

Experimental study on Biochemical and Physiological adaptation of Mercury accumulation and tolerance in *Clitoria ternatea* L.

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ABSTRACT

The effect of heavy metal stress on the activity of antioxidant enzyme was studied in the leaves of *Clitoria ternatea* plants. Seeds of *Clitoria ternatea* were collected from Kerala Agricultural University, Trivandrum. Superoxide dismutase activity, Peroxidase activity, Nitrate reductase activity (NR activity), Catalase activity, Nitrate content, Protein content, Cysteine content, Proline content, Non protein thiol content, Lipid peroxidation rates, Eugenol content were determined by standard protocols. In order to investigate the effects of heavy metal stress, chlorophyll content and total chlorophyll were calculated. It was concluded from the study that *Clitoria ternatea* could grow in mercury enriched soil and can tolerate some amount of accumulation in their leaves. Mercury induced oxidative stress was tolerated by this plant through the hyperactivity of antioxidant defence system. Mercury stress induces the production of the Eugenol (a major component of some essential oil) in *Clitoria ternatea*. The leaves with lesser concentration of mercury are very well exploited for its medicinal value though it is considered toxic if concentration is high. However, our results are laboratory based and before exploiting the results in field, a pilot scale study is recommended.

KEY WORDS: *Clitoria ternatea*, Mercury, Oxidative stress.

1. INTRODUCTION

The most common heavy metals which are hazardous and being threat to living creatures includes Mercury (Hg), Cadmium (Cd), Chromium (Cr), Lead (Pb) etc. Out of them, lead and mercury were significant contaminants leading to life threatening health issues (Boening, 2000). Mercury poses significant environmental and health concerns (Ankita suhag, 2011). The World Health Organization (WHO) has approximated that from natural and anthropogenic sources, each year 10,000 tons of mercury is released globally. (Parul Sharma, 2014). The problem with methyl mercury is that it is consumed by aquatic organisms, especially fish and they accumulate in their tissues. Biomagnifications of methyl mercury poses a serious human health risk which was first realized during the 1950 and 1960's at Minamata Bay, Japan where more than 1000 people were killed and 5000-6000 suffered irreparable neurological damage from the consumption of mercury contaminated seafood. Contamination at Minamata Bay resulted from organic mercury runoff produced by an acetaldehyde facility. Mercury poses such a huge threat to human health because once it enters the body the destruction that occurs is usually irreversible. Symptoms associated with mercury toxicity are tremors, ataxia, paresthesia, sensory disturbances, cardiovascular collapse, severe gastrointestinal damage, irreversible damage to the brain, kidneys, and developing foetuses, and even death (Raskin, 2000). Also neurological symptoms caused by methyl mercury can continue indefinitely even after exposure from the source has ceased (Jeanna, 2000). Several plant species and genotypes can colonize metal rich soils and are known for the natural ability. Mercury is known to affect various essential processes in plants also some studies highlight enhanced plant growth with lower concentration (Chibuike, 2014). Some bacteria have shown potential to tolerate the accumulation of heavy metal ions in them. As a result of these properties plant growth were induced even with the presence of mercury in the soil and in the germinating seed (Soraia, 2015, Maksymiec, 1997).

2. MATERIALS AND METHODS:

2.1. Collection of fertile soil: Fertile soil was collected from K. S. Rangasamy College of Technology (Autonomous) campus, Tiruchengode, Tamil Nadu, India.

2.2. Heavy metal for treatment: Heavy metal such as mercuric chloride (HgCl₂) was selected for the present research work.

