Journal of Scientific & Industrial Research
Vol. 80, May 2021, pp. 404-413

Impact of Tetracycline on Basil and its Remediation Potential

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Received 14 September 2020; revised 02 March 2021; accepted 05 March 2021

Over the past decade presence of antibiotics in soil and water is a major environmental concern which needs to be address on a priority basis. The present study was done to evaluate the potential of basil (*Ocimum basilicum*) for phytoremediation. A greenhouse study was conducted for removal of tetracycline from soil. The plants were grown with 200 $mgkg^{-1}$, 400 $mgkg^{-1}$, and 600 $mgkg^{-1}$ of tetracycline for four weeks. Accumulation of tetracycline in shoot and root was observed with HPTLC in plants. They showed a maximum of 97% remediation capability with 200 $mgkg^{-1}$of tetracycline treated plants. Secondary metabolites were lepoxygenase pathway products in stress condition. The same were analyzed by GCMS. Alpha-terpineol and methyl acetate completely degraded in all samples, while they were present in plants grown without tetracycline. This could be because antibiotic treatments impact the production of lipoxygenase pathway products, while in some cases secondary metabolites increase marginally as the tetracycline concentrations increased. The aim of the current work was the use of plant-based system for phytoremediation and toxicological impact of tetracycline on basil.

Keywords: Antibiotics, GC-MS, HPTLC, Phytoremediation, Secondary Metabolites

Introduction

Antibiotics are increasingly being regarded as rising pollutants of global concern.[1] The major source of antibiotics in environment is pharmaceutical waste caused by increased use of antibiotics in human and animal health care sectors. After consumption by human and animals for the prevention of diseases and enhanced meat and milk production in animals the undigested antibiotics are excreted from the host body after a short time of residence. Antibiotics are released into the environment directly through feces and urine or indirectly from animals manure when they are used in organic agriculture as a nutrient source and then active residues of antibiotics released in to soil bodies.[1] It is reported that, in many countries high level of antibiotics were found in vegetables where animal manure was used as a nutrient source in soil. Soil fertilized with sludge could result the uptake of antibiotics by food crops and could affect human and animal health.[2] Moreover, the release of these compounds into environment through other sources such as pharmaceutical waste and waste water treatment plants will lead to serious environmental problems including ecological risks, human and other livestock health damage. High utilization of antibiotics in animal and human health sector has caused increase in large amount of antibiotics production by pharmaceutical companies. Their use in the last decades has also made many pathogenic bacteria resistant to a variety of antibiotics, resulting in harmful human and animal diseases which are now untreatable. Therefore, the Indian Ministry of Environment, Forest and Climate Change, on 23 January 2020 announced standards on concentrations of antibiotics found in the waste discharged by pharmaceutical factories into rivers and other environment channels.[3,4]

Tetracycline (TC) is a broad-spectrum polyketide antibiotic and has a hydronaphthacene based structure. TC is produced from *Streptomyces aureofaciens.* TC is *used to treat* infections of the urinary tract, respiratory tract, and the intestines in human and animals. It is hydrophilic in nature and its aqueous solubility is 1000 – 1700 mgL^{-1}. Molecular weight is 444.4 $gmol^{-1}$ and it has a three Pka value 3.3, 7.7 and 9.7 respectively.[5] Tetracycline log KOW value (partition coefficient between octanol and water) is −1.25 to −1.12 and Kd value (sorption coefficient between octanol and water) is 300–2000 Lkg^{-1} represents its hydrophilic nature.[5]

Tetracycline, Chlorotetracycline (CTC) and Oxytetracycline (OTC) and has been found in levels up to 110 ngL^{-1}, 690 ngL^{-1}, 340 ngL^{-1} in wastewater.[6] Tetracycline with concentrations upto 86.2, 198.7, and 171.7 μgkg^{-1} and chlortetracycline with

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concentrations upto 4.6−7.3 μgkg^{-1} is also reported in soil by Hamscher *et al.* (2002).[7] The concentrations of oxytetracycline in soil were 305 μgkg^{-1} and tetracycline upto 216 mgL^{-1} is also reported in animal manure.[1,8,9] Moreover, upto 7.73 mgL^{-1} of chlortetracycline and 4.03 mgL^{-1} tylosin is reported in swine manure.[10] Li *et al.*[11] found 450 μgkg^{-1} to 406 $mgkg^{-1}$ range of tetracycline in soil and 820 μgkg^{-1} of OTC in swine manure. The occurrence of TC in water and soil amended with manure and exposed vegetation with it warns researchers to monitor the fate and removal of such substances using plants.

