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SEM studies of leaf surface structure changes due to Lead toxicity in *Hypericum perforatum* L.

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ABSTRACT

In the recent years, human activities such industry and agriculture promote heavy metal release into the environment. Lead (Pb) is the most common heavy metal contaminant in the environment. It is a nonessential element in metabolic processes and may be toxic or lethal to organisms even when absorbed in small amounts. *Hypericum perforatum* L is a medicinal plant is mainly due to the presence of naphtodianthrones such as hypericin and pseudohypericin. The present study was undertaken to determine the accumulation the lead in shoots of *H. perforatum* L. and it's effects on leaf surface structure changes in mature plants treated with contaminated soil in 7 treatments (75, 150, 300, 600, 800, 1000, 1500 mg/kg Pb in soil) and 3 treatments by foliar spray (0.724, 1.44, 2.9 mM Pb) with 3 repeat in per treatment for 14 days. Results indicated the Maximum observed Pb content in shoot parts was at the two treatments 600 and 1500 mg/kg Pb in soil and in in foliar spray maximum Pb content observed at 0.724 mM Pb and the deareased to 2.9 mM Pb but all treatments have significant differenc with control plants. The analysis of scanning electron micrographs of the leaf surface of *H. perforatum* grown on polluted soil showed an increase in the size of guard cells In both adaxial and abaxial surfaces with increasing Pb in soil, decrease in size of stomata aperture and closure of stomata in 600 and 1500 mg/kg Pb in soil, and alteration in structure and deposition of epicuticular waxes. In foliar spray treatments In both adaxial and abaxial surfaces size of stomata aperture decreased than control leaves but not completely closed. Also Size of guard cells in treated leaves decreased compared to the control leaves. Also Studies of leaf surface by SEM indicated that affected epicuticular waxes by Pb concentrations in foliar spray treatments. the epicuticular wax deposition in control leaf in both adaxial and abaxial surfaces was less than Pb treated leaves and it increased with increase of Pb concentration.

Key words: Pb, stomata, epicuticular waxes, *Hypericum perforatum* L.

Introduction

Heavy metal pollution of air and agricultural soils is one of the most important ecological problems on world scale. According to the environmental protection agency (EPA), Pb is the most common heavy metal contaminant in the environment (A. Brennan and Shelley, 1999; Li *et al.,* 2007). Pb contamination has resulted from mining and smelting activities, Pb containing paints and gasoline. Pb is available to plants from soil and aerosol sources (Sharma and Dubey, 2005). It is a nonessential element in metabolic processes and may be toxic or lethal to organisms even when absorbed in small amounts (Li *et al.,* 2007). Plants undergo significant morphological and metabolic changes in response to metal stress.Visible symptoms of metal toxicity in the plants are the expression of metal-induced alterations at the structural and ultra structural levels. These changes at the cell, tissue and organ levels, in turn, are the result of a direct interaction of the toxic metals with structural components at these sites (Singh and Sinha, 2004). In he avy metal stress epicuticular waxes on leaf surface and opening of stomata was affected (Mehrotra, 2005). Stomata are the principal means of gas exchange in vascular plants. Some of heavy metals such as Pb, Cd and Al have effects on plant including: decrease in total leaf number and size, a decrease in shoot biomass, inhibition of root elongation, chlorosis and necrosis of leaves leading to decreased photosynthetic activity and reduces stomata aperture (Ozyigit and Akinci, 2009; Rai *et al*., 2010). Metal induced changes in the leaf epidermis structure involved a reduction in the cell size, more abundant wax coating and an increase in the number of stomata and trichomes per unit area with simultaneous reduction in the size of the guard cells (Azmat *et al*., 2009; Ozyigit and Akinci, 2009; Rai *et al*., 2010; Weryszko-Chmielewska and Hwil, 2005). The objectives of this study are to examine changes in the leaf surface traits of *Hypericum perforatum* due to the lead toxicity.

H. perforatum L. is a perennial herbaceous plant belonging to the Guttiferae family, widely distributed in Europe, Asia, Northern Africa, and North America (Campbell *et al.,* 1997; Curtis and Lersten, 1990). The importance of *H. perforatum* as a medicinal plant is mainly due to the presence of naphtodianthrones such as hypericin and pseudohypericin (Upton, 1999). They are localized in the small black glandular structures located on flower petals, stamens, leaves and stems (Hobbs 1996; Curtis and Lersten, 1990). Despite of importance of Pb contamination, it is until unclear that what concentrations of Pb cause to decrease plant growth and toxicity mechanism of lead in plant is unknown (Kopittke *et al.,* 2007; Li *et al.,* 2007). Thus anatomical studies are very useful to understanding the mechanism of lead toxicity in plants.

