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Comparative assessment of physiological and biochemical changes in the selected plant species growing under hydrocarbon stress

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Abstract

Hydrocarbons have become a serious environmental problem due to industrialization and extensive use of vehicles. Various plant species shows a range of stress responses and adaptations to survive in hydrocarbon stress. This study was conducted to investigate the comparative phytotoxicity of polycyclic aromatic hydrocarbons (PAHs) on plants growing under hydrocarbon stress on the germination and to evaluate the response on seedling growth. For the study, two crop plant species (*Brassica juncea* L. Czern., and *Triticum aestivum* L.) and two ornamental plant species (*Tagetes erecta* L. and *Helianthus annuus* L.) were taken. Pot experiments were conducted in triplicates of 10 days old seedlings treated with 5, 20, 50 & 100 mg kg^{-1} concentrations of hydrocarbons. After 20 days, biochemical analysis and antioxidant enzyme activity of these plants were studied. Polyphenol and proline increased with increasing concentration of hydrocarbons which were maximum in *H. annuus* with 0.909 mg g^{-1} polyphenol and 0.732 µmol g^{-1} proline at 100 mg kg^{-1}. Increase in antioxidant enzymatic activities was observed with increasing concentration. *H. annuus* showed maximum activity at 100 ppm which was ascorbate peroxidase (20.37 Unit g^{-1} FW), peroxidase (0.212 Unit g^{-1} FW) and superoxide dismutase (2.13 Unit g^{-1} FW). HPLC analysis in plants and soil provided the concentration of hydrocarbons present in plants species after 20 days taken up from the treated soil. Plants cultivated in 100 mg kg^{-1} concentration were analysed and the lowest toxicity observed in *H. annuus* which was 3.013 mg kg^{-1} Naphthalene, 7.750 mg kg^{-1} Phenanthrene and 5.691 mg kg^{-1} Anthracene while highest toxicity was observed in *Tagetes* at 8.476 mg kg^{-1} Naphthalene, 0.398 mg kg^{-1} Phenanthrene and 0.416 mg kg^{-1} Anthracene. These results suggested that *H. annuus* can be adopted in phytoremediation of hydrocarbons soil.

Keywords: Antioxidant activity; *Helianthus annuus*; hydrocarbon; phytoremediation; phytotoxicity

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Introduction

Rapid urbanization and industrialization are associated with an increase in the contamination of the environment by petroleum and hydrocarbons (1). Hydrocarbons are highly hydrophobic and have low biodegradability which leads to their accumulation in the surroundings (2). Polycyclic aromatic hydrocarbons (PAHs) are a group of different chemicals which are formed due to