Phytoremediation is an emerging, eco-friendly and sustainable removal method to decontaminate soil and water from various environmental contaminants. In comparison with other remediation methods, phytoremediation is considered as efficient, cost effective and eco-friendly. The main concept of phytoremediation is that the plants uptake toxic substances from environment and converts them into simpler and less toxic forms. This has economic benefits as well. Remediation using plants is more sustainable as compared to other methods because it does not negatively impact the physical and biological properties of soil. On the contrary it improves the quality of soil. Common sweet basil (*Ocimum basilicum L.)* belongs to the genus of *Osmium L.* and family lamiaceae widely used as an edible, medicinal and industrial plant. Basil, lemon grass (*Cymbopogon flexuosus*), citronella (*Cymbopogon winterianus*), geranium mint (*Mentha sp.)* etc. are known for their tolerant capacity against various environmental stress.[12] Basil have well-developed root system which prevents the leaching of contaminants that makes it more frequently usable for phytoremediation. Basil was selected in this study for its safe, economically feasible, antioxidant producing capability and ecofriendly approach for phytoremediation. Zahedifar *et al.*[13] used basil for phytoremediation of heavy metals and Kunwar *et al.*[14] reported about the impact of heavy metals on essential oil of basil but didn't reported on antibiotics. To our best knowledge, the removal of TC from basil and its toxicological impacts has not been studied yet. Most of the plants when grown in contaminated soil and water, uptake and translocate toxic elements and entered into food chain. Whereas, Basil is well known for its antioxidant enzymes production capability in stress conditions, helps to degrade contaminants during plant metabolism without contaminating the food chain. The major environmental characteristic about the use of basil for phytoremediation is that, pollutants' entry into the food chain can be minimized. Use of basil for removal of heavy metal polluted area has been reported by many researchers in the recent past but no such data is reported in case of antibiotics. Therefore, this study was aimed at investigating the fate and toxicological impact of tetracycline and remediation potential of basil.

Material and Methods

Chemicals and Reagents

The ultra-pure grade of tetracycline with purity up to 99% (hydrochloric salt) ($\underline{C_{22}}\ \underline{H_{24}}\ \underline{N_2}\ \underline{O_8}$) (CAS NO. 64-75-5) was purchased from Hi-Media. HPLC grade methanol was used in plant extraction. All stock and working dilutions of tetracycline were prepared in distilled water having 6.1 and 7.3 pH, measured by PHM95 pH Meter (Fisher Scientific).

Plants Growth Conditions

Three weeks old uniform saplings of basil were planted in triplicate placed in porous plastic pots (15 cm diameter 14 cm deep) containing 1 kg soil. In this study silt loam type soil at pH 7.2 was selected. The plants were grown in a greenhouse for 4W in 36°C temperatures, 12:12 hr light: dark cycle with watering frequency of twice a day. The lower concentration selected was 200 $mgkg^{-1}$ as per environmentally reported range[8,11] and higher concentrations of 400 and 600 $mgkg^{-1}$ were also taken. These were then poured uniformly into soil around the root and labeled as T1, T2 and T3. These working dilutions of tetracycline (200–600 $mgkg^{-1}$) were prepared from 1000 ml of stock solution. Two control pots were also set: negative control (no antibiotics + with plant; as B) and positive control (with 600 $mgkg^{-1}$ antibiotic + no plant as; C). The negative control was prepared to compare the growth of plants with and without TC and positive control was prepared to assess the degradation (photodegradation and hydrolysis) of tetracycline in soil. Three individual sets (n=3) were established to account for error and experiment was repeated 3 times and data were recorded. All reduction data was calculated by the Eq. 1 given below;

$$\% \text{ Reduction} = \frac{A-B}{A} X100 \quad \ldots (1)$$

where A is the initial parameter of the experiment and B is final.

Toxicity Assessment

Root and Shoot Length and Biomass Analysis

All harvested plant samples were uprooted and washed after 4 weeks and root and shoot length were analyzed separately by standard centimetre scale and fresh and dry weight of root and shoot were analyzed by electrical balance for toxicological study.

Secondary Metabolites Analysis in Plants

Extraction of 5g dry plant material with 200 mL of 80% methanol was processed for secondary metabolites analysis by GC-MS (Gas chromatography mass spectrometry) method described by Redfern *et al.*[15] The GC-MS carries autosystem XL, GC fitted with Equity-5 (60 m x 0.32 mm and film thickness 0.25 μm) fused silica column coupled with Perkin Elmer turbo mass fitted with Equity- 5 capillary column. The extract was analyzed in column temperature at 290°C at 3°C/min. Hydrogen was used as a carrier gas at 10 psi column head pressure, the injector temperature was 280°C and the flame ionization detector (FID) temperature was 290°C. The column temperature was programmed from 70°–300°C at 3°C /min using helium as carrier gas. Mass spectra were taken at 70 eV in mass range from 40–450 amu with 220°C injector temperature and 0.05 μL injection volume. Secondary metabolites were identified by comparison of their Retention time (RT %) and mass spectra (MS %) compared from the NIST/NBS and Wiley 575 libraries.

Chlorophyll and Carotenoid Estimation

The chlorophyll (Chl) content was estimated according to the method of Arnon *et al.*[16] In 10 ml of 80% (v/v) acetone 0.1 gm of plant leaf (each sample in triplicate) was homogenized followed by centrifugation at 5000 rpm for 10 minutes. Optical density of supernatant was measured at 645 and 663 nm for Chl-a and Chl-b and total Chl.

Carotenoid was estimated by the method as described by Lichtenthaler *et al.*[17] Absorbance of supernatant was measured at 480 and 510 nm and the results were analyzed for the change in carotenoid content with the change in concentration of tetracycline.