2.3. Seed germination and seedlings growth: Seeds of *Clitoria ternatea* were collected from Kerala Agricultural University, Kerala. Seeds were surface sterilized for about 2 minutes with 70% ethanol, followed by 20% commercial bleach containing 0.02% of Tween-20 for 2 minutes and washed with sterilized double distilled water. Seeds were lined in petridishes of 10 cm diameter with two sterile filter papers each with 50% Hoagland solution. Seeds were stratified at 4°C under dark conditions for 3-4 days and were transformed to 24°C for another 2 days and this seeds were used for germination. *Clitoria ternatea* seeds were germinated in mercuric chloride enriched soil (T1) with the concentration of 1µg/ml and also in the soil with bio fertilizer-water mixture (T2) in concentration of 1µg/ml in pots and the control plant was also grown in the soil neither sprayed with mercuric chloride nor with bio fertilizer. Shoot and root length was measured after 10 days of germination.

2.4. Enzyme extraction: 1g of sample leaf sample (T1, T2 and Control) was grinded with acid wash sand 50 mg calcium carbonate and 10 ml of acetone (80%) on ice in dim light. The slurry was centrifuged at 10,000 rpm for 10 minutes. The supernatant collected was decanted and kept in the cold condition and the pellet was suspended and centrifuged as above in 1.5 ml chilled acetone (80%). The supernatant was then combined, made to know volume and analysed by using spectrophotometer. Chlorophyll content was found at 663 and 645 nm absorbance values.

2.5. Analysis of enzymes

2.5.1. Estimation of nitrate reductase activity: Nitrate reductase activity (NR activity) of *Clitoria ternatea* leaves analysed in control, T1 and T2 were assayed as described by Vajpayee (2000). The assay mixture consists of 50mM potassium phosphate buffer (pH of 7.5), 2.5 mM KNO₂, 3 mM methyl viologen, 20 mM sodium dithionite prepared freshly from sodium bicarbonate and 10µl of enzyme extract in a final volume of 0.1 ml. Initiation of reaction is carried out by the addition of sodium dithionite and stopped after 10 minutes by the addition of 1.9 ml of reaction stopping and colour developing reagent consisting of 0.7 ml water and sulphanimide (1% w/v in 3N HCl, 0.6 ml and 1-Naphthyl ethylenediamine Dihydrochloride). The reaction was incubated for further 15 minutes at room temperature and the pink colour was developed and measured at 540 nm.

2.5.2. Estimation of nitrate: The nitrate content in control, T1 and T2 leaves of *Clitoria ternatea* were estimated as per procedure given by Shankar (2000). 0.5 grams of *Clitoria ternatea* leaves was boiled in 20 ml double distilled water for 30 minutes and filtered. The nitrate concentration in filtrate was measured using ion selective electrode of Thermo Orion Ion Meter.

2.5.3. Estimation of protein: Protein content in leaves of *Clitoria ternatea* was estimated according to the method of Lowry (1951) for control, T1 and T2. 0.2 ml of BSA (Bovine Serum Albumin) working standard was prepared in two test tubes and make up to one ml using distilled water. The test tubes with one ml of distilled water serve as blank. 4.5 ml of Reagent I was added and incubate for 10 minutes. After incubation 0.5 ml of reagent II was added and again incubated for 30 minutes. Absorbance was measured at 660 nm.

2.5.4. Estimation of ascorbic acid: Reduced ascorbic acid (ASA) was quantified in *Clitoria ternatea* leaves of control, T1 and T2 were by means of bipyridyl method was estimated by Washko (1992). For extraction, the frozen sample was macerated with liquid nitrogen in porcelain mortars by using metaphosphoric acid at 5% weight per volume. Centrifugation of homogenate Garg was done for 10 minutes at 10,000 rpm and the absorbance was measured at 525 nm.

2.5.5. Estimation of cysteine: Cystine content in *Clitoria ternatea* leaves of control and treated plant was estimated by taking 500 mg of fresh leaves homogenized in 5% chilled perchloric acid followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. (Gaitonde, 1967). Cystine content was measured in supernatant using ninhydrin reagent. 3 ml of reaction mixture contained 1 ml each of supernatant, glacial acetic acid and ninhydrin reagent. Mixture was heated for 15 minutes and then cooled rapidly and absorbance was recorded at 560 nm.

