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Microscopic Observation of Acid Rain Induced *Bacopa monnieri* L.

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Abstract

Acid rain (AR) has been reported to induce stress in plants affecting its productivity, growth, flowering and physiology. The molecular changes induced in plants due to the effect of acid rain or acid induced orientation or chloroplast streaming remains largely unknown. Therefore, in the current study we report for the first time the static and permanent changes in the cell of the medicinal plant *Bacopa monnieri* L. due to sulphur-simulated acid rain (S-SiAR). AR induced effects witnessed by the reduction of the size of starch granules and chloroplast, amount of the granules per unit area, dissolving cell walls, breaking the normal fiber, salt-induced strain in the various components of the cell. Effect of starch granule and chloroplast due to S-SiAR was analyzed using light, confocal and scanning electron microscopic techniques. The elements viz. potassium and magnesium present in the chloroplasts reveal acidic pH due to effect of S-SiAR observed by the ionization of Mg and K (to Mg^{2+} and K^{+}), in which K^{+} induced by the effects of S-SiAR revealed a net negative Nernst potential of about −87.55 mV. Calcium is mainly present on the cell walls and responsible for binding of starch granules become ionized to Ca^{2+} on interacting with AR indicated by the altered Nernst potential of +137.04 mV. A net potential difference may cause the above streaming of chloroplast towards the large starch granules. From this study, we report AR-induced physiological changes in medicinal plant *Bacopa monnieri* L. for the first time.

Keywords

Simulated Acid Rain, *Bacopa monnieri*, Chloroplast, Starch, Microscopy, Granules, SEM, pH, LM

1. Introduction

All living things, whether terrestrial, aquatic, arboreal plants or herbs, animals or birds, are affected directly or indirectly by acid rain (AR). The AR also has an adverse impact on medicinal herbs just like other plants. It is because AR-induced changes in the cellular biochemistry and physiology of the whole plant. Biological effects of acid deposition on plants [1]-[10], are numerous and complex, and they include visible symptoms of injury (chlorosis and/or necrosis), invisible effects such as reduced photosynthesis, nutrient loss from leaves, altered water balance, and variation of several enzyme activities.

Bacopa monnieri (*B. monnieri*) has been used in traditional Indian system of medicine, the *Ayurveda*, for the treatment of anxiety, and for improving the intellect and memory for several centuries and finds importance as a medicinal plant. In addition to memory boosting activity, *B. monnieri* is also used in the indigenous system of medicine for the treatment of cardiac, nervous, respiratory and neuropharmacological disorders such as insomnia, insanity, depression, psychosis, epilepsy and stress [11]. It was reported to possess anti-inflammatory, analgesic, antipyretic, sedative, free radical scavenging and anti-lipid peroxidative activities. It exhibits potent antioxidant and free radical scavenging properties. Besides, it also possesses anticancer, hepatoprotective, antiulcer, calcium antagonistic, bronchovasodilatory, smooth muscle relaxant and mast cell stabilizing properties. It is also reported to contain brain boosting compounds, of which bacoside A and bacoside B5 are most active. The pharmacological properties of *B. monnieri* have been studied extensively and the activities have been attributed mainly to the presence of characteristic saponins called "bacosides" [12] [13]. In traditional medications, it also finds application in rebirthing therapy to accelerate trauma release and to make continuous breathing easier. *B. monnieri* is a well-known nootropic plant reported for its tranquilizing, sedative, cognitive-enhancing, hepatoprotective and antioxidant action. Ethanolic extracts of parsley, *B. monnieri* and lettuce are reported to be protective against D-galactose induced oxidative stress-induced in testes and epididymis of mice [14]. The *B. monnieri* reduces beta-amyloid deposits in the brain of an Alzheimer's disease animal model. Endogenous substances in *B. monnieri* extract (BmE) have been reported to play role in antioxidant activity manifested by reduction of divalent ions scavenging of reactive oxygen species (ROS), alterations of lipoxygenase activity and hydrogen peroxide-induced lipid peroxidation, the contents being suggestive of endogenous antioxidant activity [15].

Although like other plants *B. monnieri,* is also affected by AR, very few reports on the effect of salt-stress on *B. monniera* exists [16]. Studies with different concentrations of NaCl were shown to have an influence on the differentiation of tissues in the root and stem of *B. monniera* [16]. While higher concentration induced drastic changes in roots grown on salt-supplemented media; epidermal and cortical cells revealed altered shape, size, and orientation or disintegration. A low concentration of salt-induced a profuse development of root hairs, to-

gether with enlarged air spaces in the stem cortex and thickened xylem cell walls in the vascular ring which gradually disappeared at higher concentrations [16]. The effect of aluminum on the morphogenic response of *B. monnieri,* at the maximum concentration has been reported from the maximum tolerant cultures [17]. Sulphur-simulated acid rain (S-SiAR) have been reported to generate a number of salts in the *B. monnieri* [18] [19] thereby affecting its productivity, growth and flowering. However, the molecular changes induced in plants due to the effect of acid rain or acid-induced orientation or chloroplast streaming remains largely unknown. Therefore in this study we have tried to understand the effect of acid rain on chloroplast observed under 1) light 2) scanning electron, 3) confocal laser scanning microscope and 4) multi elemental analysis using EDS.

