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RESEARCH ARTICLE

PHYTOREMEDIATION OF HMX CONTAMINATED SOIL THROUGH *JATROPHA CURCAS*

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ABSTRACT

Phytoremediation technique can be used to clean up various contaminants viz. metals, pesticides, solvents, explosives, crude oil, polycyclic aromatic hydrocarbons, and landfill leachates. Phytoremediation is proving to be an attractive alternative to current energy intensive and very expensive cleanup methods. "Current engineering-based technologies used to clean up soils—like the removal of contaminated topsoil for storage in landfills—are very costly and dramatically disturb the landscape". Phytoremediation is a combination of technologies that use "plant-influenced biological, chemical, and physical processes that aid in the remediation of contaminated substrates". It is a potential low-cost technology that is currently being investigated for many remediation applications. In this study greenhouse pot experiment was conducted to test the natural accumulation and phytoremediation potential of HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), as contaminant under simulated conditions. The amount of explosives present in the soil before and during phytoremediation was determined by using HPLC. The result of the study reveals that *Jatropha curcas* removed upto 87% of HMX in 270 days of study thus indicating that the plant can be successfully utilized for the treatment of explosive contaminated sites. Most of the HMX was translocated in the parent form to the shoot and leaves.

INTRODUCTION

Phytoremediation may be defined as a group of innovative technologies that use plants and natural processes to remediate or stabilize hazardous wastes in soil, sediments, surface water, or groundwater (USEPA, 2001). Phtyoremediation is an effective, non-intrusive and inexpensive means of remediating soils (Alkorta and Garbisu., 2001). Phytoremediation consists of a collection of different plant-based technologies, each having a different mechanism of action for the remediation of metal-polluted soil, sediment, or water including : *rhizofiltration*, which involves the use of plants to clean various aquatic environments (Dushenkov et al., 1995; Mukhopadhyay and Maiti, 2010); *phytostabilization* (Weyens et al., 2009; Pivetz, 2001; Vangronsveld et al., 1995; Smith and Bradshaw, 1972), in which plants are used to stabilize rather than clean the contaminated soil*; phytovolatilization* (Burken and Schnoor, 1997; Ban˜uelos et al., 1997), which involves the use of plants to extract certain metals from soil and then release them into the atmosphere through volatilization; *phytoextraction (phytoaccumulation)*, in which plants absorb metals from soil and translocate them to the harvestable shoots where they accumulate (Yoon et al., 2006; Zacchini et al., 2009; Rafati et al., 2011); *phytodegradation,(phytotransformation*), where the plants breakdown the contaminants taken up through metabolic processes within the plant, or the breakdown of contaminants external to the plant through the effect of compounds such as enzymes, produced by the plants and *rhizodegradation* (*phytostimulation),* where the contaminants are broken down in the soil through microbial activity that is enhanced by the presence of rhizosphere (Burken and Schnoor, 1997; Mukhopadhyay and Maiti, 2010).Recent studies have shown that Phytoremediation is an effective low cost technology which may be used for the remediation of explosive contaminated soil. Summary of some of the previous studies related to HMX Phytoremediation have been listed below in table 1. Phytoremediation is well-suited for use at very large field sites where other methods of remediation are not cost-effective or practicable. It is cost effective than any other alternative chemical or mechanical method for removing hazardous compounds from soil. Cost effectiveness of Phytoremediation over other methods has been enlisted in table 2.

