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SCREENING AND COMPARATIVE ANALYSIS OF PESTICIDE – MICROBE DYNAMICS FROM PHYLLOSPHERE OF OKRA AND EGGPLANT

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ABSTRACT

Agroecosystems and phyllosphere microbiomes can be the potential indicators of differential segregation expected for organic and pesticide-sprayed fields. The pesticide interaction with the niche phyllosphere microbiota would serve to convey the characteristic role of the phyllosphere in biomass changes, degradation profiling, and approximation of the synthetic residues contributing towards further complexation with soil microbiome. We present the effects of differential spraying of Chlorpyrifos and Cypermethrin pesticides on the phyllosphere of Okra and Eggplant compared with control plants. Highly sensitive and discrete analysis using GC-MS and microbial enrichment followed by 16s RNA identification were conducted to report the overall biochemical and molecular quantifications on the selected plants. GC-MS studies indicate the rate at which pesticides dissipate from plants over time. In our study, dissipation was followed for 10 and 16 days, where chlorpyrifos disappeared by 95% after 16 days in okra plants, while it disappeared by 92% after 10 days in eggplants. Similarly, after 16 days, cypermethrin vanished completely from okra and it had reduced by 89% after 10 days from eggplant. The prolonged periods of pesticide dissipation indicating danger to both humans and animals led researchers to investigate the role of bacteria from polluted surfaces in accelerating the breakdown of pesticides as these are difficult to break down naturally. Scientific studies evidently configure that in contrast to plants not exposed to pesticides, bacterial proliferation on plants exposed to pesticides is enhanced. Our study design implies pesticide – microbe dynamics with survival strategies employing pesticides for their growth on plant phyllosphere.

Keywords: Phyllospheric microbes, pesticide degradation, chlorpyrifos (CP), cypermethrin (CY), okra, eggplant, GC-MS, 16S rRNA sequencing

INTRODUCTION

Pesticides have long been used in agriculture to protect crops from pests, diseases, and weeds. It is well known that pesticides play a crucial role in enhancing agricultural output by effectively controlling pests and diseases (**Popp *et al*., 2013**). Pesticide utilization by farmers has risen exponentially in the past few decades with the development of modern agriculture practices to control pests, weeds, and diseases in plants. It was found that only 0.1% of applied pesticides reach the target destination and about 10-20% retained by above ground part of plant remaining goes into soil, air, water, and various environmental components (**Uddin *et al*., 2016**). Around 45% of annual food production is thrown away due to pest infestation, which leads to increased use of different pesticides for better crop production (**Abhilash and Singh, 2009**).

The excessive and unsystematic use of pesticide has a serious effect on different biota due to high accumulation of organophosphorus compounds (OP), which are a group of highly toxic agricultural chemicals (**Varghese *et al*., 2022**). Farmers use chlorpyrifos (CP) and cypermethrin (CY), among the broad-spectrum control pesticides commonly sprayed in agricultural fields. CP is an organophosphate pesticide used to exterminate insects and worms (**Ali Mohammad Latifi, 2012**). CY is a non-systemic pyrethroid pesticide with significant insecticidal activity and sufficient air stability (**Uddin *et al*., 2016**).

The above-ground part of a plant, collectively called the phyllosphere, encompasses the aerial components such as leaves, flowers, and stems (**Ruinen, 1956; Shukuru *et al*., 2024**). Plants accommodate a diverse array of microorganisms structured at the phyllosphere constituting around 60% of the earth's total biomass, establishing it as one of the expansive habitats supporting microbial life (**Lindow and Brandl, 2003; Sohrabi *et al*., 2023**). Microbes (epiphytes and endophytes) associated with plant phyllosphere play various roles for the host plant such as nitrogen fixation, pathogen prevention, growth stimulation, and residual pesticide decomposition. These microorganisms help in the bioremediation of pesticides by the metabolic degradation ability of indigenous microbes (**Ning *et al*., 2010; Mallavarapu *et al*., 2011**). In the realms of agriculture sustenance, the phyllosphere plays a crucial role for maintaining the growth and development of crops. The investigation of phyllosphere microbiology has been notably limited in comparison to other bacterial habitats (**Remus-Emsermann and Schlechter (2018**). The aerial component of the phyllosphere, which is predominantly studied for microbial colonization, is leaves, serving as a habitat for bacteria, filamentous fungi, yeast, algae, and sometimes found protozoa and nematodes (**Lindow and Brandl, 2003**). The microorganisms colonising the phyllosphere may have beneficial, negative, or neutral consequences on the host plants (**De Mandal and Jeon, 2023**). Certain phyllobacteria, or colonising bacteria of the phyllosphere, have beneficial interactions with their host plants and enhance plant production and health by controlling the recycling of nutrients, releasing phytohormones, and defending plants from biotic and abiotic challenges (**Bashir *et al*., 2022**). Microbes associated with the phyllosphere play various roles for the host plant such as nitrogen fixation, pathogen prevention, growth stimulation and residue pesticide decomposition (**Yu Zhou *et al*., 2011**). These microbes help in phytoremediation by the degradation ability of indigenous microbes (**Montreemuk *et al*., 2024**). It has been found that several plant-growth-promoting-phyllobacteria (PGPPs) assist in plant growth and development in many ways such as the nitrogen-fixing ability on the leaf surface (**Vejan *et al*., 2016; Fürnkranz *et al*., 2008**). Compared to rhizobacteria, which have undergone extensive exploration, PGPP are still in the investigation stage. Therefore, from a scientific, agricultural, and economic perspective, it is crucial to comprehend how phyllospheric microorganisms interact with their host plants (**Nadarajah and Abdul Rahman, 2021**). This growing list of new bacterial species includes rhizobacteria, phyllobacteria, and bacterial endophytes with beneficial properties as direct and indirect biocontrol (**Orozco-Mosqueda *et al*., 2021**). However, the investigation of phyllosphere-associated microbes has been notably limited compared to rhizosphere (**Perazzolli *et al*., 2014**). With the realization of the importance of phyllobacteria's beneficial properties a considerable amount of attention this field has received.