2. Material and Methods

The field of an artificial acid rain fall garden was designed in the campus of the Institute of Physics, Bhubaneswar, India for the S-SiAR study. The experiment was carried out during the month of November 2010 to April 2011. About 60 numbers of *B. monnieri* herbs were grown and tested to evaluate the effect of acid on the morphological properties due to simulated acid rain (SiAR). These herbs were planted in 6 pots (10 herbs each). The total planted pots were divided into two groups. First group, *i.e.* G-I (2 pots), was used for normal water treatment; and second group, *i.e.* G-II (4 pots) for S-SiAR treatment. Again, this G-II was further divided into 4 sub-groups. Each sub-group was treated with acidic solution of different pH value (3.39 ± 0.02, 4.15 ± 0.02, and 5.45 ± 0.01) and another sub-group (pH 5.00 - 5.40) not given here. Acid-treatment process on the herbs every day was carried out using a syringe with least measuring unit 0.1 ml for the preparation of acidic solutions. One drop (0.1 ml) of 10% dil. H_2SO_4 in 450 ml, 700 ml, and 1100 ml of tap water produces acidic solution of pH 3.39, 4.15, and 5.45, respectively. The pHs of the above acidic solutions were set using digital pH tester. The herbs were watered regularly using normal water for 2 weeks. After that, only the young *B. monnieri* herbs of G-I (20 herbs in 2 pots) were regularly treated with normal water. However, young plants of the other group, *i.e.*, G-II, were treated with S-SiAR, after calculating the amount of acid to be added to the normal water to obtain the desired pH. Fresh samples of stem with leaf were collected after a gap of 22 weeks for the characterization.

2.1. Experimental Species

The *B. monnieri* herb was used as the test species for the experimental studies. Six number of uniform pots were selected and filled with Mud: Sand: Compost (Cow dung): 1:1:1 ratio. About 10 plantlets of around 50 mm length having 2/3 buds were planted in each of the 6 pots.

2.2. AR Treatment

The *B. monnieri* plantlets were initially treated with sufficient amount of normal

water of pH around 6.29 - 6.85 for a period of 2 weeks. A 10% standard solution of dil. H_2SO_4 was prepared. Acidic solutions of different pH (3.39 ± 0.02, 4.15 ± 0.02, and 5.45 ± 0.04) were prepared from the dil. H_2SO_4.The young *B. monnieri* herbs (2 weeks) were divided into two groups as above, *i.e.*, G-I, G-II, and were exposed to the normal environment (air, sunlight, dew) and not to the rainfall or any other means of incoming water to the pots. Every day, all the young herbs in G-I were treated with sufficient amount of normal water, and the herbs in G-II were treated with 400 ml of acidic (H_2SO_4) solution of different pH for 22 weeks.

2.3. Preparation of Extracts

The *B. monnieri* extract was extracted from its stem and leaves. A mixture of freshly prepared solution of *B. monnieri* extract (100 µl) with distilled water (5 ml) was used to study the pH of the solution. Again, the exact pH was estimated from the calibration curve of the herb extract with different proportion of water mixture, as discussed earlier. For the above sample of first kind, standard procedures to obtain the plant extracts defined by Piperno [20] were followed.

2.4. Microscopic Studies

The thin films of the raw *B. monnieri* extract were prepared on glass slides. These thin films were examined under the light microscope (SMZ-1500, NIKON, Japan) at the magnification of 10X. The cell morphology and multi-elemental in the *B. monnieri* pallet coated with platinum metal was investigated using a JEOL-JSM-T330 (JEOL, Japan) scanning electron microscope (SEM). Powder SEM analysis of sundried plant materials (*B. monneri*, leaf and stem) were carried out using an electron microscope. Plant cell components viz. starch granules, lignified fibers, chloroplasts, parenchyma etc. were clearly observed in the micrograph at two magnification, *i.e.* X500 and X1000. Relatively low magnification micrographs were taken to correlate the cell morphological data of the three microscopies techniques. Also, the multi-elemental analysis of the herb material were carried out using the SEM-EDS (JEOL-JES 6480 LV, JEOL, Japan) technique.

2.5. Confocal Microscopic Studies

Confocal microscope (LSM 780, Carl Zeiss MicroImaging GmbH, Germany) was used for confocal studies. Transverse section (T.S) of leaf, stem and root were prepared from *in-vivo* grown herb. The T.S of leaf of the plant was mounted on a glass slide and investigated under the above confocal microscope by adding the Fluoromount-G® solution for the tissue section and cell preparation.