Many Defense sites all over the world are contaminated with explosive wastes resulting from explosives manufacturing; munitions load, assembly and pack operations; explosives machining, casting, and curing; open burn and open detonation operations; and laboratory testing of munitions, and demilitarization operations (Brannon et al., 2002; Li et al., 2004; Eriksson et al., 2004). Moreover, waste disposal practices associated with military production of weapons, especially before and during World War II, have resulted in significant contamination of soils and ground water with high explosives such as TNT, RDX and HMX (Pennington *et al*., 2002). Even if only a small fraction of the total High Explosive mass within a munitions is not consumed during a detonation, repeated blasts at training ranges and impact areas result in significant High Explosive accumulation in surface

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soils, establishing a source of potentially leachable contaminants (Morley *et al*., 2006).The contamination of the environment by explosives, especially by nitro aromatics (NACs), is a worldwide environmental problem since enormous amounts of these compounds were produced during World War I and II (Nepovim *et al*., 2005). The main soil contaminants are the high explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT), although other contaminants such as 2,4-dinitrotolune (2,4-DNT), nitro-glycerine (NG), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) have also been detected (Morley *et al*. 2006). Polynitro organic explosives [hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and 2,4,6-trinitrotoluene (TNT)] are typical labile environmental pollutants that can bio transform with soil indigenous microorganisms, photo degrade by sunlight and migrate through subsurface soil to cause groundwater contamination (Halasz *et al*., 2002). The fate of High Explosive materials is of concern today not only because of possible initiation hazards in highly contaminated soils, but also because of the toxicity of these materials and their metabolites and the resulting slow, natural degradation rate (Yamamoto *et al*., 2004). Phytoremediation has proven to be effective in several applications for treatment of shallow contaminated sites. In general, plants can withstand greater concentrations of organic pollutants than most micro-organisms; they can take up the chemicals quickly and convert them to less toxic metabolites and are known to stimulate degradation of organics in the rhizosphere (Schnoor *et al*., 1999).

MATERIALS AND METHODS

Experimental set up

The present study was conducted in two phases viz.

- Site characterization
- Phytoremediation

Site Characterization

Site Selection

MOD establishments for site characterization were selected based on the processes such as manufacturing, processing and test firing of various arsenals and based on the extensive use of explosives. The extent of soil and water contamination at three different explosives manufacturing sites (Site1, Site2 and Site3) was carried out and a simulated experiment was designed based on the level of contamination. Physico-chemical properties of the soil were also studied.

Soil and Water sampling

The sites were gridded and distinctively marked based on types of soil samples viz., surface and subsurface (15-20 cm) deep were collected from different ear marked areas with the help of a screw auger and a sampling probe.

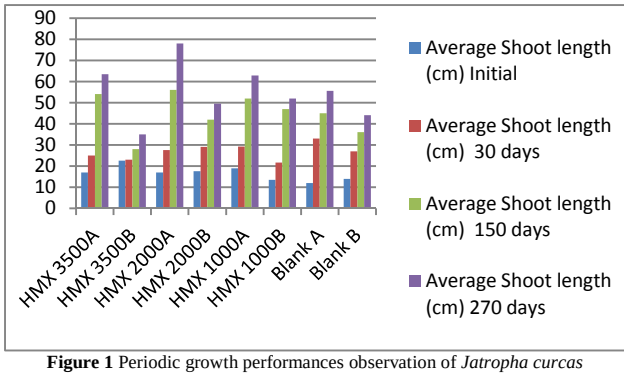

Figure 1 Periodic growth performances observation of *Jatropha curcas*

different activities being carried out in the respective site. Two Sampling locations were allocated different number codes for convenience. The soil samples were collected as per EPA method no. 540/R-97/59 (USEPA, 1997) and a composite sample from each site was obtained by quartering technique. The samples were then packed in zip lock bags and stored in ice boxes to be taken to the laboratory for analysis. Surface, sub-surface and effluent water samples were collected in sampling bottles and stored in refrigerator till analysis.

Sample Preparation

After air drying in shade for 15-20 days, soil samples were crushed gently in a ceramic pestle and mortar and sieved through 2 mm stainless steel sieve. These ground samples were stored in polyethylene bags. The ground samples were well mixed before weighing them for analysis. Duplicate samples were taken for each set to maintain accuracy and precision during analysis.

Physico-chemical analysis

Soil and water samples were analyzed for physico-chemical properties such as texture, color, pH, salinity, Nitrate-nitrogen, calcium, magnesium etc. by **DR-960 HACH soil and water analysis kit**. Respective extraction procedures were followed for analyzing the above said properties. Reagents necessary for the analysis were provided in the kit.

