Ecotoxicology and Environmental Safety 212 (2021) 111965

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Impact of particulate matter on primary leaves of *Vigna radiata* (L.) R. Wilczek

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ARTICLE INFO

Edited by: Professor Bing Yan

Keywords:
Chlorophyll
Epicuticular wax
Particulate matter
SEM
Vigna radiata

ABSTRACT

Particulate matter (PM) pollution is of great concern for human health and vegetation. In this study, we investigated the impact of PM on primary (unifoliate) leaves of *Vigna radiata* (L.) R.Wilczek by exposing leaves' adaxial surface to PM. Leaves exposed to PM showed accumulation of various metal(loid)s even after removal of epicuticular wax (EW) revealing that the metals/metalloids could penetrate through the cuticular barrier. Scanning electron microscopic studies revealed that even after thorough washing with water, a significant amount (~55%) of particles were retained on the leaf surface. Leaves did not show any particles on their surface post EW removal, revealing that particles adhered to EW. Exposing primary leaves to PM did not alter their size but gave rise to smaller sized trifoliate leaves. A decline in Chl *a/b* of PM-exposed primary leaves suggested that PM cause a shading effect on leaves. PM-exposed primary leaves also showed a decline in sugar levels. However, the trifoliate leaves did not show any variation in Chl *a/b* as well as sugar levels. Our findings furnish evidence for the negative effects of PM on plants and a probable dietary exposure of humans to PMs, warranting more in-depth studies on the potential risks of PMs in agricultural sector.

1. Introduction

Air pollution is one of the many immediate global anthropogenic threats that living beings face. Air quality is defined by six prominent criteria pollutants, namely, carbon monoxide, lead, photochemical oxidants (including ozone), nitrogen oxides, sulfur oxides, and PM (EPA, 2019). Of the various air pollutants, PM is of particular concern because of its extreme toxicity to humans; it affects humans more than any other air pollutants (WHO, 2019). Various epidemiological studies have suggested a link between PM and ill health effects which include aggravated chronic respiratory and cardiovascular diseases, impaired lung function, and premature mortality (Shaughnessy et al., 2015). PM exert its toxicity through a synergistic effect of oxidative stress and inflammation via the induced formation of reactive oxygen species and the redox-sensitive signaling pathways, respectively (Michael et al., 2013). The severeness of the effects of PM on human health varies with size, $PM_{2.5}$ being the most toxic (Gu et al., 2012; Kim et al., 2015; Du et al., 2016).

Owing to the detrimental effects of PMs on human health, it is therefore of utmost essentiality to curb PM pollution. PM pollution can be prevented by either controlling the emission sources or removing PM from the air via chemical methods, wet and dry deposition (Hirabayashi and Nowak, 2016). Numerous studies have proven the efficiency of different types of vegetation in effectively removing PMs via deposition onto leaves (Ottelé et al., 2010; Sæbø et al., 2012; Dzierżanowski et al., 2011; Przybysz et al., 2014a; Sgrigna et al., 2015; Chen et al., 2017). Hence, tree plantation and construction of greenbelts are considered as a sustainable approach to complement air pollution mitigation (Chen et al., 2017). However, the capacity of plants to accumulate PM varies from species to species and is more pronounced in (i) broad-leaved tree species with rough surface than those with smooth surface, (ii) conifers than the broad-leaved trees, and (iii) pubescent leaves than hairless ones (De Nicola et al., 2008; Dzierżanowski et al., 2011; Sæbø et al., 2012).

Despite abundant research on the quantitative aspects of the extent of PM accumulation in different plants/trees, there has not been any emphasis laid on the effects of PM on plants/trees (Ottelé et al., 2010;

Abbreviations: Chl, chlorophyll; DNSA, dinitrosalicylic acid; EDS, energy dispersive spectroscopy; EW, epicuticular wax; ICPMS, inductively coupled plasma mass spectrometer SEM, scanning electron microscope.

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https://doi.org/10.1016/j.ecoenv.2021.111965
Received 21 November 2020; Received in revised form 27 December 2020; Accepted 20 January 2021

