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DNA methylation in bryophytes as a biomarker for monitoring environmental pollution

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Rapidly growing industrialization and increased need for transportation have led to environmental pollution, particularly heavy metals. Efficient monitoring would help planning effective strategies to curb such increasing pollution. In this context, we studied the epigenetic changes in the bryophyte Greater Fork-moss, *Dicranum majus* Turner so as to use to monitor the environmental stress conditions due to accumulation of heavy metals and toxic organic compounds. The hypothesis is that the DNAm (DNA methylation) signatures reflect changes in the environmental conditions, and thus could serve as an alternate monitoring tool to study environmental pollution. The vegetative form of *D. majus* was collected from two different geographical locations where one was near the main road (MR) and another in the forest area (FS). DNAm rate was found 10.41±2.009 and 23.37±2.94 in MR and FS, respectively (P <0.005). The only difference between the two samples were traffic related pollutants. Thus, the reuslts suggest that vehicle pollution induces epigenetic changes in bryophytes, particularly DNA methylation, and could serve as a valuable biomarker to assess pollution risk due to vehicle traffic.

Keywords: Abiotic stress, Bioindicator, Bryomonitoring, Epigenetic, Greater Fork-moss, Heavy metal, 5-Methycytocine, Moss, Vehicle pollution

Plants respond to diverse environmental conditions without changing the DNA content by epigenetic modifications that control gene expression by histone methylation, histone phosphorylation, lysine methylation, and genomic imprinting. In light of our knowledge today, it shows that these mechanisms are involved in almost every aspect of plant life, including critical agricultural traits such as flowering time[1-3], fruit development[4-6], responses to the environment[7-12] and plant immunity[13,14]. DNA methylation is one of the mechanisms by which plants regulate gene expression at both transcriptional and translaslational levels under non-optimal conditions. Both environmental and genetic stimuli are known to alter methylation. Several lines of evidence suggest that DNA methylation is a potental biomarker that evaluates both toxicity assessments and biotic/abiotic stress conditions on plants[10,12,15-17].

Bryophytes are widely preferred in biomonitoring surveys because of their high specificity and sensitivity; however, none of the studies were performed to analyze stress-related epigenetic changes. Lacking roots and a vascular system, they draw water from all surfaces and accumulate more toxic compounds than flowering plants. The use of mosses to monitor atmospheric accumulation has been widely used in Europe since 1970. One of the favourable moss genus for biomonitoring is *Dicranum* Hedw. comprising 95 species considered legitimate by MBG (2019), mainly distributed across the holarctic. *D. majus*, commonly called as Greater Fork-moss, is one of the most prominent members of the genus, both on humid to wet soil and on the ground rocks, in the forest, and in more open habitats. According to these properties, they are widespread all over the world.

In this study, we tried, for the first time, to illustrate the epigenetic alterations in bryophytes in response to environmental stress. Thus, we evaluated the link between the DNA methylation profiles of *Dicranum majus* in two different environmental conditions: one on the main road side, and another in the forest area away from vehicle traffic. We compared the effects of anthropogenic sources and transport pollution on global methylation levels between samples from such different conditions.

Material and Methods

Sample collection and preparation

The process of selecting and collecting the vegetative form of *Dicranum majus* was performed

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from two different geographical locations in Çan, Çanakkale, in Turkey. Two different samples were collected in each sampling area, and the global methylation experiment was performed triplicate for each sample. The location of the samples was 40°0'39"N attitudes, 27°0'52"E, and 27°0'47"E longitude for the main road and forest area, respectively (Fig. 1). We randomly selected each sample, all taken at the same altitude above sea level. The climatic conditions were the same for all samples. Therefore, the climatic factors affecting our study are excluded in that way. The samples were apart, approximately 50 and 70 m in the main road (MR) and the forest site (FS), respectively. The samples' location is illustrated in ArcGIS-Arc Map 10.4.1(©2015 ESRI, Digital Globe). Both *D. majus* samples were transferred to our laboratory into a large inner pocket, with a small pocket inside containing each bryophyte species in a specific place. All samples were air-dried at room temperature (approx. 20°C) in shadow, then pulverized and kept at +4°C until DNA extraction.

DNA extraction

The DNA isolation was performed by the method described by Sika and colleagues previously[18]. Briefly, 50 mg of dried samples were dissolved in 1200 µL extraction buffer (1% SDS, 0.5 M NaCl) for homogenization. This step has been followed by removing supernatant after centrifugation of homogenate (13,500 rpm, 4 min at room temperature). The supernatant was resuspended with an equal volume of isopropanol and replaced on ice for 5 min. The supernatant was removed after the mixture was centrifuged again (13,500 rpm, 4 min at room temperature). Next, the DNA pellet was resuspended with 70% ethanol and removed by centrifugation (13,500 rpm, 2 min at room temperature). The DNA pellet was air-dried for a while and then dissolved in dH_2O. The genomic DNA was quantified using a spectrophotometer and stored at –20°C.