Metal analysis

All chemicals used were of A.R. grade. All samples were analyzed for metal content by AAS using Standard method: 2gm. soil sample taken in washed glass flasks. Soil sample digested with 9:1 Nitric acid and perchloric acid. Solution fumed for a final step to Normality / ml. Diluted and analyzed for residual total heavy metals by Graphite Furnace AAS.

Phytoremediation Experiments

Plant Selection

While selecting a species for phytoremediation several factors have to be taken into account. The species should be fast growing, high biomass producing, competitive, hardy, with profuse root system, tolerant to adverse environmental

Table 1 Summary of Phytoremedition Studies

Plant used	Explosive removed	Reference
Periwinkle (*Catharanthus roseus*)	HMX	Bhadra *et al*. (2001)
Poplar	HMX	Yoon(2002)
Bean, alfalfa, canola	HMX	Groom (2002)
Lolium perenne	HMX	Groom (2001)
Lettuce, corn stover and yellow nutsedge	HMX	Price *et al*. (1997)
Lolium perenne	HMX	Rocheleau *et al*. (2008)

conditions, non edible and economically beneficial (Garbisu *et al*., 2002; Alkorta *et al*., 2004, Pilon-Smits, 2005). Taking all these factors into consideration we have chosen *J. curcas* and tested its suitability for phytoremediation of HMX. *Jatropha curcas* is a large shrub or small tree belonging to the family Euphorbiaceae. It is regarded as a potential biofuel crop for future due to its low moisture demands, pure hardiness and

Table 2 Cost Advantage of Phytoremediation over other Technologies (Schnoor, 1998)

Type of Treatment	Range of Costs $/Ton
Phytoremediation	$10-35
In-situ Bioremediation	$50-150
Soil Venting	$20-220
Indirect Thermal	$120-300
Soil Washing	$80-200
Solidifcation/Stabilization	$240-340
Solvent Extraction	$360-440
Incineration	$ 200-1,500

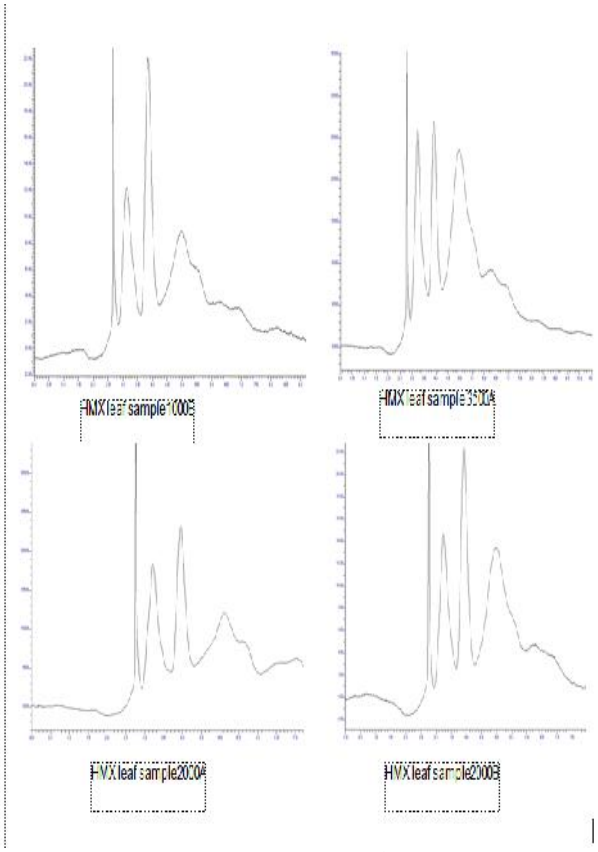

Figure 2 Chromatograms showing the peaks of various secondary products formed during the treatment of HMX inside the plant leaves and stem

stress handling ability (Kaushik *et al*., 2007; Singh, 2007). It is a drought resistant and a perennial plant yielding 5-12 ton per hectare oil seed and produces 2-4 tones of biodiesel (Debnath *et al*., 2008). It grows fast with little maintenance and can reach a height of 3-8 m (Kaushik *et al*., 2007; Gunaseelan, 2009). It has been identified in India as the most suitable oil bearing plant and has been recommended for plantation on waste land as it requires minimal inputs for its establishment (Singh, 2007; Gunaseelan, 2009).