2.5.6. Estimation of proline: Proline content in Control and treated leaves of *Clitoria ternatea* was measured by rapid colorimetric method (Garg, 1998). 0.5 gram of dry leaf samples was grinded in 10 ml sulphosalicylic acid (3%) and the mixture was then centrifuged at 10,000rpm for 10 minutes. 0.2 ml of extract was taken in to the test tube (incubated in a water bath at 90°C for 30 minutes) to which 2ml of freshly prepared acid ninhydrin solution was added. Termination of the reaction is done in ice bath and extraction of reaction mixture is done with 5 ml of toluene and vortexed for 15 seconds. Tubes were allowed to stand at least for 20 minutes in darkness at room temperature to allow the separation of toluene fraction (measured at 520 nm) and aqueous phase.

2.5.7. Estimation of non-protein thiol content: Non protein thiol content was measured for Control, T1 and T2 in the supernatant by diluting it with Ellman reagent (1:9 containing 5mM EDTA, 0.6mM DTNB in 120 mM phosphate buffer pH 7.5 was adjusted). Absorbance recorded at 412 nm after 15 minutes. 700 mg of sample was homogenized in 3 ml of 6.67% 5- sulfosalicylic acid and centrifuged at 13,000rpm for 10 minutes at 4°C. Non-protein thiol content.

2.5.8. Estimation of lipid peroxidation: Lipid peroxidation rates of Control, T1 and T2 in *Clitoria ternatea* leaves were estimated by measuring the malondialdehyde equivalents according to Dinar *et al.*, (1986). 0.5 gram of *Clitoria ternatea* leaves was homogenised with 80% ethanol and the homogenate was subjected to centrifugation at 3,000 rpm for 10 minutes at 4°C. The pellet was extracted with twice with the same solvent. 1 ml of this sample was added in each test tube with an equal volume of the solution comprised of 20% trichloroacetic acid, 0.01% butylated hydroxyl toluene and 0.65% thiobarbituric acid. Samples was heated at 95°C for 25 minutes and cooled to ambient temperature to measure the absorbance value at 600 nm.

2.5.9. Estimation of Thin Layer Chromatography (TLC) for eugenol content: The eugenol content in *Clitoria ternatea* leaves was determined according to the procedure of Zayed and Terry (2003). Control, T1 and T2 was extracted by shaking one gram powdered leaves in dichloromethane for 15 minutes. The clear filtrate was evaporated to dryness after filtering the suspension. The residue was dissolved in one ml toluene and 25 µl of aliquot was applied on TLC plates in triplicates. The eugenol content in control and treated plant was estimated in Spectrophotometer by setting wavelength at 540 nm.

2.6. Effect of heavy metal stress on molecular weight determination in SDS-PAGE: Discontinuous SDS PAGE was carried out by using 12% resolving gel (pH 8.8) and 5% stacking gel (pH 6.8) in tris glycine buffer (pH 8.3) for control, T1 and T2 leaves of *Clitoria ternatea*. It is carried out using the method of Laemmli (1970).

2.7. Procedure: A Glass plate measuring the size of 15 X 15 cm was used for this study. To make uniform thickness of gel, two clean plates and three one mm spacers were assembled to make glass plate sandwich. The sandwich was locked using stand and metal clamps. Appropriate volume of solution containing the required acrylamide concentration for the resolving gel was prepared in an Erlenmeyer flask and components were mixed thoroughly.

