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Histological and ultrastructure changes in *Medicago sativa* in response to lead stress

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In the recent years, human activities such industry and agriculture promote heavy metal release into the environment. Lead is the most contaminant metals in environment which adversely affects both plant and human life. The present study was undertaken to determine the effects of Pb on structural characteristics in *Medicago sativa* L. 30 days plants treated with Pb in 5 treatments (0,120, 240, 500, 1000µM Pb) with 3 repeat in per treatment for 10 days. Histochemical method of lead detection revealed significant accumulation of this metal in cortex and xylem tissues in roots and in stems lead deposits on cell wall of collenchymas tissues. The analysis of scanning electron micrographs of the leaf surface of *M. sativa* grown on hydroponic culture treated with Pb showed an increase in the size of guard cells in adaxial surface and decrease in abaxial surface, decrease in size of stomata aperture and closure of stomata in 1000 µM Pb in medium. Alternation of epicuticular waxes is one of the most important of pollution symptoms, in our study, alteration in structure and deposition of epicuticular waxes were observed. Also anatomical characteristics of stem and root affected by Pb contamination. Under Pb toxicity, anatomical symptoms including increase the diameter of stems and root as well as amplified vascular bundles and pith area were obsreved.

Keyword: Lead , stomata, epicuticular waxes, anatomical changes, *Medicago sativa L.*

1. Introduction

Heavy metals pollution of soil and water is a very serious environmental problem with potentially harmful consequences for agriculture and human. 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. Immobilization of metal in the cell wall and detoxification of its cytoplasmic pool by sequestration in vacuoles are important processes influencing plants' tolerance to lead; both processes protect the cell metabolism from its toxic effects (Seregin and Ivanov, 2001).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 heavy 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). *Medicago sativa* L. (alfalfa) is a flowering plant in Fabaceae family. Previous studies demonstrated that alfalfa plants have the ability to germinate and grow in polluted soil with heavy metals (Bali *et al*., 2010; Gardea-Torresdey *et al.,* 1998; Gardea-Torresdey *et al.,* 1999; Peralta-Videa *et al,*. 2002; Singh *et al.,* 2008). 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. (Gardea-Torresdey *et al.,* 1998; Gardea-Torresdey *et al.,* 1999). Thus anatomical studies are very useful to understanding the mechanism of lead toxicity in plants. The objectives of this research were to investigate the lead localization in and it's effects on structural changes in roots, stems and leaves of *M. sativa.*

2. Material and methods

2.1 Growth of the Plants and Pb Treatment:

M. sativa L. cv. Hamedani seeds were sterilized with 5% NaClO for 10 min, then rinsed several times with distilled water and germinated on perlite and vermiculite for 7 days. After germination, young seedlings were transferred to 3 L polyetheylene containers with 1/2- Hoagland nutrient solution in a growth room (temperature: 25 ± 2 °C and relative humidity: 60 ± 5%). and after one month treated with 0,120, 240,500, 1000µM Pb. After 10 days, plants were collected and the parts of leaves, separated and fixed in FAA (formaldehyde: acetic acid: alcohol) for 24 h and then preserved in 30% alcohol for anatomical studies.

2.2 Pb analysis: Dry plant samples were digested with nitric acid, percholoric acid and solphoric acid (40:4:1)and impurities were removed by filtration on Whatman 42 (Gupta, 2000). After digestion, Pb concentrations in plant samples were measured by atomic absorption (SHIMADZU AA- 6709).

2.3 Location of lead: Histochemical detection of lead in plant roots and stems was performed using dithizone . This reagent form product with lead that range in color from red to blue-black. To locate lead using dithizone, roots and stems rinsed in deionized water were placed in a solution of the reagent (30 mg dithizone dissolved in 80 ml 80% acetone containing a few drops of acetic acid) for 1.5 h. The roots and stems were viewed after being rinsed several times with deionized water. The slides were viewed with an Olympus BH2 light microscope.

2.4 Anatomical Methods: Cross-sections of stems and roots were taken by hand. Sections were cleared in sodium hypochlorite and stained by carmine-vest (1% w/v in 50% ethanol) and methyl green (1% w/v, aqueous) and mounted in gelatin. Then well-stained sections were photographed with an Olympus BH2 and all the measurements and observations were performed10 times on different slides were performed by measurement software with 5 repeats at each part.