Pot culture study

Site characterization studies were carried by CFEES to assess the level of contamination at defense facilities. The same level of contamination was simulated in pot culture studies to study the potential of treatment of HMX by *Jatropha curcas.* Physico-chemical characteristics of HMX were presented in table 3.Weight of empty pot - 4.5 Kg, weight of pot with soil - 11 Kg, weight of soil in the pot – 6.5 Kg.

- ***Preparation of 3500mg/Kg solution of HMX*** Dissolve 22.75g HMX in 2 litres (250ml water+1750ml acetone).Solution added to 6.5 Kg of soil and left for air drying so that acetone vaporises and only HMX remains in bound form. Two replicas to be prepared for two replications of plants.
- ***Preparation of 2000mg/Kg solution of HMX*** Dissolve 13g HMX in 2 litres (500ml water+1500ml acetone).
- ***Preparation of 1000mg/Kg solution of HMX*** 6.5g of HMX dissolved in 600ml water and 1400ml acetone.
- ***Control*** 6.5 kg of air dried soil put into the pot without mixing anything.

The above 1000mg/Kg, 2000mg/Kg and 3500mg/Kg solutions were added to the 6.5Kg of soil sample, mixed well and allowed to dry well on a sheet. After drying, the soil was put into the pots in 2 replications of each concentration and *Jatropha curcas* was transplanted. Two controls were also set up to match the effect of HMX on the plant. After setting up the experiments, soil samples from the pots were taken at fixed intervals of time to see the general physicochemical parameters and amount of HMX remaining in the soil. Soil samples were prepared by EPA method 8330 and were analyzed by HPLC.

Explosive analysis in soil and water

Chemicals/ Solvents

Analytical standard for HMX obtained from Terminal Ballistic Research lab were dried to constant weight in a dessicator. Methanol used in preparation of the eluent was Baker HPLC grade. Acetonitrile used was Merck, HPLC grade and Water used for preparation of effluent was of Merck HPLC grade. The mobile phase was prepared by combining appropriate portions of each component and was degassed with Helium before use.

Preparation of standards:

$$RF = R/C$$

Individual stock Standa

Individual stock standards HMX were prepared by weighing out approximately 250 mg of each analyte material to the nearest 0.01 mg, transferring to individual 250 ml volumetric flask and diluting to volume with acetonitrile. Stoppered joints were wrapped with parafilm to retard evaporation and solutions were stored at 4°C. Concentration of the analytes in these stock solutions was approximately 1000 mg/L (USEPA, 1997).

Working standards

5 ml of stock solutions of each analyte was transferred to 100 ml volumetric flask to obtain 50 mg/Kg concentration. From

$$\text{Concentration of analyte} = \text{Response (peak area unit)}/\text{Response factor } (R\ F_a)$$

Calibration

Daily working standards were analyzed in triplicate at the beginning of each day of analysis, once at the midpoint and once at the end of each day of analysis. Response factors for each analyte are obtained from the mean peak area. A linear

model with a zero intercept is used for calibration (USEPA, 1997).