Flavonoid and Proline Estimation

Total flavonoid content (TFC) was analyzed by the method describe by Chang *et al.*[18] Standard was prepared with the quercetin. Pigments were extracted using the chlorophyll extraction method. Supernatant was further treated with 0.15 mL of 5% $NaNO_2$, 0.15 mL of 10% $AlCl_3$, 1 mL of 4% NaOH and 3.2 mL of distilled water. After 30 min of incubation absorbance was measured at 510 nm and TPC was calculated in treated plant and plant grown without TC (n=3).

Proline content was estimated in plants (in triplicate) method described by Bates *et al.*[19] In 5 mL of 3% aqueous sulfosalicylic acid 0.5g leaves were homogenized, shaken, and then filtered. Into 2 mL of supernatant, 0.2mL glacial acetic acid and 2 mL acidic ninhydrin was added. To 1.25 gm ninhydrin, 30 mL glacial acetic acid and 20 mL of 6M orthophosphoric acid was added heated with constant stirring till dissolution. Add 4 mL of Toluene into reaction mixture and proline was estimated at 520 nm using toluene as a blank.

Remediation Analysis

Standard Preparation and Quantification of Tetracycline in Plants

Stock solution of TC (0.1 mg/mL) was prepared in 80% methanol for standard and applied on aluminum plates backed with silica gel RP-18W Merck HPTLC plate (size 200.0 mm × 100.0 mm, thickness 0.2 mm, Length: 8.0 mm) (Catalogue no. 5554). TLC plate was coated with 10% aqueous EDTA for TC bands visualization in plates (silica gel and RP-18W layer). TC injected volume was 1.0 μL in HPTLC system. For scanning of chromatogram, χ-position was reserved at the lanes from 20.0 mm to 72.0 mm, and y-position scanned from 28.1 mm to 27.6 mm. The linear regression determined by UV-absorption where wave length range was 190 nm to 700 nm. Different concentrations of TC were taken for standard preparation. Relationship between the concentrations of antibiotics and peak area was achieved for antibiotics range from 200 $mgkg^{-1}$ to 600 $mgkg^{-1}$ (from T1 to T3) with coefficients of determination (R^2). After Chromatographic analysis concentration of TC was calculated on the basis of R_f value. Same method was applied for the TC content in plant parts. All roots, shoots of harvested plant samples were air dried at 36 – 40 °C for 3 – 4 days. 20 mg of dry root and shoot were macerated in 100 mL of 80% methanol in falcon tubes, and then shaken at 2000 rpm for 20 min.[20] The extraction process was done 3 times and 0.2 μL of resulting extract were applied to HPTLC (High performance thin layer chromatography) plates and R_f was calculated.

Soil Analysis

Remaining tetracycline content was also analysed by HPTLC in all treated soil samples and in control

setup. After four weeks, 5.0 g of soil was collected from the pots at 0 to15 cm depth near the root (rizosphere area). Soil samples were then processed by crushing, mixing and then air drying at 36–40°C till constant weight. To 10 mL of 100% methanol, 0.5 g of soil sample was added in volumetric flask and after manual shaking, 0.2 µL samples for different concentration were applied in HPTLC plates and R_f was calculated.

Statistical Analysis

All experiments were performed in triplicate (n=3). The mean and standard error (SE) of plant growth and tetracycline concentration in plants and soil for all samples were calculated and mean values of all samples were presented. One way ANOVA was performed by excel to test the significant difference of the means of the control and treated variables (all samples) at 95% confidence intervals. The t-test ($p < 0.05$) was also performed to assess the significant difference between antibiotic content in control and treated soils and plants.

Results and Discussion

Toxicity Analysis of Tetracycline (TC) in Plants

Two basic parameters Toxicity and Remediation were estimated after 4 weeks of starting the experiment. Significant decrease in root and shoot length of plant was seen in plants grown with tetracycline. On comparing with plant grown without tetracycline, approximately 22.2% decrement was observed in root length of plant at T1 and T2 (200 and 400 $mgkg^{-1}$) while 11.11% root reduction was observed in T3 (600 $mgkg^{-1}$). Similarly, 11.76% reduction observed in shoot length at T1 and T2 and 25.4% reduction was observed at T3. Several studies have shown the impact of antibiotics on growth reduction of seed, length of plant, generation of oxidative stress and inhibition of photosynthetic rate (Fig. 1).[21] Statistical analysis showed significant difference (P= 0.01) for root length between untreated and treated plant and no (P= 0.62) significant difference was observed in case of shoot. Brain *et al.*[22] found that tetracycline was phytotoxic to plants as tetracycline binds irreversibly to the 30S subunit of ribosomes, blocking the binding of aminoacyl transfer to DNA, inhibit protein synthesis also.

