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# BIOSORPTION OF Hg: II. REPAIRING OF EPIDERMAL LAYER OF LEAVES THROUGH NUTRIENTS OF SEAWEEDS IN HG DISTORTED SEEDLINGS OF *CICER ARIETINUM*

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**Abstract**

Seaweeds are the fascinating groups of marine plants gorging enormous important micro and macro nutrients. Due to their ability to concentrate minerals and trace elements from the sea, render them a potent source of nutrients for crop farming. This article discusses the credible of the green seaweed *Codium iyengrii* to concentrate non-essential trace element mercury (Hg). The plants were cultivated in randomized block design, exposed to varying concentrations of Hg in the soil separately and simultaneously with seaweeds. It was found that Hg damages the upper epidermal layer of leaves trait. Application of dry seaweeds powder in the Hg contaminated soil showed a repairing condition of epidermal layer of leaves. The role of epidermal layer on leaf surface is important because it protect internal tissues of leaves, involves in exchange of gases and transpiration through stomata. A direct relation between stomatal density and water contents due to the accumulation of Hg in epidermal layer was accountable for photosynthetic destruction. While repairing of leaves with substantial enrichment in % germination, relative water contents (RWC), plant growth with respect to length, biomass, and in opening and closing of stomata in seaweed amended Hg contaminated plants. Scan electron microscopy (SEM) was used to verify the results of Phase contrast microscopy.

## Introduction

The toxic metal pollution dynamics depends greatly upon the uptake and accumulation of chemically active elements and compounds and its mobility is greater while entering through stem or leaf. It accumulates biologically and enters into the food chain system generating long term health problems. Trace element Mercury exists in several forms such as metallic mercury, inorganic and organic mercury (Thangavel *et al.*, 1999) caused significant reduction in growth parameters, (Chaudhry & Khan, 2007). Husain *et al.,* (2010) reported patterns of bioaccumulation of mercury as root> stem> leaves revealing feeble translocation to the shoot system and inhibition in seed germination and hypocotyl elongation of *Cymopsis tetragonoloba* under mercury stress (Azmat *et al.*, 2006a ; Parmar & Chanda., 2005) due to the inhibition in cell division in the apical meristem, thus affecting directly the auxin synthesis, which in turn affected the degradation of xyloglucan (Ilza *et al.*, 2002). Gas exchange between tobacco leaves and atmosphere was blocked under Hg pollution, and net photosynthesis rate was modulated by stomatal and non-stomatal factors (Jian-jun *et al.*, 2008). A significant decrease in length and width of stomata was observed by Nidhi *et al.*, (2004). Mercury ($HgCl_2$) acts as an efficient blocker of most aquaporins and has been used to experimentally demonstrate the significant contribution of water channels to overall root water transport. Aquaporin-rich membranes may be needed to facilitate high-rate water flow across the trans-cellular path. Aquaporins are considered to be crucial for radial water transport in roots (Bramley *et al.*, 2007). Stomata can be regarded as hydraulically and chemically driven valves in the leaf surface, which open to allow $CO_2$ uptake and close to prevent excessive loss of water. Movement of these valves is regulated by environmental signals, mainly light, $CO_2$ and atmospheric humidity. Stomatal response to humidity is of special interest with respect to plant water use in harsh environments (Fletcher *et al.*, 2007). Stomatal guard cells can sense environmental signals and they function as motor cells within the stomatal complex.

The objective of this research to develop a cost effective technique which should be easy to handle and associated no risk in it. *Codium iyengrii*, a marine algae found abundantly at Karachi coastal areas, was used directly into Hg contaminated soil at laboratory scale in form of dry powder manure to study the growth of the plant and credible of marine algae in concentrating the metal on their surface. This paper will discuss the effect of Hg on leaf surface and the survival of the plant in presence of seaweeds.

## Material and Methods

Sterilized seeds were sown in polythene bags containing soil. The polythene bags were divided into two sets, one set of control supplied with Hoagland solution, the other set was again divided into two, one set of experimental plants supplied with different concentrations of mercury based Hoagland solution and other of treated plants supplied with green seaweeds (3gm/kg) along with Hg based Hoagland solutions (5ppm, 10ppm, 15ppm, 20ppm, 25ppm Hg). Rate of seed germination in percentage was calculated after $5^{th}$ day. Visual symptoms, relative water contents, measurements, and biomass were recorded after 15 days.