This solution was degassed for five minutes and TEMED was added just before pouring. Acrylamide solution was poured into the gap between the glass plates without any air bubble by a Pasteur pipette. Distilled water was layered slowly onto the gel solution and the entire set was placed in a vertical position at room temperature for 30 minutes. After complete polymerization, the water layer was removed. To remove any unpolymerized acrylamide, the top of the gel was washed several times with deionized water. Then finally remaining water was removed with the edge of a paper towel. The appropriate volume of solution containing the desired concentration of acrylamide for the stacking gel was prepared, mixed and degassed. TEMED was added to the mixture prior to pouring. Stacking gel was directly poured onto the surface of the polymerized resolving gel. Teflon comb was carefully inserted into the stacking gel solution. In order to fill the spaces of the comb completely more stacking gel was added. Gel was placed in vertical position at room temperature for 30 minutes. While the stacking gel is polymerizing, the sample were prepared by heating at 100°C for 3 minutes in SDS gel loading buffer to denature the protein (1:1 v/v). After polymerization of the gel, Teflon comb was removed carefully from the wells. The wells were washed with the tank buffer and half of the well was filled with the same buffer, the bottom spacer in the glass sandwich was removed carefully. Gel was mounted in the electrophoresis apparatus. This glycine electrophoresis buffer was added to the top and bottom reservoirs and the air bubble that were trapped at the bottom of the gel between the glass plates were removed carefully. A known amount of mercuric chloride protein sample (T1) and biofertilizer protein sample (T2) with equal amount of SDS gel loading buffer along with molecular marker was loaded into the bottom of the wells by pipette man. The electrophoresis apparatus was attached to an electric power supply by connecting the positive electrode with the buffer reservoir at the bottom. Initially, a voltage of 8V/cm was applied to the gel, then after that the dye front had moved in to the resolving gel, the voltage was increased to 15 V/cm until the bromophenol blue reached the bottom of the resolving gel (about 4 hours). Then the power supply was turned off.

From the electrophoresis apparatus, the glass plates were removed and placed on a paper towel. Using spatula, the plates were separated apart. Cutting a corner from the bottom of the gel that was closet the leftmost marked the orientation of the gel well. After the completion of the electrophoresing, the gel was immersed in 5 volume of staining solution and placed on a slowly rotating platform for a minimum of 48 hours at room temperature. The gel was de-stained with the same relevant system without the dye (CBB R- 250) till the disappearance of blue colour background. Stained gel was stored at 4°C in acetic acid. The band was visualized in gel documentation system.

3. RESULTS

3.1. Effect of heavy metal stress on nitrate reductase activity: A concentration of nitrate reductase activity in *Clitoria ternatea* leaves was observed in control and treated plants. The maximum inhibition of 69.69% in nitrate reductase activity was recorded when plants was exposed to 1µg/ml of extract. Nitrate reductase activity for control plant was 2.03, T1 plant was 3.03 and T2 plant was 3.05. The percentage of Nitrate reductase activity in *Clitoria ternatea* leaves slightly decreased for both T1 and T2. The percentage of Nitrate reductase activity of T1 was 65.71 and T2 was 69.69 (Figure 1).

3.2. Effect of heavy metal stress on nitrate: The nitrate accumulation due to Hg toxicity was found in *Clitoria ternatea* leaves of control and treated plants. A maximum reduction of 96.66% in nitrate content was observed within 24 hours. Nitrate content for control plant was 2.09, T1 plant was 3.01 and T2 plant was 3.00. The percentage of protein content in T1 was 93.54 and T2 was 96.66 (Figure 2).

3.3. Effect of heavy metal stress on protein: The protein contents of the treated plants of *Clitoria ternatea* leaves was also found affected by Hg level in control and treated plants. A maximum reduction of 107.14% in protein content was observed within 24 hours. Protein content for control plant was 3.0, T1 plant was 2.08 and T2 plant was 3.02. The percentage of protein content in T1 was 93.75 and T2 was 107.14 (Figure 3).

3.4. Effect of heavy metal stress on ascorbic acid: Mercury significantly reduced the ascorbic acid of *Clitoria ternatea* leaves of control and treated plants. The maximum inhibition of ascorbic acid was 79.31% and was recorded when the plants are treated with HgCl₂ for 72 hours. Ascorbic acid content for control plant was 2.03, T1 plant was 2.09 and T2 plant was 3.0. The percentage of ascorbic acid content in T1 was 76.66 and T2 was 79.31 (Figure 4).