Changes in Biomass (FW, DW of Root and Shoot) of Basil due to Toxicity

FW (Fresh weight) and DW (Dry weight) of root and shoot was also estimated for toxicity analysis of TC where highest FW of root and shoot was observed in blank sample of plant and significant decrement was observed in T1 and T2 followed by T3 (Fig. 2). Highest DW of root was found in blank sample of plant and due to tetracycline toxicity 52.8%, 16.9% and 18.8% reduction was found in T1, T2 and T3 similarly highest DW of shoot also found in plants grown without tetracycline (B) and due to tetracycline toxicity 56.2%, 1.59% and 2.6% reduction was found in T1, T2 and T3. Reduction of physical parameter in TC exposed crop depicted the toxicological impact of tetracycline. Similar results have been observed by Makhijani *et al.*[23] during their research where the weight of Cicer arietinum got affected by tetracycline exposure. Statistical analysis showed significant difference (P= 0.02) for fresh and dry weight of root and no (P= 0.23) significant difference was observed in case of fresh and dry weight of shoot

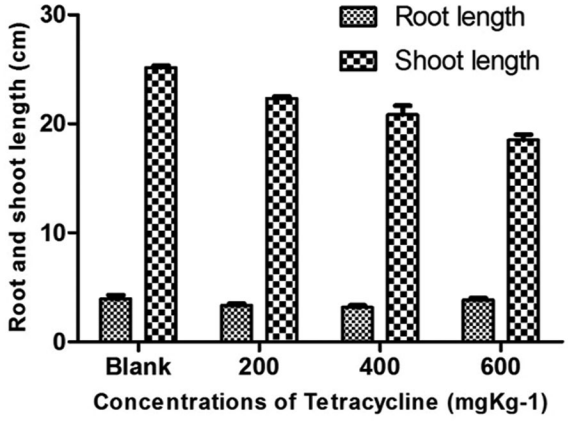

Fig. 1 — Root and shoot length (mean ± SD, n=3) of basil exposed to different concentration of tetracycline in soil

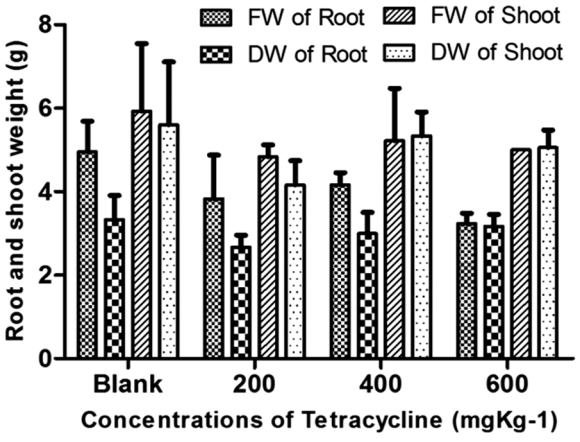

Fig. 2 — Fresh weight (FW), dry weight (DW) of root and shoot (mean ± SD, n=3) in basil exposed to different concentration of tetracycline in soil

Secondary Metabolite Degradation Analysis in Basil

This study uses GC-MS for the identification of terpenes (monoterpenes and sesquiterpenes) and other volatile organic compounds in stress conditions. It is reported that the destruction in free fatty acids responsible for emission of green leaf volatiles in stressed plants takes place in lipoxygenase pathway and aromatic volatiles production by shikimic acid pathway,[24] isoprenes and monoterpenoids are produce by methylerythritol pathway and production of volatile sesquiterpenoids takes place by mevalonic acid pathway.[25]

Phytochemical analysis of the basil after TC uptake showed degradation of secondary metabolites in all samples. While only 23–29 compounds were identified in tetracycline treated plants (200 $mgkg^{-1}$ to 600 $mgkg^{-1}$) around 35 were present in the ones grown without it (Table 1). It was seen that monoterpene and terpene alcohol ranged from 11.039% to 16.08% and 22.89% to 24.24%, respectively. However, hydrocarbons of sesquiterpene and sesquiterpenes oxygenated form ranged from 24.955 to 26.786% and 27.684% to 28.68%, respectively.

Monoterpenes like Sabinene, D-limonene and Beta-ocimene were completely degraded in T2 and T3 though they were 6.427%, 8.121% and 8.796% present in Blank and 6.43%, 8.127% and 8.808% in T1. 6-Methyl-5-Hepten-2-one, alpha-terpineole and spathulenol were totally degraded in all samples and they were 6.696%, 15.359% and 28.537% present in

Table 1 — Secondary metabolites analysis in Basil in response to various concentrations of Tetracycline