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Glob. J. Biodivers. Sci. Manag., 3(2): 256-263, 2013

Material and Methods

Preparation of seed bed and pot plants:

The seeds were planted on top of moist potting soil and watered with tap water (pH 6.0) by a sprinkler for one month. Following germination, plants (about 1.0 cm high) were transferred to pots (9 cm in diameter, one plant per pot) containing 200 g of soil mixture. The experiment was conducted in a greenhouse June to July 2008 and the mean irradiance at midday in the greenhouse was 377 ±64 mm s^{-1} cm^2, and temperature was 28 ± 7 ºC. The soil (pH 7.17, 5.5% organic matter, 4. 8 mg kg_1Pb).

Pb treatments:

Mature plants transferred to pots (20 cm in diameter) with contaminated soil in 7 treatments (75, 150, 300, 600, 800, 1000, 1500 mg/kg Pb in soil) and 0.724, 1.44 and 2.9 mM Pb as foliar spray with 3 repeat in per treatment. After 14 days plants emitted from soil and the parts of leaves, separated and fixed in FAA for anatomical studies.

Pb analysis:

Dry plant samples was digested with Nitric acid, percholoric acid and solphoric acid (40:4:1) and impurities were removed by filtration. After digestion, Pb concentrations in plant tissues were measured by ICP-OES (vista-pro, varian).

SEM (Scanning Electron Microscopic) studies:

Epidermal surface were studied with SEM microscope (XL30, Philips) for which the samples were covered by gold. All morpho-anatomical measurements were done by measurement software with 3 repeat for each part.

Statistical analyses:

The design of all experiments was a complete randomized design and treatments consisted of three replications. The data were analyzed with SAS Version 9. Significant differences between means were assessed by a Duncan test at P<0. 01. All the values are mean of 5 replicates. a, b, c, d indicates Significantly difference by Duncan grouping(P<0.01).

Results:

Metal accumulation:

The uptake and accumulation of Pb in leaves, varied depending on Pb concentrations. Maximum content of Pb in leaves observed in 600 and 1500 mg/kg Pb in soil (Fig1). The foliar applications significantly increased Pb concentration in the leaves sprayed with Pb over the control. Pb concentration extremely increased in 0.724 mM Pb than control but with increasing of Pb concentration to 1.44 and 2.9 mM uptake and accumulation of Pb decreased (Fig2).

Scanning electron microscopic studies:

The effects of various concentrations of Pb on stomata aperture and size of guard cells in leaves of *H. perforatum* after 14 days was presented in Table 1, 2.

Table 1: Effects of soil pollution of Pb on stomata aperture and size of guard cells in *H. perforatum L.* Means labeled with different letters differ significantly (P < 0.01).

Size of guard cell in abaxial surface	Size of guard cell in adaxial surface	Stomata aperture in abaxial surface	Stomata aperture in adaxial surface	Size of cell (**µ**m) / treatment (mg\kg)
6.76 c	8.5c	1.33a	1.66a	0
7.16 b	10 b	0.83 b	0.05c	300
7.16 b	10 b	0.33c	0 d	600
10 a	14.16 a	0 d	0 d	1500

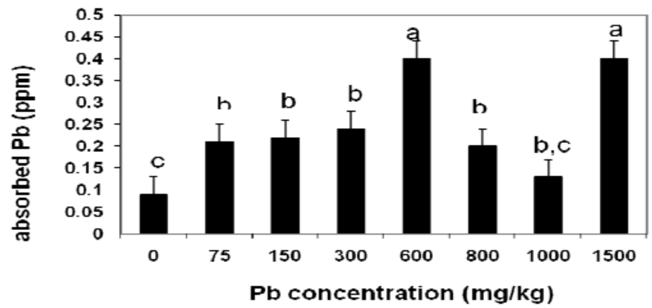


Fig. 1: Pb absorption in leaves in soil treatment. Data represent the mean of 3 replicates ± SD and values having different letter are significantly different according to duancan test.