Table 3 Physico-chemical characteristics of HMX

S. No	Parameters	Values for HMX
1	CAS Number (Chemical Abstract Services Registry Number)	2691-41-0
2	Molecular formula	$C_4H_8N_8O_8$
3	Molecular Weight	296.2
4	Melting point (^oC)	285-287 °C
5	Boiling point (^oC)	no data
6	Water solubility	1.14 @5°C 4.42@10°C 6.62@20°C 11.6@30°C 17.4@35°C 140@83°C
7	Vapour Pressure (torr@ 25^oC)	3.33 x 10^{-14}
8	Henry's Law Constant KH (atm-m3/mole, 25oC)	2.60x10^{-15} atm.m^3/mole
9	Octanol / water partition coefficient (log K ow)	0.26, 0.06
10	Log Koc	0.54
11	Soil water partition Coefficient K_d (l/kg)	
12	Physical form @ 20 ° C	Crystalline
13	Colour	White
14	Odour	no data
15	Density (g/cm3)	1.90

this intermediate standard a series of working standards viz. 2,4,6,8 and 10 mg/Kg were prepared (USEPA, 1997).

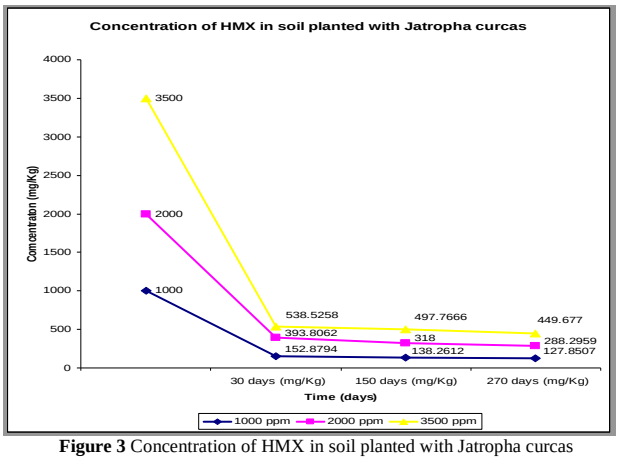

Figure 3 Concentration of HMX in soil planted with Jatropha curcas

Calculation

(a) Response factor

Since a linear calibration curve with zero intercept is to be expected, calculations of daily results were obtained using response factors calculated for each analyte.

The concentration of each analyte (C_a) are obtained by dividing the response for each analyte by the appropriate response factor (R F_a)

Table 4 Physico-chemical properties of soil to be filled in the pots for the treatment

S. No.	Parameters	Concentration
01.	Texture	Sandy Loam
02.	Ph	7.2
03.	Nitrate	12.236mg/Kg
04.	Bicarbonate	91.5 mg/Kg
05.	Chloride	99 mg/Kg
06.	Lead	218.7 mg/Kg
07.	Iron	24300.3 mg/Kg
08.	Chromium	120 mg/Kg
09.	Phosphorus	107.8 mg/Kg

Explosive analysis in Plants

5g of finely cut plant samples were suspended in 10-20 ml of

The mean response (R) for each analyte from repeated determinations of the daily calibration solution is obtained in peak area units. The response factor (RF) for each analyte is obtained by dividing the mean response by the known concentration (C) in mg/L.

where, R= mean response and C= Known concentration

(b) *Analyte concentration*

RESULTS AND DISCUSION

Physico-chemical properties of soil

The purpose of soil characterization was to adjudge the soil quality for the growth of *Jatropha curcas*. After selecting the most suitable site soil was dug out to obtain virgin soil and subjected to extensive physico-chemical analysis. Various physical parameters, which influence root establishment in soil and determine water and air movements within, were determined such as texture, pH, conductivity, salinity; chemical parameters, which extracts elements from the soil and determine their respective quantities available to plants.