3. Results and Discussion

In *Ayurveda*, the raw extract or the dried powder sample of *B. monnieri* (leaf and stem) is habitually used as medicine. This is the reason why we are interested to analyze the extract and the powder of *in-vivo* grown and S-SiAR treated *B.*

monnieri medicinal herb. For the above sample of the first kind, standard procedures were followed to obtain the plant extracts as described by Piperno in his book [20]. In this process, starch granules from mature plant specimens can be removed for the direct analysis. The thin films of the raw *B. monnieri* extract were prepared on glass slides. The above thin films were examined under the light microscope (LM) at the magnification of 10X. The starch grains (amyloplast) of both simple and compound type along with lignified (simple) fiber were clearly seen in the photograph, **Figure 1**. The average grain size measures using standard method [21] are found out to be 28.98 μm in case of *in-vivo* grown plant. The starch grain size of the *B. monnieri* herb treated with S-SiAR (pH = 5.45) shows increased grain size (35.83 μm) with large amount of compound starch grains. The average grain sizes of plants treated with S-SiAR at pH 4.15 was found out to be 44.26 μm. The grain size of starch slightly decreases with high concentration of acid. Again, the amount of grains/unit area is also found out to decrease. The plant treated with S-SiAR of higher pH (=3.39) shows very small grain size of about 24.29 μm (**Figure 1(d)**), with decreased number of grains/unit area.

To overcome this confusion related to size of the starch granula and the size of the chloroplast, an attempt has been made to measure the exact size of the chloroplast using a special microscope called "confocal laser scanning microscope or CLSM". Transverse section (T.S.) of leaf, stem and root were prepared from the *in-vivo* grown herb. The T.S. of leaf of the plant was mounted on a glass slide and investigated under the above special-type microscope by adding the special Fluoromount-G® solution for the tissue section and cell preparation. The T.S of

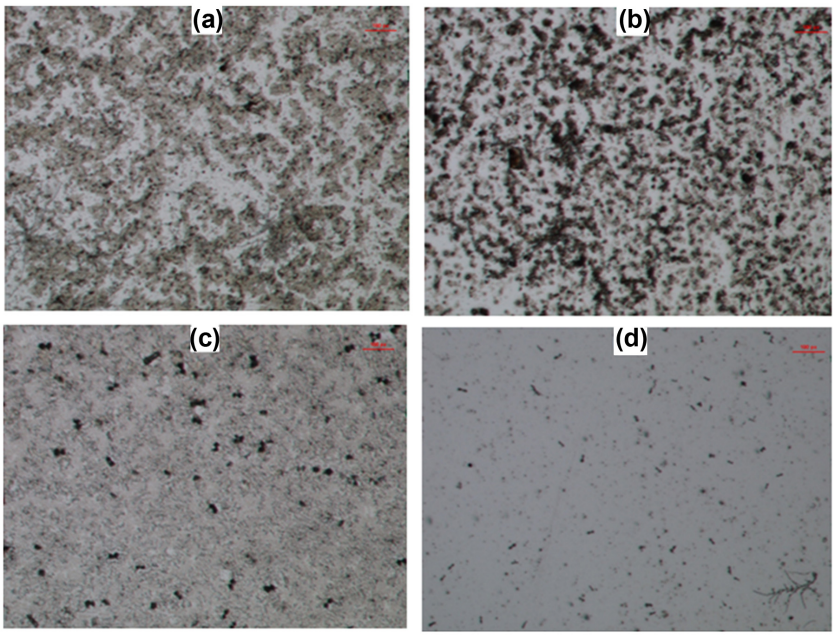

Figure 1. Photograph of thin film prepared from extracted fluid materials showing starch granule of *B. monnieri* using light microscope: (a) *in-vivo* grown plant, and acid rain affected (b) S-SiAR of pH, 5.45, (c) S-SiAR of pH 4.15, and (d) S-SiAR of pH 3.39.

the leaf sample are shown in Figure 2(a) & Figure 2(b). Similarly, T.S. of stem and root were investigated under the same microscope and are shown in Figures 2(c)-(e) and Figure 2(f), respectively. Fluorescence emission from the chloroplast (ovoid shape red particles) makes it visible in the thin-walled *B. monnieri* cells arranged in the honeycomb-like structure as shown in Figures 2(a)-(e). The fluorescence and the movement of the particles with respect to time (in a time scan) due to interaction with laser light confirmed the identified particles as chloroplasts. The size of the chloroplasts of *B. monnieri* was measured to be 5 μm. The amount of chloroplast has been found to be more in the leaf sample compared to that of the stem. The cell dimension of *B. monnieri* varies from 60 - 75 μm. It was observed that the chloroplast pigments are present in leaf and stem samples along with starch grains (Figure 2(a)) and oil-globules: white circular particles (Figure 2(b)). Usually, in most of the cases, the chloroplasts are absent in the root of the plants. The absence of the above fluorescence particles in the root (Figure 2(f)) confirmed the particles are non-other than chloroplast.

