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Effects of As, Cd, Hg and Pb Mixture on Growth and Yield of Fenugreek (*Trigonella foenum- graecum* L.) in a Semi-Hydroponic System

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Abstract

Nutrient rich sewage water (SW) is used for crop irrigation and disposal worldwide. Treated (SW) contains several crop toxic heavy metals (HMs). In this study, effects of mixture of four noxious HMs (As, Cd, Hg and Pb) present in Aligarh Muslim University, SW has been determined on the growth, root nodulation and yield of fenugreek (*Trigonella foenum-graecum* L. cv. PEB) grown in 5" polyvinyl pots filled with composted soil (1:3). 15 days old plants were suspended half way through a pierced lid in 5 rectangular polyvinyl hydroponic systems each filled with 16 L nutrients solution and aqueous mixture of As, Cd, Hg, and Pb in the ratio 1:3:1:15 mg/L (specific to average concentrations present in AMU sewage sludge) in 5 varying concentrations as 0%, 50%, 100%, and 200%, and 300% (T_0, T_1, T_2, T_3, T_4, respectively). Plants were treated for 105 days from sowing. Growth and biochemical parameters were recorded in 90 days old plants and yield at 120 days. Growth, yield and nodulation of the fenugreek treated with 50% concentration (T_1) increased significantly without any adverse effect on root nodulation leading the host crop to continue symbiotic benefits. Effects of T_2 - T_4 treatments on all plant growth parameters, yield and nodulation were adverse. Dose-effects correlations were strong in curvilinear regression patterns.

***Key words*:** *Trigonella foenum- graecum,* Arsenic (As), Cadmium (Cd), Mercury (Hg), Lead (Pb)

Agriculture is the base of Indian rural economy fulfilling major part of food, oil, fibres and other similar requirements of India, in addition to exports. Fast growing human population, urbanization and proportionate increase in noxious pollutants from a number of anthropogenic sources are consistently polluting cultivated lands and water bodies worldwide [1-2]. HMs released from anthropogenic sources leads to a major global environmental issue [3-4]. HMs containing sewage sludge or fly ash amended soil are beneficial for soils by improving their physical, chemical, and biological characteristics [5], when used in optimum doses. But most of these HMs are highly toxic for plants, animals and micro-organisms exposed to them [4], [6-9]. Some most common noxious heavy metal pollutants of urban and industrial origin include cadmium (Cd), mercury (Hg) and lead (Pb) and arsenic (As)- chemically a metalloid [1], [10-12]. These HMs get bio-transferred in plant- animal food chain and accumulate in plants at sub- phytotoxic level, but often biomagnifies at higher trophic levels [7-8], [13-16]. Initially, they accumulate in plants at sub-phytotoxic levels, meaning the concentrations are not high enough to cause visible toxicity to plants. However, as these metals move through the food chain, from plants to herbivores and then to higher trophic levels (carnivores and omnivores), their concentrations increase significantly. This process, known as biomagnification, can lead to toxic effects in organisms at higher trophic levels, including humans. Thus, uptake, accumulation and bio-transfer of heavy metals from contaminated soil and/or water to food chain organisms via plants cause these toxicants to persist for a time longer than their half- life in addition to toxicities [3], [7-9], [17-20]. Some of these HMs are reported to be carcinogenic [21-22], mutagenic and teratogenic in nature [3], [19], [23-25].

Research reports also revealed that the HMs interferes with symbiotic microbial activities in the soil [4], [26-27] and thus adversely affect the soil fertility and crop productivity [8]. Sewage water reportedly contains a number of toxic HMs [1], [29]. Types of heavy metals and relative proportions in sewage water vary with the cities, types of polluting industries: and varies their uptake in plants, bio-transfer, accumulation and effects in food chain organisms [3], [7-9], [15], [19]. [20], reported that plants exposed to higher concentrations of arsenic overproduced reactive oxygen species (ROS) and resulted into oxidative stress in plants as a secondary outcome of heavy metal toxicity and caused skin diseases [30]. Cadmium, uptake and accumulation in plants interferes with various biological processes in plants. Uptake and accumulation of Cd and Pb reduced photosynthesis [1], [31]. Intake of both these HMs leads to high prevalence of upper gastrointestinal cancer [22],