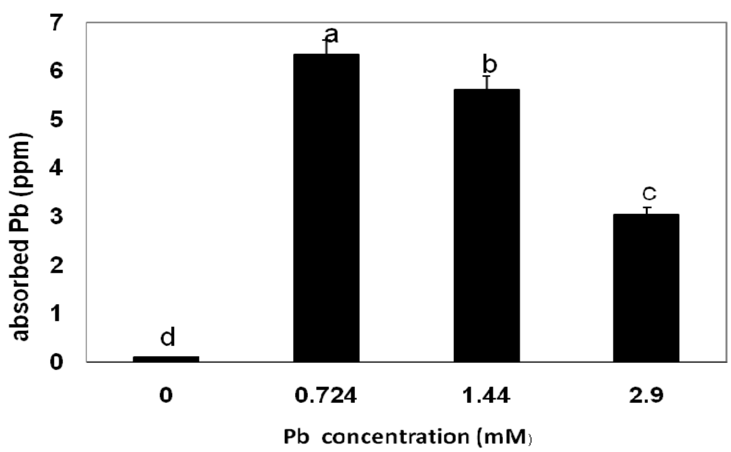


Fig. 2: Pb absorption in leaves in foliar spray treatment. Data represent the mean of 3 replicates ± SD and values having different letter are significantly different according to duancan test.

Table 2: Effects of foliar spray of Pb on stomata aperture and size of guard cells in *H.* perforatum . Means labeled with different letters differ significantly ($P < 0.01$).

Size of guard cell in abaxial surface	Size of guard cell in adaxial surface	Stomata aperture in abaxial surface	Stomata aperture in adaxial surface	Size of cell (**µ**m) / treatment (mM)
14.16 a	14.16 a	1.66 a	1.66 a	0
7b	7.4 c	1.33 b	0.83 b	0.724
5 c	7.5b	1.16 c	0.83 b	1.44
4.5 d	7.5b	0.83 d	0.83 b	2.9

The analysis of the results revealed that in soil treatment with increasing Pb concentration in soil opening of size of stomata aperture slightly decreased and in 600 and 1500 mg/kg Pb completely closed in adaxial surface. Similar effects also observed in abaxial surface but completely closed stomata only observed in 1500 mg/kg Pb in soil (Fig3).

Size of guard cells in treated leaves significantly different as compared to the control leaves. In both adaxial and abaxial surfaces size of guard cells increased with increasing Pb in soil (Fig3). In both leaf surfaces largest guard cell observed in 1500mg/kg Pb. In both adaxial and abaxial surfaces in foliar sprayed leaves of H. *perforatum* size of stomata aperture decreased than control leaves but not completely closed. Also Size of guard cells in treated leaves decreased compared to the control leaves (Fig4).