Powder SEM analysis of the sun-dried plant materials (*B. monneri*, leaf and stem) was carried out using an electron microscope. Plant cell components, viz. starch granules, lignified fibers, chloroplasts, parenchyma, etc., were clearly observed in the micrograph at two magnifications, viz. X500 and X1000. Relatively low magnification micrographs were taken to correlate the cell morphological data of the three microscopic techniques. Micrographs of *in-vivo* grown plants are shown in Figure 3(a) & Figure 3(b). Spherical starch grains of dimension 7.7 μm to 27.5 μm were observed. Lignified phloem fiber of around from 1.1 - 11 μm thickness was clearly visible. The ovoid shape chloroplast was found out to be of 4.5 - 5.8 μm length. An interesting phenomenon was observed when S-SiAR of low acidic value (pH = 5.45) treated *B. monnieri* plant was investigated under

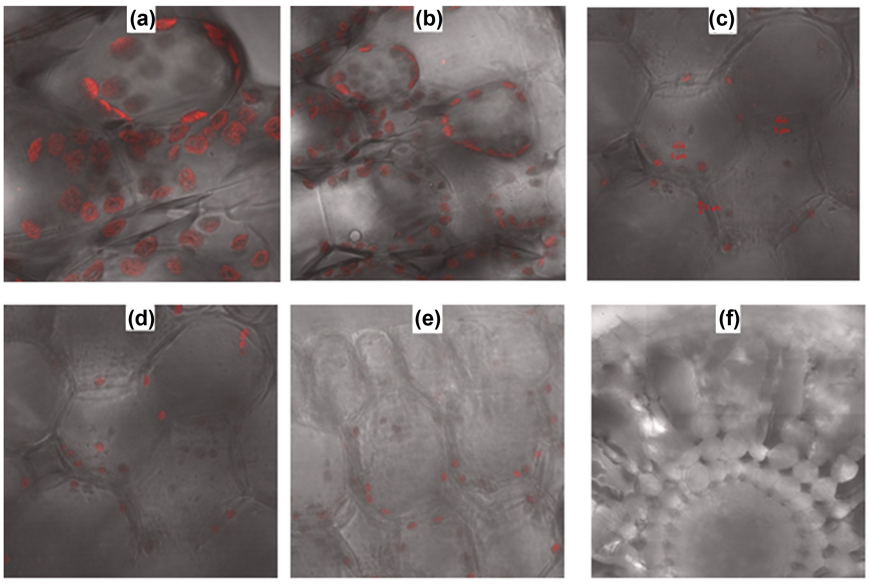

Figure 2. Confocal fluorescence micrographs (a)-(b) T.S of leaf, (c)-(e) T.S of stem and (f) T.S of root of *in-vivo* grown *B. monnieri*.

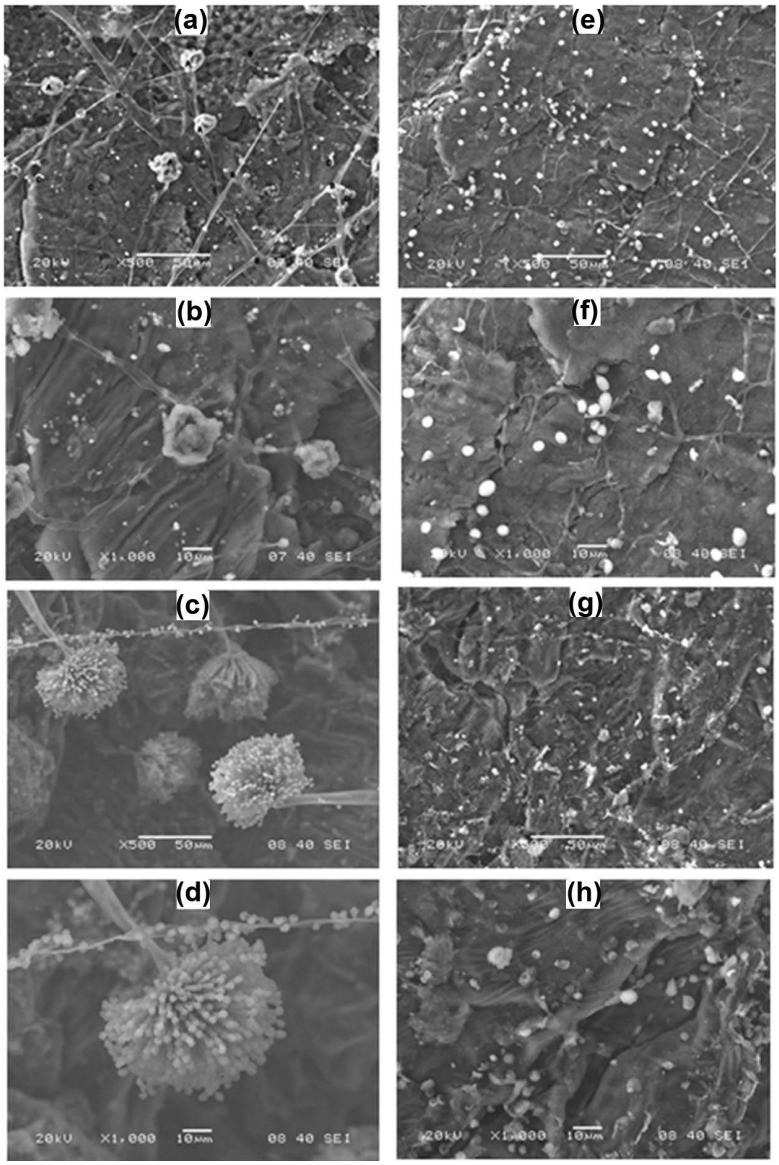

Figure 3. SEM micrographs of *B. monnieri* (a) (b) *in-vivo* grown (A—Starch granola, B—lignified phloem fibre, C—Parenchyma, D—Chloroplast), and acid rain affected (c) (d) S-SiAR of pH 5.45, (e) (f) S-SiAR of pH 4.15, and (g) (h) S-SiAR of pH 3.39. Micrographs of *in-vivo* grown plants were shown in (a) & (b).