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[32]. Excessive accumulation of Cd and Pb in food crops induce cancer [22], cardiovascular, renal, and neurological disorders in human beings [33-34]. Crop failure, fragile bone, skeletal deformities, kidney failure in humans are generally the result of complex alterations brought about by Cd at the biochemical, physiological, and genetic levels [35]. Cadmium, reduces activities related with δ-aminolevulinic acid synthetase, arylsulfatase, alcohol dehydrogenase, and lipoamide dehydrogenase enzymes [32]. Lead (Pb) is highly phyto- and zootoxic non-essential elements [3], and damage the structure and function of chloroplasts by binding with the -SH group of enzymes [15]. Heavy metal ions prevent some essential metals like Mn, Zn, and Fe from being taken up and transported in leaves and eventually inhibit chlorophyll synthesis or directly damage carotenoid and chlorophyll pigments [4], [36]. Chlorophyll contents in lead (Pb) stressed fenugreek plants reduced, either by the peroxidation of membrane lipids, pigments, and chloroplasts or by oxygen radicals as a result of oxidative stress [37] due to the inhibition of enzymes like δ aminolevulnic acid dehydratase (ALA dehydratase) and protochlorophyllide reductase, linked with chlorophyll biosynthesis [38-39]. High amounts of mercury reportedly increase the synthesis of free amino acids and eventually reduce the amounts of protein and RNA, dry weight, activities of catalase and protease as well as chlorophyll content in leaves [9]. All four selected heavy metals (As, Cd, Pb, and Hg) of the present study are phytotoxic and reduce protein content, germination rate, and photosynthetic contents and also generate excessive reactive oxygen species [20,40,41]. All these four selected heavy metals damage membrane lipid peroxidation, and degrade antioxidant enzymes [4], [42-43].

In present study, we have studied the effects of four highly toxic HMs, namely arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb), as detected in the sewage water released from the Aligarh Muslim University, Aligarh. In this sewage water As, Cd, Hg, Pb and Ni have been detected in first and second stage treated sewage waters of the university treatment plant [44]. The treated sewage water is released in agricultural fields for irrigation. The crops are irrigated 3 to 4 times in a session. In this study, we have treated 15 days old fenugreek with the mixture of four HMs (As, Cd, Hg and Pb) in the ratios (1:3:1:15) close to ratio as worked out by Kaifiyan [44] and studied the effects on the growth at 90 days and yield at 120 days after sowing. For the present study Indian fenugreek (*Trigonella foenum- graecum* L.) was chosen as a model plant to determine the effects of four most hazardous HMs (As, Cd, Hg, Pb) discharged through sewage water and eventually released into the environment or agricultural fields after sewage treatments. The objective of the present study was to estimate effects of the mixture of four selected heavy metals on growth and yield of the selected host plant and root nodulation of symbiotically associated *Rhizobium*.

MATERIALS AND METHODS

Authentic seeds of fenugreek (*Trigonella foenum-graecum* L. cv. PEB, family fabaceae) were procured from IARI New Delhi. The seeds were surface sterilized with 0.5% mercuric chloride for 5minutes and washed thoroughly with DDW and soaked for 24 hours in DW. The authentic and suitable *Rhizobium* of fenugreek were mixed with aqueous jaggery paste and kept overnight and mixed next day with the DW soaked seeds of fenugreek and left overnight. Ten *Rhizobium* inoculated seeds of fenugreek were sown in 15 polyvinyl pots (150 mm diameter; Fig 1a) filled, with garden soil mixed with compost in 3:1 ratio (pre-stabilized for 15 days). Thinning was done 15 days after sowing to have 3 seedlings of equal growth vigor in each pot.