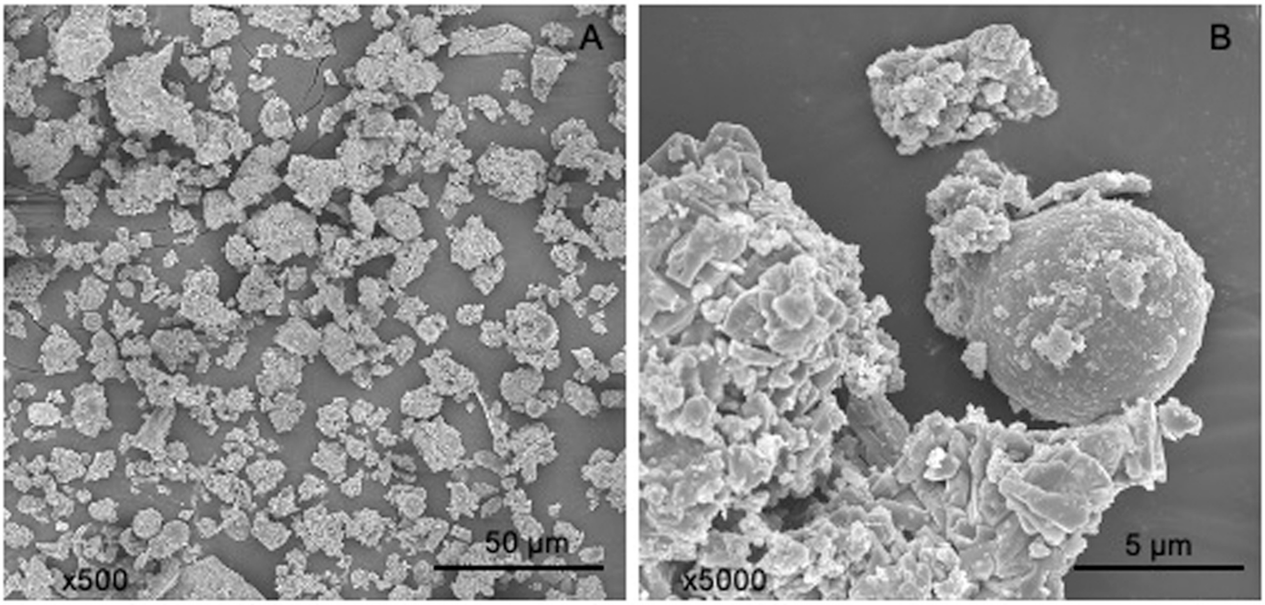

Fig. 1. The SEM pictures of PMs used in the exposure study with primary leaves of *Vigna radiata*.

Sæbø et al., 2012; Dzierżanowski et al., 2011; Przybysz et al., 2014a; Sgrigna et al., 2015; Chen et al., 2017). Popek et al., (2017, 2018); Przybysz et al. (2014b) reported negative effect of PM deposition on photosynthetic efficiency of leaves of the woody trees. Nevertheless, there are numerous reports of foliar exposure of dust or nanoparticles or ions or other non-gaseous pollutants and their subsequent negative effects on plant physiology (Walker and Everett, 1987; Hirano et al., 1995; Kumar et al., 2008; Kuki et al., 2008; Saunders and Godzik, 1986; Hong et al., 2014; Larue et al., 2014b). Extensive research has been done to study the fate of transport, speciation and accumulation of ions/nanoparticles/solutes inside the leaves and other parts of plants upon foliar exposure (Eichert et al., 2008; Larue et al., 2014a; Hong et al., 2016; Rossi et al., 2019). However, similar studies have not been reported for foliar exposure to PM. In this study, we investigated impact of PM on the primary (unifoliate) leaves of *Vigna radiata* (L.) R.Wilczek (Mung bean) by studying (i) role of epicuticular wax in retention of PM and the associated metal(loid)s on leaves, (ii) leaf morphology, and (iii) chlorophyll and sugar contents. *V. radiata* was chosen for this study as it is an important legume crop whose annual production is ~ 3 million tonnes and has high potential to grow under water stress and nitrogen deficient conditions and improve the soil fertility (War et al., 2017).

2. Materials and methods

2.1. Plant material and experimental design

Seeds of *Vigna radiata* (L.) R.Wilczek (cv-SML-668) were procured from the Indian Agricultural Research Institute, New Delhi, India. Particulate matter (SRM 1648a - Urban Particulate Matter) were obtained from the National Institute of Standards and Technology (Department of Commerce, USA). PM powder was black in colour. The particles were irregularly sized (Fig. 1); they sized between 1.35 µm and 30.1 µm with the mean diameter of 5.85 µm. More details about the PM standard can be found at https://www-s.nist.gov/srmors/certificates/1648a.pdf. All the chemicals used in this study were analytical grade and were procured from Sigma -Aldrich (St. Louis, Missouri, USA).

Seeds were inoculated in pots containing soilrite soil mix (consisting of peat, perlite, and vermiculite at the ratio of 2:1:1) and kept in a growth chamber (25 °C, 16 h photoperiod, 120 µmol photons $m^2 s^{-1}$). 7-day old seedlings, with fully expanded primary leaves, were used for the PM-exposure study. A PM suspension of 10,000 mg L^{-1} (black in colour) was prepared in deionized water (pH 7.0) and sonicated before use. The PM-solution was drop-coated on the adaxial surface of primary leaves using a 20-µL micropipette. The total quantity of PM drop-coated on the leaves was 3 mg. Deionized water was used for control plants (untreated). Plants were kept in the plant chamber under the condition described above and watered daily with Hoagland media. Further investigations were carried with leaves after ~ 2 weeks of PM-exposure. Prior to any investigation, leaves were washed with deionized water using a soft brush and then sonicated in deionized water in a beaker for 30 s and rinsed thereafter several times.

2.2. Metal(loid) content in leaves

Leaves, with and without epicuticular wax, were dried in an oven at 90 °C for 24 h. Leaf samples were digested using HNO_3 for 10 min in a BUCHI Digest Automate K-438 (New Castle, DL, USA) at 100 °C. After cooling, the samples were diluted with deionized water and filtered using a GF/F glass microfiber filter (0.7 µm). To measure the quantity of metal(loid)s leached from PM, the suspension was prepared in distilled water (pH 7.0). After 7 days, the suspension was centrifuged at 15,000 x *g* and filtered. The samples were analyzed for metal(loid) content using an inductively coupled plasma mass spectrometer (Schimadzu ICPMS-2030, Kyoto, Japan). For quality control, readings of the blank as well as a standard were taken after the measurement of every batch of 10 samples.

2.3. Quantification of particulate matter accumulation on primary leaves

The protocol made by Dzierżanowski et al. (2011) was followed to quantify PM accumulation on the leaf surface. Leaves were washed as described above. To remove epicuticular wax, leaves were sonicated in chloroform for 30 s. The washed-off solutions were then filtered (0.7 µm) using a pre-weighed filter paper, which was then dried at 80 °C to a constant weight.