For phase contrast microscopy of leaves, fresh leaves were immersed in 10% KOH for two to three days so that they became soft and decolorized. The leaves were then placed on a glass slide with a drop of glycerin, covered with a cover slip and observed under a phase contrast microscope at the power of 100x. Their images were saved. Three replicates of each group were observed for the number of open and closed stomata. For scanning electron microscopy, small pieces of fresh clean and dried leaves were placed on stubs in scanning electron microscope (Joel, JSM-6380A), the images were observed and saved.

## Results and Discussions

Mercury injuring has develop a problem of current interest as a result of environmental pollution on a universal scale. The results of current research would certainly be helpful in governing the prospects of Hg and other toxic metal pollutants contamination with specific reference of control of toxic metals by seaweeds. Once understood the concept and its reasons a farmer will be in a position to understand and apply the safeguards and preventive measures to a contaminated soil in a better way. The research covers the following aspects of growth which would be beneficial to the food safety.

**1. Physicomorphological history of plant:** The effect of Hg was observed in a series of the experiments where plants in Hg contaminated soil pose deleterious morphological and structural changes in whole seedlings such as decline in overall plant length, weak and hair less stem and severely chlorotic and necrotic stem and leaves was similar to the work reported by earlier worker (Prasad *et al.*, 1986). A significant decline in rate of germination was observed from 100 to 70, 80, 80, 60 and 40% respectively at all experimental levels of applied mercury (Table 1), as Dhasarathan *et al.*, (2005) found 94% inhibition in seed germination by $HgCl_2$ (Azmat *et al.*, 2006a). Seeds Injury was directly proportional to the increasing rates of applied Hg (Munzuroglu *et al.*, 2008). Several former workers reported that Hg has most inhibitive effects on germination (Bandana *et al.*, 2008). Reduction in shoot length of *Cicer arietinum* was proportional to the varying concentration of applied mercury, it declined from 18.2 cm to 13, 11, 10, 9, and 8.3 cm respectively. Inhibition in root length was found as 17.5, 15, 14.4, 12.43, 11.66, 10.66 cm respectively. Literature revealed that an increase in the concentration of mercury caused severe damage to plasma membrane (Zhang & Tyerman1999, Singh & Singh 1992) which may attributed to decline in rate of mitosis that in turn results in reduction of overall seedlings growth. It may be related to a greater Hg affinity for biomolecules like sulfydryl, phosphate, carboxyl, amide, and amine groups that may cause cell wall loosening and reduced rate of mitosis, attributed to that inhibition in root formation and the shoot length according to Goyer (2001). Mean leaf area/$cm^2$ inhibited significantly up to 1.52, 1.42, 1.25, 1.20, and 1.11 over control which was found to be 2.4 cm. Reduced leaf area is a xerophytic character acquired by leaves to control rate of transpiration. The dwindling of water was also associated with accumulation of Hg in cellulose of cell wall which results in the elongation of the cell. The same phenomena were observed by earlier workers who reported that Hg caused a significant elongation in hypocotyls. It was observed that the elongated hypocotyls were very week and unable to stand in an erect position (Azmat *et al.,* (2006b). The seedlings were experienced more rapid death which may be due to depolarization in plasma membrane and replacement of major cations by Hg (Patra, 2000). The inhibition in water uptake due to Hg accumulation was observed in this study, related to the aquaporins in plasma membrane in seedlings as reported earlier by Zhang & Tyerman (1999). An application of green seaweeds as a nutrient soil conditioner, in Hg contaminated soil resulted, dramatic healthy features like considerable improved seed germination, maximum root/shoot length; erect, herbaceous and hairy stem which was yellowish green in color. The leaves were dark green, compound, hairy and attained maximum surface area. The growth of seaweed treated plants showed marked perfection which may be attributed to ligands formation between seaweed nutrients and Hg, due to which mobility of metal was controlled (Table 1) and overall good growth were observed in contaminated soil. Substantial development in seed germination, morphology and physiological processes which were observed in this study was the consequences of the nutrients of seaweeds that were helpful for survival in stress conditions (Azmat *et al.*, 2006a; Patra *et al.,* 2000).