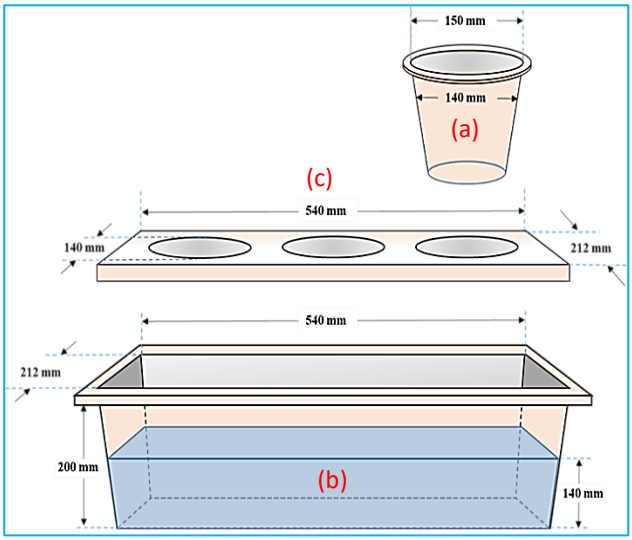

Fig 1 Semi-hydroponic set-up

Four rectangular polyvinyl pots of 540×212×200 mm, (length × width × depth; Fig 1b-c) were filled with 16L of fresh water containing Hoagland nutrient solution to a depth of 140 mm (Fig 1b). In the nutrient solution of four hydroponics, aqueous mixture of As, Cd, Hg, and Pb {prepared in the ratio of 1:3:1:15 mg/L, respectively as average concentration in AMU sewage water closed to this ratio [44] were added in four varying concentrations (50%, 100%, 200%, and 300%; of the HMs mixture T_1, T_2, T_3, and T_4, respectively). The 5th hydroponics of nutrient solution was maintained as control (T_0) without HM mixture. The top of all five hydroponics was covered with a lid of water proof PVC foam board of 15 mm thickness, having 3 equidistant circular holes of 140 mm diameter (Fig 1c). The pots with 15 days old fenugreek seedlings were inserted through the holes in the lids so as their 10 mm basal part remained dipped in the hydroponics. Water level in each rectangular hydroponic pot (Fig 1b) was maintained constantly at 16L with deionized water throughout the experiment. The water of hydroponics was aerated for 30 minutes at alternate days. The overall concentrations of all four selected HMs (As, Cd, Hg, Pb) in T_0, T_1, T_2, T_3, and T_4 were, (1) nil in T_0, (2) 50% concentration as 0.5:1.5:0.5:7.5 mg/L in T_1, (3) 100% as 1:3:1:15 mg/L in T_2, (4) 200% as 2:6:2:30 mg/L, and (5) 300% as 3:9:3:45 mg/L in hydroponics of respective treatments. Thus, T_2 had concentration of HMs in single irrigation, 200% concentration as applied in crops irrigated twice, and 300% in crop irrigated thrice with the sewage water.

15 days old plants of fenugreek were treated in hydroponics for next 105 days in hydroponic pots for a total age of 15+105 = 120 days after sowing (DAS). Growth parameters were recorded in 90 days old plant samples, and yield parameters after final harvest of 120 old plants. The vegetative growth recorded in present study are, length, fresh and dry weight of root and shoot, counts and area of leaf, counts of branch and counts of *Rhizobium* nodules per plant. Length of shoots and roots of three randomly selected plants per treatment were measured with a measuring scale after uprooting and gentle cleaning with tap water. Number of leaves, branch and root nodules were also recorded in triplicates. Leaf area was determined by graph sheet method as per laboratory practice [4]. Plants were dried for 48 hours at 80°C in an oven and weighed for dry biomass.

All biochemical parameters were determined in fenugreek plants at 90 DAS. Chlorophyll and carotenoid contents in fresh leaves were determined following the method of Arnon [45] and Mackinney [46], respectively as described by [4].

NR enzyme activity enzyme was determined with the method of Jaworski [47]. 200 mg fresh and finely chopped leaf tissues of each sample were placed in plastic vials and 2.5 ml phosphate buffer (pH 7.5), 0.5 ml potassium nitrate solution, and 2.5 ml of 5% isopropanol were added. The samples were incubated in a BOD incubator for 2 hours at 30 ± 2°C in dark. 0.4 ml of the incubated mixture was taken in a test tube and 0.3 ml 0f 1% salphanilamaide solution and 0.02% NED- HCL were added. The combination was left in the test tube for 20 minutes for optimal color development and was diluted to 5 ml with DDW. Color absorbance at 540 nm was recorded against a blank reagent on the spectrophotometer (T70 UK). Standard curve was plotted using known graded concentrations of sodium nitrite solution. The absorbance of each sample was compared with the calibration curve to record NR activity and expressed as µM NO^2 g^{-1} FW h^{-1}.

The protein content in leaf samples was determined by the method of Lowry [48] method. 300 mg of leaf tissue were ground with 10 ml of 20% trichloroacetic acid (TCA) in a prechilled mortar and pestle. The samples were centrifuged at 600 rpm for 15 minutes. The supernatant was discarded. 5.0 ml of 0.1 N NaOH was added to the pallet, mixed thoroughly and again centrifuged for 15 minutes. The protein fraction was taken from the residue. 5.0 ml alkaline copper solution was added to 0.5 ml protein extract and left to stand for 10 minutes in the dark. The Folin- Ciocalteau reagent was then added to this solution and mixed immediately. The blue color developed. The solution was kept again in dark for 30 minutes for maximum color development. The absorbance of solution was recorded at 660 nm in a spectrophotometer (T70 UK). The protein contents were calculated by comparing the optical density of each sample with standard curve plotted taking known graded dilutions of standard solution of bovine serum albumin and expressed in mg g^{-1} fresh leaf tissue.