SEM as shown in **Figure 3**. The dried powder indicated that permanent changes had taken place in the sample. Chloroplast streaming mechanism was observed at pH 5.45, as shown in **Figure 3(c)** & **Figure 3(d)**. The size of starch granules and number of the granules per unit area also decreases as shown in **Figure 3(e)** & **Figure 3(f)**. Increasing acid concentration (pH = 3.39), the normal fiber and the cell walls start dissolving and breaking, which can be seen in the **Figure 3(g)** & **Figure 3(h)**. Acid-induced strain in the various cell components have also been observed and can be seen in the respective micrographs. Few reports only are available regarding the streaming of chloroplast, although chloroplast migration [22] due to light transmission has been observed long back. The acid-induced

orientation or chloroplast streaming has not yet been reported. Usually, mitochondria eject H^+ ions during electron transport, hence the interior becomes alkaline. During the day time, under the action of light, the above H^+ ions are absorbed and the interior becomes acidified [23]. The ions such as K^+ and Mg^{2+} are ejected into medium. Again, during the night time (dark), H^+ ions are ejected and K^+ and Mg^{2+} are absorbed by the chloroplasts.

In the same way, the acid stress due to S-SiAR leads to ionizes the potassium and magnesium present in the chloroplasts to make it acidic without depending on light and dark. Therefore, it is expected that the Mg and K become ionized (Mg^{2+}, K^+), when induced with S-SiAR, and possess net negative Nernst potential of about − 87.55 mV (=−96.81 mV + 9.26 mV). The Nernst potential of the minor elements are calculated theoretically and compared with the standard value. The Nernst equation can be expressed as $V = \frac{RT}{zF} \ln \frac{\left[X^+\right]_0}{\left[X^+\right]_i}$, where the symbol V represents the Nernst potential which is the equilibrium potential of a particular ion, R is the universal gas constant (8.314 $J \cdot mol^{-1} \cdot K^{-1}$), T is the temperature in the absolute scale, z is the valence of that particular ion of interest, F is the Faraday's constant (9.649×10^4 $C \cdot mol^{-1}$), $\left[X^+\right]_0$ is the concentration of the ion outside the cell and $\left[X^+\right]_i$ is the concentration of the ion inside the cell. Binding of starch granules is mainly due to the presence of calcium, and plays a key role in plant growth and development [24]. Hence the respective Nernst potential for the above ions such as K, Mg and Ca can be expressed as:

$$V_{\mathrm{K}} = \frac{RT}{(+1)F} \ln \frac{\left[\mathrm{K}^+\right]_0}{\left[\mathrm{K}^+\right]_i}, \quad V_{\mathrm{Mg}} = \frac{RT}{(+2)F} \ln \frac{\left[\mathrm{Mg}^+\right]_0}{\left[\mathrm{Mg}^+\right]_i}, \text{ and } \quad V_{\mathrm{Ca}} = \frac{RT}{(+2)F} \ln \frac{\left[\mathrm{Ca}^+\right]_0}{\left[\mathrm{Ca}^+\right]_i}.$$

The calcium present in the cell walls and starch granules becomes ionized to Ca^{2+} due to acid interaction. The Nernst potential of Ca^{2+} is about +137.04 mV. A net potential difference may cause the above streaming of chloroplast towards the large starch granules. Previous workers have noted that the size of the chloroplast decreases due to acid induction [25] [26]. The size of chloroplast of the *in-vivo* plant has been found out to be 4.5 - 5.8 μm length and 3 - 4 μm width, which matches with the fluorescence microscopy data. However, the size of the chloroplast of the S-SiAR treated plants at different pH values such as 5.45, 4.15 and 3.39 has been found out to be 3.6 μm, 3.2 μm and 3.2 μm, respectively as shown in **Figure 4**. Again, acid rain caused salt of elements viz Mg, Al, K, Ca etc. can cause strain in various cell components.

The SEM-EDS-based quantative analysis of elements has been carried out using the INCA Energy Software developed by Oxford Instruments Analytical, UK [27] [28]. The method used in the INCA Energy Software suppresses the background, using a filtering method which avoids any specific shape calculation. A profile can be considered as a fingerprint for an element. It can be either for K, L or M series lines and is calibrated both in energy and resolution. Profiles are