Eggplant and okra are two highly valued crops that are essential in global agriculture and culinary traditions. These versatile vegetables are not only delicious and nutritious but also offer numerous health benefits. Eggplant (*Solanum melongena*), also known as aubergine, is a member of the nightshade family and is widely used in Mediterranean, Middle Eastern, and Asian cuisine. Okra (*Abelmoschus esculentus*), however, is a popular vegetable in tropical and subtropical regions known for its unique texture and ability to thicken dishes. Okra

and Eggplant are the major vegetables extensively cultivated with a total area of nearly 530.80 thousand hectares for okra and 7.11 million hectares for eggplant across India (**Mayannavar *et al*., 2017; Reddy and Kumar, 2022**). Both eggplant and okra have distinct seasonality, with peak production occurring during specific times of the year. Okra grows in the rainy season; it is sown during June-July and for the spring season, it is cultivated in February – March. Eggplant can be grown in both winter and summer seasons. As a winter crop, it is better to sow brinjal between June and July. As a summer crop, it is better to sow brinjal between December and January month. Understanding the factors that influence the growth and productivity of these crops is crucial for farmers, researchers, and consumers alike. Therefore, it is crucial to study the effects of various factors on the phyllosphere of eggplant and okra to develop sustainable farming practices and maximize crop productivity. However, the impact of CY and CP on the phyllosphere of these two distinct plants remains poorly understood. Understanding the effects of CY and CP on the phyllosphere of these two crops is particularly important due to their widespread cultivation and economic importance.

In the current study, we analytically quantified the pesticide residues of CP and CY in the phyllosphere of two distinct vegetable plants *A. esculentus* and *S. melongena*. With the dissipation of the residues, we also analyzed the bioremediation ability of phyllosphere microorganisms.

MATERIALS AND METHODS

Chemicals and reagents

The commercial formulation of CP and CY, reference standards of CP and CY, Ethyl acetate, sodium sulphate (Na_2SO_4), sodium chloride (NaCl), Sodium citrate tribasic dehydrate, sodium citrate dibasic sesquihydrate, anhydrous sodium sulfate, graphitized carbon black, acetic acid, methanol, peptone, Plate count agar (PCA) media, KH_2PO_4, K_2HPO_4, NH_4NO_3, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$ and $Fe(SO_4)_3$, $NH_4SO_4.2H_2O$, $MgCl_2.7H_2O$, Vitamin B1.

Selection of site for collecting samples

Two plants were chosen: okra (*A. esculentus*) and eggplant (*S. melongena*). Samples were collected from a farm field in a village in the district of Muzaffarnagar (Latitude and longitude coordinates were 29.471397, 77.696732). For each plant species separate field area was chosen and each area was further divided into two-control field and experimental field. The experimental field was sprayed with a pesticide (CP and CY), and the other portion was not sprayed, was kept as experimental control.

Persistence and dissipation behavior of selected pesticides

Crop seedlings were sown on approximately 1.6 acres of land in mid-March. The field was separated into two sections (experimental and control). After 25 days, the commercial formulation of pesticide, CP and CY were sprayed on both the crops grown in experimental fields. The untreated sections of fields were used as control where only water was sprayed. Samples of leaves 25 grams approximately were randomly collected from each treated field on days 0 (2 hours), 1, 3, 5, 10, 16, and 20 following spraying (**Chauhan R *et al*., 2018**)

The samples were cleaned up and extracted by using QuEChERS method (**Prodhan *et al*., 2017**). The sample weighing 0.25 Kg was compressed and homogenized thoroughly in a homogenizer, from which, 10 g sub-sample of leaves was transferred in a 50 ml polytetrafluoroethylene (PTFE) centrifuge tube. Add 10 ml water and 10 ml ethyl acetate (HPLC grade), mix by shaking manually and then vortex for 1 minute. Thereafter add Na_2SO_4: NaCl: Sodium citrate tribasic dehydrate: sodium citrate dibasic sesquihydrate 4g: 1g: 1g: 0.5 g and mix manually for 1 minute and then vortex for 10 minutes. The sample was then centrifuged for 10 minutes at 5000 rpm. Separate 6 mL of the ethyl acetate layer in a new centrifuge tube of 15 mL capacity containing 150 mg of PSA (Primary Secondary Amine), 900 mg of anhydrous sodium sulfate, 45 mg graphitized carbon black (GCB). The sample was mixed properly by vortexing for 1 minute. Centrifuge at 10000 rpm for 5 minutes. For analysis using GC-MS technique, take out 2ml of aliquot and filter through a syringe filter. This extract was evaporated to dryness under nitrogen at 35 °C. Reconstitute the residue in 2ml of 0.1% acetic acid in water and methanol in the ratio of 1:1.

The residues of CP and CY were quantified by gas chromatograph mass detector triple quadruple (GC-MS/MS) (**Chandra *et al*., 2014**), model Shimadzu TQ 8030 fitted with a 60m x 0.25 i.d. x 0.25 µm film Rxi-5MS column (Restek International, USA) and operated in MRM (Multiple Reaction Monitoring) mode. Instrument conditions included spitless injection at 250 °C and injector at 260 °C. For carrier gas, Helium was used with a flow rate of 1 $mLmin^{-1}$. Under these conditions, the retention time of CP and CY isomers were found to be 23.3,42.6, 42.8, 43.0, 43.1 minutes with parent mass transition as 313.9>257.9;181.1>152.1; 181.1>152.1; 181.1>152.1; 181.1>152.1 respectively.

Isolation of Pesticide-degrading bacteria through enrichment method

Initial screening of phyllosphere microbes present on Okra and Eggplant.

Leaves (20 g) were collected from the phyllosphere of plants exposed to the pesticides in a sterile sample collection bag from each site. To recover microbial communities from leaves, 10 g of leaf samples were added directly to the 100 mL, 0.1% peptone in the sterilized bag. Bags containing peptone and sample were manually rubbed for 5 to 10 minutes so that microbes present over the sample surfaces were transferred to the diluent. With this extract serial dilutions were prepared upto 10^{-5} dilutions. For total bacterial count, each dilution was poured into the Petri plate along with PCA media. This was done in duplicates. Plates were incubated at 30 °C for 72 hours (**Naphade *et al*., 2012**).