Proline content in fresh leaves were measured following Bates [49]. 300 mg of fresh leaf tissues were crushed in mortar with 5.0 ml of 3% sulphosalicylic acid. The homogenate was filtered with Whatman No. 1 filter paper. In 2 ml of the filtrate, 2 ml glacial acetic acid and 2 ml of freshly prepared acid ninhydrin was added and heated in a boiling water bath for 1 hour. The reaction was stopped by transferring the test tubes to an ice bath. 4 ml of toluene was added and vigorously stirred for 20-30 seconds until a pink chromophore layer is seen. The absorbance of the pink color chromophore layer was measured at 520 nm on spectrophotometer (referred above) against a reagent blank. The amount of proline in the sample was calculated by a standard curve prepared from pure proline (range, 0.1-36µmol).

µ moles of proline g^{-1} tissue = µg proline ml^{-1} × ml toluene/115.5 × 5/g (sample)

Where, 115.5 is the molecular weight of proline

Pod counts were recorded in three freshly harvested 120 days old plants of each treatment. Length and fresh weight of 10 pods, fresh seed weight was also recorded and plants were dried in an oven at 80 °C for 24 hours to record dry weight of other yield parameters.

RESULTS AND DISCUSSION

Data of all growth parameters (plant length, fresh and dry weight, leaf number and area, branch number, nodule counts) of 90 DAS fenugreek treated with varying concentrations of the heavy metal's mixture are summarized in Fig (2a-d). Plant length, fresh, dry weight, leaf number and area, number of branches and root nodules increased significantly on treatment with lower dose (T_1) of the heavy metals as compared to control (Fig 2a-d). But, higher doses of the HM mixture (T_2-T_4) reduced all these plant growth parameters in proportion to doses of HMs mixture

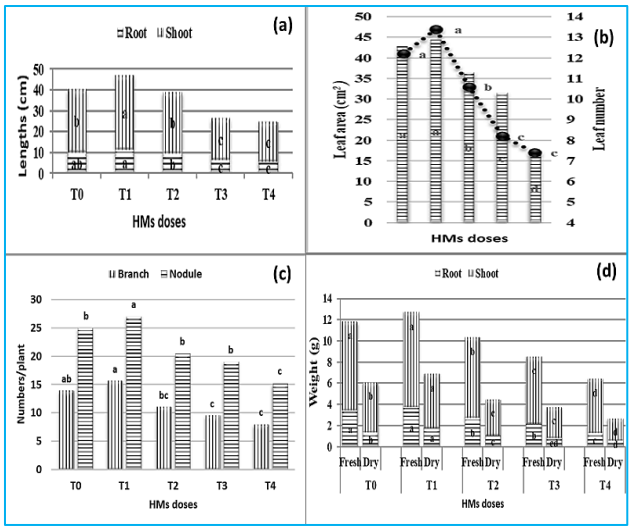

Fig 2 (a-d) Effects of varying doses of HMs concentration on selected growth parameters of fenugreek plants

Responses of total chlorophyll content, carotenoid content, protein content, nitrate reductase activity and proline accumulation in heavy metal treated fenugreek recorded 90 DAS (Fig 3 a-c) were analogous to responses of growth parameters showing significant increase on treatment with lower dose (T1) of the heavy metal mixture and consistent reductions with increasing doses (T2-T4) of the heavy metal mixture (Fig 3 a-c). The proline content in the plants increased linearly with the concentration of HMs indicating a dose dependent plant stress (Fig 3c).

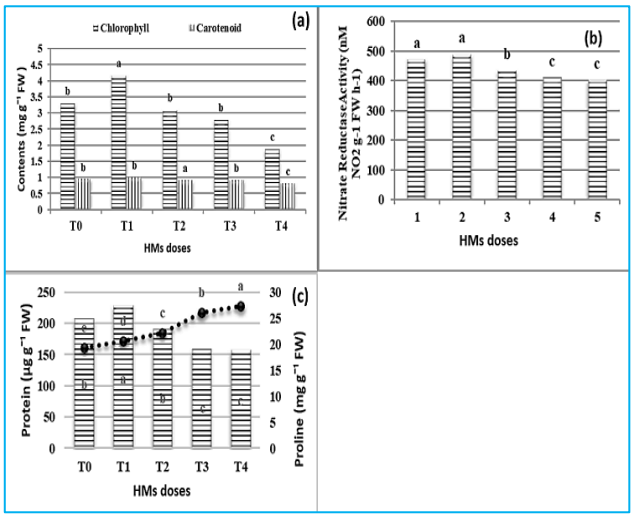

Fig 3 (a-c) Responses of selected biochemical parameters om treatment of fenugreek with varying doses of HMs mixture

The yield parameters of the heavy metal treated fenugreek plants were recorded at 120 DAS (final harvesting at maturity). Yield of fenugreek as evident from the data (Fig 4 a-d) increased on application of lower dose (T_1) of the HM, but higher doses of the heavy metal mixture (T_2-T_4) reduced all yield parameters studied (pod number and length, pod fresh and

dry weights, seed number per pod and per plant, seed fresh and dry weights).