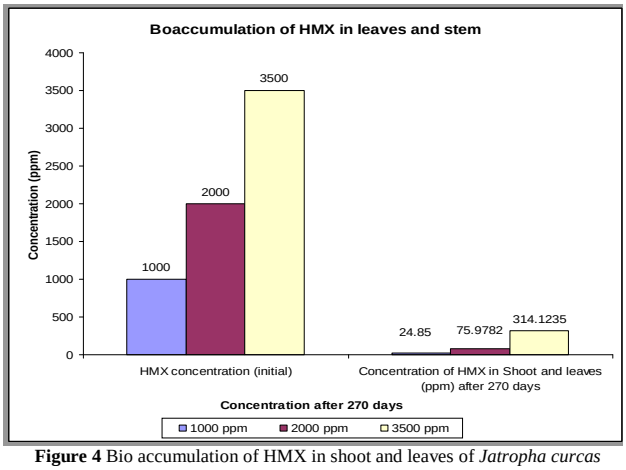

Figure 4 Bio accumulation of HMX in shoot and leaves of *Jatropha curcas*

Table 5 Percent uptake of HMX by Jatropha curcas

S. No.	Initial concentration (mg/Kg)	Concentration after 30 days (%)	Concentration after 150 days (%)	Concentration after 270 days (%)
01	1000	84.72	86.18	87.22
02	2000	80.31	84.1	85.86
03	3500	84.62	85.78	87.153

These elements also known as "available nutrients" are important indicators of soil fertility viz. nitrate, phosphorus. The respective concentrations in the soil were found as shown in table.1, concentrations were also determined. Soil texture, which was found to be sandy loam, has profound effect upon the properties of soil including its water supplying power, rate of intake, aeration, fertility and ease of tillage. The pH was 7.2, which lies within the recommended value for proper growth and efficient uptake of nutrients and compounds from soil takes place at this pH. Macronutrients including metals were also present in substantial amount as shown in the table.4. These parameters along with the climatic conditions and concentrations of explosive wastes (HMX, TNT, and DNT) directly influence plant establishment, growth and development.

Growth performance of J. curcas

Qualitative and quantitative observations on shoot length and visual stress symptoms were recorded periodically. Periodic observations of shoot length of the plants for *Jatropha curcas* have been given in the figure 1 which shows that there was no negative effect of the higher concentration of HMX on the shoot length as no retardation in growth of the plant was observed thus leading to the conclusion that the accumulating concentrations of HMX are not toxic to the plants. Biometric observations reveal that the plant has endurance up to the concentration of 3500 mg/Kg of HMX. A 21-day exposure to HMX in soil had no adverse effects on ryegrass growth; HMX was translocated to ryegrass shoots, with bio concentration factors (BCF) of up to 11, thus concluding that HMX can accumulate in plants and may potentially pose a risk of biomagnification across the food chain (Rocheleau *et al*., 2008). However, no critical symptoms such as chlorosis and leaf loss were observed.

HMX uptake by Jatropha curcas

Several authors have reported bioaccumulation of HMX by plants (Price *et al*., 1997; Best *et al*., 1997; Groom *et al*, 2002; Halasz *et al*., 2002; Rocheleau *et al*., 2008). Rye-grass (*Lolium perenne*) present in field samples was found to extract and accumulate HMX from soil without further degradation (Groom *et al*, 2002); the predominance of HMX (0.10 m*M*) was noted, along with the presence of its reduction product octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazine (MN-HMX); no degradation occurred although the transport of the explosive to the plant tissue might constitute a potential future *in situ* remediation technology (phytoextraction); RDX and HMX are reportedly degraded by anaerobic sludge in liquid culture via a sequential reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso- 5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5- trinitroso-1,3,5-triazine (TNX). Studies conducted by Halasz *et al*., 2002 concluded that HMX was detected in all tested plants and accumulated mainly in the blade tissue; sufficiently irrigated wheat and rye-grass cultivars accumulated HMX in their senescent leaf tissue to over 500 mg/kg (plant dry mass basis) from soil with an average HMX concentration of 30 mg/kg (dry weight basis); only the predominance HMX (0.10 m *M*) was noted, along with the presence of trace amounts of its reduction product octahydro1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazine (mn-HMX) as determined by LC–MS; the deprotonated mass ion $[M_2H]$ was 16 μ less than that of HMX indicating a difference of one oxygen atom between HMX and its nitroso derivative (mn-HMX). Interestingly mn-HMX was also found in the soil, indicating that HMX did not bio transform but was extracted by all tested plants. Substantially less data are available on the sorption of HMX (Pennington *et al*., 2002). In column studies HMX sorption was approximately described using a linear equilibrium model (Myers, 1998). HMX is apparently sorbed less than is TNT by soils (Townsend 1996, Price, 1998).