Enrichment and isolation of pesticides degrading bacteria

Okra and Eggplant leaves were put into two separate 250 mL flasks containing 150 mL of sterile liquid Mineral Salt Medium (MSM) with 10 ppm of CP and incubated at 28±2 °C for 7 days on static condition, was used for isolating CP degrading bacteria. The MSM has the following composition in (g/L): KH_2PO_4, 4.8; K_2HPO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4.7H_2O$. 0.2; $Ca(NO_3)_2.4H_2O$, 0.04; and $Fe(SO_4)_3$, 0.001 with pH 7.0. For isolating CY-degrading bacteria, the leaf samples were added in a sterile Minimal Medium with 10ppm of CY incorporated into it. The medium has the composition of (in gL^{-1}): K_2HPO_4, 10.5; KH_2PO_4, 4.5; $NH_4SO^4.2H_2O$, 1.0; $MgCl_2.7H_2O$, 1mL from the autoclaved stock of 200 gmL^{-1}, Vitamin B1 (Thiamine), 0.5 mL from 1% stock and CY, 10ppm. All the above flasks were incubated at 28±2°C for 7 days on static conditions (enrichment 1). From every flask, 5mL was re-inoculated to the flask with the same medium composition aseptically and further incubated at 28± 2°C for 7 days on static conditions (enrichment 2). Then from every flask a loopful of culture was streaked on the sterile Nutrient agar plate and the plates were incubated 28±2°C for 48 hours to get isolated colonies of bacteria. The well-isolated colonies were grown on sterile nutrient agar slants as pure cultures and maintained at 10°C as stock cultures. The colony characters were identified based on the colony morphology and staining characters (**Naphade *et al*., 2012**).

Identification and Molecular characterization of pesticide-degrading bacterial isolate

The isolates were examined for morphological, cultural and biochemical studies which included gram staining, oxidase, catalase, casein hydrolysis, starch hydrolysis, and gelatin liquefaction. Some specific biochemical tests included Indole, Methyl red, Vogues Prousker and Citrate Test, Nitrate Reductase, Hugh Leifson test, and growth on SMA (Standard Method Agar) (**Chauhan and Jindal 2020**). Bacterial cultures isolated from 8 different colonies were preserved in NA slants. DNA was isolated from the bacterial pellet drawn from 8 different cultures using kit Add gene, cat no. GF-BA100.16S rDNA region of isolated DNA was amplified using Emerald Amp GT PCR Master mix. Using primers of 16S 1F and FGPSR, we amplified 1300 bp amplicon and purified by gel elution. The purified product was sequenced by Sanger's method of DNA sequencing. The sequencing results were assembled and compared with the BLAST at NCBI database.

Growth of the isolates and biodegradation of pesticides in Liquid culture

The bacterial isolates were grown in separate 250ml flasks containing 50mL of mineral salt medium (MSM) at 120rpm and 37°C on a rotary shaker for 24 hrs. From these flasks, 1 mL of the 24 hrs culture was then used as inoculum. This inoculum was used to inoculate 250mL flasks containing 100mL MSM and 10 ppm of pesticides, CP and CY in different flasks in duplicate. The flasks were incubated in a rotary shaker at 120 rpm and 30°C for 14 days. The growth (optical density) of the isolates was determined every day using a Spectrophotometer (Model UV 1800 Schimadzu) at 600 nm.

RESULTS AND DISCUSSION

Organophosphates like CP are used to kill insects and worms on crops. WHO (World Health Organization) classifies CP as moderately hazardous to humans. CY is a pyrethroid insecticide with significant insecticidal activity and sufficient air stability. It is utilized extensively in agricultural regions. Although efficient degradative microbial strains for CY and CP are difficult to obtain, the pesticide has been identified as a degradable chemical for environmental bacteria. Thus, bioremediation technology using indigenous bacteria is an effective way for CY and CP remediation. Phyllosphere is the window towards monitoring the growth promotion or alteration through the resonating diverse microbiota habitats. Similarly, the composition of the phyllosphere microbiome colonizing the plant communities would be giving insights towards the functional role in plant productions and its overall health. The organic practices in comparison to pesticides regulation of synthetics used commonly while cultivation would give the differential details of the varied biochemical, physiological and overall plant health outcomes. The highly dynamic processes in direct relation to such environmental changes, aka the foliar community microbiota and the resultant

interactions with these chemicals reposition the symbiotic relationships, antibiosis and competition for resources amongst the phyllosphere microbiome and its plant host. There are limited functional omics studies available for studying the architecture of the phyllosphere microbiome, pathobiomes that are quintessential towards impact on overall agroeconomics and agroecosystems (**Dastogeer *et al.*, 2020**). The studies of the bio-control agents, functional analyses of the phyllosphere, composition of the phyllosphere microbial population, and pesticide degradation can correlate the technical agriculture practices for sustaining the crop quality and quantitive yields in the future.

After foliar application of the pesticides, the leaves of the plants were collected and pesticide residues in plants were analyzed by GC-MS/MS. A wide analytical scope provided by QuEChERS method used to extract samples followed by gas chromatography tandem mass spectrometry (GC-MS/MS) method to analyze residues. (**Lehotay *et al.*, 2010**).