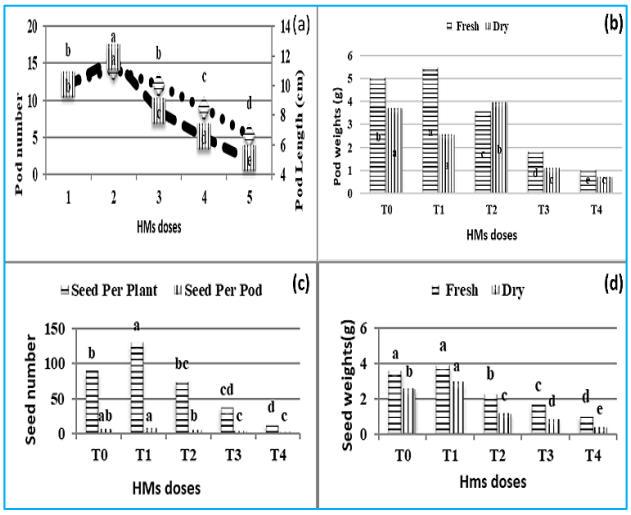

Fig 4 (a-d) Effects of varying doses of HMs mixture on selected yield parameters of fenugreek plant

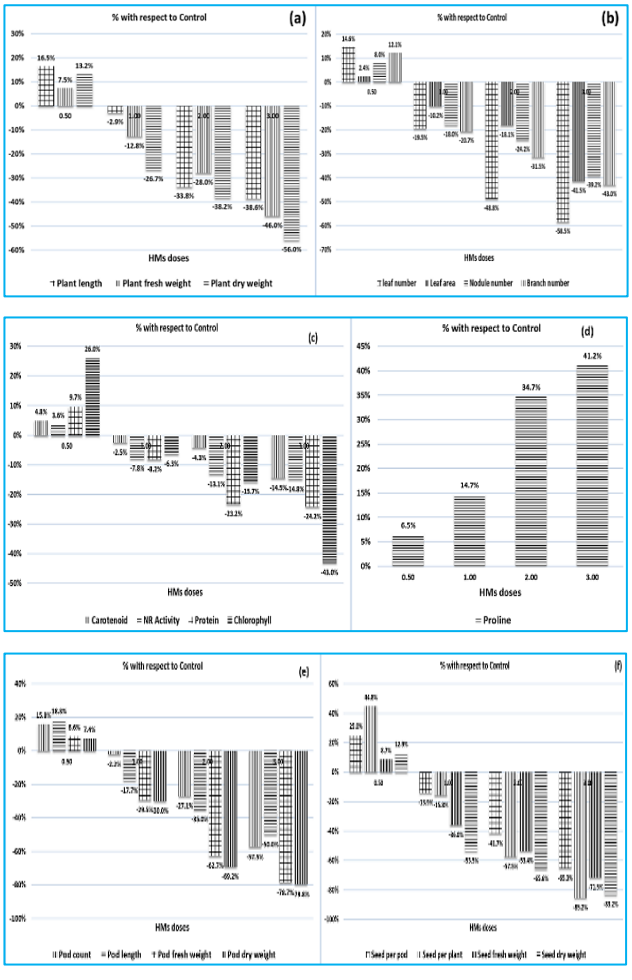

Fig 5 (a-f) Percent variation in plant growth, biochemical and yield parameters with respect to control plants of fenugreek treated with varying doses of HMs. Variation above zero are positive and below zero are negative

Percent variation of all studied parameters with their respective controls were determined to compare the relative sensitivity of the parameters to varying concentrations of the heavy metal mixture (Fig 5a-f). The lowest concentration (T_1) of the heavy metal mixture increased all studied growth parameters of fenugreek in the order of amplitude as plant length> leaf number >plant dry weight> branch counts> nodule numbers> plant fresh weight> leaf area. Application of higher doses (T_2, T_3, and T_4) had adverse effects on all growth parameters in the order of magnitude as leaf number >plant dry weight> branch counts> plant fresh weight> plant length> nodule number> leaf area. Among biochemical parameters, the degree of reductions was in the order, chlorophyll content> protein content> NRA> carotenoid contents. The order of increase in proline accumulations were in dose dependent manners showing proportionate increase with the concentration of the HMs (T_1-T_4). The percent variation in yield parameters in T_2-T_4 treated fenugreek were in the order as seed per plant> pod length> pod number> seed dry weight> seed fresh weight> pod fresh weight> pod dry weight> pod dry weight, indicating that seed setting and development (weight) benefitted from lower concentrations of the HMs.