3.5. Effect of heavy metal stress on cysteine: Mercury reduced the cysteine content in *Clitoria ternatea* leaves for control and treated plants. The maximum inhibition of cysteine was 73.33% and was measured when the plants are

treated with HgCl_2 for 24 hours. Cysteine content of control plant was 2.02, T1 plant was 3.00 and T2 plant was 3.4. The percentage of ascorbic acid content in T1 was 64.70 and T2 was 73.33 (Figure 5).

3.6. Effect of heavy metal stress on proline: In *Clitoria ternatea* leaves mercury reduced the proline content of control and treated plants. The maximum inhibition of proline was 120.00% and was measured when the plants are treated with HgCl_2 for 72 hours. Proline content for control plant was 2.04 and T1 plant was 2.00 and T2 plant was 2.9. The percentage of proline content in T1 was 82.75 and T2 was 120.00 (Figure 6).

3.7. Effect of heavy metal stress on non-protein thiol content: Non protein thiol content in *Clitoria ternatea* leaves was reduced in control and treated plants. The maximum inhibition of non-protein thiol content was 76.92% and was measured when the plants are treated with HgCl_2 for 36 hours. Non protein thiol content for control plant was 2.04 and T1 plant was 2.06 and T2 plant was 2.08. The percentage of non-protein thiol content in T1 was 71.42 and T2 was 76.92 (Figure 7).

3.8. Effect of heavy metal stress on lipid peroxidation: During present study a significant increases in malondialdehyde (MDA) content of *Clitoria ternatea* leaves was observed initially at mercury level after 24 hours exposure. The maximum inhibition of Lipid peroxidation content was 55.55% was measured when the plants are treated with HgCl_2 for 42 hours. Lipid peroxidation content for control plant was 1.00, T1 plant was 1.08 and T2 plant was 2.03. The percentage of lipid peroxidation content in T1 was 43.47 and T2 was 55.55 (Figure 8).

3.9. Effect of heavy metal stress on eugenol content: Mercury significantly reduced the eugenol content of *Clitoria ternatea* leaves of control and treated plants. The maximum inhibition of eugenol content was 69.10% was recorded when the plants are treated with HgCl_2 for 42 hours. Eugenol content for control plant was 2.00, T1 plant was 2.04 and T2 plant was 3.20. The percentage of eugenol content in T1 was 56.646 and T2 was 59.11 (Figure 9).

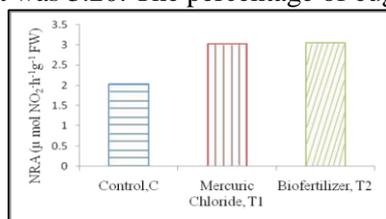


Figure.1. Impact of HgCl_2 on nitrate reductase activity of *Clitoria ternatea* plants