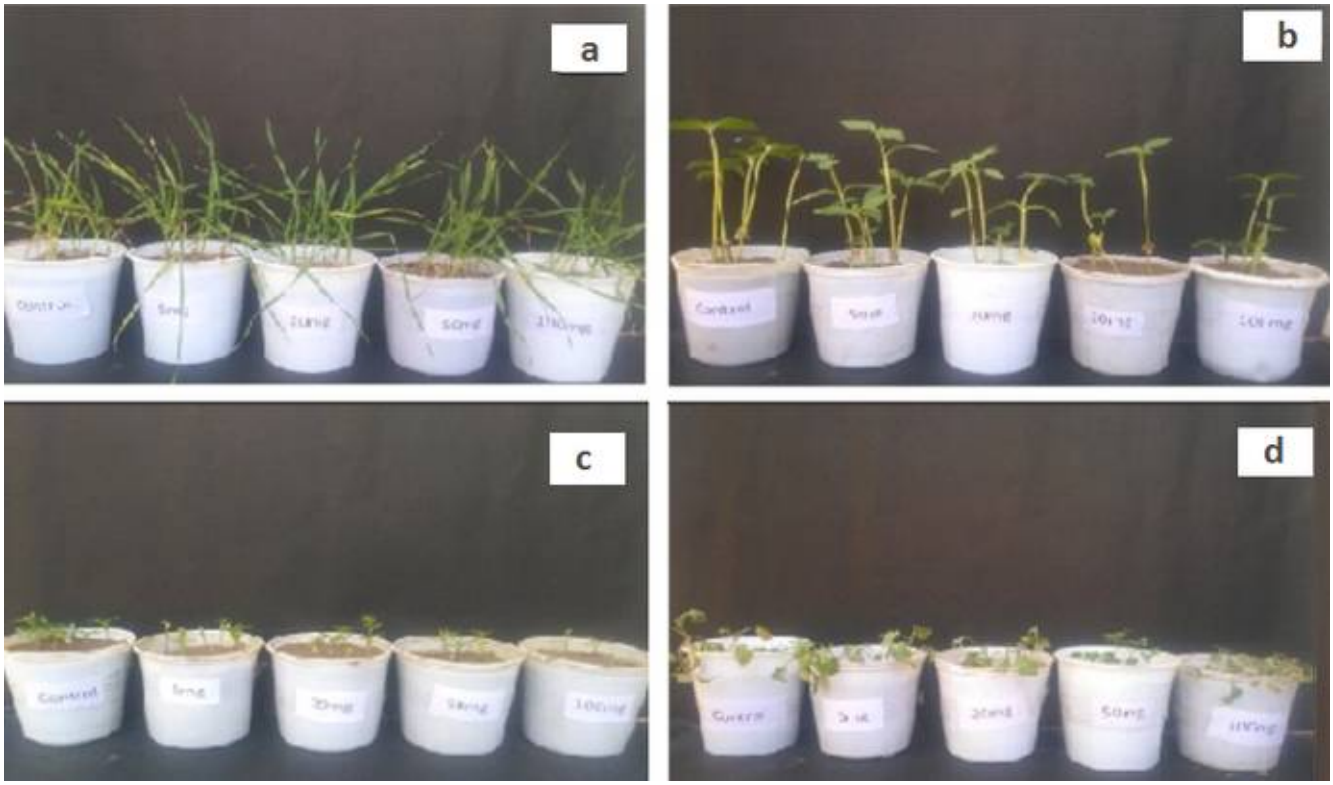

Fig. 1. Effects of hydrocarbons on plants after 20 days. **a**) *Triticum aestivum*, **b**) *Helianthus annuus*, **c**) *Tagetes erecta*, **d**) *Brassica juncea*.

incomplete combustion, oil and gas, soot, burning of coal and other organic substances like tobacco (3). HCs are used in agricultural as well as, photographic products, pharmaceuticals, plastics and in lubricating materials (2). Soil contamination with hydrocarbons occurs from various sources such as refineries, pipelines and tankers, storage tanks and oil spillage accidents (4). PAH from all these sources ultimately mixed with the soil majorly affecting plants growing in contaminated soil.

The major negative morphological and anatomical alterations in plants caused by PAH were morphological symptoms on the growth of root and shoot, chlorosis, formation of white spots, trichome malformations and reduction of root hairs (5-6). PAHs initiate changes in anatomical structure and the architecture of roots. The induction of oxidative stress by PAHs cause peroxidation of membrane's lipids, tissue damages, as well as DNA and RNA damage (7).

The most important symptoms observed in the plants contaminated with hydrocarbons were chlorophyll degradation (8), alterations in the stomata metabolism and reduction in respiration and photosynthesis rate, enhanced production of phytohormones related to stress (9), and accumulation of pollutants and their byproducts in vegetal tissues. Rye-grass biomass in soil with 5000 mg kg^{-1} hydrocarbons was reduced by 46 % (10, 11). Hydrocarbons adversely disturbed the germination rate and growth of plants in the soil (12). PAHs uptake cause carcinogenic and mutagenic effects on humans and they are strong immunosuppressants (13-14). The effects of higher exposure of PAHs on humans are nausea, eye irritation, decreased immune function, breathing problems, diarrhea, skin inflammation and lung function abnormalities (15-17).

Uptake of contaminants by plants resulted in the entry of the same to the food chain which causes various diseases in humans. Many plant species are sensitive towards the pollutants, their growth is affected and it is time consuming to create enough biomass for meaningful soil remediation. Although PAHs cause many deleterious effects on plant growth but different groups of plants vary in their sensitivity and tolerance under hydrocarbon stress. Sunflower has already reported for high degree of tolerance to the multicontaminated heavy metals and hydrocarbons (18). *Helianthus* enhanced the biodegradation of hydrocarbons in the soil. Taking this into account, we have investigated the survivability and toxic effect of hydrocarbons on four different plants such as wheat, mustard, marigold and sunflower growing in the soil contaminated with phenanthrene, anthracene and naphthalene.

Materials and Methods

Experimental treatments

For the present study, two crop plants *Triticum aestivum* L. (wheat), *Brassica juncea* L. (mustard) and two ornamental plants *Helianthus annuus* L. (sunflower), and *Tagetes* L. (marigold) were collected from Krishi Vigyan Kendra, Banasthali

Vidyapith, Rajasthan, India. Ten seeds per pot were sown containing 1 kg of soil under controlled conditions. Pots were placed in green house with a photoperiod of 13 h light and 11 h dark at 35±2 ºC. Ten days old uniform seedlings were selected for the treatment of hydrocarbons (anthracene, phenanthrene & naphthalene) at concentrations 5, 20, 50, and 100 mg kg^{-1} in triplicates along with control (0) in triplicates (Fig. 1). Changes in morphology, bioconstituents and induced antioxidant enzymes [catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD) and superoxide dismutase (SOD)] activity were recorded on the 20th day after treatment. The accumulated concentration of hydrocarbons in plant and soil was detected by HPLC.