It was found that pesticide residues take a period to get eliminated from the ecosystem. In figure 1, GC-MS chromatogram had been presented showing the single peak of CP and four peaks of 4 isomers of CY. The highest concentration of CP was recorded at around 20 ppm on the day of sprayed (Day 0) in okra plant which gradually dissipated to 43 %, 57 %, 62 %, 80 %, and 95 % on days 1,3,5,10, and 16 respectively (Table 1). Till the 20th day, it was observed almost disappeared form the leaf samples. Similar results were obtained from eggplant phyllosphere samples. Initially, 23ppm of CP on day 0 was dissipated as 32 %, 64%, 84%, and 92% on days 1, 3,5, and 10 respectively (Table 2). It was found in eggplant leaves dissipation rate of CP is fast as compared to okra. It is presumed that the dissipation rate of pesticides may depend upon the surface area and characteristics of the leaves such as leaf roughness (**Lu *et al.*, 2014**). When same experiment was conducted with pesticide CY, from initial concentration of around 14 ppm on day 0 its dissipation rate was 12 %, 31%, 54 % and 87 % on day 1, 3, 5 and 10 respectively in okra plant. Four isomers of CY are present as CY1, CY2, CY3 and CY4. A comparable pattern was noticed in eggplant as shown in table 2. With the primary concentration of 14 ppm CY dissipated to 18 %, 36 %, 53 % and 89 % on day 1, 3, 5 and 10 respectively. As can be seen in Figure 2 and 3, the concentration curve of CP residues dissipated remarkably in the first a few days and persisted in the crops for long period of time.

Table 1 Concentration and dissipation of pesticide residues at different intervals from the day of application in okra plant.

Days	0	1		3		5		10		16		20	
Pesticide	Conc. (ppm)	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.
CP	20.11	10.91	45.75	8.59	57.28	7.6	62.21	4.0	80.11	0.91	95.47	BDL	100.0
CY1	14.31	12.44	13.07	9.69	32.29	6.28	56.11	1.88	86.86	BDL	100.0	ND	100.0
CY2	14.21	12.29	13.51	9.57	32.65	6.39	55.03	1.97	86.14	BDL	100.0	ND	100.0
CY3	13.86	12.07	12.91	9.66	30.30	6.29	.54.62	1.73	87.52	BDL	100.0	ND	100.0
CY4	14.19	12.49	11.98	9.64	32.06	6.69	52.85	1.61	88.65	BDL	100.0	ND	100.0
CY	14.14	12.32	12.87	9.64	31.84	6.41	54.66	1.80	87.29	BDL	100.0	ND	100.0

Note: CP = Chlorpyrifos, CY = Cypermethrin, Conc. = Concentration, Diss. = Dissipation, BDL = Below Detection Limit, ND = Not Detected

Table 2 Concentration and dissipation of pesticide residues at different intervals from the day of application in eggplant.

Days	0	1		3		5		10		16	
Pesticide	Conc. (ppm)	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.	Conc. (ppm)	% Diss.
CP	23.84	16.09	32.51	8.48	64.43	3.77	84.19	1.87	92.16	BDL	100.0
CY1	14.82	11.98	19.16	9.21	37.85	6.76	54.39	1.61	89.14	BDL	100.0
CY2	14.85	12.18	17.98	9.43	36.50	6.82	54.07	1.65	88.89	BDL	100.0
CY3	14.40	11.94	17.08	9.15	36.46	6.66	53.75	1.55	89.24	BDL	100.0
CY4	14.99	12.14	19.01	9.51	36.56	6.99	53.37	1.60	89.33	BDL	100.0
CY	14.77	12.06	18.32	9.33	36.84	6.81	53.89	1.60	89.15	BDL	100.0

Note: CP = Chlorpyrifos, CY = Cypermethrin, Conc. = Concentration, Diss. = Dissipation, BDL = Below Detection Limit

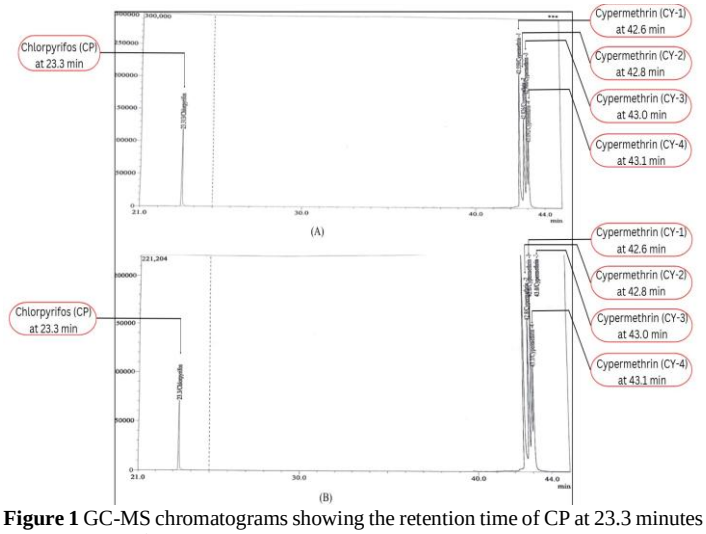
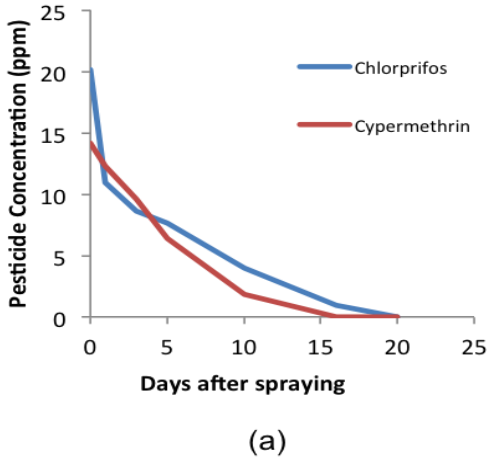


Figure 1 GC-MS chromatograms showing the retention time of CP at 23.3 minutes and 4 isomers of CY, CY-1 at 42.6 minutes, CY-2 at 42.8 minutes, CY-3 at 43.0 minutes and CY-4 at 43.1 minutes analyzed in Okra plant samples (A) and eggplant samples (B).