Regression analysis and correlation coefficient between heavy metal concentrations (x- axis) and growth parameters (y-axis) show significant strong correlations (express as R^2). The regression lines best fitted to data are curvilinear indicating increase in selected growth parameters on treatment with 50% of the selected HMs mixture as compared to control and then a consistent decline with the increase in the concentrations of HMs ratio (T_2-T_4). The regression analysis affirmed a dose-related response of the growth of fenugreek (Fig 6 a-f).

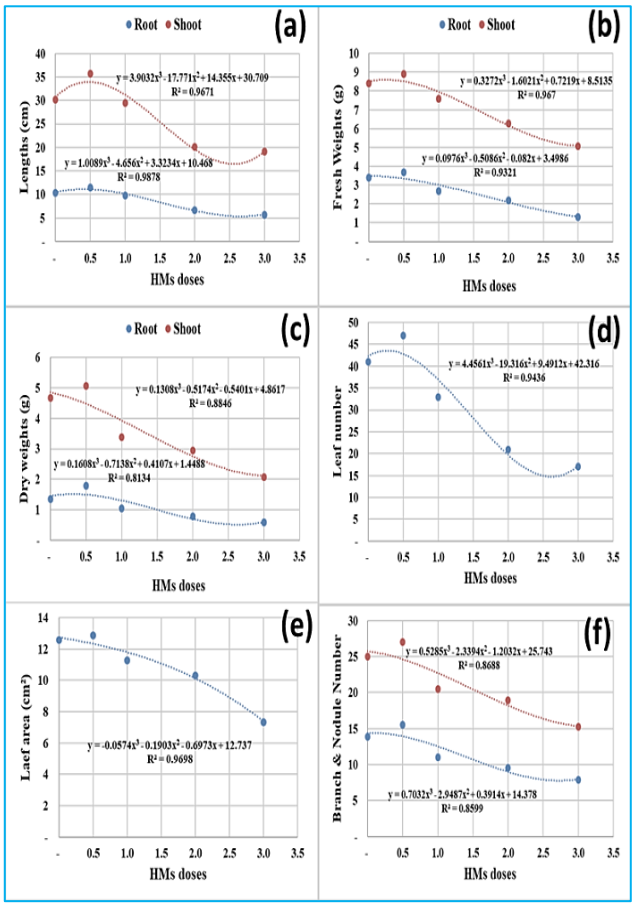

Fig 6 (a-f) Regression lines with equations and square correlation coefficient between treatments (x-axis) and selected growth parameters (y-axis) of fenugreek treated with HMs mixture

The regression lines and correlation coefficients of HMs concentrations (x- axis) with respect to chlorophyll content (Fig 7a), NRA (Fig 7b), Protein (Fig 7c), and Proline (Fig 7d), were significantly correlated showing a curvilinear response. The degree of correlations (R^2) was high and significant indicating a dose- dependent response.

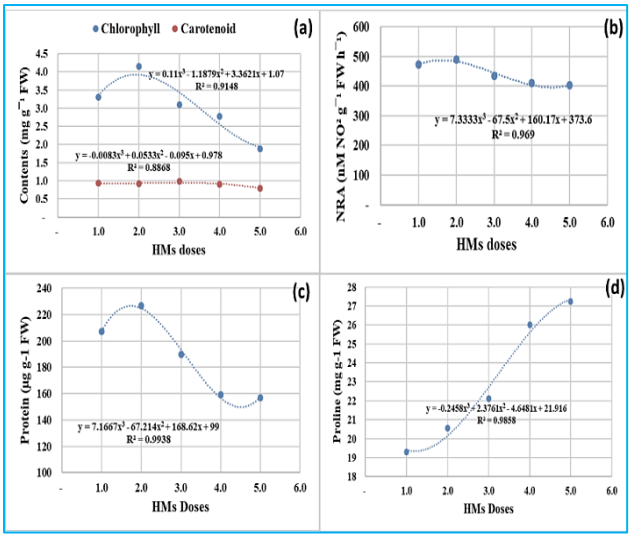

Fig 7 (a-d) Regression curves with equations and correlation coefficients between HMs doses (x-axis) and biochemical parameters (y-axis) of fenugreek plants

The yield parameters were also strongly related in curvilinear order with doses of HMs mixture in addition to strong correlation coefficient (R^2) (Fig 8a-f).