Analysis of the morphological and biochemical parameters

Morphological analysis of root-shoot length was recorded immediately in control and treated plantlets after 20 days. Total chlorophyll (19) and proline (20) were estimated. Proline concentration was analysed at 520 nm by double beam spectrophotometer. Polyphenol content was analyzed (21) and determined spectrophotometrically at 725 nm.

Fourier transform infra-red spectrophotometer (FTIR)

FTIR studies were conducted for determination of functional groups in plant samples. Plant sample (0.2 g) was taken and crushed using pre-chilled mortar and pestle. Petroleum ether was used as a solvent for FTIR analysis and sample was incubated for 24 h. The region used for infrared spectroscopy is 4000 ~ 400 cm^{-1} because the absorption of most of the organic and inorganic compounds lies in this region. Data analysis was done by the transmittance frequency peaks in the sample and the vibrations of molecules. The results were analysed using Opus 7.0 software tool.

HPLC analysis

The samples (0.5 g) were homogenized with mixture of benzene and methanol (3:1, *v*/*v*). Solvents evaporation was done through vacuum concentrator. 1 ml of solvents (benzene and methanol) was supplemented and vortexed for 30 min at 100 rpm. Sonication was carried out for 30 min at 4 °C and 40 W. Then the mixture was centrifuged at 10,000 g for 40 min. The supernatant was evaporated by using a vacuum evaporator and dissolved in acetonitrile (300 µl). Samples were stored at -20 °C.

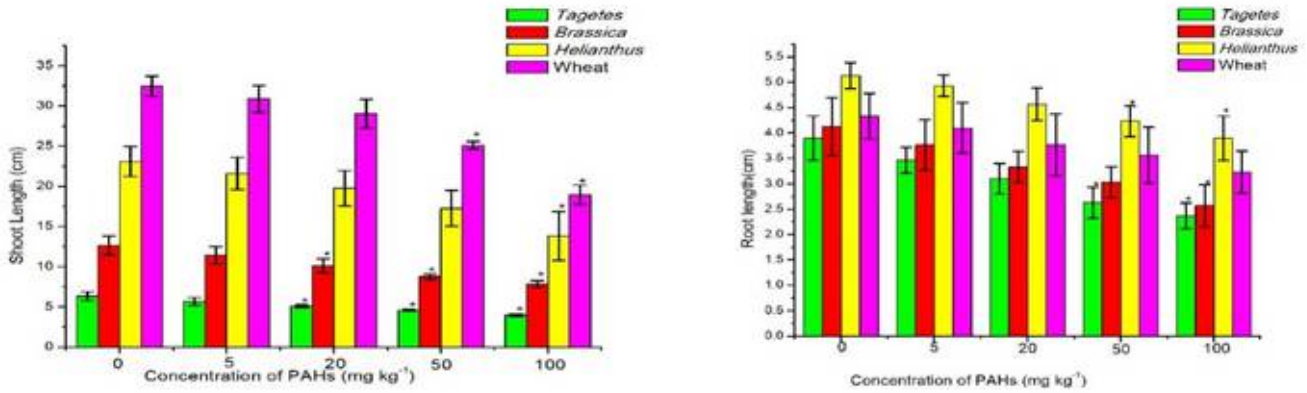

Fig. 2. Effect of hydrocarbon concentrations on (a) Shoot length (b) Root length. Values indicate the mean ± SD (n=3). Values followed by * are significant by $p \leq 0.05$.

Ascorbate peroxidase (APX) activity in control *vs* treated plantlets was measured (22). The rate of oxidation of ascorbic acid was determined by the change in the absorbance at 290 nm after every 25 sec for 3 min with the help of the molar extinction coefficient 2.8 $mmol^{-1}$ cm^{-1}. The catalase (CAT) activity was determined (23). The molar extinction coefficient of H_2O_2 is 39.4 $mmol^{-1}$ cm^{-1} at 240 nm. Standard method (24) was used for the determination of superoxidase dismutase (SOD) activity. The reaction mixture was visible to light at 70 mol photons m^{-2} s^{-1} for 10 min. The absorbance was observed at 560 nm. The activity of peroxidase (POD) in hydrocarbons treated and control leaf samples were determined. Absorbance was measured at 436 nm.