2.5 SEM 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 5 repeat for each part.

2.6 Statistical Analysis: The design of all experiments was a complete randomized design and treatments consisted of three replications. Data were evaluated using ANOVA followed by a duncan stest (MsTATC Version 2.1) at $P < 0.05$.

Table 1: The effect of Pb treatments on root and shoot growth of *M.sativa*

Shoot dry weight (g)	Root dry weight (g)	Root length (cm)	treatment (µM)
2.89 a	1.337 a	26 a	0
1.94 b	0.923 b	22 b	120
1.72 c	0.513 c	18 c	240
1.7 c	0.483 c	18 c	500
1.65 c	0.427 c	17 c	1000

Means labeled with different letters differ significantly (P < 0.05).

3. Results

3.1 Growth parameters: The effect of Pb treatments on the root and shoot growth of *M. sativa* after 10 days was presented in Table 1. The effects of Pb on root growth of *M. sativa* varied with the different concentrations of lead in medium culture. The analysis of the results revealed that the root and shoot dry weight and root length had significant differences with control plants and decreased progressively with increasing Pb concentration (P<0.05).

Table 2: Effects of Pb tretment on stomata aperture and size of guard cells in *M.sativa*

Size of guard cell in abaxial surface)m(µ	Size of guard cell in adaxial surface)m(µ	Stomata aperture in abaxial surface)m(µ	Stomata aperture in adaxial surface)m(µ	Treatment (µM)
19.7 a	12.5 b	8.3 a	1.66 a	0
18.3 b	9.1 c	8.3a	0.66 b	240
16.6 c	16.6 a	0 b	0.33 c	1000

Means labeled with different letters differ significantly (P < 0.05)

3.2 Pb uptake and Accumulation

Fig. 2 shows the concentration of Pb found in root and shoot of plants exposed to the different treatments containing Pb.The uptake and accumulation of Pb in the shoot and roots varied depending on Pb concentrations. Pb content in roots increases with rising Pb concentration in medium and positive linear relation were found between Pb concentration in medium and root tissue concentration during the loading period. Maximum of Pb accumulation observed in 1000 µM Pb in. The obtained results indicated the significant difference in the shoot parts. Maximum Pb content in shoot was observed in treatments containing 1000 Pb in. Lead concentration in roots was higher than that in shoot.

3.3 Scanning Electron Microscopic Studies:

The scanning electron microscopic studies of the leaves indicated that epicuticular waxes, size of guard cells and stomata ostiole were influenced by Pb concentrations .The effects of various concentrations of Pb on stomata aperture and size of guard cells in leaves of *M. sativa* after 10 days was presented in Table 2. The analysis of the results revealed that size of stomata aperture slightly decreased with increasing Pb concentration in medium. In leaves of the treated plants, most of the stomata were found closed as compared to the control plants. Stomata ostiole decreased in both adaxial and abaxial surfaces (Fig 3), also stomata completely closed on abaxial surface in the treatment containing 1000 µMPb (Fig 3. F). Size of guard cells also affected by Pb in both surfaces in treated leaves. In adaxial surface increased with raise in Pb concentration and in abaxial surface decreased. Deposition of leaf epicuticular waxes also

affected by Pb treatment. In SEM study, epicuticular wax was observed starshaped in control and treated plants, but The epicuticular wax deposition in control leaf was less than that in Pb-treated leaves and it increased with enhancement of Pb concentration. Also Deposition of epicuticular waxes around the stomata in treated plant was more than controls (Fig 4).

Table 3: Changes in some anatomical parameters of *M.sativa* stem under Pb stress.

Pith)m(µ	cortex)m(µ	epiderm)m(µ	Stem diameter)m(µ	Pb Treatment (µM)
234e	51e	14d	649e	0
310d	62d	16c	833d	120
316c	65c	18.5b	864c	240
581.5b	68b	22a	874b	500
590a	86a	22a	1005a	1000

Means labeled with different letters differ significantly ($P < 0.05$).