**Table 1. Effect of Hg on physical parameters of *Cicer arietinum*.**

| S. No. | Hg [ppm] | Experimental plants | | | | Treated plants | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Germination (%) | Shoot length (cms) | Root length (cms) | Leaf area ($cm^2$) | Germination (%) | Shoot length (cms) | Root length (cms) | Leaf area ($cm^2$) |
| 1. | 0 | 100 | 18.2 | 17.5 | 2.4 | - | - | - | - |
| 2. | 5 | 70* | 13* | 15 | 1.52* | 80** | 14.1** | 17.67 | 1.88** |
| 3. | 10 | 80* | 11* | 14.4 | 1.42* | 80** | 14** | 15.5 | 1.46** |
| 4. | 15 | 80* | 10* | 12.43* | 1.25* | 90** | 12.4** | 14.21** | 1.36** |
| 5. | 20 | 60* | 09* | 11.66* | 1.2* | 70** | 11.53** | 13.23 | 1.3** |
| 6. | 25 | 40* | 8.3* | 10.66* | 1.11* | 50** | 9.5 | 12.83 | 1.26** |

Marked effects are significant at $p<0.05000$ (*indicated significant values of experimental plants over control ones, whereas ** shows significant values of treated plants over experimental. e = experimental plants (Hg contaminated), t = treated plants (Hg + seaweeds)

**2. Biomass and relative water content:** Metal hyper accumulation is a characteristic; present in majority of plant species and its tolerance are genetically inherited traits. Plants possess a range of potential cellular mechanisms that may be involved in the detoxification of heavy metals and thus develop tolerance to metal stress. The fresh development of current techniques has helped to improve the plant adaptation to extreme metallic environments. This study showed that seaweeds corrected the contaminated soil and helpful in plant growth due to additional nutrients in it and thus almost nullified the effects at lower degree. Mercury induced a significant deterioration in fresh weight, dry weight and relative water content of plants at all studied doses (Table 2) (Shiyab *et al*., 2009). The highest Hg dose (25 ppm) reduced biomass production especially in the shoots (Elvira, *et al.*, 2008). Reduced dry weight showed a failure in all metabolic activities under Hg stress, fresh and dry weights were inhibited by 69%, 88%, 66% and 71% respectively after 5 days of growth. (Dhasarathan *et al.*, 2005). Heavy metals like Pb and Hg have been widely reported to inhibit germination, shoot and root length elongation thereby decreasing fresh and dry mass of shoot/root in various plant species (Mukherji & Maitra, 1977). Declined biomass and RWC (relative water content) may be attributed to the damage in root cells due to depolarization of plasma membrane and reduced metabolic activities at all applied doses (Table 2). In root cells high osmotic potential might be developed due to the presence of Hg in soil. Leaf area indicates the water status of plants, reduced relative water content seems to have disturbed homeostasis in experimental plants due to mercury. However, fresh weight, dry weight and relative water content of treated plants showed an enhancement due to the presences of seaweed as they have capability to hold water and make it available to plants (Tab: 2) (Askari *et al*., 2007). Fresh weight was improved non-significantly at all applied doses except at 20 ppm Hg, dry weight was enhanced non-significantly in all applied concentrations of Hg except at 20 and 25 ppm whereas relative water content increased considerably at 15 and 25 ppm Hg and non-significantly at 5 ppm, 10 ppm, and at 20 ppm Hg. Seaweed contains all trace elements and major and minor plant nutrients such as alganic acid, vitamins, auxins, gibberllins and antibiotics (Azmat *et al.*, 2006a) which were found to be effective in controlling the mobility of metal within contaminated soil.

**Table 2. Effect of Hg on biomass of *cicer arietinum*.**

| S. No. | Hg [ppm] | Experimental plants | | | Treated plants | | |
|---|---|---|---|---|---|---|---|
| | | Fresh weight (gms) | Dry weight (gms) | RWC % | Fresh weight (gms) | Dry weight (gms) | RWC % |
| 1. | 0 | 2.4 | 1.3 | 95.18 | - | - | - |
| 2. | 5 | 2.02* | 0.9* | 73.01* | 2.3 | 1.2 | 80 |
| 3. | 10 | 1.92* | 0.8 | 65.64* | 2.2 | 1.1 | 75.15 |
| 4. | 15 | 1.78* | 0.6* | 60.66* | 2 | 0.9 | 69.54** |
| 5. | 20 | 1.71* | 0.5* | 53.96* | 1.9** | 0.8** | 65.44 |
| 6. | 25 | 1.5* | 0.3* | 45.49* | 1.8 | 0.8** | 60.55** |