The instrument HPLC (SCL-10AVP Shimadzu) analysis consisted of a solvent delivery pump operating in a range from 0.001 to 9.999 ml min^{-1} and an UV-VIS detector. 20 µl was injected using an autosampler. Mobile phase consisted of A: acetic acid (50 mM) and B: 100% methanol. Compounds were eluted by linearly increasing gradient: 0 → 6 min (70% B), 6 → 10 min (100% B), and 10 → 15 min (100% B). Detection was carried out at 275 nm.

Statistical analysis

Biochemical and morphological observation were analyzed by standard statistical methods to determine the mean values and standard deviation (SD). The values in the figures were expressed as the mean ± SD of the three replicates.

The result was analyzed using SPSS version 20 by one-way ANOVA. The treatment means were employed to Tukey's multiple comparisons to determine significant ($p<0.05$) difference in treatment.

Results and Discussion

Plant growth and morphology

Variation in root and shoot length of *T. aestivum*, *H. annuus*, *B. juncea* and *T. erecta* was observed with increasing hydrocarbon concentration on 20 days (Fig. 2a and b). Visual symptoms of growth reduction, chlorosis and necrotic lesions had been observed in all plant species (6). Significant ($p<0.05$) decline in root-shoot length was recorded in treated seedlings. Among all the treated plants, *Tagetes* is most affected in terms of shoot-root length reduction at 100 mg kg^{-1} concentration while wheat has shown least toxicity symptoms in their morphological character. With increasing concentration of hydrocarbon, tolerance index in roots and shoot appeared to be decreasing.

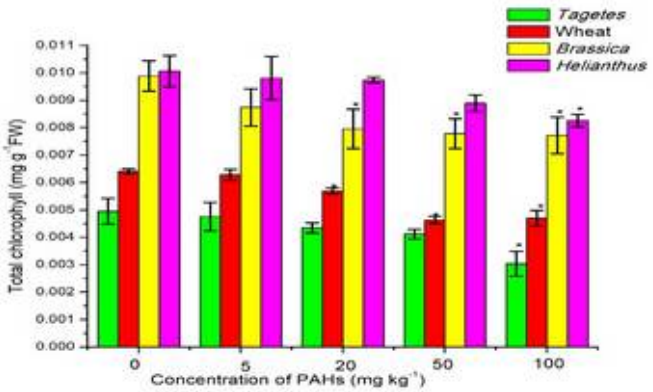

Fig.3. Effect of hydrocarbon concentrations on total chlorophyll content of 20 days old seedlings. Values indicate the mean ± SD (n=3). Values followed by * are significant by $p \leq 0.05$.

ultimately the photosynthetic rate in plants. A significant decrease of chlorophyll 0.0049 mgg^{-1} FW to 0.0030 mgg^{-1} FW observed in *Tagetes* whereas *Helianthus* species was less affected as there was not much difference in total chlorophyll 0.010 mgg^{-1} FW to 0.008 mgg^{-1} FW (Fig. 3). Similarly, mustard and wheat showed slight decrease in chlorophyll content as compared to control. The concentration of hydrocarbon tolerance index in roots and shoot appeared to be decreasing.

Proline content: Proline which is involved in antioxidative defense mechanism found to be enhanced significantly ($p<0.05$) with an increasing hydrocarbon concentration (Fig. 4a). The highest proline content 0.353 µmol g^{-1} to 0.732 µmol g^{-1} was recorded in *Helianthus* ($p<0.05$) and minimum content 0.227 µmolg^{-1} to 0.499 µmol g^{-1} in *Tagetes*.

Polyphenol content: Polyphenol content was found to be enhanced significantly ($p<0.05$) with increasing phenanthrene: naphthalene: anthracene ratio (Fig. 4b). Based on the absorbance of samples reacted with the Folin–Ciocalteu reagent and taking the standard of gallic acid, total polyphenol shows in the graph 4b. The highest polyphenol 0.311 mg g^{-1} to 0.909 mg g^{-1} was recorded in *Helianthus* ($p<0.05$) while comparing with other three plant species.

Antioxidant enzymes activity: The significant increase in APX, CAT, POD and SOD activities were observed in hydrocarbon treated plants as compared to control. For 20 days, APX activity was noted highest in *Helianthus* as 20.37 Unit g^{-1} FW at 100 mg kg^{-1} of hydrocarbons compared to activity in the other three plants species (26). In the comparative account activity was *Tagetes*>*Brassica*>wheat>*Helianthus* (Fig. 5a). The CAT activity was found to be enhanced in leaves of treated seedlings as the concentration increases.