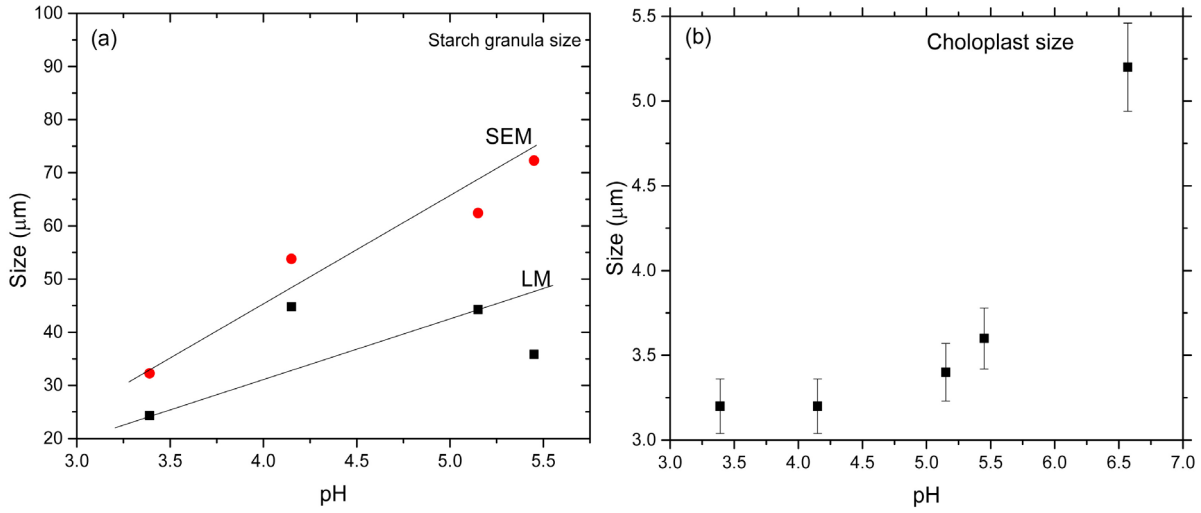

Figure 4. S-SiAR affected size variation of (a) Starch granula and (b) Chloroplast.

filtered in the same way as the unknown spectrum and are subsequently fitted to the filtered spectrum using a least squares routine. The filtering and fitting technique is collectively known as the Filtered Least Squares or FLS. The concentration of element x in the unknown sample can be approximated as: $C_x^{\text{sam}} = C_x^{\text{std}} \left[\frac{I_x^{\text{sam}}}{I_x^{\text{std}}} \right]$ and is often referred to as the "apparent concentration" or the "uncorrected concentration".

Elements (minor), viz. Na, Mg, Al, P, S, Cl, K, Ca, and Fe in *B. monnieri* herb have been determined by the EDS technique. Usually, various active elemental constituents of the medicinal plants are the metabolic products of the plant cells. Again, minor and trace elements play an important role in the metabolism. These important elemental constituents of the medicinal plant possess different curative capability of human diseases. Elemental constituents in *in-vivo* grown *B. monnieri* herb and the herb grown in S-SiAR environment are shown in **Figure 5**. The weight percentages (W%) of *in-vivo* grown *B. monnieri* herb were calculated by the comparator method of analysis and are recorded in **Table 1**. Similarly, S-SiAR treated samples at pHs 5.45, 4.15, and 3.39 are tabulated in **Tables 2-4**, respectively. The JEOL-standards (element) used for the above comparison purpose are: Albite 1(Na), MgO 1(Mg), Al_2O_3 1(Al), GaP 1(P), FeS_2 1(S), KCl 1(Cl), MAD-10 Feldspar 1(K), Wollastonite 1(Ca), and Fe 1(Fe). The more precise estimation of the concentrations of elements have been found out after 2-successive iterations, when a self-consistent set of concentrations and correction factors is obtained. The W% values listed in the **Table 1** show a precision of ±1.63 - ±2.50 for *in-vivo* grown *B. monnieri* herb. The sensitivity of the EDS technique as estimated from the present work was found to be at a level of 10^{-3} g. Uncorrected or apparent concentrations C_{app}, corrected intensity of individual elements I_{corr}, and atomic percentages a% of each element are also listed in the above tables.