3.4 Light microscope studies:

Anatomical features of the stressed and unstressed plants based on transverse sections of the root and the stem were studied (Table 3). Several tissues of root and stem of *M. sativa* have changed in Pb treated plant than control. Anatomical changes in root include: diameter, size of xylem , phloem, cortex and epidermis, which are all affected by Pb treatment. Diameter of root, size of xylem and phloem increased with enhancement of Pb concentration but size of cortex tissue decreased with increasing Pb (Fig 5). Epidermis consists of small cells in control plants but in Pb treaeted ones consists of large and disordered cells. Also in 1000µM Pb, sclerenchyma cells observed in cortex.Transverse sections taken from the stem of both control and treaeted plants were observed in Figure 6. Epidermis consists of monolayer of thin-walled and rectangular cells. Cortex is consisting of 4-5 rows in both plants. Phloem contains 2-3 rows in both plants but sclerenchyma fibres are arranged continuously in phloem tissue of the treated plants with Pb. Also diameter of stem and pith, size of xylem, phloem and cortex, are increased with Pb treatment (Fig 6).

Table 4: Changes in some anatomical parameters of *M.sativa* root under Pb stress.

cortex)m(µ	phloem)mµ	xylem)m(µ	root diameter)m(µ	Pb treatment (µM)
27 a	43 d	107 b	256.64 c	0
23 c	47 c	109 a	282 a	120
26 ab	52 a	107 b	224 d	240
24.5 bc	49 b	108 ab	219 e	500
19 d	53 a	108 ab	277 b	1000

Means labeled with different letters differ significantly ($P < 0.05$).

3.5 Histochemichal Detection of Lead in Plant Tissues: The presence of lead in the tissues of the analyzed plants was confirmed by the appearance of a strong stain ranging in color from red-brown through brown to black. In roots of treated plants, lead deposits were found in epidermis, cortex and xylem. The first site of intense staining, indicating intense accumulation of lead, was in the cell walls. The most accumulation of lead in root was observed in cortex cells in both 1000

and 500 µM Pb (Fig 3 A, B). In roots of treated plants there is a positive correlation between intensity of lead deposits and absorbed Pb with treated roots. Therefore the intensity of lead deposits increasing with enhancing of absorbed Pb (data for 120 and 240 µM Pb not shown).

In stem of treated plants, like roots, lead was detected mainly in cell walls of different tissues. In 500µM Pb treated plants most lead deposits were found in collenchymas cells in cortex of stem (Fig 3, C). In 1000µM Pb treated plants lead deposits were observed in cortex, xylem and pith but most deposition were found in cell wall of xylem (Fig 3, D). In 120 and 240 µM Pb treated plants lead deposits were not observed in stems.

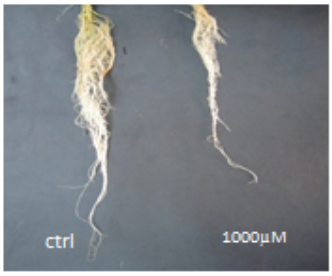

Fig1.Effects of Pb treatments on root length of *M.sativa*

4. Discussion

Plants have developed a variety of protective mechanisms against heavy metals, allowing them to develop and grow on stands strongly polluted by them. In this research we focused on localization of lead in plant tissues and anatomical and structural alterations in *M. sativa* plants under Pb treatments. Our results indicated that lead deposited in root tissues included epidermis, cortex and xylem and in stems Pb deposits observed in xylem and collenchymas tissue. One of the main sites of nontoxic heavy metal sequestration is the cell wall. This applies particularly to lead, since it has the highest affinity for the polygalacturonic acids that are part of the cell wall (Seregin and Ivanov, 2001). Accumulation of Pb in cell walls may be passive process related to apoplectic transport of it and to its retention by numerous cell wall components. Also collenchymas tissue is one of the most significant sites of heavy metal accumulation (Vollenweider *et al*., 2006).

In our study, 10 days 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).

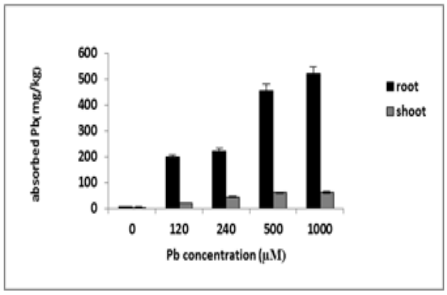

Fig 2. Pb accumulation in roots and shoots of *M.sativa*. Vertical bars represent standard errors (n = 5).

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 sur-vive 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 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). Another tolerance mechanism in response to Pb toxicity is the increase in diameter of stems with increasing Pb concentration in soil, whose cause is the amplified vascular bundles and pith area.