	RT %				MS %				FID %			
Tetracycline treatment ($mgkg^{-1}$)	0	200	400	600	0	200	400	600	0	200	400	600
COMPOUNDS NAME												
ALPHA-PINENE	5.46	5.46	5.46	5.266	0.06	0.06	0.06	0.06	0.07	0.06	0.06	0.06
SABINENE	6.427	6.43	NIL	NIL	0.02	0.02	NIL	NIL	0.02	nn	NIL	NIL
BETA-PINENE	6.542	6.542	6.5420	6.296	0.06	0.06	0.06	0.06	0.06	0.08	0.08	0.09
6-METHYL-5-HEPTEN-2-ONE	6.696	NIL	NIL	NIL	0.02	NIL	NIL	NIL	0.02	NIL	NIL	NIL
D-LIMONENE	8.121	8.127	NIL	NIL	0.06	0.05	NIL	NIL	0.05	0.04	NIL	NIL
1,8-CINEOLE	1.19	8.19	8.19	7.875	0.54	0.55	0.55	0.56	0.58	0.59	0.58	0.6
BETA-OCIMENE	8.796	8.808	NIL	NIL	0.03	0.02	NIL	NIL	0.02	0.02	NIL	NIL
LINALOOL OXIDE	9.712	9.706	9.712	9.969	0.62	0.58	0.6	0.61	0.63	0.61	0.61	0.68
LINALOOL	11.039	10.971	11.016	10.581	14.32	14.55	14.42	13.07	17.52	17.36	17.52	17.45
L-MENTHONE	13.299	13.294	13.294	12,830	0.16	0.14	0.15	0.12	0.16	0.13	0.14	0.12
ISOMENTHONE	14.278	14.255	13.809	13.537	0.9	0.87	0.06	0.01	0.92	0.9	0.01	0.02
LEVOMENTHOL	14.278	14.255	14.272	13.769	0.9	0.87	0.88	0.88	0.92	0.9	0.91	0.9
ALPHA-TERPINEOL	15.359	NIL	NIL	NIL	0.06	NIL	NIL	NIL	0.05	NIL	NIL	NIL
ESTRAGOLE	16.08	15.932	16.035	15.434	77.53	78.14	77.8	80.17	74.69	74.74	75.03	75.7
CARANE	17.591	19.96	17.585	NIL	0.04	0.05	0.03	NIL	0.31	0.04	0.02	NIL
Z-CITRAL	17.677	17.66	17.671	18.518	0.18	0.15	0.17	0.39	0.21	0.18	0.2	0.61
D-CARVONE	17.751	17.734	17.745	NIL	0.1	0.14	0.1	NIL	0.14	0.12	0.1	NIL
E-CITRAL	17.677	18.947	18.958	NIL	0.18	0.33	0.38	NIL	0.21	0.49	0.44	NIL
METHYL ACETATE	19.965	NIL	NIL	19.554	0.06	NIL	NIL	0.05	0.05	NIL	NIL	0.04
ALPHA-COPAENE	22.889	22.884	22.889	22.512	0.04	0.04	0.04	0.04	0.03	0.05	0.03	0.03
BETA-ELEMENE	23.41	23.41	23.41	NIL	0.05	0.05	0.05	NIL	0.06	0.06	0.05	NIL
CARYOPHYLLENE	24.24	24.24	24.24	23.862	0.22	0.19	0.21	0.16	0.2	0.21	0.2	0.15
TRANS-ALPHA-BERGAMOTENE	24.755	24.749	NIL	24.4	0.05	0.75	NIL	0.84	0.05	0.68	NIL	0.64
BETA-SESQUIPHELLANDRENE	24.955	24.955	24.955	24.612	0.05	0.04	0.05	0.04	0.05	0.07	0.05	0.05
ALPHA-HUMULENE	25.241	25.241	25.241	24.875	0.11	0.1	0.11	0.19	0.1	0.13	0.1	0.08
TRANS-BETA-FERNESENE	25.356	25.362	25.356	25.035	0.11	0.2	0.21	0.16	0.15	0.29	0.24	0.21
BETA-BISABOLENE	26.786	26.786	26.786	26.46	0.08	0.07	0.07	0.05	0.08	0.08	0.08	0.94
DELTA-CADINENE	27.169	27.17	27.169	26.826	0.02	0.02	0.03	0.02	0.02	0.03	0.03	0.01
CIS-ALPHA-BISABOLENE	27.684	27.673	27.679	27.556	1.56	1.43	1.54	1.31	1.17	1.19	1.16	0.94
PARA-METHOXY CONNAMIC ALDEHYDE	28.177	28.188	NIL	27.856	0.23	0.59	NIL	0.48	0.61	0.51	NIL	0.08
SPATHULENOL	28.537	NIL	NIL	NIL	0.06	NIL	NIL	NIL	0.06	NIL	NIL	NIL
CARYOPHYLLENE OXIDE	28.68	28.675	28.674	28.314	0.4	0.33	0.41	0.37	0.32	0.31	0.31	0.31
UNKNOWN					0.22	0.41	1.03	0.34	0.16	1	1.25	1.17

blank. Carane did not degrade in T1 while in case of T2 1% degradation was seen as compared to blank and is completely degraded in T3. Similarly, maximum D-Carvone content was found in T1 and is degraded in T2, T3. Moreover, Isomenthone and levomenthol shows 2% to 4% degradation from T1 to T3 as compared to blank where it is present in higher percentage (14.287% and 13.299%). Linalool 2% decreased in T1 and T2 but slightly increased in T3. Linalool oxide (Oxigenated terpenoids) increased from 9.712%–9.969% (B to T3). Alpha-pinene (5.46%) and beta-pinene (6.542%) remains unchanged from Blank to T2 but slightly decreased in T3. 1, 8-Cineole showed its maximum (8.19 %) content in T1 while in case of T2 and T3 it was degraded similarly E-Citral was degraded in T3 but in case of B and T2 it was increased and shows its maximum percentage in T2 setup (17.677%). Relative content of terpenoids like caryophyllene, Alpha –humulene and Beta–bisabolene remained unchanged from B to T2 but in case of T3 a slight degradation was observed. Methyl acetate degraded in T1 and T2 and almost 10% degradation was also observed in T3 and showed its maximum percentage in blank. Trans-alpha-bergamotene showed complete degradation in T2 and 2%–6% degradation was also observed in case of T1 and T3 as compared to Blank. Whereas, para-methoxy cinnamic aldehyde also got completely degraded in T2 but in case of T1 and T3 2% to 6% enhancement was observed as compared to blank. Estragole (16.08%), Alpha-copaene (22.889%), Beta-sesquiphellandrene (24.995%), trans beta fernesene (25.563%), Delta-cadinene (27.169%) and Cis-alpha-bisabolene (27.684%) also showed its highest percentage in blank and showed 2%–8% degradation from T1 to T3. The present study is the first study to show that the presence of TC can change the synthesis of secondary metabolites in plants.