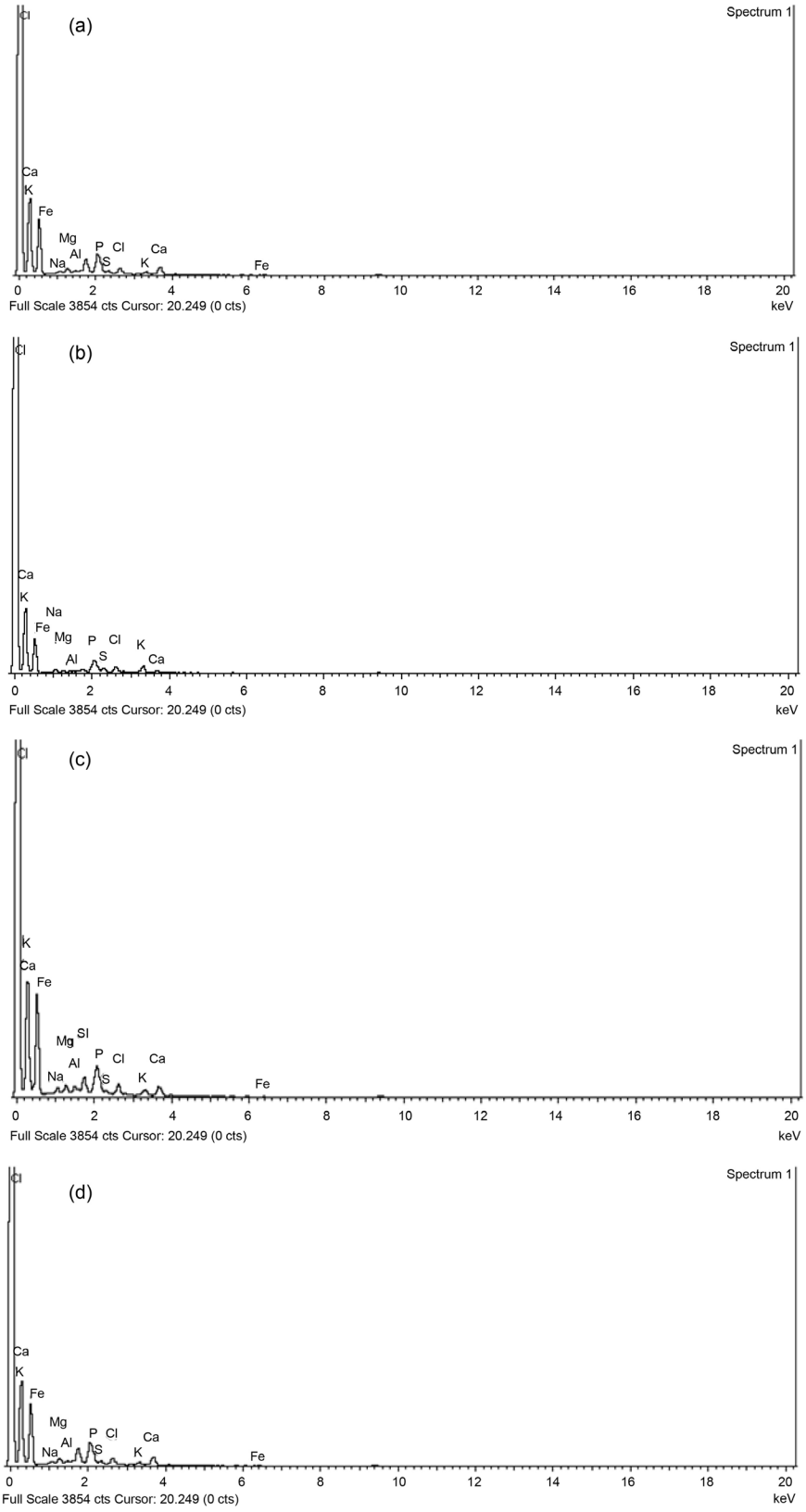

Figure 5. EDS analysis of *Bacopa monnieri* herb (a) *in-vivo* grown in uncontrolled habitat environment with tap water (pH = 6.29 - 6.85), (b) S-SiAR of pH 5.45, (c) S-SiAR of pH 4.15, and (d) S-SiAR of pH 3.39.

Table 1. EDS data of various elements obtained from *in-vivo* grown *Bacopa monnieri* (Normal (pH= 6.29 - 6.85) in Controlled condition.

Sl. No	*Element*	*Z*	C_{app}	I_{corr}	*W*%*	*a*%	C_{corr} (mg/g)
1	Na	11	0.90	1.0047	5.36 ± 2.10	7.67	8.72 ± 3.41
2	Mg	12	1.29	0.8288	9.36 ± 1.84	12.67	15.22 ± 2.99
3	Al	13	0.36	0.8186	2.62 ± 1.63	3.19	4.26 ± 2.65
4	P	15	5.34	1.3527	23.66 ± 2.50	25.14	38.47 ± 4.07
5	S	16	1.07	0.8245	7.80 ± 1.79	8.00	12.68 ± 2.91
6	Cl	17	1.87	0.7197	15.57 ± 2.04	14.45	25.32 ± 3.32
7	K	19	1.25	0.9670	7.78 ± 1.68	6.55	12.65 ± 2.73
8	Ca	20	3.84	0.9067	25.45 ± 2.27	20.90	41.38 ± 3.69
9	Fe	26	0.34	0.8439	2.40 ± 1.42	1.42	3.90 ± 3.58
Total			16.26		100		

*1% = 10,000 ppm = 10 mg/g.

Table 2. EDS data of various elements obtained from *Bacopa monnieri* (pH= 5.45).

Sl. No	*Element*	*Z*	C_{app}	I_{corr}	*W*%*	*a*%	C_{corr} (mg/g)
1	Na	11	1.89	1.064	10.43 ± 2.49	14.96	17.58 ± 4.2
2	Mg	12	0.35	0.798	2.60 ± 1.97	3.51	4.38 ± 3.32
3	Al	13	0.18	0.885	0.57 ± 1.68	0.69	0.96 ± 2.83
4	P	15	4.55	1.453	18.52 ± 2.83	19.61	31.22 ± 4.77
5	S	16	2.11	0.908	13.76 ± 2.24	14.07	23.2 ± 3.78
6	Cl	17	2.53	0.736	20.07 ± 2.70	18.84	33.84 ± 4.55
7	K	19	4.25	0.941	26.12 ± 2.93	22.40	44.04 ± 4.94
8	Ca	20	1.14	0.824	7.92 ± 2.47	6.48	13.35 ± 4.16
9	Fe	26	0.14	0.846	0.96 ± 3.14	0.56	1.62 ± 5.29
Total			16.86		100		

*1% = 10,000 ppm = 10 mg/g.