The obtained results also revealed increased diameter of roots with increasing Pb concentration in soil. This increase is resulted from increase in volume of vascular bundles. These results are similar to those of previous studies (Vollenweider *et al*., 2006; Bosabalidis *et al.,* 2004; Soares *et al.,* 2011). Increasing of xylem area as observed in this study can be associated to the promoted root maturation by heavy metals as a result of the hormone balance alteration. Heavy metals can affect the balance of root hormones, which in turn can affect tissue morphogenesis, also influencing the number of cells in these tissues (Soares *et al.,* 2011).

Also in high metal concentrations, reduction of root growth is compensated by the increase in diameter (Bosabalidis *et al.,* 2004). In present research, increasing Pb concentration in medium results in reduction of the dry weight of roots and shoots and root length , which is similar to the stress yielded from other heavy metals. In many of previous studies, reduction of growth of root and shoot is reported which is the consequence of reduction in cell division, toxic effect of heavy metals on photosynthesis, respiration and protein synthesis (Sharma and Dubey, 2005; Bosabalidis *et al.,* 2004; Vollenweider *et al*., 2006; Vijayaragavan *et al*., 2011; Heidari *et al*., 2011).

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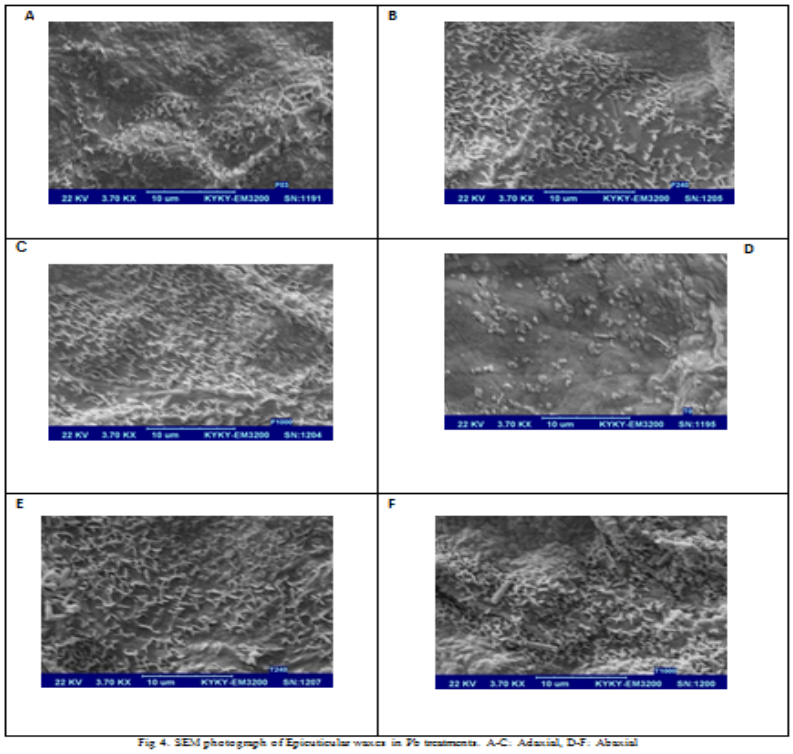

Fig 4. SEM photograph of Epicuticular waxes in Pb treatments. A-C: Adaxial, D-F: Abaxial