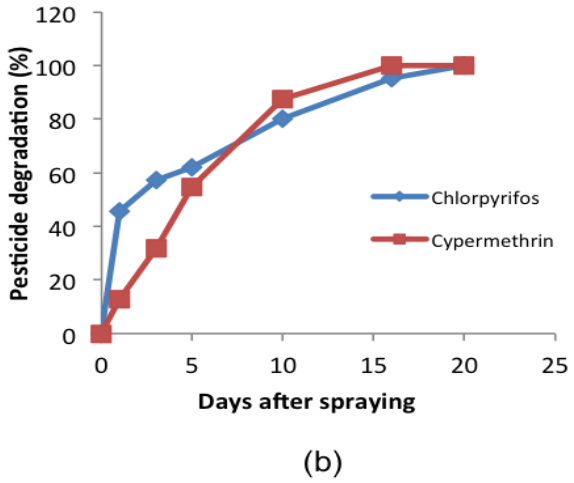


Figure 2 Trend of pesticide residue concentration and degradation in okra plant sprayed at 2mLL^{-1} at different days after spraying. (a) Graph displayed was plotted between pesticide concentration (in ppm) on y-axis and days after spraying on x-axis. (b) Graph represents pesticide degradation (in %) on y-axis and days after spraying on x-axis. In both the graphs solid blue line represents chlorpyrifos and dark red line represents cypermethrin.

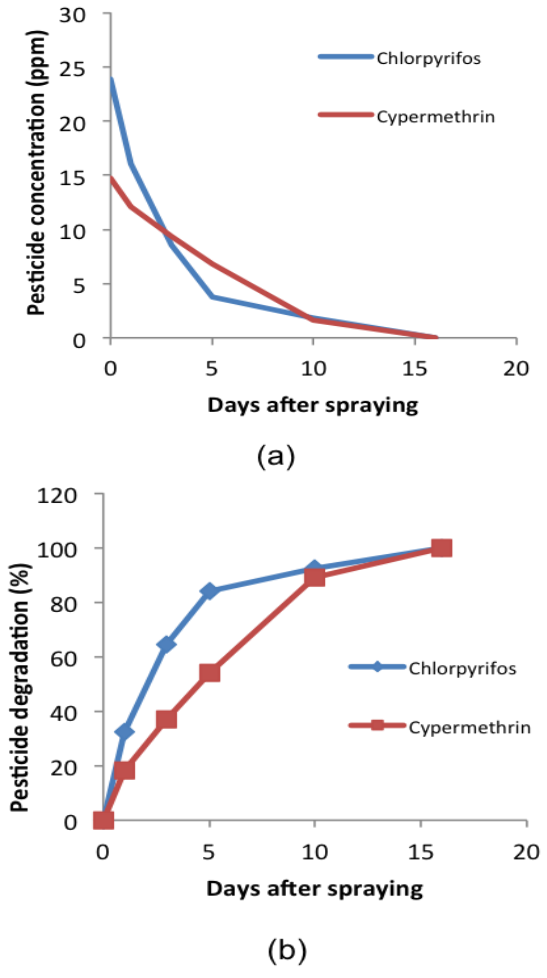


Figure 3: Trend of pesticide residue concentration and degradation in eggplant sprayed at 2mLL^{-1} at different days after spraying (a) Graph displayed was plotted between pesticide concentration (in ppm) on y-axis and days after spraying on x-axis. (b) Graph represents pesticide degradation (in %) on y-axis and days after spraying on x-axis. In both the graphs solid blue line represents chlorpyrifos and dark red line represents cypermethrin.

Different crops harbor different microbial compositions in their phyllosphere (**Perazzoli *et al.*, 2014**). These microbial compositions can have significant effects on crop health and productivity. For example, studies have shown that certain microbial species in the phyllosphere can promote plant growth by enhancing nutrient uptake and increasing resistance to pathogens (**Batool *et al.*, 2016**). On the other hand, some microbial species in the phyllosphere may harm crop health by causing diseases or reducing nutrient availability (**Gouda *et al.*, 2016**). Microorganisms capable of degrading selected pesticides (CP and CY) were screened by the enrichment technique and several bacterial strains were isolated from the phyllosphere of okra plant and eggplant when exposed to these pesticides. For total bacterial count, different dilutions prepared from the leaf surface extract were poured with the PCA in the petri plates. In Figure 4 (a) bacterial colonies acquired from eggplant leaf samples on PCA plates of different dilutions can be seen. Figure 4 (b) shows the countable colonies of bacteria at 10^{-5} dilution in control and pesticide-treated samples. Likewise, Figure 5 (a) displayed the serial dilutions of bacterial counts from the okra plant. In Table 3, the contrast of the number of bacterial colonies in control and pesticide-treated plants was exhibited. In eggplant, at 10^{-5} dilution we obtained 175 CFU (colony forming unit) in the pesticide-treated sample as compared to 105 CFU in the control, where the pesticide was not sprayed over the plants. Similarly, in the okra plant number of bacterial colonies is higher in the pesticide-treated plant (50 CFU at 10^{-4} dilution) in contrast to the control sample (12 CFU at 10^{-4} dilution). It is speculated that the microbiome in the phyllosphere may be able to metabolize pesticides resulting in the accumulation of their increased number. Our observation shows similarity with the study carried out with an organophosphorus compound Fenitrothion (o,o-dimethyl o-(3-methyl-p-nitrophenyl) phosphorothionate, abbreviated as MEP. Similar study was done by **Itoh H *et al.***, in the year 2014, he or she demonstrated that the succession and adaptation processes of microbial communities under MEP application, which were critically affected by insecticide spraying.

Table 3 Bacterial count was done on colony counter, the number of colonies of bacteria grown on PCA (Plate count agar) plates of dilutions from10^{-1} to 10^{-5} were counted in both control and samples collected from pesticide treated samples okra and eggplant.

Dilutions	Eggplant		Okra Plant	
	CFU in Control	CFU in Treated Sample	CFU in Control	CFU in Treated Sample
10^{-1}	TNTC	TNTC	TNTC	TNTC
10^{-2}	TNTC	TNTC	TNTC	TNTC
10^{-3}	TNTC	TNTC	113	TNTC
10^{-4}	TNTC	TNTC	12	50
10^{-5}	105	175	1	5

*CFU-Colony Forming Unit **TNTC-Too Numerous to Count

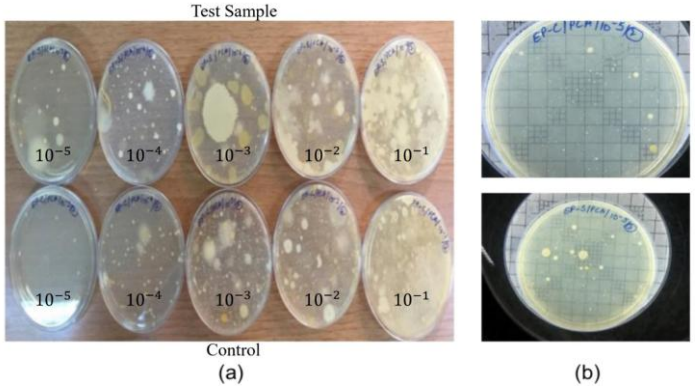


Figure 4: (a) Bacterial colonies from Eggplant sample exposed to pesticides on PCA media plate of different serial dilutions; (b) comparison of control and pesticide treated dilutions on PCA media plate.