Glob. J. Biodivers. Sci. Manag., 3(2): 256-263, 2013

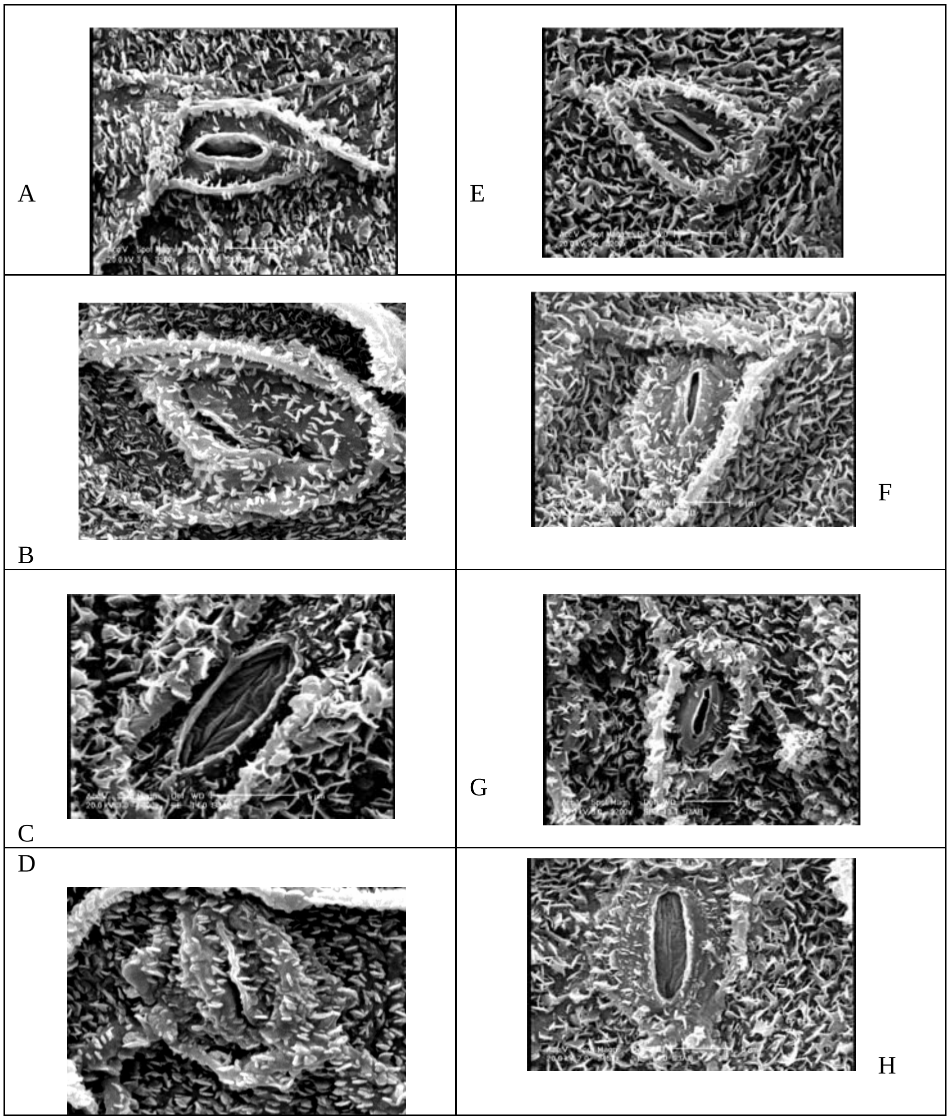
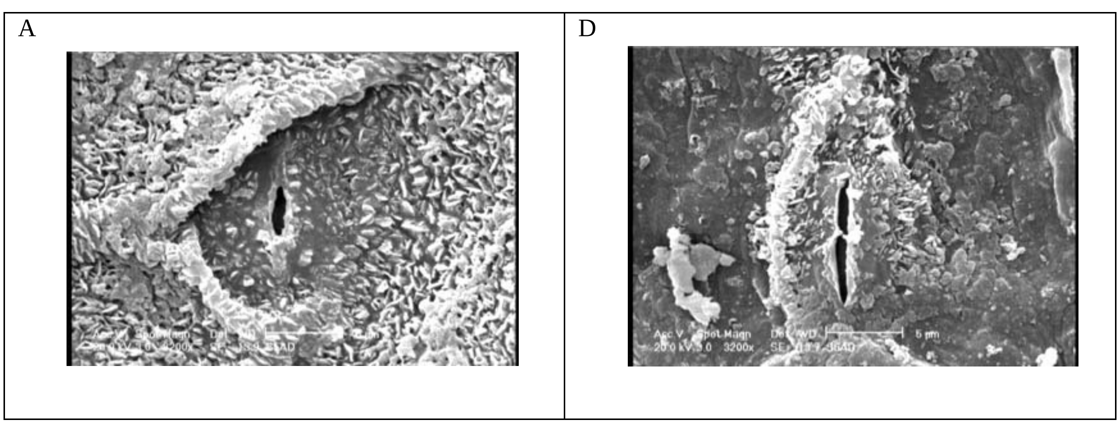


Fig. 3: SEM photograph of leaf stomata in soil pollution. A-D: Adaxial, E-H: Abaxial A,E:control, B,F: 300 mg/kg Pb, C,G: 600 mg/kg Pb, D,H: 1500 mg/kg Pb.