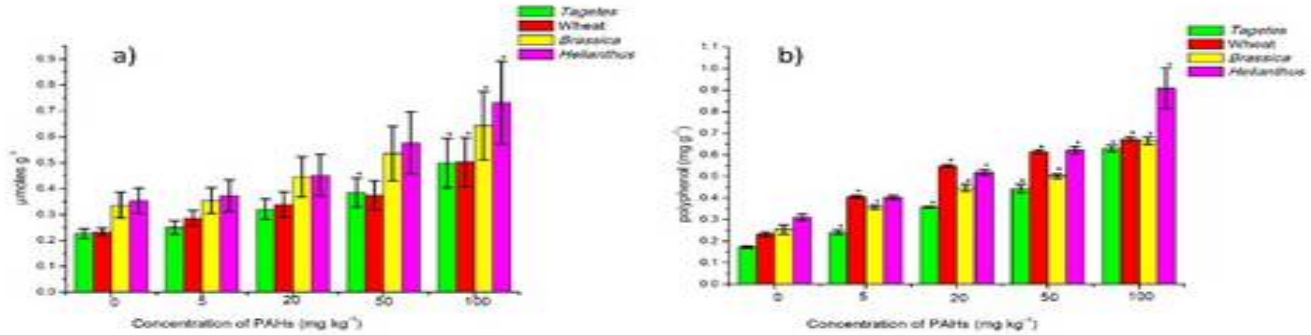

Fig.4 (a-b). Effect of hydrocarbon concentrations on a) proline content b) polyphenol content of 20 days old seedlings. Values indicate the mean ± SD (n=3). Values followed by * are significant by $p \leq 0.05$.

Total chlorophyll

Chlorophyll content was found to be decreased with the increased concentration of hydrocarbons. Similar results were observed in *Arabidopsis thaliana* after hydrocarbon stress (25). Control showed maximum total chlorophyll content as compared with other concentrations. It leads to disruption of photosynthetic machinery and

Among all stressed plant species, response of *Helianthus* to increasing hydrocarbon concentration was 1.76 Unit g^{-1} FW to 21.66 Unit g^{-1} FW (Fig. 5b), measured significantly high against other hydrocarbon treated plant species. Like APX and CAT enzymes, SOD and POD enzyme activities also showed a significant increase in concentration and time depended manner. The results were verified according to earlier reports where similar