Marked effects are significant at $p<0.05000$ (*indicated significant values of experimental plants over control ones, whereas ** shows significant values of treated plants over experimental. e = experimental plants (Hg contaminated), t = treated plants (Hg + seaweeds)

**3. Stomatal function and Hg:** Plants carry on the process of photosynthesis by combining together several ingredients in their leaves like carbon dioxide water and solar energy (sunlight). It indicates that the anatomy of a leaf, play a crucial rule that, how these materials are brought to the leaf so that they can form the food (glucose). The stomata is the way in which this transfer of ingredients can occur, transferring the $CO_2$, light and water from the surrounding atmosphere into the plant. In light the guard cells swell, causing the pore to be at its widest, and $CO_2$ diffuses into the leaf to be assimilated in photosynthesis. In the dark or under drought conditions the guard cells are not turgid, the stomata are closed and no photosynthesis takes place. Opening of the stomata not only allows $CO_2$ to diffuse into the leaf, but allows water vapor to diffuse out of the leaf. The alteration in the number, opening and closing of the stomata was reported in response to Hg accumulation as shown in the (Table 3) which indicated that alteration in stomatal morphology and reduced number was the adaption for survival in stress condition to carry on photosynthesis for continuous metabolic processes and control the evaporation of water. Results of seaweed amended plants showed perfection in morphology of stomata, this clearly indicates the positive role of seaweed surface nutrients for controlling the mobility of Hg.

**Table 3. Effect of Hg on stomata of leaves of *Cicer arietinum*.**

| S. No. | Hg [ppm] | Experimental plants | | | Treated plants | | |
|---|---|---|---|---|---|---|---|
| | | Total no. of stomata | No. of open stomata | No. of closed stomata | Total no. of stomata | No. of open stomata | No. of closed stomata |
| 1. | 0 | 67 | 65 | 2 | - | - | - |
| 2. | 5 | 60* | 50* | 10* | 65 | 62** | 3** |
| 3. | 10 | 51* | 39* | 12* | 60** | 55** | 5** |
| 4. | 15 | 40* | 21* | 19* | 55** | 47** | 8** |
| 5. | 20 | 35* | 13* | 22* | 50** | 35** | 15 |
| 6. | 25 | 34* | 9* | 25* | 50** | 32** | 18** |

Marked effects are significant at $p<0.05000$ (*indicated significant values of experimental plants over control ones, whereas ** shows significant values of Treated plants over experimental. e = experimental plants (Hg contaminated), t = treated plants (Hg + seaweeds)

**4. Study of stomata under phase contrast microscope:** Phase contrast microscopy of leaves by clearing technique showed drastic effects of mercury, on epidermal layer and on stomatal morphology. Mercury reduced total number of stomata, number of open and closed stomata (Godbold & Huttermann 1988: Mishra & Pradhan 1972) significantly and proportionally along with its increasing concentrations. Water stress caused an increase in abscisic acid level which might be involved in the closure of stomata. All these drastic effects were removed by green seaweeds significantly at all applied doses of mercury except at 15 ppm Hg where number of closed stomata were improved non-significantly (Tab: 3). Accumulation of mercury in the form of black spots can clearly be seen on the surface of leaves (Shiyab *et al*., 2009) which is certainly responsible for the reduced leaf area, damaged epidermis, absence of trichomes and closed stomata (Fig. 1b, d, f, h, j). Thus effects on the rate of photosynthesis which is a vital process for any plant life. Absence of black spots of mercury on epidermal layer of treated plants exhibited that mercury fail to accumulate in roots and seaweeds blocked the entrance of mercury into the plants (Fig. 1; c, e, g, i, k.). Aquatic plants have shown to be bio-accumulators of mercury (Patra & sharma 2000).