Glob. J. Biodivers. Sci. Manag., 3(2): 256-263, 2013

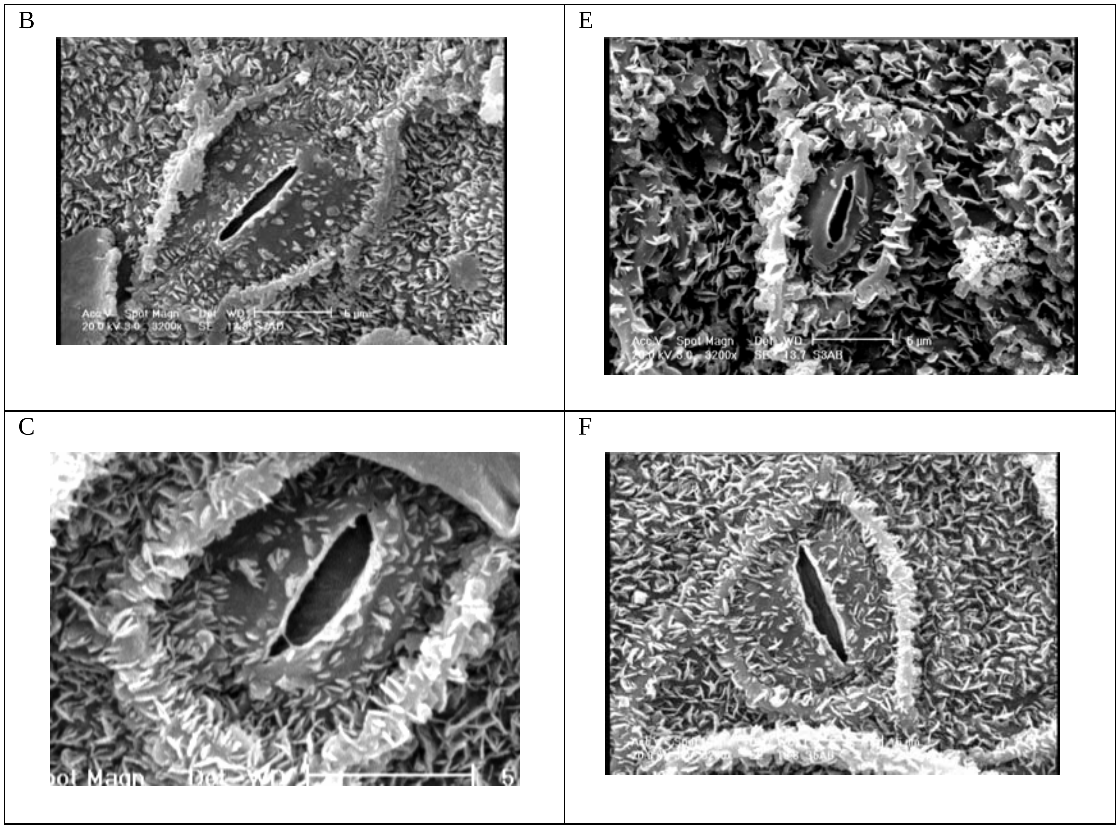
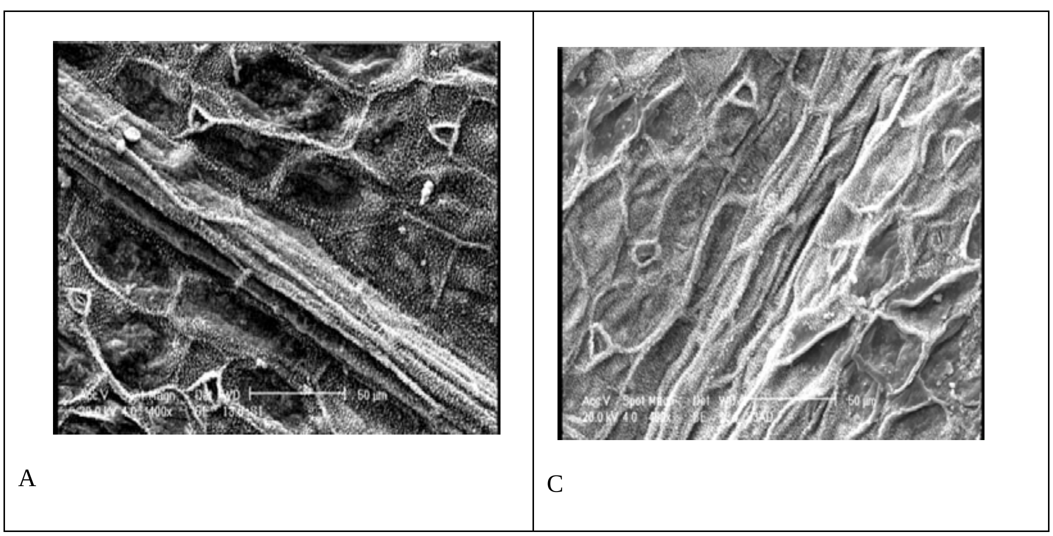


Fig. 4: SEM photograph of leaf stomata in foliar spray. A-C: Adaxial; D-F: Abaxial A, D: 0.724 mM Pb, B, E: 1.44 mM Pb, C, F: 2.9 mM Pb.