Table 3. EDS data of various elements obtained from *Bacopa monnieri* (pH= 4.15).

Sl. No	*element*	*Z*	C_{app}	I_{corr}	*W*%*	*a*%	C_{corr} (mg/g)
1	Na	11	2.17	1.121	9.64 ± 1.59	11.08	17.72 ± 2.92
2	Mg	12	1.57	0.852	9.13 ± 1.41	9.92	16.78 ± 2.59
3	Al	13	0.89	0.854	5.17 ± 1.29	5.07	9.50 ± 2.37
4	P	15	4.08	1.134	17.98 ± 2.21	15.32	33.05 ± 4.06
5	S	16	1.05	0.781	6.44 ± 1.43	5.44	11.84 ± 2.63
6	Cl	17	2.57	0.702	18.20 ± 1.60	13.55	33.45 ± 2.84
7	K	19	2.23	0.946	11.73 ± 1.40	7.92	21.56 ± 2.57
8	Ca	20	3.59	0.886	20.42 ± 1.68	13.33	37.53 ± 3.00
9	Fe	26	0.23	0.844	1.29 ± 1.52	0.61	2.37 ± 2.79
Total			18.38		100		

*1% = 10,000 ppm = 10 mg/g.

Table 4. EDS data of various elements obtained from *Bacopa monnieri* (pH= 3.39).

Sl. No	*Element*	*Z*	C_{app}	I_{corr}	*W*%*	*a*%	C_{corr} (mg/g)
1	Na	11	2.94	1.1494	11.33 ± 1.47	15.24	22.77 ± 3.09
2	Mg	12	2.38	0.8211	12.83 ± 1.40	16.31	26.96 ± 2.94
3	Al	13	1.75	0.7647	10.13 ± 1.33	11.61	21.28 ± 2.79
4	P	15	3.59	1.2088	13.17 ± 1.62	13.14	27.67 ± 3.40
5	S	16	1.77	0.8313	9.44 ± 1.29	9.10	19.83 ± 2.71
6	Cl	17	1.67	0.7165	10.34 ± 1.28	9.02	21.72 ± 2.69
7	K	19	4.41	0.9757	20.00 ± 1.44	15.81	42.02 ± 3.025
8	Ca	20	2.43	0.8677	12.40 ± 1.36	9.57	26.05 ± 2.86
9	Fe	26	0.07	0.8456	0.36 ± 1.34	0.20	0.76 ± 2.82
Total			21.01		100		

*1% = 10,000 ppm = 10 mg/g.

4. Limitations

The present study have some limitations and are as follows:

1) Preparation of pH solution for S-SiAR with an exact round up number (pH = 1, 2, 3, 4, 5 etc.) every day is a difficult and time consuming task for the researcher. Again, direct measurement of pH of the herb extract using the standard pH meter is a challenging because of the amount of the extract is small. Since, getting 100 ml extract from medicinal herb like *B. monnieri* grown in a simulated-acid rain laboratory required bundles of herb.

2) *In-situ* analysis of plants such as *B. monnieri* herb and acid rain treated *B. monnieri* herb, using a traditional SEM is one of the difficult procedure. Unless an environmental SEM with special attachment, it is quite complicate to carry out the *In-situ* analysis of living plants and acid rain effected herbs. There is a possibility for radiation(electron beam)-induced ionization inside the plant cell, which effects the pH value of the medium inside the plants, hence plant cell materials drastically effected. This causes confusion between the effect due to power concentration of Hydrogen atom (pH) or due to radiation-induced ionization mechanism.

3) Similar to SEM, possibility of Laser-induced ionization and heat based effect cannot be neglected in the *in-situ* analysis of the acid rain-treated *B. monnieri* herb. Since, pH vary with the temperature (Laser-induced heat) of the plant medium. So, it is difficult to estimate the exact value of pH inside the plant cell and the contribution of above change in pH on various cell materials. So, Laser based confocal microscopy, *i.e.* CLS have applied to measure the size of the chloroplast and the thin-walled of the *in-vivo* grown *B. monnieri* cells. This is the reason, why we have not prefer to take the confocal study of S-SiAR treated *B. monnieri* herb.

5. Conclusion

Acid rain is well known to affect adversely the plants and material objects. Al-

though few reports exist over the effects of acid rain in plants, no report exists on the effect of acid rain on medicinal plant *B. monnieri*. Prompted by the urge of understanding how acid rain affects the physiology of the medicinal plants, we have taken up the study on the plant *B. monnieri* known for its myriads of medicinal property in an artificially induced simulated acidic environment that maintained growth of the plant and was not cytotoxic to the plant growth. However, due to the acid induction, the biological effects, cellular effects, etc. cannot be ignored. The reduction in the size of chloroplast and starch granula has confirmed the above-said effects using CLSM and SEM techniques. The present study is very much interesting and important to understand the acid rain-caused effects and defects arising in plants in general and medicinal herbs like *B. monnieri* in particular. In this report, we have successfully shown that simulated acid rain could cause alteration in the physiology of the plant.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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