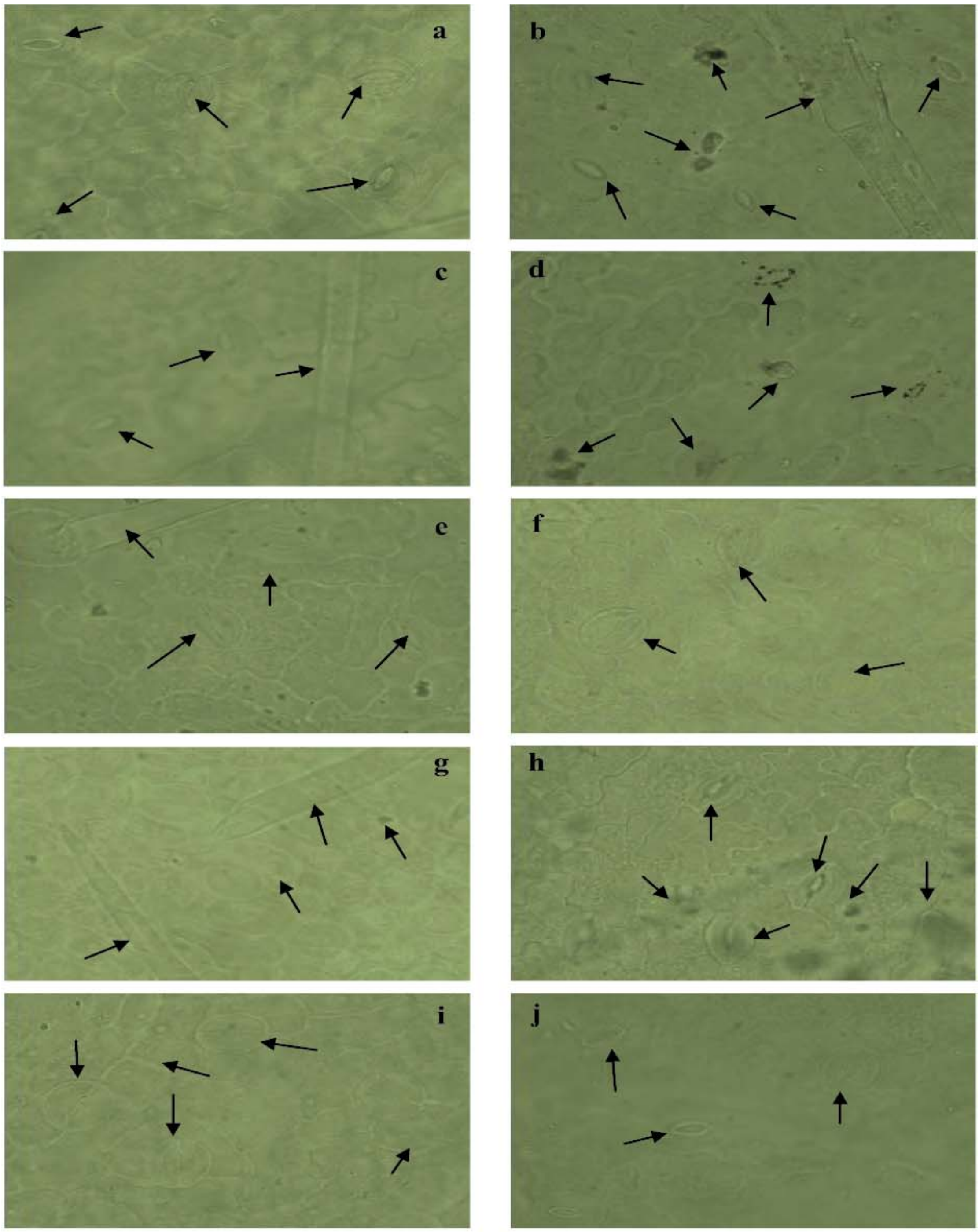
**5. Scan electron microscopy of the leaves:** Leaves of all experimental and treated plants were analyzed by scanning electron microscopy to characterize the structural changes caused by the exposure to Hg. Pressure potential of mercury was decreased in seaweed treated plants as observed earlier by Askari *et al*., (2007). Disintegration of epidermal layer, loss of trichomes and reduction in number of stomata was observed in leaves exposed to Hg (Fig. 2b, d, f, h, j). Xeromorphic characters of leaves were clearly observed in SEM micrograph of the leaves. Nature has assigned two duties to stomata, one is transpiration (liberation of water vapours from leaves to atmosphere) and second is exchange of gasses i.e., $CO_2$ and $O_2$. Total number of stomata reduced from 67(control) to 34 (25ppm Hg), whereas number of open stomata declined from 65(control) to 9 (25ppm Hg) (Tab: 3). The same phenomena was reported by earlier researchers who observed that photosynthetic rate, stomatal conductance of $CO_2$, transpiration rate was significantly reduced by Hg with morphological changes in a plant that include reduced number of stomata per leaf.