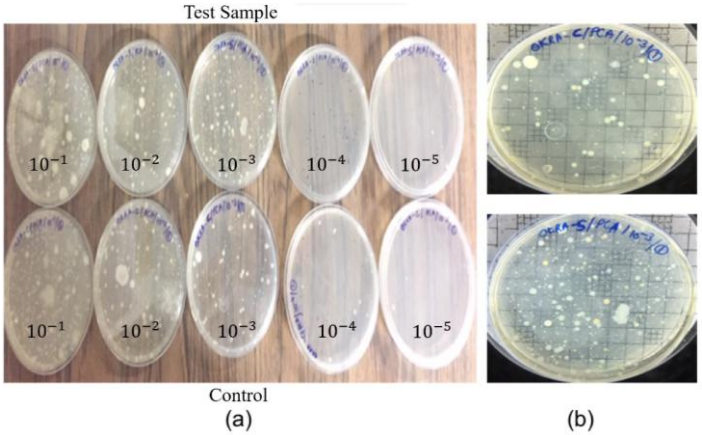
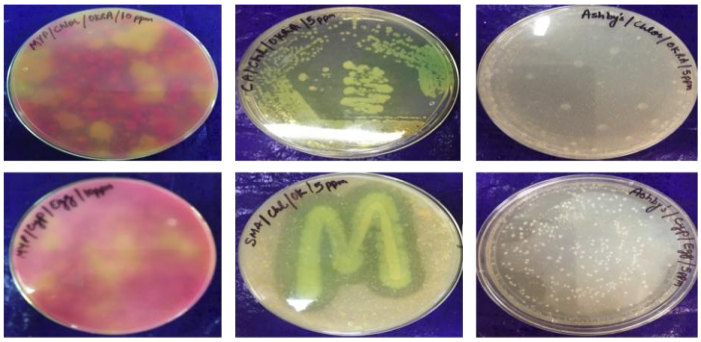


Figure 5: (a) Bacterial colonies from Okra plant sample exposed to pesticides on PCA media plate of different serial dilutions; (b) comparison of control and pesticide treated dilutions on PCA media plate.

For the isolation of potential pesticide-degrading bacteria, an enrichment culture technique was used in which microbes inhabiting in phyllosphere of the plants were grown *in vitro* in a minimal salt medium with pesticide as a carbon source. These isolated bacterial colonies were investigated for cultural and morphological characteristics. Colony characteristics used for identification include colour, elevation, margin, and size. As observed under a compound microscope all of the 8 colonies are rod-shaped, 3 of them are gram-positive and the rest 5 were gram-negative (**Rayu *et al*., 2017**). The isolated colonies were grown on selective media plates like Mannitol egg Yolk Polymyxin agar (MYP agar), Cetrimide Agar (CA), Skim Milk Agar (SMA) and Ashby's mannitol agar. Figure 6 shows the growth of isolated bacteria on selective media plates, in (a) pink and yellow colonies were observed on MYP agar plate indicating the presence of *Bacillus sp*., (b) indicates presence of *Pseudomonas sp.* as green colour colonies were observed on CA and SMA plates in (c) white coloured opaque colonies were observed on Ashby's agar shows the presence of *Azotobacter sp.*. Subsequently, biochemical tests were performed and Table 4 summaries the results which signify the isolated bacterial colonies belong to the genera of *Bacillus, Pseudomonas and Azotobacter*.



(A) (B) (C)

Figure 6 Selective media plates of Isolated CP and CY Degrading bacteria (identified as (A)-*Bacillus* sp., (B)- *Pseudomonas* sp., (C)- *Azotobacter* spp.)

Table 4 Summary of biochemical tests performed with isolated colonies of bacteria.

Test Parameter		For *Bacillus Sp.*		For *Pseudomonas sp.*		For *Azotobacter sp.*	
		Sample (S1,S2,S3)	Positive control (ATCC-6633)	Sample (S4)	Positive Control (ATCC-9027)	Sample (S5,S6,S7,S8)	Positive Control (ATCC-9043)
Gram Staining		+, Rods	+, Rods	-, Rods	-, Rods	-, Rods	-, Rods
Hemolysis (on Blood Agar Plates)		+	+	NA	NA	NA	NA
Catalase		+	+	+	+	+	+
Oxidase		NA	NA	+	+	NA	NA
Indole		NA	NA	NA	NA	+	+
Urease		NA	NA	NA	NA	-	-
Voges -Proskauer		+	+	NA	NA	+	+
Citrate utilization		+	+	NA	NA	NA	NA
Methyl red test		NA	NA	NA	NA	+	+
Casein Hydrolysis		+	+	+	+	NA	NA
Starch Hydrolysis		NA	NA	-	-	NA	NA
Gelatin Liquefication		+	+	+	+	NA	NA
Carbohydrate fermentation	**a) Glucose**	+	+	NA	NA	NA	NA
	b) Mannitol	+	+	NA	NA	NA	NA
Hugh Leifson Test		NA	NA	+	+	NA	NA
Nitrate Reduction		NA	NA	+	+	NA	NA
Growth on SMA	**a) At 42°C**	NA	NA	+	+	NA	NA
	b) At 4°C	NA	NA	-	-	NA	NA

+ -Positive, - -Negative, NA- Not Applicable

Genomic DNA was isolated from all 8 isolated bacterial cultures (S1, S2, S3, S4, S5, S6, S7 and S8). Figure 6 shows the gel image of DNA bands obtained from 8 different cultures. In well numbered 1, 2, 3, 7 and 8 single band was obtained, and no RNA contamination was there. However, in well numbered as 4, 5 and 6 along with DNA marginal contamination of RNA was there. After PCR amplification, the isolates were shown to have fragments of the amplified DNA of around 1300 bp (Figure 7). Through DNA sequencing isolates were identified based on the similarity to sequences in the GenBank.