Changes in Chlorophyll and Carotenoid Content in Response to Tetracycline

The photosynthetic pigments were included as a biomarker of toxicity, an important parameter for primary productivity and photosynthetic potential of plant exposed to antibiotics.[26] In this study highest total Chl and Chl a was observed in T1 and T2 plant samples while Chl b was highest in blank. Total Chl and Chl a increased in T1 and T2 while due to TC toxicity these were degraded in T3. In Chl b content about 38.9%–13.5% reduction was found (T1–T3) due to TC toxicity and T1 > T2 > B > T3 reduction trend was found (Fig. 3). Statistical analysis showed significant difference for Chl a, Chl b and total Chl between untreated and treated plant. Several research data has shown that low antibiotic concentrations could impact chlorophyll biosynthesis by affecting nucleic acid and protein in the plant cell. It reduced chlorophyllase activity to delay chlorophyll degradation in cell, and hence showed increased chlorophyll content in plants.[27,28] Basil did not show any visible symptoms of chlorosis but in T3 mortality was found due to TC exposure after four weeks.

In this study increasing concentration of TC caused a decrease of total carotenoid content. Highest carotenoid was observed in blank samples of plant and showed decrease with increasing concentration of tetracycline (7.228–3.897 $\mu g g^{-1}$ DW) and reduction trend observed was B >T1 >T3 > T2 (Fig. 4). Statistical analysis showed significant difference for total carotenoid between untreated and treated plant. A recent study showed the effect of tetracycline in

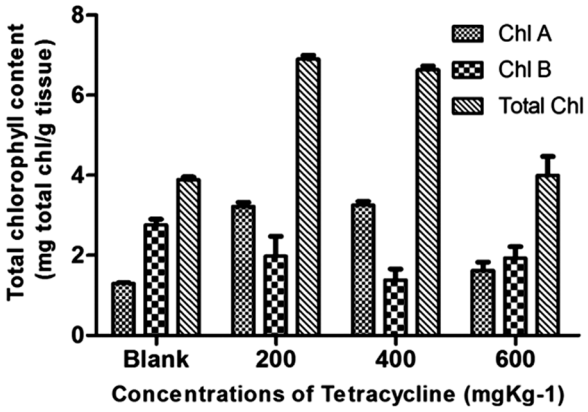

Fig. 3 — Photosynthetic pigments: Chl a, Chl b and total Chl (mean ± SD, n=3) (P = 0.02, P=0.02, P=0.01) in *Ocimum basilicum* exposed to different concentration of tetracycline in soil

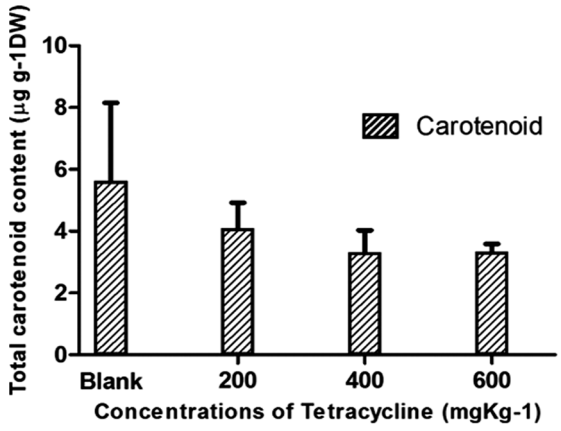

Fig. 4 — Total carotenoid content (mean ± SD, n=3) (P = 0.03) in basil exposed to different concentration of tetracycline in soil

carotenoids and chlorophyll of *L. Gibba*[22,29] with the reduction in pigment-binding complexes content.

Flavonoid (TFC) and Proline Content in Response to Tetracycline

Total flavonoid content (TFC) (Fig. 5) was also calculated as an important part of secondary metabolites and showed degradation with increasing concentration of TC. Standard was prepared with quercetin and y= 0.0004x + 0.2199 regression equation was derived with R^2 = 0.997. Highest TFC was found in blank sample of plant and significant decrease was found from T1 to T3 and 20.6% to 51.2% reduction was observed with B>T1>T2>T3 reduction trend. Statistical analysis showed no significant difference for total flavonoid between untreated and treated plant.

The effect of TC on proline (Stress protein) content was observed. Since it is a stress protein, it increases in every abiotic and biotic stress factor. In present study highest proline content (Fig. 6) was found in blank and it decreased with increasing concentrations of TC and in which B > T2 > T1 > T3 reduction trend was seen. Statistical analysis showed no significant difference (P=0.09) for total proline between untreated and treated plant.