Increased osmotic potential and decreased water content caused reduction in number of stomata and reduced number of stomata lowered the rate of transpiration (Table 3). As transpiration is useful in maintaining a flow of solutes through the plants, decline in rate of transpiration also reduced the availability of solutes for all metabolic activities which caused adverse effects on growth and development of plants (Tables 1 & 2). Rough and deformed leaf surface was also observed in experimental plants (Fig. 2; b, d, f, h, j). The adverse conditions were significantly improved by the application of green seaweeds (Fig. 2; c, e, g, i, k). Highest adsorption ability of green marine macroalgae *Chaetomorpha sertularioides* and *codium iyengrii* for Hg was observed by (Kumar *et al.*, 2009, Igwe *et al.*, 2008; Askari *et al*., 2007).

## Conclusion

The experimental results revealed that soil and aquatic resources are now contaminated with uncontrolled industrial activities. These are accountable for the reduced and poisonous food, safety measurements through application of seaweeds as a cost effective biosorbent, can provide us a better technique to check the mobility of toxic metal by adsorption within contaminated soil to get safe food. Investigation revealed that seaweeds proven itself a good biosrbent which results in good, healthy growth of seedlings with all improved biomorphological history include surface morphology of leaf with vital water contents. It is concluded that mercury is most hazardous heavy metal. It is bioaccumulative and non biodegradable i.e. once enters the living body, it accumulates there and cause drastic effects as are revealed in all tables and charts. Mercury harms human not only by direct exposure from atmosphere, water, or cosmetics but also by eating mercury containing fruits, vegetables and fishes. People should be well informed and well aware about hazardous nature of mercury. It is the duty of our government to control unnecessary and careless use of mercury at industrial level. It is also concluded that the process of Biosorption is economically significant and most efficient technique. It is an economical and competitive practice for environmental applications in detoxifying effluents mainly heavy metals. Seaweeds are the best source for the removal of heavy metals like mercury from soil. It may be collected from the Karachi coast abundantly and free of cost. It may be used as a good fertilizer with no side effects as compare to other pesticides and insecticides. Seaweed can improve the soil condition as well as productivity of crop.

**Phase contrast microscopy of mercury and seaweed treated plants:**



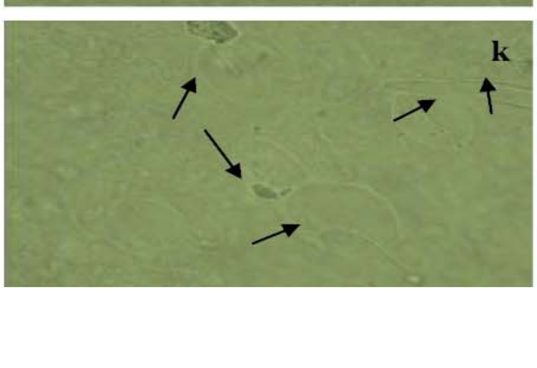




Fig. 1. Phase contrast microscopy of *Cicer arietinum* a=Leaf of control plant, Light green in color, arrows are showing open stomata. b=5ppm Hg, c=5ppm Hg+sw, d=10ppm Hg, e=10ppm Hg+sw, f=15ppm Hg, g=15ppm Hg+sw, h=20ppm Hg, i=20ppm Hg+sw, j=25ppm Hg, k=25ppm Hg+sw. Dark green color, black spots and closed stomata are obvious in all concentrations of applied mercury (b,d,f,h,j). Whereas leaf of seaweed (sw) treated plants showing light green color, trichomes and numerous open stomata(c,eg,i.k.).