2.4. Scanning electron microscopic investigations

Leaves were washed as described above. The epicuticular wax of the leaves was removed following the protocol designed by Zeisler and

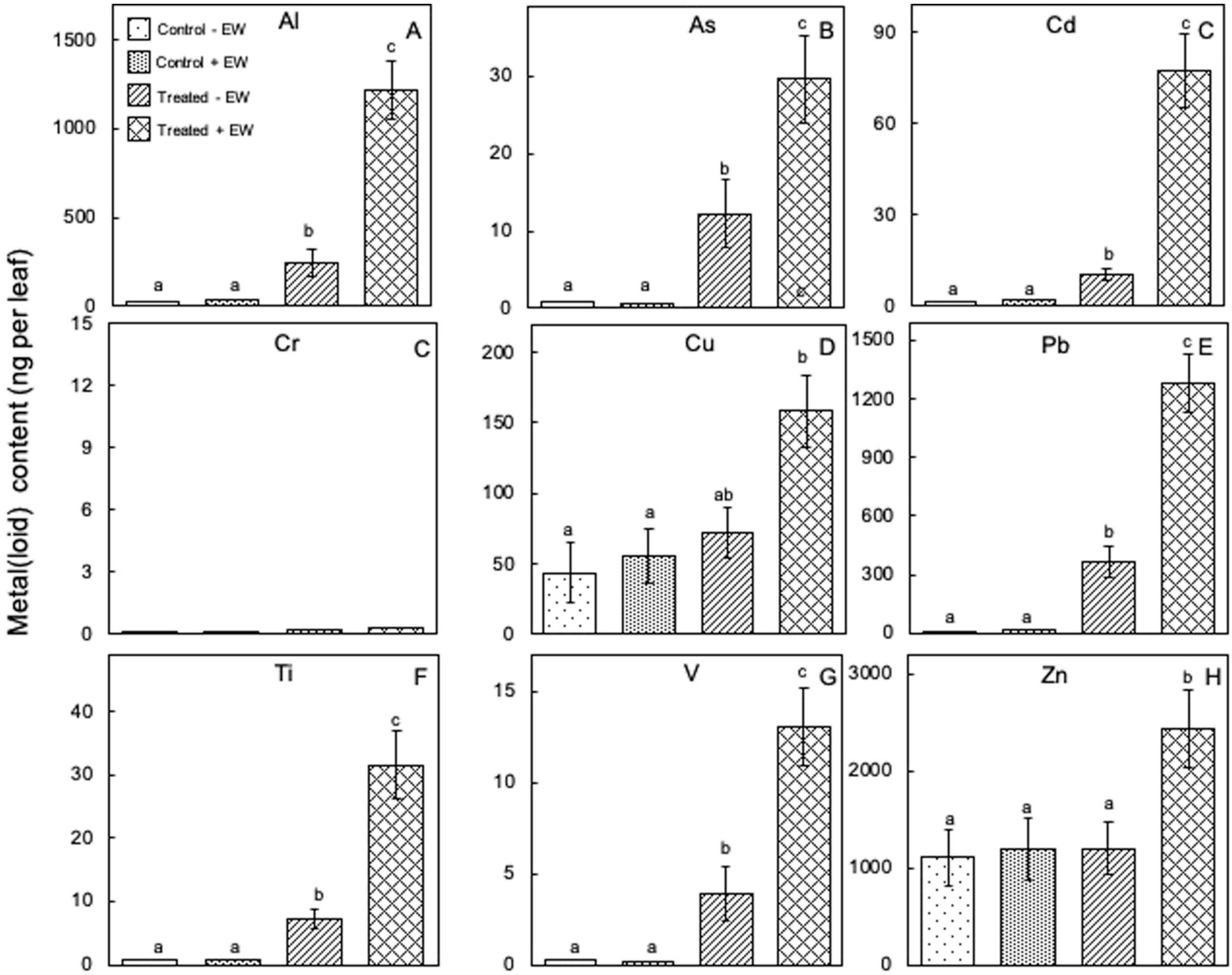

Fig. 2. The contents of various metal(loid)s in control and the PM-treated primary leaves of *Vigna radiata* before and after the removal of epicuticular wax (EW). The vertical lines on data bars represent standard errors. The different small letters above the bars depict that the values recorded for samples differ significantly ($P \leq 0.05$ level) (Duncan's multiple range test).

Schreiber (2016). Cellulose acetate (5% in acetone) was gently applied using a brush to completely cover the adaxial surface of the primary leaves. After the evaporation of acetone (~ 3 min), the strip of cellulose acetate was removed using a fine tweezer. Leaves, with and without epicuticular wax, were cut into small segments and air-dried. The dried leaf segments were coated with Pt-Pd using HITACHI MC1000 ion sputter (Hitachi, Tokyo, Japan) and then viewed under Hitachi S-8220 field emission scanning electron microscope (Hitachi, Tokyo, Japan) with associated hardware for energy dispersive spectroscopy (EDS).

2.5. Leaf morphology

After 14-d exposure, primary (unifoliate) and trifoliate leaves were removed. The leaves were scanned using a scanner, and the leaf area was calculated using the ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). Leaves were then weighed to determine the fresh weight. For the measurement of dry biomass, leaves were oven-dried at 80 °C to a constant weight.