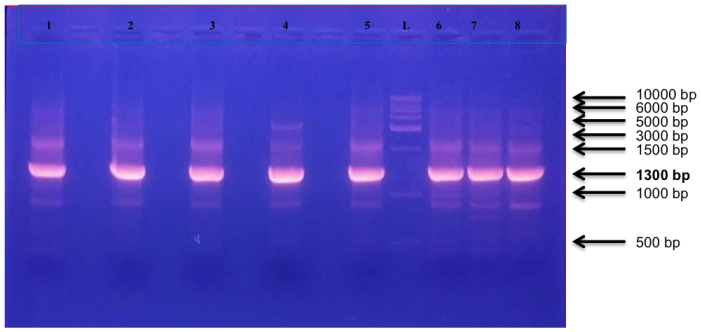


Figure 7 Gel image of PCR amplified 16S rDNA of the bacteria isolates in agarose gel. Lane L, 1kb DNA ladder; Lanes 1–5 and 6-8, genomic DNA of isolated strains (S1 -S8).

16S rRNA sequencing at Biologia Research India Pvt. ltd identified a total of 8 bacterial isolates (S1, S2, S3, S4, S5, S6, S7 and S8). During microbial identification of the isolated microbial colonies, three different bacterial species are identified. The isolates belonged to the genera *Bacillus, Pseudomonas and Azotobacter* (Table 5). S1, S2, and S3 shares the most sequence alignment with *Bacillus sp*.; S4 shares the sequence alignment with *Pseudomonas* sp.; S5, S6, S7, and S8 share the sequence alignment with *Azotobacter sp.*.

Table 5 Identity of isolated Bacteria from phyllosphere samples based on similarity to the 16S rDNA sequence.

Microbes Identified	Okra Plant		Eggplant	
	Chlorpyrifos	Cypermethrin	Chlorpyrifos	Cypermethrin
Bacillus Sp.	+ve	+ve	-ve	+ve
Pseudomonas Sp.	+ve	-ve	-ve	-ve
Azotobacter Sp.	+ve	+ve	+ve	+ve

+ve- Positive, -ve- Negative

The phylogenetic grouping tree (Figure 8) was constructed using MEGAX Software (MEGA Software Company). Strains were clustered together based on the similarity of sequences. Moreover, 16S rRNA gene sequences formed the basis of the Maximum Likelihood phylogenetic tree. Table in Figure 8 displays bacterial colonies S1, S2 and S3 belong to genera *Bacillus*, S4 to *Pseudomonas* and S5, S6, S7 and S8 to *Azotobacter*. Table 6 summarizes the culture extracted from okra plants which was exposed to CP were similar to genera *Bacillus* (S1), *Pseudomonas* (S3) and *Azotobacter* (S5), while exposure to CY shows similarity with *Bacillus* (S2) and *Azotobacter* (S7). However, isolates from eggplant which were exposed to CP shows similarity with *Azotobacter* (S6), while exposure to CY shows similarity with *Bacillus* (S3) and *Azotobacter* (S8).

Table 6 Summary of microbial identification of bacterial colonies isolated from Okra plant and Eggplant when exposed to Chlorpyrifos and Cypermethrin.

Bacterial isolates	Species (16S rRNA gene analysis)	Accession no.	Identity (%)
S1	Bacillus sp. SKM33	LM655316	94.59
S2	Bacillus subtilis 16S rRNA	AY631853.1	95.66
S3	Bacillus sp. StrainVPS50	MH819978.1	94.33
S4	Pseudomonas aeruginosa strain Y 12	MH997635.1	97.90
S5	Azotobacter chroococcum strain 76A	KX1088661.1	94.27
S6	Azotobacter sp. Strain N9	MN658515.1	91.79
S7	Azotobacter sp. Strain N9	MN658515.1	93.57
S8	Azotobacter sp. Strain N9	MN658515.1	97.11

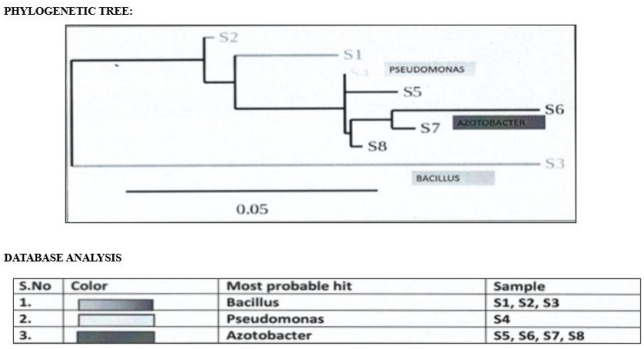


Figure 8 Phylogenetic tree showing the relationship of the 8 isolated bacteria strains (S1-S8).