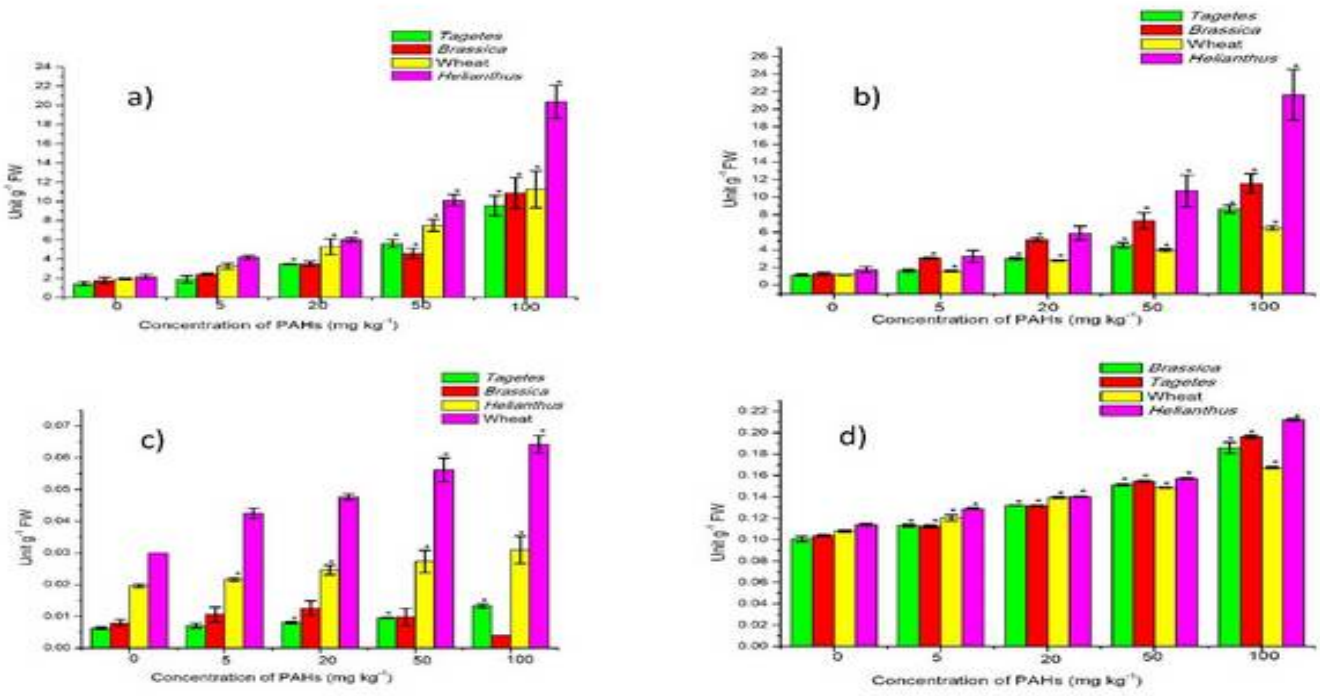

Fig. 5 (a-d). Effect of hydrocarbon concentrations on (a) APX, (b) CAT, (c) SOD, and (d) POD enzyme activities. Values indicate the mean ± SD (n=3). Values followed by * are significant by $p \leq 0.05$.

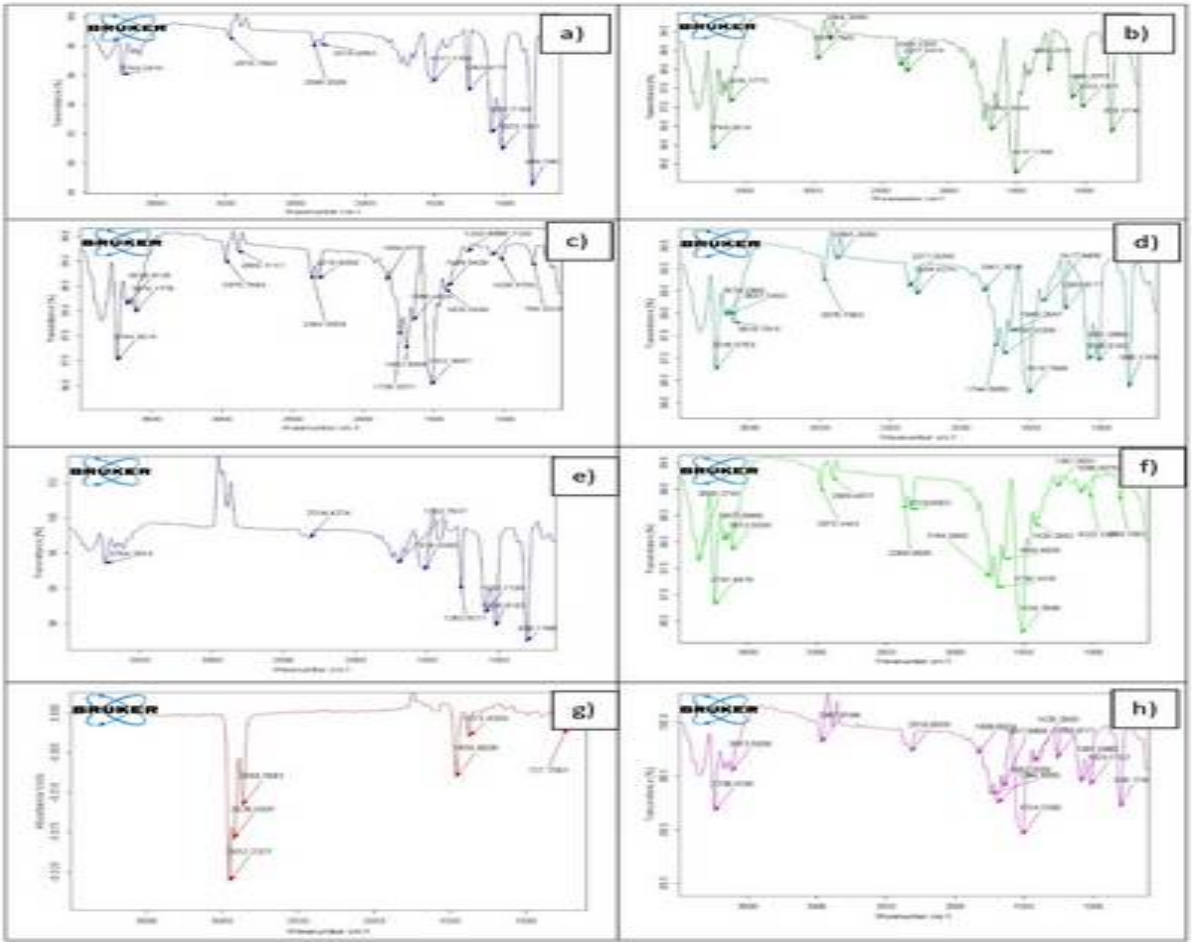

Fig. 6 (a-h) Peaks showing functional groups present in plants at concentration a) control, b) 100 mg kg^{-1} of *Brassica juncea*, c) control, d) 100 mg $kg^{-1}$of *Triticum*, e) control, f) 100 mg kg^{-1} of *Helianthus*, g) control, and h)100 mg kg^{-1} of *Tagetes*.