In both adaxial and abaxial surfaces size of guard cells decreased with increasing Pb sprayed on leaves (Fig4). The smallest guard cell is observed in 2.9Mm Pb concentration. Studies of leaf surface by SEM indicated that affected epicuticular waxes by Pb concentrations in soil and foliar spray treatments. In soil and foliar spray the epicuticular wax deposition in control leaf in both adaxial and abaxial surfaces was less than Pb treated leaves and it increased with increase of Pb concentration (Fig 5, 6).



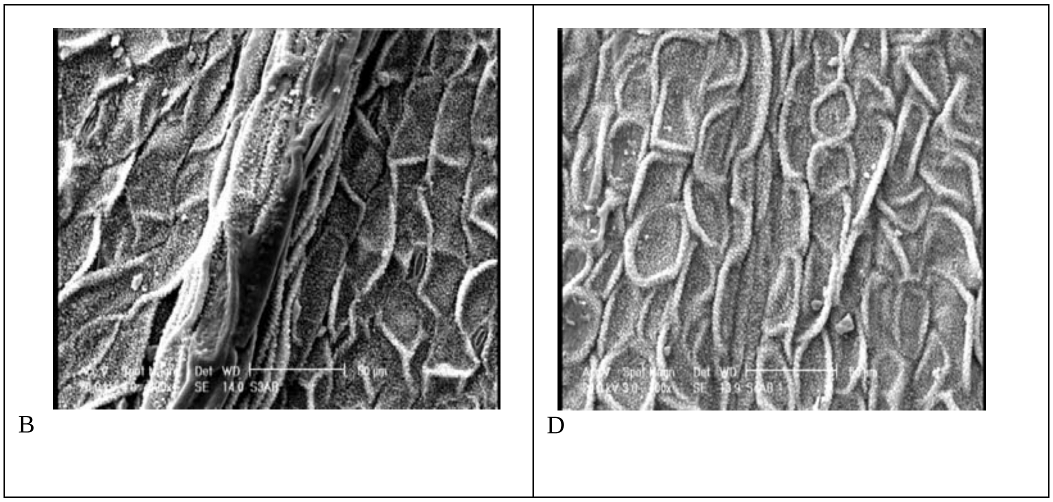


Fig. 5: Epicuticular waxes in soil treatments. A,C control; A:abaxial, C: adaxial B,D:1500 mg/kg Pb; B:abaxial, D: adaxial.