2.6. Chlorophyll and sugar content

After washing of leaves, chlorophyll pigments were extracted and determined using the method and equations given by Arnon (1949). For the determination of sugar content, washed leaf samples were homogenized in 80% (v/v) ethanol in a chilled mortar and pestle and centrifuged at 1000 x *g* for 15 min at 4 °C. The supernatant was used for estimating sugars. For reducing sugar content, the protocol of Miller (1959) was followed. To 3 mL of sample, 3 mL of reagent containing DNSA (1%), NaOH (1%), phenol (0.2%) and sodium bisulphite (0.05%) were added. After boiling the contents for 15 min, 1 mL of Rochelle salt (40%) was added. Absorbance was recorded at 575 nm. For total sugars, the protocol proposed by Dubois et al. (1956) was followed. To 1 mL sample, 1 mL phenol (5%) and 5 mL of concentrated H_2SO_4 was added and mixed. After cooled, absorbance was recorded at 490 nm. D-glucose was used as the standard.

2.7. Statistical analysis

All experiments were repeated 4 times, each with triplicates. Data are presented as mean ± standard error. The level of significance between mean values of control and treated plants was determined using the Student *t*-test. To determine the level of significance between means of a single metal(loid) content of different leaf treatments, ANOVA was performed, followed by Duncan's multiple range test.

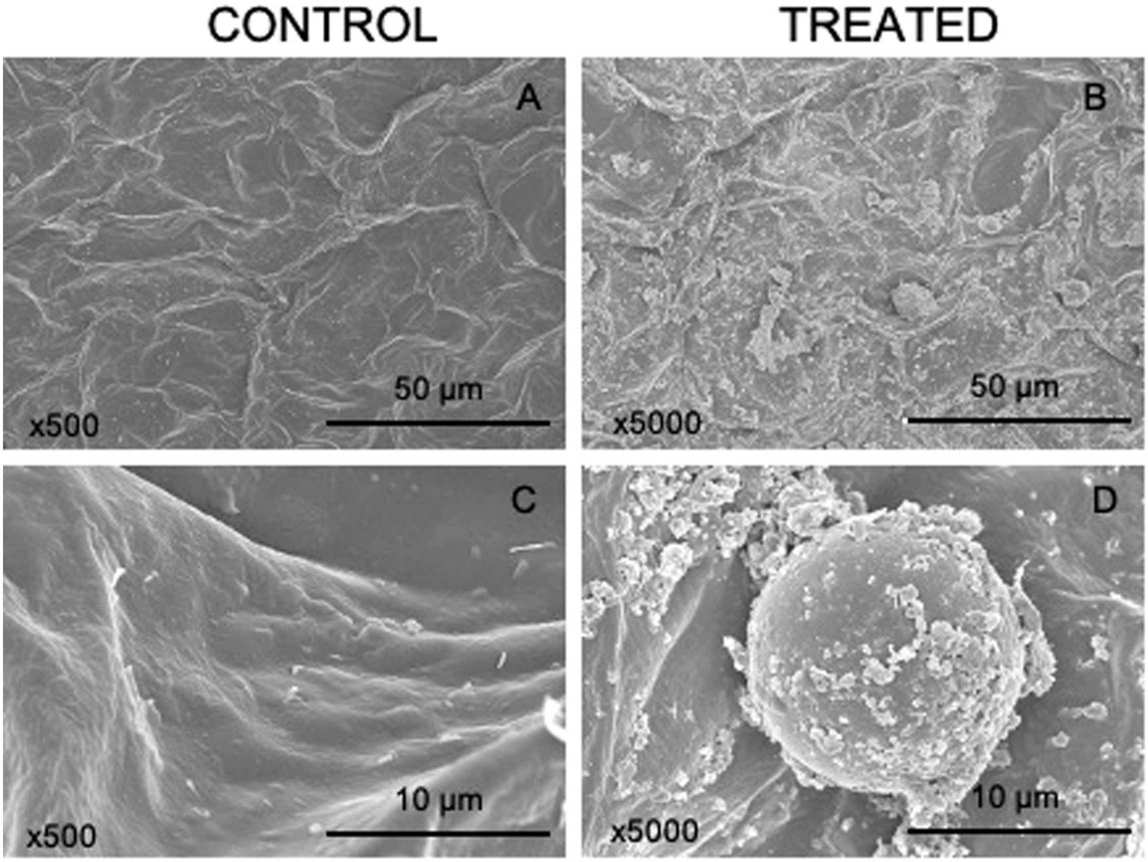

Fig. 3. Scanning electron micrographs of a portion of the adaxial surface of the primary leaves of *Vigna radiata* before (A, C) and after being exposed to PMs (B, D).