results were analyzed in oxidative activities (25-27). SOD also showed an increase in activity with increasing concentration in hydrocarbon treated *Helianthus* exhibited 0.032 to 0.064 Unit g^{-1} FW (Fig. 5c). Comparing to *Triticum>Tagetes>Brassica*. peroxidase (POD) showed increased activity with increasing concentrations in all the four-plant species. *Helianthus* concentration showed POD activity 0.114 to 0.212 Unit g^{-1} FW from control to 100 mg kg^{-1} phenanthrene: naphthalene: anthracene ratio (Fig. 5d). POD activity in *Helianthus* was greater than activity observed in *Triticum>Brassica>Tagetes>Helianthus* (27).

FTIR analysis in treated and controlled plants

FTIR analysis had peaks at various wavelengths in which carboxylic acid ranges from 4000 - 3400 with O-H group (28). Absorbance in 900 - 700 cm^{-1} region hold aromatic C–H bond in out-of-plane and were strongly reflected by the peak at 806 cm^{-1} can be allocated to aromatic rings; peak at 808 cm^{-1} were assigned to benzene ring with 1,4-disubstituted hydrogens and peak near 790 cm^{-1} can be allocated to benzene rings *i.e.* 1,2-disubstituted per ring (29). The bonds are stronger due to C-H stretching of peripheral hydrogen atoms bonded to aromatic carbon atoms. At higher wavelengths, a low frequency mode appeared that may be associated with skeletal distortions around 800 cm^{-1} in samples (Fig. 6a-h). The skeleton vibration peak of benzene ring appears between 1200 – 1600 cm^{-1} (30). At lower frequency, a skeletal bend may account for peaks at 806 and 808 cm^{-1}. Peaks from 1600 - 1000 cm^{-1} attributed to the C-H plane bending and aromatic carbon skeletal stretching (31). FTIR results showed the breaking of aromatic rings in samples which were present in the PAHs.

HPLC

With the help of HPLC analysis the amount of hydrocarbons present in plants were analysed after 20 days of the treatment. *Helianthus* was acting as hyper-accumulator plant as it degrades the hydrocarbons in its best way then other plants species. HPLC analysis in plants provided concentration of hydrocarbons present in the plant species after 20 days taken up from the treated soil (32). In Fig. 7 showing at 100 mg kg^{-1} concentration *Helianthus* had 3.013 mg kg^{-1} Naphthalene, 7.750 mg kg^{-1} phenanthrene and 5.691 mg kg^{-1} anthracene. *Tagetes* had 0.416 mg kg^{-1} Naphthalene, 8.476 mg kg^{-1} phenanthrene and 0.398 mg kg^{-1} anthracene. Wheat had 0.700 mg kg^{-1} Naphthalene, 6.7750 mg kg^{-1} phenanthrene and 2.102 mg kg^{-1} anthracene while *Brassica* had 0.945 mg kg^{-1} Naphthalene, 7.480 mg kg^{-1} phenanthrene and 1.779 mg kg^{-1} anthracene (33). Naphthalene was sequestered more in the tissues as compared to other two compounds in plants. Analysis