Global DNA methylation analysis

According to the manufacturer's instructions, the global DNA methylation profile was determined by an ELISA-based commercial kit (5mC DNA ELISA Kit (Zymo Research, D5326). Briefly, 200 ng of genomic DNA and calibrators were adjusted to 100 µL by 5- mC coating buffer, then denatured at 98°C for 5 min. After all, samples were immediately placed on ice. The denatured DNA samples were transferred to the well strips and incubated at 37°C for 1 h. After the incubation period, all wells were washed thrice by 200 µL 5 mC ELISA Buffer. The reaction was followed by adding a total of 100 µL antibody mix to each well, and again one-hour incubation was performed. At the end of incubation, all weels were washed (by 200 µL of 5mC ELISA buffer). Then 100 µL HRP developer was added to start color development, and that reaction was analyzed by spectrophotometer within 30-60 min. The colour development was measured at 405 nm absorbance using Microplate Spectrophotometer (Multiscan-GO,Thermo Scientific). Calibrators generated the standard curve with a known methylation profile.

Statistical analyses

All data were analyzed by IBM SPSS (version 18) and PRISM-Graphpad (version 9.0) software package. All data were represented as mean ± standard error of the mean (SEM), and P <0.05 was considered significant. The second-order logarithmic regression was used to determine the 5mC percentage depending on absorbance.

Results

The sampling period of moss was determined to depend on the growth cycle of the mosses. The meteorological parameters for Canakkale were obtained from meteorological measurement station records of Turkey's General Directorate of Meteorology (TUMAS 2020). The real-time

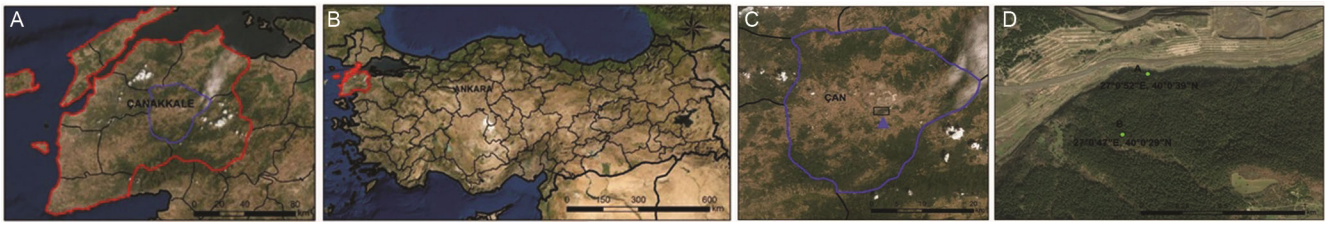

Fig. 1 — The global methylation status is compared between two locations to evaluate if the traffic-related pollutants affect methylation status. (i) Showing the location and areal ratio of the town (Çan) in Çanakkale. (1.25 times zoom of B); (ii) City's location on Turkey; (iii) location of the collection area within the town (14.4 times zoom of A); and (iv) The sampling area of *Dicranum majus* on the main road (A) and inside of the forest (B) at Çan, *Turkey* (20 times zoom of C).

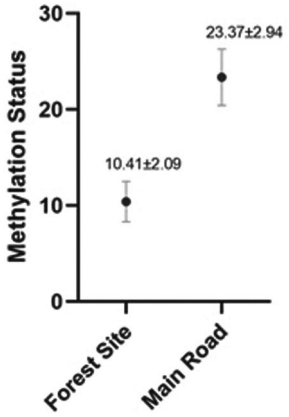

Fig. 2 — The global DNA methylation status of two samples from the forest site and main road. The difference between the samples is clearly shown that traffic-related pollutants change epigenetic marks.

temperature results were recorded at sampling time. The soil and air temperatures in April were 14.3°C and 23.4°C, respectively. The average of air temperature were estimated 12.5°C (min/max: 8.2°C /17.9°C).The relative humidity was determined as 7.5% and 56% in soil and air, respectively. The average sun duration was estimated at 7.2 h.

The global methylation status was found statistically different in the two sample sites. The samples were obtained in two different locations to emphasize the hypothesis. The first sample was picked from the landed area near the main road (MR), while another sample was taken from the gate of the forest site (FS).The global methylation rate (median level of 5mC and 5mC%) for MR and FS samples was 10.41±2.009 and 23.37±2.94, respectively (Fig. 2).

Discussion

These results can be explained with the following potential mechanisms: stress reactivity alters global methylation to enhance the adoption of new stress conditions. Global methylation, especially on C (cytosine) base, which occupies specific positions in the DNA molecule, is the best-understood mechanism among epigenetic regulations. The current literature knowledge indicates cytosine methylation is related to many genetic functions, including DNA replication and repair, gene transposition and transcription, cell differentiation and gene silencing, imprinting, bio-defense, transgene expression, and foreign gene expression in the cell[19-22].