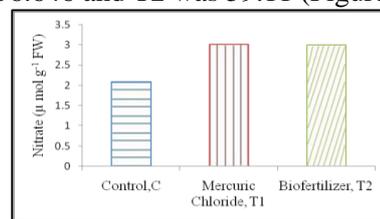


Figure.2. Impact of HgCl_2 on nitrate of *Clitoria ternatea* plants

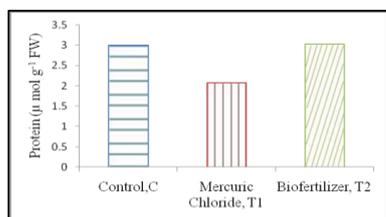


Figure.3. Impact of HgCl_2 on protein of *Clitoria ternatea* plants

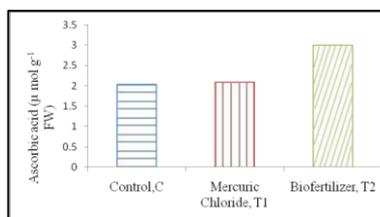


Figure.4. Impact of HgCl_2 on ascorbic acid of *Clitoria ternatea* plants

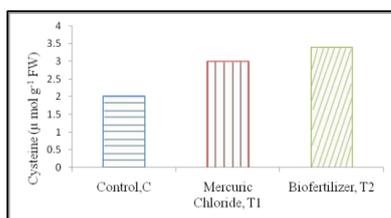


Figure.5. Impact of HgCl_2 on cysteine of *Clitoria ternatea* plants

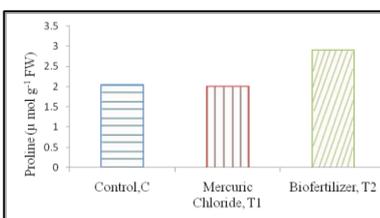


Figure.6. Impact of HgCl_2 on proline of *Clitoria ternatea* plants

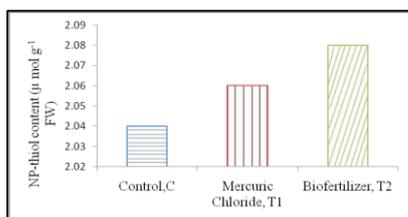


Figure.7. Impact of HgCl_2 on Non protein thiol content of *Clitoria ternatea* plants

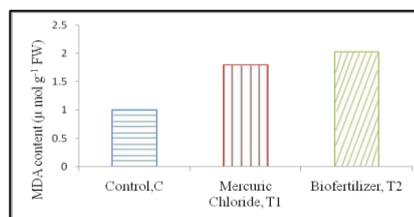


Figure.8. Impact of HgCl_2 on Lipid peroxidation of *Clitoria ternatea* plants

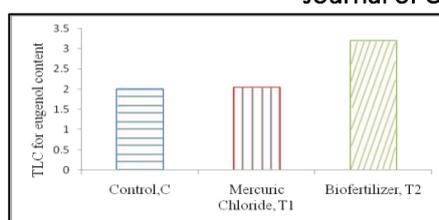


Figure.9. Impact of HgCl_2 on TLC for Eugenol content of *Clitoria ternatea* plants

3.10. Effect of heavy metal stress on Sodium dodecyl sulphate: This is the simple and the basic method used for the separation of proteins. Since protein has a high molecular weight it is found that the current given to the electrophoresis should be high (to the maximum of 40 mA) for the movement of the enzyme. If the current applied is very low the enzyme cannot travel beyond the stacking gel which was observed from (Figure.10). If the maximum current of 40mA (~150V) is applied in SDS-PAGE, the band will be visualized near the anode. This is due to its oxidizing property and its high molecular weight (~130KDa). The samples were loaded in the gel and the electrophoresis was run at constant current of 40 mA. Then the gel was stained with coomassie brilliant blue G-250 for overnight and the bands was visualized in UV- transilluminator.

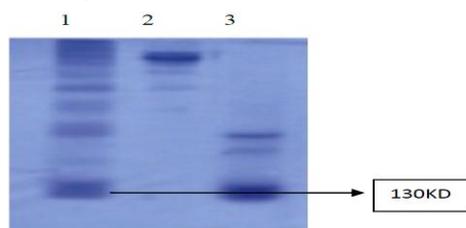


Figure.10. Protein profiling of Protein from *Clitoria ternatea* leaves by SDS-PAGE

Lane-1: Protein Marker, Lane-2: Mercuric Chloride (T1), Lane-3: Biofertilizer (T3)