3. Results and discussion

3.1. Particulate matter induced accumulation of metal(loid)s in leaves

For assessing the metal(loid)s accumulation in leaves, the metal(loid)s of PM which are non-essential for plant growth and development were chosen (except for Cu and Zn because these were present in high quantities in PM). Primary leaves exposed to PM showed significant accumulation of all metal(loid)s tested (except Cr), compared to control (Fig. 2). The quantity of metal(loid)s accumulated in the primary leaves exposed to PM (in comparison with control) followed the order: Pb>Al>Zn>Cu>Cd>Ti>As>V. Various reports have mentioned the important role of waxes, particularly epicuticular wax (EW), on the surface of leaves in capturing the PM (Dzierżanowski et al., 2011; Chen et al., 2017). Epicuticular wax, present on the surface of the cuticle membrane of leaves (stem, and fruits also), provides resistance to water and heat stress, chemical penetration, high irradiation, pest and pathogen attacks, physical and mechanical forces, and various other biotic and abiotic stresses (Zeisler-Diehl et al., 2018). To understand the role of epicuticular wax in restricting the metal(loid)s (present in PM) uptake by leaves, the metal(loid) content was determined in leaves after the removal of epicuticular wax. Interestingly, a drastic decline was noted in the content of these metal(loid)s after the removal of epicuticular wax (Fig. 2). The amount of metal(loid)s retained post removal of epicuticular wax in these leaves was 38%, 20%, 50%, 45%,14%, 23%, 42%, 30%, for Pb, Al, Zn, Cu, Cd, Ti, As, V, respectively, compared to the values before removal of epicuticular wax. This data clearly shows that washing of the leaves does not necessarily remove the various metal(loid)s associated with PM. Moreover, the epicuticular wax, which is a barrier against the PM, does not work that effectively against the metal(loid)s which can be toxic to plants above threshold levels. Even in the presence of epicuticular wax, the metal(loid)s could effectively cross the cuticle barrier. These findings further add to the apprehensions of dietary exposure of humans to PM via leafy vegetables or fruits.

3.2. Role of epicuticular wax in particulate matter accumulation on primary leaf's surface

Scanning electron microscopic investigations showed the presence of particles all over the surface of primary leaves, even after repeated washings and sonication (Fig. 3). This clearly reveals that washing does not essentially remove all the PMs attached to the surface. In the present study, ~45% of the particles applied to leaves were washed off after sonification and rinsing with water; the rest 55% were still attached to the surface of the leaves. Similar findings were also reported in *Lactuca sativa* L., *Petroselinum crispum* and *Lolium perenne* L., grown in the courtyard of a secondary Pb smelter, where Pb particles were found on the surface after washing with deionized water (Schreck et al., 2012). In another study by Xiong et al. (2014), CdO and Sb_2O_3 particles were noted on washed leaves of *Spinacia oleracea* and *Brassica oleracea* after exposure to CdO and Sb_2O_3 PM. EDS analysis revealed that the particles accumulated on the surface of primary leaves of *V. radiata* were composed of various elements, Al, Cu, Fe, Si, As, Cd, Cr, V, and Ti being the major ones (Supplemental Information, Fig. S1). We believe that the particles seen on the surface of primary leaves after washing are, most probably, the ones adhered to the epicuticular wax.

To confirm if the particles retained on the surface of the primary leave after thorough washing are the ones trapped in the wax portion of leaves, SEM investigations were carried with leaves devoid of epicuticular wax. The scanning electron micrographs of the leaves after removal of epicuticular wax did not show any particles on the surface (Fig. 4E-H). These results clearly indicate that the epicuticular wax acts as an effective barrier against PM. Sæbø et al. (2012) reported a positive correlation between PM accumulation and wax content. In the same