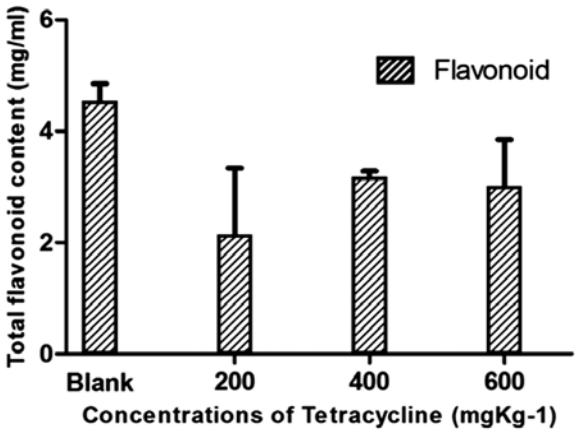

Fig. 5 — Total flavonoid content (mean ± SD, n=3) (P = 0.23) in basil exposed to different concentration of tetracycline in soil

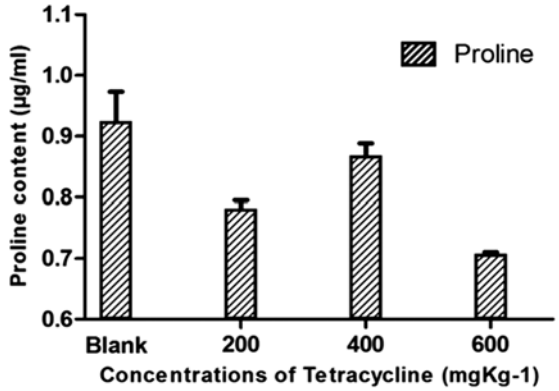

Fig. 6 — Total proline content (mean ± SD, n=3) in basil exposed to different concentration of tetracycline in soil

Accumulation of Tetracycline in Basil

Standard was prepared for TC where highest peak was observed at λ=372 nm and 0.32 R_f was calculated (Fig. 7). A linear relationship between the concentrations of tetracycline and peak area was derived for range from T1 to T3 (200–600 $mgkg^{-1}$) and correlation coefficient R^2=99.890 was calculated.

The presence of TC in root, shoot and in control sample was analyzed to see the remediation potential of basil. Highest accumulation of TC was observed in T1 (200 $mgkg^{-1}$) and it decreased with increasing TC concentrations. That means with higher concentrations, the reduction of tetracycline was less and hence will need more time for its complete removal. Furthermore, accumulation of TC was observed in root and shoots separately and high accumulation was observed in root as compared to shoots except T1, where high accumulation was

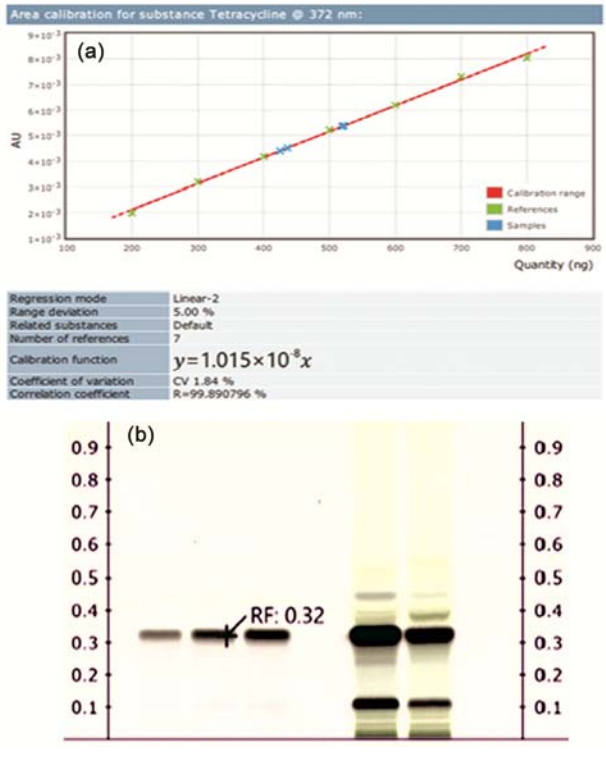

Fig. 7 — [A] Standard graph of Tetracycline (X-Axis is concentration of tetracycline and Y-Axis denoted area under the peak, Near-UV region tetracycline shows strong absorption in standard graph); [B] HPTLC of tetracycline on silica gel layers

observed in shoot. Similarly highest % remediation was found in T1 (97%) and 38.9% and 22.2% remediation was recorded in case of T2 and T3. No tetracycline was found in negative control (set without antibiotics) while in positive control (set without plants + with 600 $mgkg^{-1}$ of TC) negligible decrease (3.11%) was found. Trend of % remediation recorded was T1> T2 >T3 > C and hence showed remediation potential of basil. Three replicates (n=3) were done for assessing remediation potential in control and treated plants and average values were presented. After statistical analysis significant difference (p=0.03) was found in between control and treated plant samples. These indicate a comparative reduction of the antibiotics in control and treated samples which can be considered to be transported to roots and shoot of the plant. Large amount of tetracycline was accumulated in basil. After greenhouse experiment it was confirmed that basil holds a great potential for phytoremediation of antibiotics. In shoot, total 64.5, 25.8 and 15.6 mg were accumulated and in root part 32.5, 52 and 52.1 mg of TC was found. TC was not found in soil samples at the end of experiment. This shows basil is a good remediator for tetracycline from soil. The development of basil in treated soil indicates the tolerance capacity of plant for antibiotics. Basil uptook tetracycline via the diffusion process and converted it into a less toxic form via metabolic processes like transformation, conjugation and stabilization. Our results are encouraging and further research will be validated by similar work at actual sites in near future. Furthermore, as per the Indian Ministry of Environment, Forest and Climate Change, rule issued on January 2020, large amount of antibiotics are discharged by pharmaceutical factories into rivers and other environment channels. Further research will be made in this field which can make it viable, promising and cost effective in coming future, though large variety of aquatic plants and weeds are already reported for removal of these substances from aquatic channels.[29]