**SEM of leaves of mercury and seaweed treated plants:**

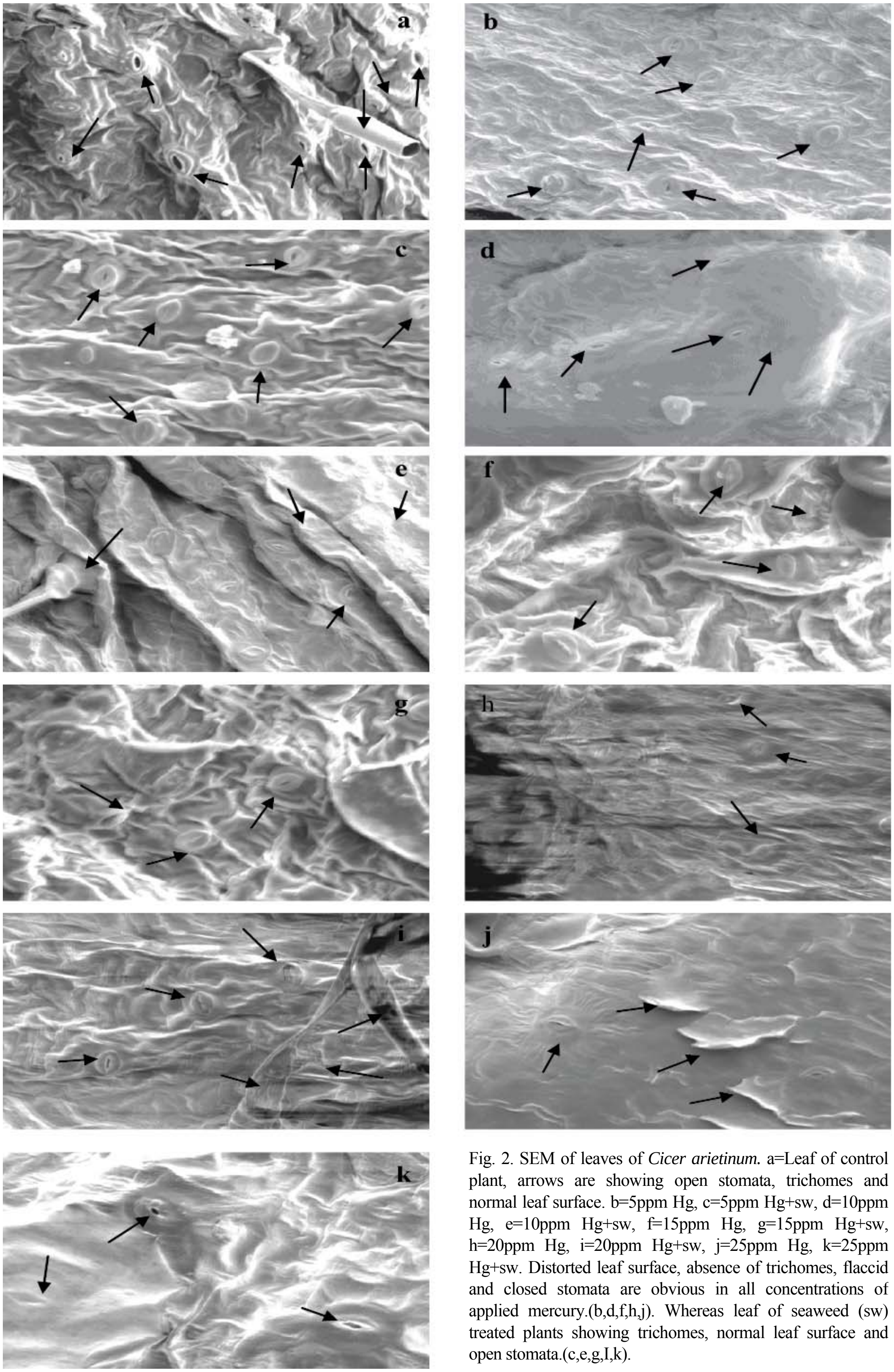



Fig. 2. SEM of leaves of *Cicer arietinum.* a=Leaf of control plant, arrows are showing open stomata, trichomes and normal leaf surface. b=5ppm Hg, c=5ppm Hg+sw, d=10ppm Hg, e=10ppm Hg+sw, f=15ppm Hg, g=15ppm Hg+sw, h=20ppm Hg, i=20ppm Hg+sw, j=25ppm Hg, k=25ppm Hg+sw. Distorted leaf surface, absence of trichomes, flaccid and closed stomata are obvious in all concentrations of applied mercury.(b,d,f,h,j). Whereas leaf of seaweed (sw) treated plants showing trichomes, normal leaf surface and open stomata.(c,e,g,I,k).

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