doses in the samples with UV radiation. Accordingly, the effect of UV stress on lichen DNA stability is genetically thought to be higher than heavy metals. On the other hand, heavy metal samples are epigenetically considered to have less stability of the methylation pattern.

Several studies have been published to determine the genotoxicity with DNA molecular markers of heavy metals exposure in lichens. Hamutoğlu et al. [6] observed that there were 19, 45 and 51 bands in *P. furfuracea* (control band number 83), respectively, after the 1st, 2nd and 3rd regions, located 50, 100 and 200m away from the cement factory that was exposed to contaminants. Sorrentino et al. [42] investigated with ISSR molecular markers in moss *Sphagnum palustre* for showing both Cd and Pb salts a genotoxic effect in a dose-dependent manner. They observed a total of 169 reproducible bands using 12 primers, ten of which yielded polymorphisms, pointing out a clear genotoxic effect caused by the metals. Batir et al. [43] determined the effect of different concentrations of copper (Cu) solutions on maize (*Zea mays* L.) seedlings by using physiological parameters and RAPD analysis. In this study revealed band increase and or loss in the RAPD profiles of the samples. They found that the RAPD band profiles of the samples and the GTS ratios were compatible with each other. Our study significantly determined the maximum change of band intensities. The highest number of the band that appeared and disappeared was observed in the *R. farinacea* for 120 mg/L. We determined a total of 42 bands using three primers to evaluate Cr^{6+} heavy metal samples in *R. farinacea*. It could be easily said that these species could be used as bioindicator organisms for genotoxicity. As a result of the present study, it could be used as a novel organism for remediation of polluted sites.

Bajpai et al. [44] revealed that the exposure of Cr^{6+} (0, 10, 25, 50, 75, and 100 μM) for several days under controlled conditions caused a major reduction in physiological parameters with increasing metal stress in *Pxine coces* lichen

specimen. They pointed out that genetic changes could be used as a tool to investigate ecological stress and polymorphisms because of genotoxicity [44]. When this lichen specimen was compared with *R. farinacea* according to their genotoxic capacity and band differences, *R. farinacea* was a higher accumulator of Cr^{+6} than *P. coces*. In the RAPD band profile created after exposure to Cr^{+6} heavy metal in *R.farinacea* lichen specimen, differences in the number of band were detected compared to the control.

As observed in the present study that *R. farinacea* has a considerably higher accumulation compare to the other organisms studied on heavy metal accumulation and this result is compatible with many remarkable studies in the literature [10,45,46,47]. In our previous study, *Hypogymina physodes* lichen specimen was evaluated with the genotoxic effect of pollutants. It was concluded that changes in RAPD assay and DNA methylation analysis observed that homologous nucleotide sequences in the genome from untreated and treated species with pollutants showed different band and methylation patterns [16]. It revealed that thallus heavy metal accumulation in the contaminated areas was particularly higher for Cr^{6+}, Cd^{2+} and Pb^{2+}, and UV radiations compared to the control [16]. However, it was determined that *R. farinacea* had more heavy metal accumulation capacity than *H. physodes*. Chetia et al. [48] also reported that Pb, Cd, Zn, Cu, Co, Ni and Cr concentrations were found to be higher in the lichen species collected from the polluted areas compared to the control. This may be due to the wider surface of the lichen tallus.

Zulaini et al. [5] evaluated two lichen species as a bioindicator for the accumulation of heavy elements in Malaysia. Their results imply that *P. tinctorum* was found suitable bioindicator of air pollution due to the higher capability to accumulate heavy metals compared to *U. diffracta*. Sujetoviene et al. [10] also investigated the physiological response of lichens *Evernia prunastri* and *R. farinacea*, which were transported near a landfill in the center of Lithuania, and evaluated airborne contamination of heavy metals using these lichens. They found that the concentrations of heavy metals (Cd, Fe, Cr, Mn) varied between the study sites. The study showed an increased accumulation of some heavy metals in lichens transplanted to the sites downwind from the factory. The measured values in the samples collected in the study sites showed moderate air pollution. In the current study, we showed that we collected lichen samples from the clean area and treated with different heavy metal. In particular, Cr^{6+}, Cd^{2+}, and Pb^{2+} examined at the highest concentrations caused a GTS reduction of about 25-40 %. This result determined us the level of genotoxic effect depending on GTS level.

It is cellular DNA that is most affected by UV radiation [49] and UV radiation mainly causes mutations to occur in DNA [49, 50]. The RAPD-PCR assay is applied to analyze the genetic damage caused by UV and X-rays in plants and macroalgae species [51]. The results obtained show that determining the genotoxic effect with the RAPD and MSAP-AFLP techniques in *R. farinacea* has enabled us to get detailed information on the level of pollution.

Garty et al. [52] evaluated the effects of UV-B radiation combined with $NaHSO_3$ solution on stress ethylene production of two lichen species, including the same genus, under laboratory conditions. *Ramalina lacera* was found to be more sensitive to the effect of UV-B radiation combined with $NaHSO_3$ compared to *R. maciformis*, an epiphytic Mediterranean lichen. The adaptation of *R. maciformis* to UV-B radiation appears to be related to the photoreactive abilities of lichen components. The findings show that *R. lacera* is in greater danger due to the lack of photoreactive components involved in UV-B radiation compared to *R. maciformis*. *R. lacera* has been found to be less at risk from intense air pollution and severe UV-B radiation increase than *R. maciformis*. With the data obtained as a result of Garty's study [52] and our study, it was confirmed that both epiphytic lichen species that belong to *Ramalina* genus are a good bioindicator organism in determining the genotoxic effect.

5. CONCLUSION

This study determined that the *R. farinacea* lichen sample can be predicted as a key organism to monitor the genotoxic effect at the molecular level against different stress sources. It is the first study to determine the genotoxic effect by using *R. farinaceae*, which is a cheap, more easily obtainable, useful biological organism to determine the negative effects of environmental pollutants. However, the results of the study need to be investigated in detail with the use of advanced molecular biological methods.

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Declaration of Competing Interests The authors declare no conflict of interest.

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