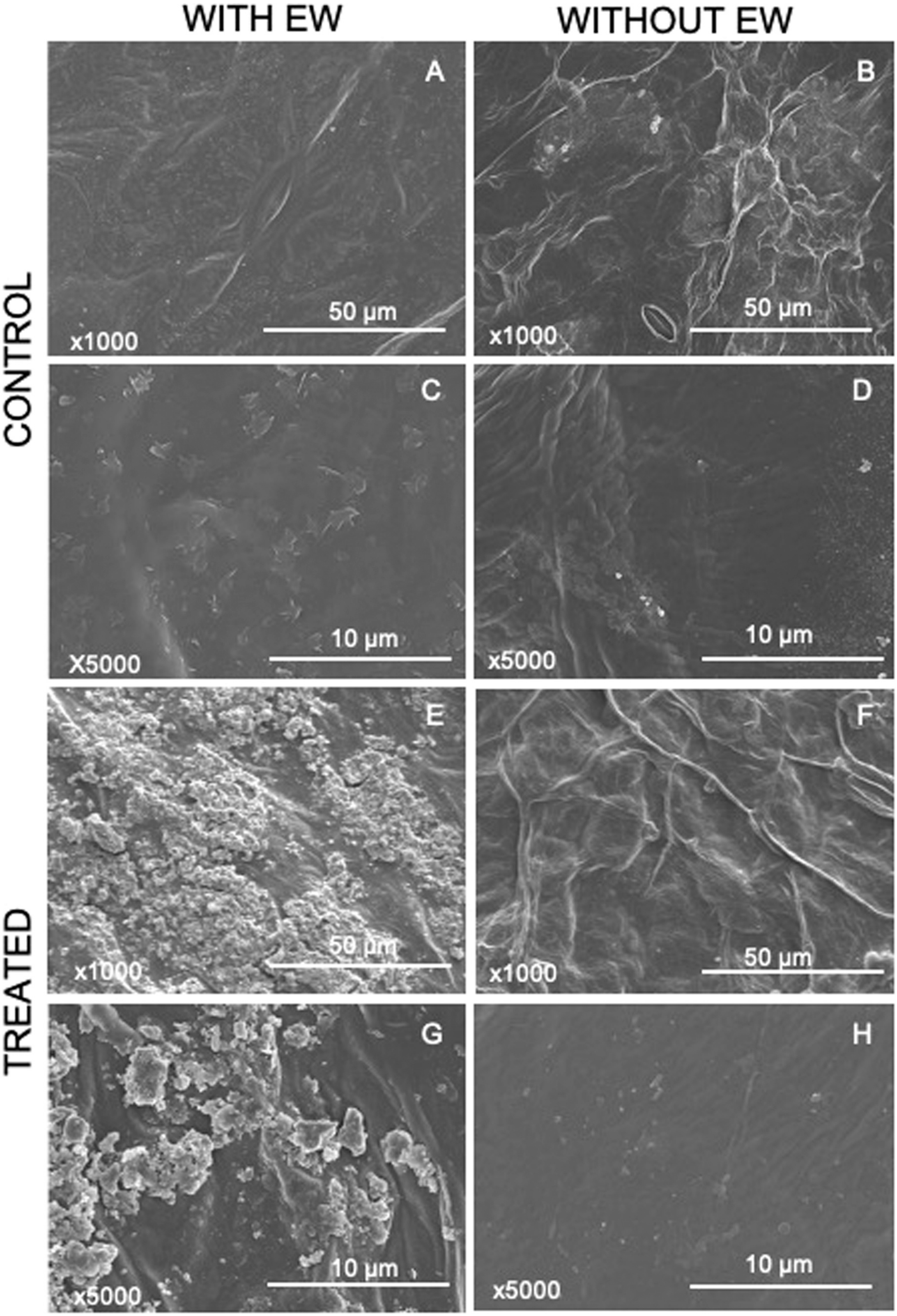

Fig. 4. Scanning electron micrographs of a portion of the adaxial surface of control (A-D) and the PM-treated (E-H) primary leaves of *Vigna radiata* before (A, C, E, G) and after the removal of epicuticular wax (EW)(B, D, F, H).

Table 1

Chlorophyll pigments in primary leaves and trifoliate leaves of *Vigna radiata* after exposure of primary leaves to particulate matter. Data is mean ± standard error. Values denoted by different small letters (in superscript) between control and treated leaves differ significantly at $P \leq 0.05$ (Student *t*-test).

	Concentration ($\mu g\ g^{-1}$ fresh weight)	Control	Treated
Primary leaves	Chl *a*	1136.5 ± 69.6^a	1206.7 ± 22.1^a
	Chl *b*	417.3 ± 35.6^a	647.1 ± 5.8^b
	Chl *a*+*b*	1622.8 ± 105.2^a	1854.9 ± 27.8^a
	Chl *a*/*b*	2.35 ± 0.04^a	1.86 ± 0.02^b
Trifoliate leaves	Chl *a*	1162.6 ± 155.7^a	1287.1 ± 62.3^a
	Chl *b*	330.9 ± 58.57^a	584.9 ± 45.6^a
	Chl *a*+*b*	1612.6 ± 214.2^a	1864.8 ± 102.5^a
	Chl *a*/*b*	2.59 ± 0.05^a	2.25 ± 0.12^a

study, these authors also reported the range of retention of PM by the wax portion of leaves to be as high as 82.6% in *Betula pendula* to as low as 25% in *Fagus silvatica* (Sæbø et al., 2012).

Various studies focused on the foliar application of metal nanoparticles have revealed the uptake of respective metal ions/nanoparticles by leaves (Hong et al., 2014; Larue et al., 2014a, 2014b). However, the mechanisms of such uptake have been poorly explained; both cuticular penetration and stomatal uptake were credited for the uptake of metal ions/nanoparticles (Schreck et al., 2012; Hong et al., 2014; Larue et al., 2014a, 2014b). None of these researchers took into account the role of epicuticular wax in restraining the metal ions/nanoparticles. In fact, we believe that the metal ions/nanoparticles accumulated in the leaves, as reported by these researchers, could be the ones restricted to the epicuticular wax of the cuticle. Eichert et al. (2008) reported that while particles of size 1.1 μm could never be detected in the leaves after foliar application, particles sized 43 nm occasionally penetrated inside the leaf through stomata. Moreover, the radii of the