In the present study, after 270 days of treatment, HMX was found in the leaves and shoot samples in the form of the parent compound as well as three more peaks were observed in the chromatogram one of them for the solvent front and the other two suggesting the transformation of the parent compound into two other by-products. Figure 2 shows the Chromatograms showing the peaks of various secondary products formed during the treatment of HMX inside the plant leaves and stem (sample collected after 270 days of the study). Rate of explosive uptake was observed to be the maximum during the first thirty days, then decreasing sharply and after the period of thirty days the uptake was gradual and at a uniform rate over a period of 270 days. The results of the experiment in the form of decreasing concentration in the soil have been presented in graphical form as figure 3. It can be observed that the efficiency of taking up of HMX by the plants was almost equal and there was no toxicity effect of higher concentration. Maximum bio accumulation was observed in shoot and leaves of *Jatropha curcas*.

The concentration of HMX in the leaves and shoot were found to be quiet high; 24.28 mg/ Kg in 1000 mg/Kg concentration pots, 75.9782 mg/kg in 2000 mg/Kg concentration pot and the highest was in 3500 mg/Kg concentration pot i.e. 314.1235 mg/Kg. The results obtained were in accordance with earlier studies and supported by studies conducted by Halasz *et al*., 2002 which concluded that HMX was detected in all tested plants and accumulated mainly in the blade tissue; sufficiently irrigated wheat and rye-grass cultivars accumulated HMX in their senescent leaf tissue to over 500 mg/kg (plant dry mass basis) from soil. The respective results have been represented in figure 4.

The efficiency of removal of HMX by *Jatropha curcas* was quiet high as supported by the decrease in soil concentration. Maximum concentration of the contaminant was removed from the first thirty days only as seen from table 5, during the first thirty days the removal from the 1000, 2000 and 3500 mg/Kg pots were 84.72%, 80.31% and 84.62% respectively. The efficiency of removal for 1000mg/Kg concentration and 3500 mg/Kg concentration were almost equal thus suggesting that there was no toxicity caused by the high concentration of the contaminant on the plant. During last phase of the experiment, the uptake was slow.

CONCLUSIONS

In all phytoremediation is the combined effect of soil, plant, abiotic processes and microbial interaction mechanisms in the soil and thus we can say that phytoremediation is a synergistic phenomenon encompassing all favourable features and effects. In this study we use Jatropha curcas, a biofuel plant for the Phytoremediation of HMX contaminated soil and the observations and conclusions are as follows:

- Maximum bio accumulation was observed in shoot and leaves of *Jatropha curcas*.
- Rate of explosive uptake was observed to be the maximum during the first thirty days, the decreasing sharply and after the period of thirty days the uptake was gradual and at a uniform rate over a period of 1 year.
- The efficiency of taking up of HMX by the plants was almost equal and there was no toxicity effect of higher concentration.
- The concentration of HMX in the leaves and shoot were found to be quiet high; 24.28 mg/ Kg in 1000 mg/Kg concentration pot, 75.9782 mg/kg in 2000 mg/Kg concentration pot and the highest was in 3500 mg/Kg concentration pot i.e. 314.1235 mg/Kg as supported by other studies conducted by Halasz *et al*., 2002 which concluded that HMX was detected in all tested plants and accumulated mainly in the blade tissue; sufficiently irrigated wheat and rye-grass cultivars accumulated HMX in their senescent leaf tissue to over 500 mg/kg (plant dry mass basis) from soil.

Jatropha curcas considered as a smart biofuel crop ensures energy and environmental security. This study reveals that *Jatropha curcas* is tolerant to HMX and can thus be successfully used for the phytotreatment of defence sites contaminated. The results suggest the potential applicability and advantage of using Jatropha species for phytoremediation of explosives in an eco friendly and efficient manner for environmental cleanup.

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