A,D:control, B,E: 240 µMPb, C,F: 1000 µMPb

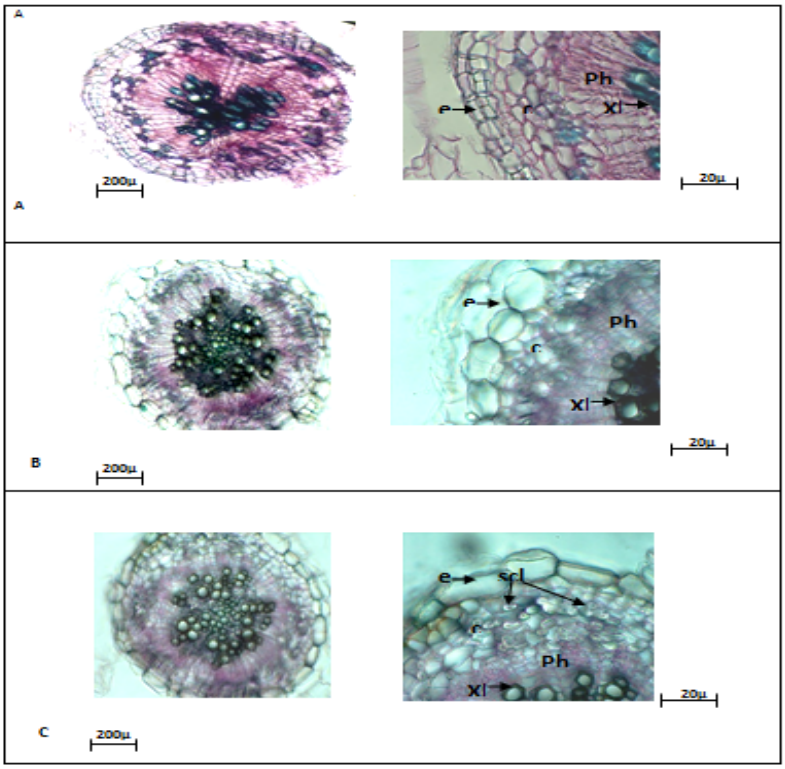

Fig 5.Root cross sections of *M.sativa* under Pb treatments. A: control, B: 240µM, C:1000µM

c: cortex, ph: phloem, xl: xylem, e: epidermis, scl: sclerenchyma

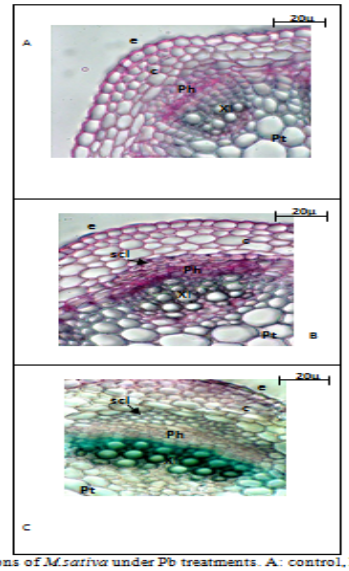

Fig 6.Stem cross sections of *M.sativa* under Pb treatments. A: control, B: 240µM, C: 1000µM.

c: cortex, ph: phloem, xl: xylem, e: epidermis, scl: sclerenchyma, Pt: Pith.

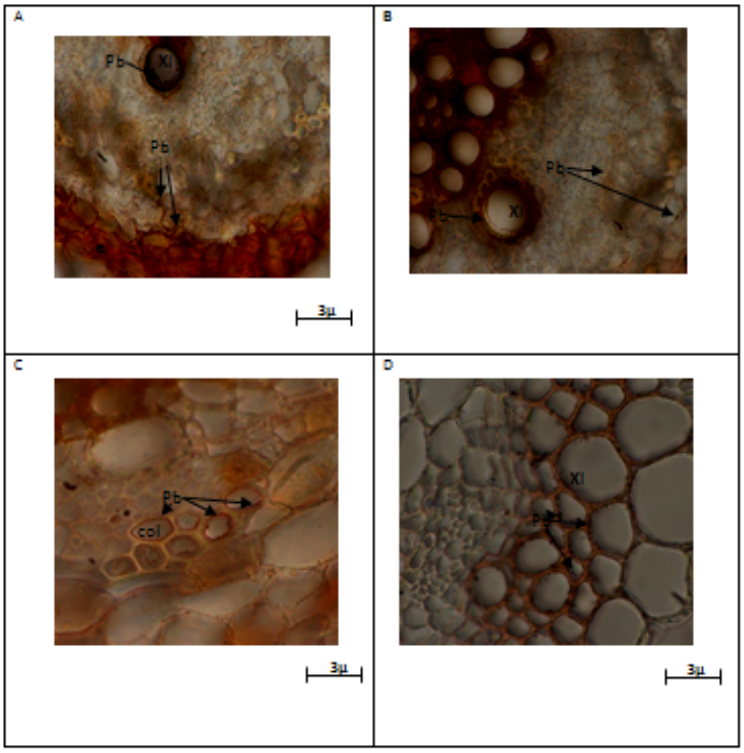

Fig 7. Lead localization in root and stem tissues *of M.sativa*. Lead deposits (Pb, arrows).A: root cross section treated with 500µM Pb, B: root cross section treated with 1000µM Pb, C:stem cross section treated with 500µM Pb, D: stem cross section treated with 1000µM Pb. c: cortex, xl: xylem, e: epidermis, col: collenchyma

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