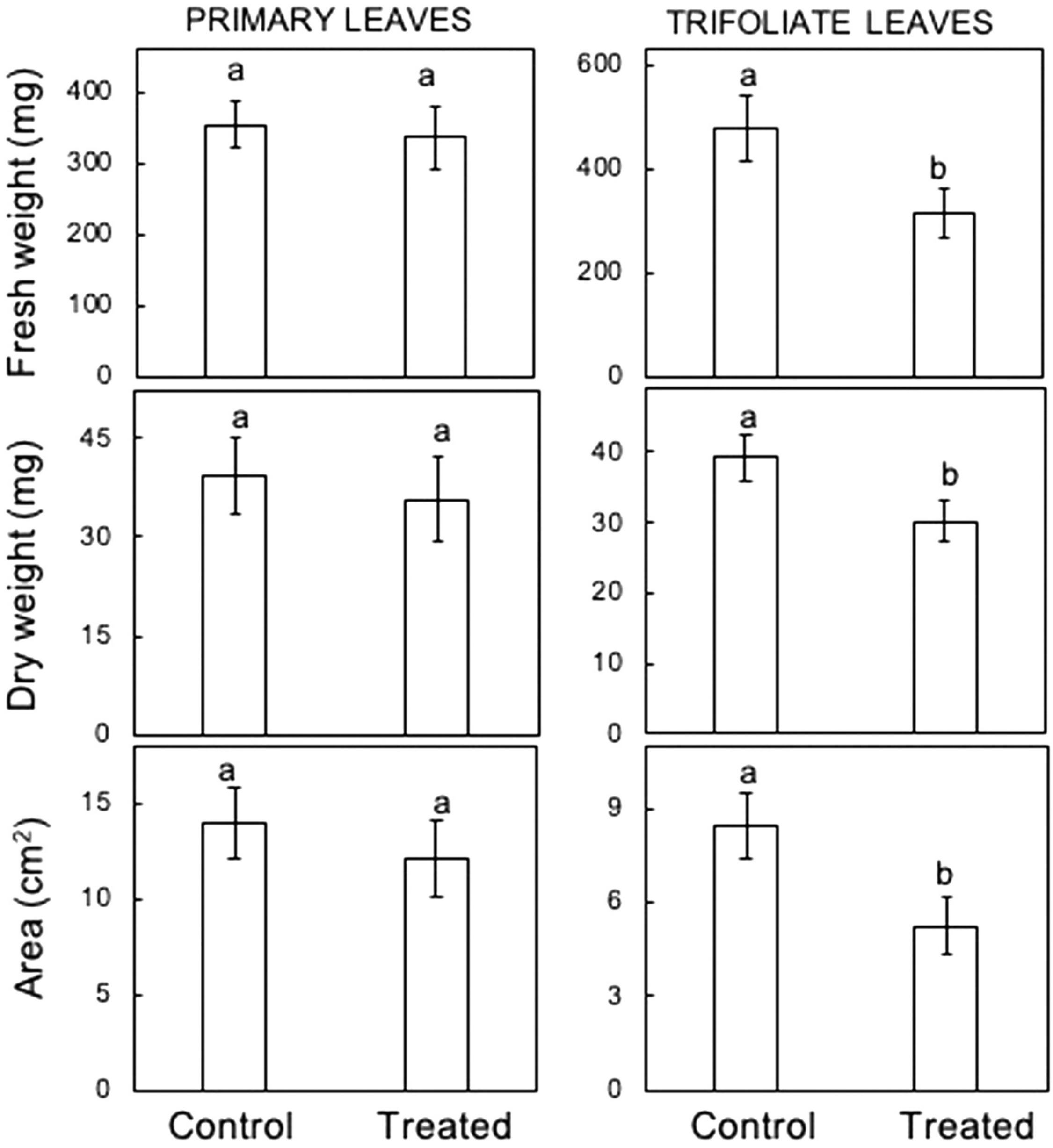

Fig. 5. The fresh weight (A, D), dry weight (B, E), and area (C, F) of the primary (A-C) and trifoliate (D-F) leaves of *Vigna radiata* after the exposure of the primary leaves to PMs. The vertical lines on data bars represent standard errors. The different small letters above the bars depict that the values recorded for control and the treated samples differ significantly ($P \leq 0.05$ level) (Student *t*-test).

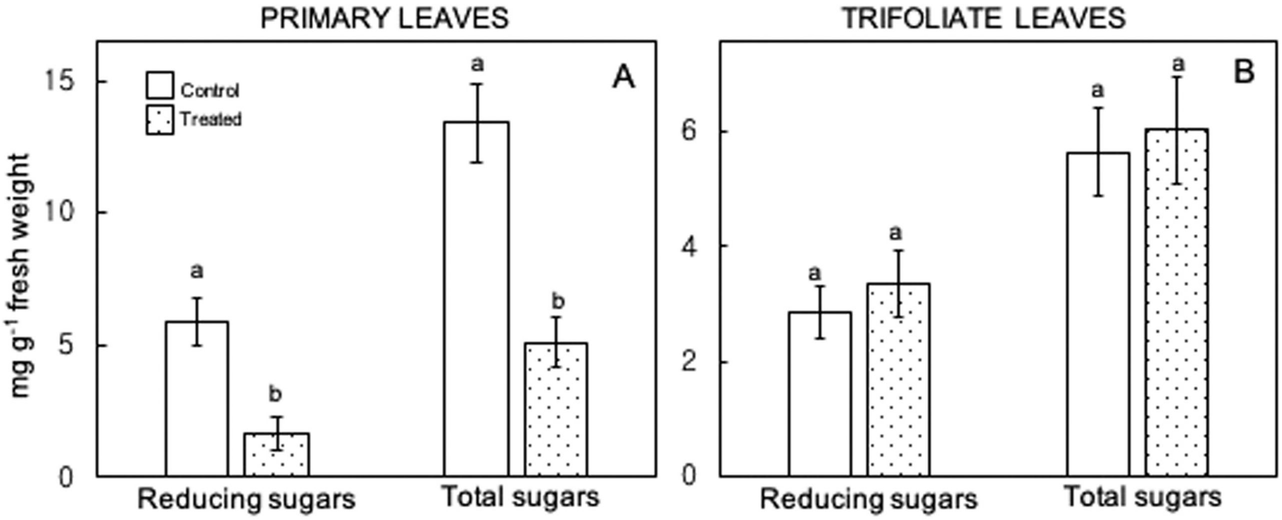

Fig. 6. The reducing sugar and total sugar contents of the primary (A) and trifoliate (B) leaves of *Vigna radiata* after the exposure of the primary leaves to PMs. The vertical lines on data bars represent standard errors. The different small letters above the bars depict that the values recorded for control and the treated samples differ significantly ($P \leq 0.05$ level) (Student *t*-test).

aqueous pores in the cuticle that allow transport of hydrophilic substances ranges between 0.6 and 4.8 nm, thus restricting the penetration of substances larger than 4.8 nm. In the present study, the particles (1.35–30.1 μm) are too large to penetrate the leaves via either cuticular pathway or stomatal pathway. Since no particles were observed on the leaf surface after the removal of epicuticular wax, we believe that it were the ions of the metal(loid)s that were able to penetrate through the cuticle surface. Various metal(loid)s were leached out from the PM in the suspension (Supplemental Information, Table 1). We believe that the ions of these metal(loid)s that were leached out from the PM were able to cross the cuticular barrier. Hence, although the epicuticular wax can restrict the PM, it does not work effectively to restrict the metal(loid) ions uptake by the leaves.