DISCUSSION

3.11. Effect of heavy metal stress on nitrate reductase activity, nitrate and protein contents: Nitrate reductase is a key enzyme of nitrogen metabolism. Nitrate reductase catalyses the first step of reaction by assimilatory reduction of nitrate into ammonia thereby reducing nitrate to nitrite. During present study it was found that NRA severely affected by mercury treatment. Further, a decline in NRA was found which was correlated to the decrease in total chlorophyll content which could affect photosynthesis process. It was suggested that NRA depends upon photo synthetically generated reductant and energy. Hence, reduction in NRA in mercury treated *Clitoria ternatea* plants could be due to reduced photosynthesis as a result of inhibition of chlorophyll biosynthesis. It was also observed that the nitrate content of leaves was found to be more due to the impaired nitrate reductase activity. Mercury has been reported to reduce foliar protein content in plants. In this case, also a reduction in protein content was also observed in mercury treated *Clitoria ternatea*. As suggested by earlier workers protein degradation might be either due to the increased activity of protease or due to other catabolic enzymes which were activated under mercury stress. It is also likely that mercury induced lipid peroxidation in *Clitoria ternatea* and fragmentation of proteins led to reduce protein content in T1 and T2 plants which may be due to the adverse effect of reactive oxygen species.

3.12. Effect of heavy metal stress on Ascorbic acid, Cysteine and non-protein Thiol contents: Cellular antioxidants in plants also play major role by offering resistance to metals (free radicals formed due to metabolic activity which leads to oxidative stress) by protecting labile macromolecules (Kumar, 2011). During the present study, reduced levels of NP-SH, Carotenoids and Cysteine were observed in Hg treated plants. Ascorbic acid, a primary antioxidant also plays major role in Tocopherol regeneration and Ascorbate in the antioxidant metabolism. Thus the lipid peroxidation effect is limited in *Clitoria ternatea* which was observed by the decline in ascorbic acid content of the leaves. Some of the environmental stress factors reported to increase the level of Proline in plants includes heavy metals, UV radiation, temperature and drought (Alia, 1991). During the present study, higher accumulation of Proline in mercury treated plants of *Clitoria ternatea*, has been observed which might be attributed to the strategies adapted by plants to cope up with mercury toxicity as Proline has multiple functions, such as, Osmoticum, scavenger of free radicals etc. (Zhao Sheng Zhou, 2009). Which also acts as sink for energy to regulate redox potential in T1 and T2 plants in our case.

3.13. Effect of heavy metal stress on Lipid peroxidation: The mercury accumulation in *Clitoria ternatea* results in physiological and biochemical characteristic changes. Further, peroxidation of membrane lipids resulted in potassium leakage, indicating a loss of membrane integrity. In T1 and T2 plants mercury induced loss of membrane permeability along with increased MDA production was observed in *Vallisneria spiralis* (Ladislav, 2001).

3.14. Effect of heavy metal stress on Eugenol content: The secondary metabolites are formed under various stresses as a defence mechanism (Cresswell, 1995). The secondary metabolites are formed under various stresses as a defence mechanism. During the present investigation, all the mercury concentrations increased Eugenol (a Phenyl

propanoid) content respectively after 24 hours. However, in our case mercury induced the eugenol content in treated plants in comparison to control. This might be a part of defence strategy adapted by *Clitoria ternatea* against chromium toxicity to protect them in T1 and T2 plants.

4. CONCLUSION

From the present study it was concluded that mercury contamination found in plants (*Clitoria ternatea*) did not impose any significant effect in the development and growth of the plants. There was a constant increase of metal concentration in the plant tissue (roots and leaves), which was sufficiently correlated with the metals in the watering solutions and not with the metals in the substrate. This toxicity was significantly correlated with the soil pattern and not the substrate. Thus *Clitoria ternatea* could grow in mercury sprayed soil and accumulate high amount of mercury in roots followed by leaves. Mercury accumulation also affected some physiological processes as discussed above. Mercury stress induced the production of the eugenol (a major component of essential oil) in *Clitoria ternatea*. Therefore, *Clitoria ternatea* could be grown in mercury polluted soils for higher yields of essential substances. However, our results are laboratory based and before exploiting the results in field, a pilot field study is recommended.

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