As noted earlier, under abiotic stress, the different varieties usually show inherited phenotypic variation at the DNA methylation level accompanied by an epigenetic mechanism. That way controls abiotic stress tolerance through epigenetic alterations during plant breeding programs. Abiotic stress can cause an increase or decrease in cytosine methylation throughout the genome at the specific locus[23] together with stress-induced changes able to inherence to new generations epigenetically[16,24,25]. One of the first examples is that drought stress causes cumulative effects on two rice varieties and triggers drought resistance by epigenetic inheritance[26]. Moreover, none of the studies analyzed the stress factor and DNA metylation level in bryophte. Considering this, we suggest that the increase in global DNA methylation activity and the involvement of new pathways as a response to stress depend on methylation.

Mosses have been used as passive bioindicators of their ubiquitous geographical distribution[27], high cation exchange capacity[24], and accumulation rate of metals during biomonitoring studies studies[28-31]. Hence, we chose bryophytes for biomonitoring and checked the pollutant depending on the global methylation level. The bryomonitoring technique has wide applicability depending on two essential properties of mosses. First is the pollutants' accumulation capacity, and thereby binding of trace metals and toxic compounds prevents cumulative accumulation on both higher plants and environments. To give an excellent example of this from the field, Zechmeister *et al*.[32] recommended using mosses as a cost-effective alternative to technical particle filters to prevent CO_2 and NO_2 emissions depending on traffic in tunnels[32].

Secondly, a powerful indicator of long-term pollution control and management is the efficient uptake of pollutants and reflection of metal concentrations in moss tissue[31]. The accumulated metal composition of the bryophyte is used to evaluate the pollution status, but all of these data are the results of cumulative results during the years[27,28,32].

Although the bryomonitoring technique has been used for many years in our country and Europe, the main limitation is the determination limits to contaminants' threshold value[31]. Together, the bryomonitoring technique lacks equivalent results; thus, detection/correction methods are required[28]. Thus, none of the current methods for environmental monitoring can show an early response to environmental stressors. Also, bryomonitoring often

considers species widely distributed in the geographic area of interest and ignores possible species differences in tolerance to elements or compounds.

In another limitation of the classic biomonitoring technique, the heavy metal content of the moss depends on environmental conditions[29], the ion exchange capacity, hydro capacity, and the surface area of the moss tissue[30]. The tick cell wall and higher protoplast-to-wall ratio help to accumulate more heavy metals, which is helpful for bioremediation but not for monitoring.

Several lines of evidence suggest that DNA methylation is a potential biomarker that evaluates toxicity assessments. However, none of the studies was performed on the hypothetical relationship between the DNA methylation status of mosses in biomonitoring. Our alternative and novel epi-bryomonitoring technique, bryophyte type, or tissue-related properties (surface tickness or area of the moss tissue) never limitate the results.

In the literature, two common moss species, *Hylocomium splendens* and *Pleurozium schreberi* are widely used for aero-monitoring. *Isothecium stoloniferum* was also reported as effective as common moss species recently[33]. Along with *Hypnum cupressiforme*[27], other bryophytes such as *Marchantia polymorpha*[34], *Pottia truncata*, *Dicranella heteromalla* and *Bryum argenteum* have been reported to accumulate high concentrations of Cd, Cr and Zn[35]. In contrast, all bryophyte species are suitable for epi-bryomonitoring because this technique is a natural reflection of the organisms. In other investigation techniques, it is essential to know the contaminant type in the environment. Aside, the contaminant must be reached to a threshold value to determine. Global methylation status is a natural reaction of biological organisms and reflects the environmental pollution stress response. Thus, the pollution type or passing threshold value for determination lack importance in epigenetic monitoring.

Overall, in this study, we compared effects of anthropogenic sources and transport pollution on global DNA methylation levels between two samples taken from relevant sites. The results of our data clearly show that epigenetic alterations occur due to pollution, and global methylation status may be used to evaluate the risk level. This study is possibly the first approach to demonstrate epigenetic-based bryomonitoring approach, "epi-bryomonitoring" for monitoring environmental conditions.

Conclusion

In this study, we hypothesized that if DNA methylation status is related to stress factors, environmental stress induces hypo/hypermethylation, and these changes may be a valuable tool for monitoring the abiotic stress caused by vehicle emissions. Our result has shown that road traffic emissions alter the DNA methylation status of the *Dicranum majus*. This study is possibly the first attempt to use the DNA methylation state of mosses as a biomonitoring tool. Therefore, the methylation status could be a suitable replacement for the classic bryomonitoring tool. Global methylation status changes two-fold, which means aggressive alterations depend on the pollutant — this quick response to traffic-related pollutants such as vehicle exhausts, vehicle emissions, and non-combustion. Overall, epigenetic monitoring is an early monitoring tool and reduces costs for government agencies or local institutions.

Conflict of Interest

Author declares no competing interests.

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