3.3. Effect of particulate matter on leaf morphology

The exposure of primary leaves of *V. radiata* plants to PM did not result in any alteration in their fresh weight, dry weight, and area. However, exposure of primary leaves to PM caused a marked decline in the size of the first trifoliate leaves. The fresh weight, dry weight, and surface area of the trifoliate leaves reduced by ~34%, 23%, and 37%, respectively (Fig. 5). The delcine in the first trifoliate leaves' area has been reported in *V. radiata* plants under salinity (Wignarajah et al., 1975). Similar findings have also been reported in *Glycine max* plants exposed to shade or low light conditions (Wu et al., 2017). Researchers have postulated that the development of new leaves is dependent on the light environment of the mature leaves. This signal from the mature leaves to the newly developing leaves is called the systemic irradiance signal (Wu et al., 2018). In leaves of *Viburnum tinus*, black coloured dust caused shading thus reducing the utilization of the incident light and causing decline in overall photosynthetic efficiency (Thompson et al., 1984). Similarly, Hirano et al., (1995) also reported reduced photosynthetic efficiency in leaves of *Cucumis sativus* and *Phaseolus vulgaris* due to dust induced shading effects. The PM used in this study, which was black in colour, can thus reduce incident light on the surface of leaves thus negatively affecting photosynthetic capacity and sugar content which all together decrease the size of the trifoliate leaves.

3.4. Effect of particulate matter on photosynthetic pigments

Exposure of PM to primary leaves did not cause any significant change in the Chl *a* and Chl *a*+*b* levels, compared to control. However, a marked increase was noted in Chl *b* (~33%) and a decrease in Chl *a/b* ratio (~20%) of primary leaves exposed to PM compared to the control leaves (Table 1). Nanos and Ilias (2007) also reported a decline in Chl *a/b* ratio due to shading effects of inert dust on leaves of *Olea europaea.* Exposure of primary leaves to PM did not result in any significant change in Chl *a*, Chl *b*, Chl *a*+*b,* and Chl *a/b* ratio of the trifoliate leaves, compared to control. A low Chl *a/b* ratio corresponds to shade adapted features of leaves (Shabnam et al., 2015; Shabnam and Pardha-Saradhi, 2016; Porra and Scheer, 2019). This suggests that the exposure of PM (which is black in colour) to the adaxial surface of primary leaves reduces the incident light and causes a shading effect, which results in a decrease in Chl *a/b* ratio. The alterations in Chl *a/b* ratio reflects the potential of chloroplasts to optimize their photosynthetic efficiencies to fluctuating light intensities by regulating (i) the amount of photosystem (PS) II to PSI; and (ii) the size and amount of Chl composition of the light-harvesting complex of each photosystem (Porra and Scheer, 2019). This suggests that the low Chl *a/b* ratio in primary leaves exposed to PM is a mechanism of the leaves to maximize the absorption of light under conditions of PM-induced shading effects.

3.5. Effect of particulate matter on sugar levels

PM exposure to primary leaves caused a significant decline in both reducing (~71%) and total soluble sugars (~63%), compared to the control (Fig. 6A). However, the levels of sugars remained unaltered in trifoliate leaves of *V. radiata* after exposure of primary leaves to PM (Fig. 6B). A similar finding was also reported in *Phaseolus vulgaris* plants where carbohydrate content of primary leaves decreased upon shade treatment, but the same in trifoliate leaves were unaffected (Araya et al., 2008). Carbohydrates are the direct products of photosynthesis- light and dark reactions. The reason behind PM-induced decline of the sugar content of primary leaves can be attributed to the reduced photosynthetic efficiency of primary leaves due to shading, thus generating less reducing power and/or disruption of dark reactions of photosynthesis due to less availability of reducing power. Heavy metals have been reported to decrease the sugar levels in *Pistia stratiotes*, *Spirodela polyrrhiza*, *Eichhornia crassipes*, *Lagerstomia speciosa* and *Acacia moniliformis* (Mishra and Tripathi, 2008; Gupta et al., 2011). Thus, we believe that heavy metals accumulated in the leaves as well as shading induced decrease in photosynthetic efficiency, could be responsible for decline in sugar content in the leaves thus giving rise to smaller sized trifoliate leaves.

4. Conclusions

In the present investigations, we studied the impact of PM on primary leaves of *V. radiata*. Our findings revealed that even after rigorous washing with water, the surface of leaves could retain ~ 55% of the applied PM, which was attributed to epicuticular wax. Primary leaves exposed to PM showed a significant amount of metal(loid)s even in the absence of epicuticular wax, suggesting that they could cross the cuticular barrier. PM exposed primary leaves gave rise to smaller sized trifoliate leaves. Exposure of PM to primary leaves decreased the sugar content and Chl *a/b* ratio suggesting that PM exposure caused a shading effect on the leaf surface. These findings raise a concern for the harmful impact of PM on crop plants as well as dietary exposure of humans to PM, especially in leafy vegetables and fruits. These findings would call for better analysis and evaluation of the risk of PM in the agricultural sector.

CRediT authorship contribution statement

Nisha Shabnam: Investigation, Formal analysis, Writing - original draft. **Joosung Oh:** Methodology, Resources. **Sangwon Park:** Data curation. **Hyunook Kim:** Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was funded by the Ministry of Agriculture, Food and Rural Affairs, Korea Govt.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.111965.

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