The difference in the amount of tetracycline accumulated in the plant and present in soil could have degraded in plant into transformation products (Table 2). It is reported that at initial level plant uptakes tetracycline by gas exchange, aqueous channel and lipid channel uptake, etc. and then degrades them in plant tissues through mixed function oxidases and mono oxygenases enzymatic degradation in plant cell processes.[30] The antioxidants/secondary metabolites producing capacity of basil also plays a major role in degradation of organic pollutant in plant metabolism (phase (II)). According to green liver model tetracycline can combine with enzyme/ secondary metabolites and either completely degraded or converted into products which can comparatively less toxic to plant than the parent compound.[31]

Translocation and Bioaccumulation Factor

TF and BCF was calculated by method of Michelini *et al*.[31] TF shows translocation of antibiotics from root to shoot while BCF showed accumulation of content in plant/content in soil (C_{plant}/C_{soil}). Highest TF was observed in T1 (1.6) which decreased with increasing TC concentrations. Plants with TF value less than 1 accumulated TC more in roots and TF values > 1 more in shoots (Table 3). Bioaccumulation factor was calculated to assess the potential of basil to accumulate antibiotic in plants. In this case maximum BCF with a maximum value of 176 was seen in T1 plant sample and high

Table 2 — Tetracycline degradation in basil

Plant samples (Con. of tetracycline)	Initial amoxicillin content in soil (mg)	Total TC accumulated in plant (mg)	Degraded tetracycline content (mg)
Blank	0.00	0.00	0.00
T1(200 $mgkg^{-1}$)	100	97	3
T2(400 $mgkg^{-1}$)	200	77.8	122.2
T3(600 $mgkg^{-1}$)	300	67.7	232.3

Table 3 — Accumulation of tetracycline in roots and shoots (mg), translocation factors (TF), bioaccumulation factors (BCF)

Samples (Conc. of TC)	Initial TC content in Soil (mg)	After 4W accumulation in shoot (mg)	After 4W accumulation in root (mg)	TF	BCF	% Remediation
Blank	0.00	0.00	0.00	0.00	0.00	0.00
T1 (200 $mgkg^{-1}$)	100	64.5	32.5	1.6	176	97 %
T2(400 $mgkg^{-1}$)	200	25.8	52	0.29	36.05	38.9 %
T3 (600 $mgkg^{-1}$)	300	15.6	52.1	0.17	19.5	22.5 %

Data presented in mean (n=3) and ± SE.

bioaccumulation represents the remediation potential of basil. Some TC residues (Table 2) were not accumulated in plant could have degraded due to photolysis and hydrolysis. The secondary metabolites/antioxidant producing capacity of basil play a major role in degradation of TC in plant metabolism (phase (II)). According to green liver model TC can combine with enzyme/ metabolites and degraded in plant and converted into their transformation products which can comparatively less toxic to plant than parent compound.[32]

Conclusions

Present study is the first study to confirm that the basil has a greater potential for phytoremediation of TC. In toxicity analysis selected concentrations of TC showed significant impact on plant growth, biomass, Chl a, Chl b, total Chl and carotenoids, etc. as compared to blank, suggesting dose-specific impact in different plant setups of TC. TFC also showed significant impact of TC in its content while in case of proline slight enhancement was observed in T2 plant setup. Further study undertaken the remediation potential of basil for TC and highest percentage remediation was observed at T1 (200 mgkg−1) which was significantly higher than control setup (antibiotic without plant). This study estimated that the Antioxidant/secondary metabolites producing capacity and cytochrome P-450 enzyme in hydroxylation and oxidation process and glutathione-mediated conjugation in phase II can degrade tetracycline content in plant. Terpenoids and sesquieter penoids were also affected by TC toxicity in basil. It has been found that tetracycline stress enhances the production of secondary metabolites percentage of certain samples. Therefore, we can say that economic benefits can also be achieved by growing this plant in polluted sites. Although many remediation methods are available, these are costly and time consuming and not very efficient. Therefore, there is need to adopt a more effective process to complete removal of these pollutants. Phytoremediation has been proven to be economical and environmental friendly method. These results are encouraging and undertaking large efforts in order to have more comprehensive research to understand the uptake mechanism of antibiotics by basil and their translocation in plant parts will be useful. Basil showed higher tolerance level for TC and can be used for remediation. In conclusion, the sensitivity of basil towards antibiotics could be taken as a bioindicator of the harmful impact of medical waste on environment and its sustainability.

Acknowledgement

The authors are grateful to Jaypee Institute of Information Technology for providing the necessary laboratory facilities and encouragement. Essential oil association and Anchrom laboratory are also acknowledged. Animal work was not done in this study.

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