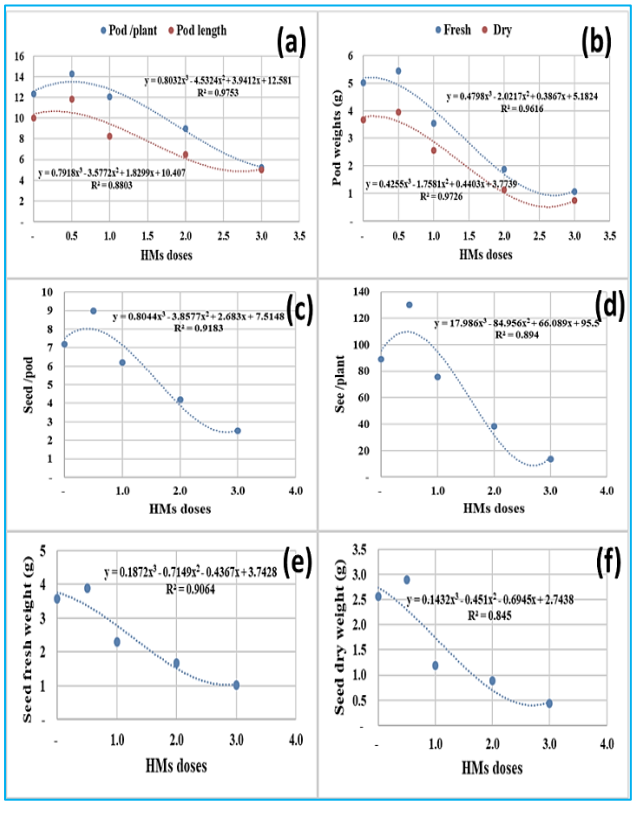

Fig 8 (a-f) Regression curves with equation and correlation coefficients between HMs doses (x-axis) and yield parameters (y-axis) of fenugreek plants

In present study, root and shoot length, plant fresh and dry weights, leaf count, leaf area, branch number and nodule numbers of fenugreek, increased as compared to the control on application of 50% concentrations of HMs (T_1), but increase in HM concentration (100-300%; T_2-T_4) reduced these measures significantly (Fig 2a-d). Significant reductions in growth and yield of some crop plants treated with some HMs or HMs laden sewage sludge and fly ash have been reported in *Cucumis sativus* under Cd stress [50], *Vigna radiata* [51], *Trigonella foenum- graecum* treated with Cd [52], *Brassica juncea* [53-54], Nicotiana tabacum [55], *Vicia faba* treated with Cd [16], mung reduced with Cd stress [56], mung with fly ash amended soil [44], and barley [3] and in *Vicia faba* treated with Cd [57].

Some HMs detered root growth and lateral root formation and ultimately decreased plant fresh weight and dry weight (Fig 2d) as reported in *Vicia faba* [16]. HMs toxicity also causes chlorosis and imbalance in water uptake to repair the injuries and loss of biomass in *Vicia faba* [16]. In line to our findings, [58] also recorded significant decrease in shoot height and leaf area in response to treatment with some HMs. Application of HM laden sewage sludge in plant triggers the translocation of a significant amount of photosynthates to plant organs under stress at the cost of plant biomass. Nodule numbers, in our findings (Fig 2c) increased on application of lower dose (T_1) of HMs, but decreased with higher doses (T_2-T_4). This shows that HMs in higher doses affected the nodulation adversely but lower doses had a positive effect (Fig 2c). Similar effects showed by Vigna radiata treated with lower dose of sewage sludge [52], Treatment with optimum Ni concentration enhanced nodule numbers in Glycine max and *Trigonella foenum-graecum* as reciprocal outcome of increase in nodule biomass [59-60]. Also, Ni deficiency, delayed nodule formation in *Glycine max* [61-60]. Ni has been added to the list of essential plant nutrient as a component of urease enzyme of vital importance in nitrogen metabolism in higher plants [62]. [63] reported that increasing soil pollution with Cd, Cr and Pb inhibited root mass and nodule development (in terms of brighter nodules) without any effect on nodule number in *Vicia sativa* and *Pisum sativum*. In this finding [63], inferred from their findings that excessive soil pollution with these HMs (Cd, Cr and Pb) reduced symbiotic efficiency due to reduction in nodule development. Similarly, the growth of plant biomass decreased in Brassica [64] and *Cymbopogon citratus* [65] treated with fly ash, red mung treated with Cr [66], *Lens culinaris* [44] and *Trigonella foenum-graecum* with Ni [67]. These variable responses of crops may be due to species, soil structure and environment related changes including hydroponic system. The regression curves in our studies between HMs doses (x- axis) and varying growth parameters (y-axis) of fenugreek were curvilinear and strongly correlated (Fig 6) affirming that only lower HMs dose enhanced the growth parameters but further increase in HMs doses reduced the growth of plants. In contrary, a linear regression relationship between dose and response has been reported in some species treated with some HMs showing dose- dependent response [16], [36], [68-69].