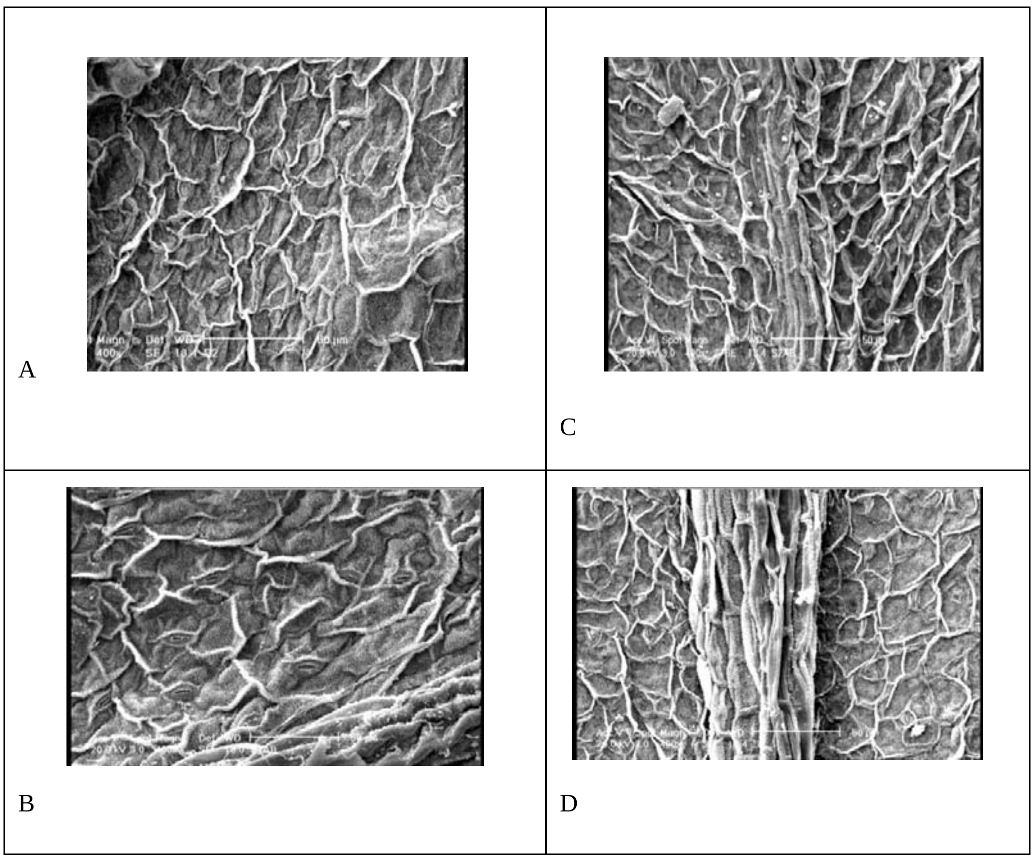


Fig. 6: Epicuticular wax in foliar spray. A,C:Adaxial, A; 1.44 mM Pb, C: 2.9 B,D:Abaxial, B1.44 : mM Pb, D: 2.9 mM Pb.

Discussion:

In this study, 14 d's treatment with Pb significantly affected stomata opening, size of guard cells and epicuticular waxes. Leaves are more sensitive but more flexible to environmental stresses (Cai and Shi, 2009). The results of SEM studies indicated that more important effect of Pb toxicity was decreasing the stomata ostiole and increasing size of guard cells. Closure of stomata in the leaves of Helianthus annulus L. grown on tannery sludge amended soil also reported (Singh and Sinha, 2004). Pb is available to plants from soil and aerosol sources. Therefore Pb enters the leaf through stomata openings and their toxicity may disturb the physiological activity of plants (Sharma and Dubey, 2005) .

Heavy metal treatments deal to appearance the symptoms similar to water stress including increase leaf thickness, palisade mesophyll and size of epidermal cells, increase the number of stomata, reduction of the stomata opening and increase the size to guard cells (Cai and Shi, 2009). The reason of decreasing the stomata opening seem is increased cell wall thickness and reduction of turgor pressure. The decrease in the size of stomata aperture in the leaves is in line with the hypothesis that metals induce water stress (Singh and Sinha, 2004).

Closed stomata of the leaf result in a slower rate of diffusion due to greater diffusion gradient of water vapors (Bondada and Oosterhuis, 2000). The various effects of water deficit seen on stomata structure are clearly mechanisms to enable plants to survive in stress conditions (Comstock, 2002).

The main reason behind the stomata closure under metal stress may be a strategy to prevent the water loss through transpiration as the translocation of water and solutes get disturbed in the presence of metals. The effect of metals on stomata opening was thought to be due to either metal-induced inhibition of an energy system or the alterations of K^+ fluxes through membranes (Singh and Sinha, 2004). Pb physically block the uptake of water and water stress led to substantial losses in dry weight, leaf area, root dry weight and length (Azmat *et al.*, 2006).

The structure and morphology of epicuticular waxes is a indicator of plant health and regulate the resistance to pollution stress. Changes in leaf wet ability, rate of transpiration, and loss of solutes from leaf cells are some of the effects that result from disruption of the epicuticular wax layer. The results of SEM studies indicated that in soil and foliar spray the epicuticular wax deposition in control leaf in both adaxial and abaxial surfaces was less than Pb treated leafs and it increased with increase of Pb concentration. A well-developed epicuticular wax layer may be crucial in protecting them from water loss, and any change in the original morphological structure make these plants more sensitive to water loss (Rai *et al*., 2010; Shepherd and Griffiths, 2006).

Conclusion:

As a well-known pollutant Pb affected on morphology, anatomy and ultrastructtutre of plants. Based on our results in *H. perforatum* plants Pb toxicity in both soli and foliar spray treatments significantly affected stomata opening, size of guard cells and epicuticular waxes. The results of this study suggest that changes of stomata and epicuticular waxes in both soil pollution and foliar spray on leaves of *H. perforatum*. caused by Pb toxicity and these alterations in foliar spray may be a probably protecting mechanism of Pb entrance to leaves.

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