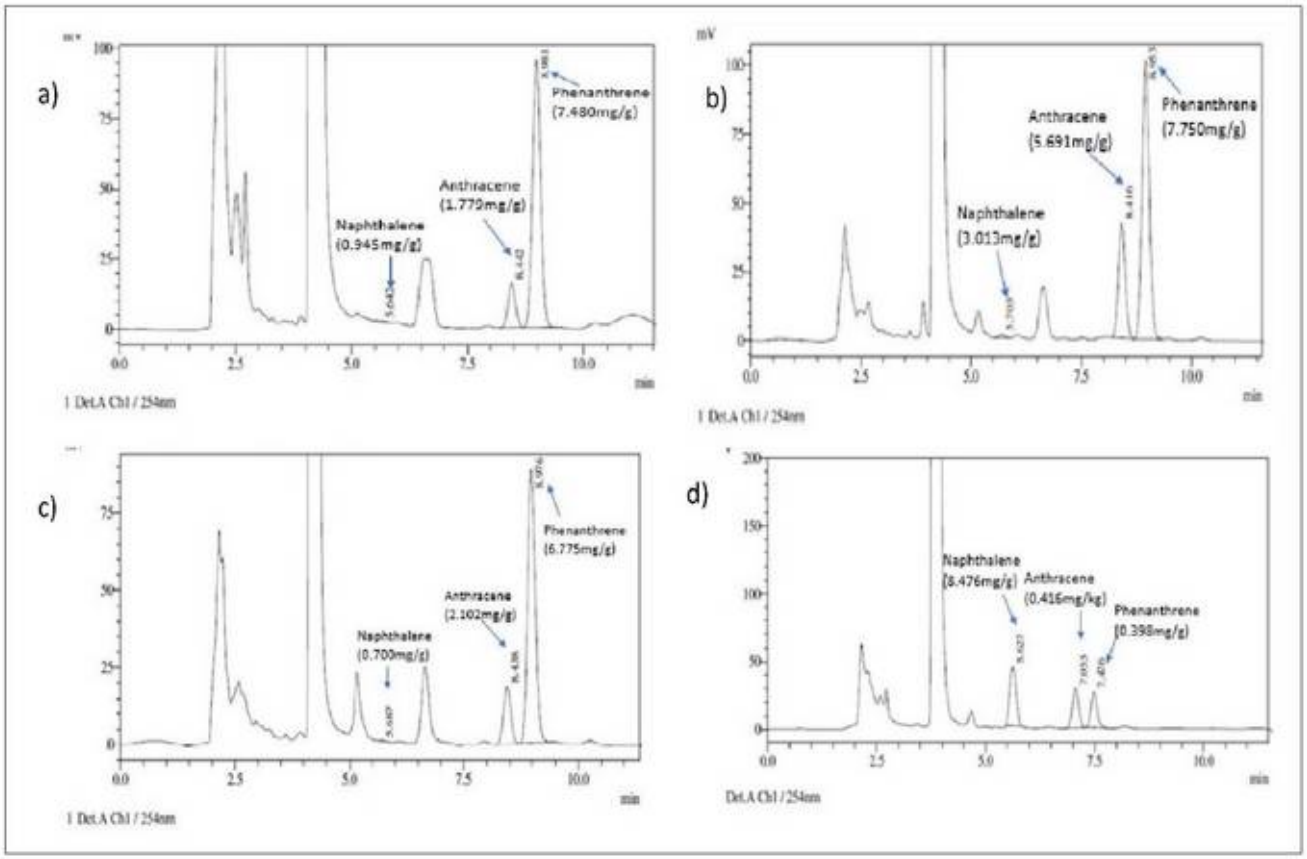

Fig. 7 (a-d). HPLC-UV chromatograms showing the separation of hydrocarbon (Naphthalene, anthracene and phenanthrene) in plant samples a) *B. juncea* (100 mg kg^{-1}) b) *Helianthus* (100 mg kg^{-1}) c) *T. aestivum* (100 mg kg^{-1}) and d) *T. erecta* (100 mg kg^{-1}).

revealed that naphthalene and phenanthrene fractions were more than anthracene in *Tagetes* suggesting that accumulation is less in *Tagetes* and it showed highest toxicity because of anthracene. PAHs uptake by plants has been demonstrated in some papers; however detailed studies are still missing. It is well known that plants are able to uptake these class of compounds via root system. This way of uptake is complicated due to low solubility of PAHs in water. In the case of PAHs uptake via aerial parts, they are deposited in lipophilic substances on the plant surface (cuticle, waxy layer), or they are able to enter the plants via stomata as integral part of gas (28).

Conclusion

Plant species has its own mechanisms of resistance against the negative effects of hydrocarbons. The most common effect of hydrocarbons toxicity in plants is short growth due to chlorosis, vulnerable nutrient uptake and alteration in the activity of enzymes in metabolic pathways. Contamination by PAHs has negative impact on the plant species under the study. But, *H. annuus* and *T. aestivum* exhibited the highest growth and germination at 100 mg kg^{-1} of hydrocarbons so, these can be used for the remediation of contaminated soils. On the other hand, using plants like *B. juncea* and *T. erecta* reflected sensitivity towards the soil pollution.

On the basis of the results it can be concluded that morphological and biochemical responses of plants could be served as potential indicators of hydrocarbon concentration and its effect in soil. To diminish the hydrocarbons induced oxidative effect and free radicals, level of proline, polyphenol, ascorbic acid and antioxidative enzymes were elevated in plant species. For survival, plants are having some defense mechanism against hydrocarbon stress as ascorbate, catalase, peroxidase and superoxide dismutase are increasing as the hydrocarbon concentration increases. The results indicate an extensive possibility for using *H. annuus* in remediation of contaminants from polluted soil.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author's contribution

JM conceived the idea of present research work and supervised overall findings of this work. RP carried out the experimental work, compiled the results and drafted the manuscript along with support of JM. Both the authors discussed the results and contributed to the final manuscript.

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