Moving on to the biochemical features, we found that contents of total chlorophyll, carotenoid, protein content and NR activities in leaves also increased on application of lower dose (T_1) of the HMs combination as compared to control (Fig 3a-c). In lines to the findings of our study, some reports revealed increase in chlorophyll contents in *Lens culinaris* treated with HMs rich sewage sludge [43], *Shorea robusta* with HMs [70], *Brassica juncea* [69], *Hordeum vulgare* treated with sewage sludge [3] *Vicia faba* treated with Cd [59] and in variety of other plants under HM stress [71], significant ($p < 0.05$) reduction in chlorophyll and carotenoid contents has also been reported in *Vicia faba* treated with Cd [16]. Carotenoids are non-enzymatic antioxidants, which protect the chlorophyll molecules against oxidative stresses. Increase in carotenoid content in plants treated with lower doses of HMs may thus be attributed to defence strategy of the stressed plant. But, at higher HMs concentrations plants loses this defensive efficiency via carotenoid production. There may several reasons of increase in plant sensitivities on HM exposures.

The effects of mercury on proteins content and suppression of important enzymes involved in photosynthesis can be related to changes in energy absorption, dissipation, and trapping efficiency of plants due to adverse effects on their PSII reaction center [9]. We have also recorded reductions in NR activity in fenugreek plants treated with higher concentrations of HMs mixture (Fig 3b). In our study, Protein content decreased at higher concentration (T_2-T_4) in dose- dependent manner (Fig 3c). Similarly, Protein decreased in *Vigna radiata* with increase in sewage sludge amounts [51]. Proline increased with doses (T_1-T_4) of HMs mixture (Fig 3c). Adverse effects of some HMs on nitrate reductase enzyme had been reported in maize [72], chickpea [35], [73], tomato [74-75], soybean [76]. Excess accumulation of proline in numerous plant species is a general indicator of various environmental stresses [19], [69]. Likewise, in our findings, application of heavy metals mixture significantly increased ($p<0.05$) proline content in the leaves of *Trigonella foenum- graecum* L. as compared to control in dose dependent manner (Fig 3c) fenugreek with Ni [67-77]. The oxidative stress triggered by any environmental adversity including heavy metal load in sewage sludge or fly ash treated plants brings in excessive proline production as defensive strategy [4], [69], [78]. This indicates that plants facing stress due to either a single HM or a range of HMs results into production of this osmolyte at the cost of plant growth [67]. This metabolic change in stressed plants leads to generation of excessive proline to scavenge free radicals and ROS [15].

In our study pod and seed number and their weights of fenugreek plants initially increased on treatment with lower HMs concentration (T_1) and decreased at subsequent higher concentration of HMs in curvilinear order (Fig 4a-d). In contrary, increase in pod and seed numbers in [79] reported significant increase in seed weight in *Lens culinaris* medic cv sazar-91 treated with varying amounts of sewage sludge and *Vigna radiata* on treatment with sewage sludge [51]. But, decreased in *Vicia faba* treated with Cd [16]. The losses in yield parameters may have been caused by the persisting stress of HMs mixture on seed setting. [80] also reported significant increase in yield of lady's finger grown in varying amounts of HMs laden sewage sludge. Yield of *Vigna radiata* increased with doses of sewage sludge applied in soil and decrease with higher dose [51]. But decreased in *Oryza sativa* [81] and *Vicia faba* [16] on exposure of elevated heavy metal levels.

CONCLUSION

We inferred from our findings that occasional application of treated sewage water in the crop may enhance the growth and yield of fenugreek due to presence of nutrients and lesser amounts of HMs as evident from responses o fenugreek treated with 50% (T_1) of HMs mixed nutrient solution. But application of untreated sewage water containing all these four HMs in quantities as in (T2) and repeated irrigation twice or thrice (T_3 and T_4), may adversely affect the growth and yield and rhizobial nodulation fenugreek (*Trigonella foenum-graecum* L. cv. PEB) due to toxic concentrations of all four HMs usually present in the treated sewage water.

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Conflict of interest

None.

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