In the earlier studies related to degradation of pesticides, it was found out that strains like *Enterobacter*, *Pseudomonas*, *Klebsiella*, *Bacillus* could degrade the chemical and its metabolite (**Wolfenden, R and Spence, G (1967); Bhadbhade *et al*., 2002b; Mrozik and Piotrowska-Seget, 2010**). Phyllosphere presents as the interface between the host, microbiota, and atmosphere, coordinating unequivocally the crosstalk of biochemical and physiological responses and tuning the overall dynamics of ecosystem health and development (**Kumar *et al*., 2017**). During incubation of 14 days, when periodic monitoring of bacterial growth in MSM enriched with pesticides was done using UV-VIS Spectrophotometer there was an increase in cell mass (OD 600 nm) in most flasks, reflective of bacterial growth. In negative controls, the OD levels remained consistent. The bacterial growth curve in the media exhibited the typical four phases of lag, exponential, plateau, and decline. Figure 10 demonstrates the growth curve of *Bacillus sp*., *Pseudomonas sp*. and *Azotobacter sp.* isolated from Okra sample in 10 ppm CP enriched MSM and figure 11, shows the growth curve of *Bacillus sp.* and *Azotobacter sp.* isolated from 10 ppm CY enriched MSM. Figure 12, shows the growth curve of *Azotobacter sp*. isolated from Eggplant sample in 10 ppm CP enriched MSM and figure 13, shows the growth curve of *Bacillus sp*. and *Azotobacter sp.* in 10 ppm CY enriched MSM. Increase in the cell biomass reveals the consumption of these pesticides as the source of carbon since no other carbon source was present in the MSM media. In previous studies, it was reported that for degradation of hazardous pesticide compounds a number of microorganisms were used and some of the soil-borne bacterial strains such as *Arthrobacter spp*., *Burkholderia spp*., *Bacillus spp*., *Azotobacter spp*., *Flavobacteria spp*., *Pseudomonas spp*., and *Rhodococcus spp*. are widely used in the degradation and bioremediation studies (**Chennappa *et al*., 2019; Castillo *et al*., 2011**). These bacterial genera possess enzymes and functional genes, which are responsible for the degradation of toxic pesticide. According to research, pesticides are broken down by bacteria on plant leaves over a two-week period, indicating that the microbes may be using the pesticides as food. This gives rise to environmental issues, but it also presents opportunities for bioremediation and the study of chemical resistance and degradation by microbial communities.

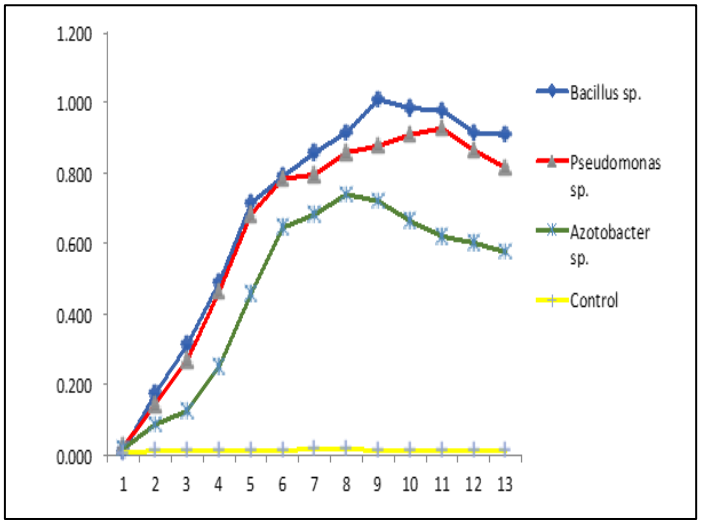


Figure 10: Growth Curve of *Bacillus sp.* (blue), *Pseudomonas sp*. (red), *Azotobacter sp.* (green) and control (yellow) isolated from Okra sample in 10 ppm Chlorpyrifos enriched MSM. In the graph, x-axis represents the absorbance at 600 nm (OD) and y-axis represents the days of incubation.

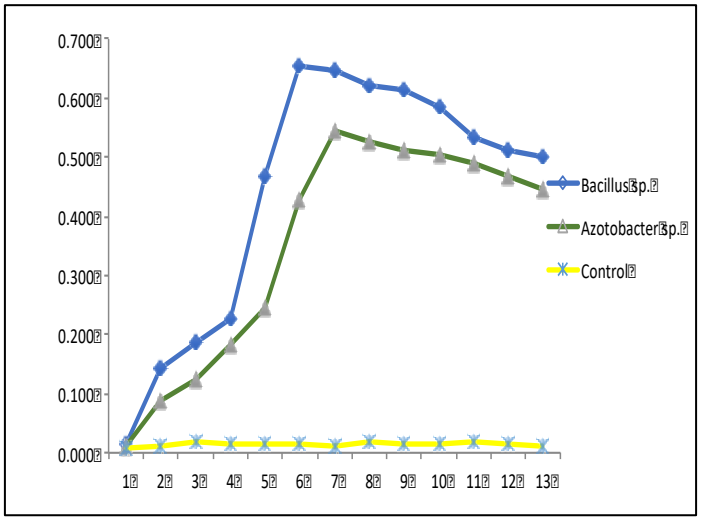


Figure 11 Growth Curve of *Bacillus sp.* (blue), *Azotobacter sp.* (green) and control (yellow) isolated from Okra sample in 10 ppm Cypermethrin enriched MSM. In the graph, x-axis represents the absorbance at 600 nm (OD) and y-axis represents the days of incubation.

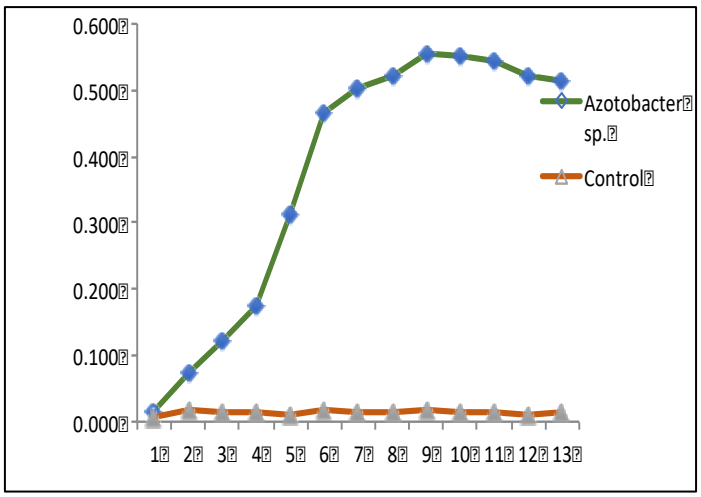


Figure 12 Growth Curve of *Azotobacter sp.* (green) and control (red) isolated from Eggplant sample in 10 ppm Chlorpyrifos enriched MSM. In the graph, x-axis represents the absorbance at 600 nm (OD) and y-axis represents the days of incubation.

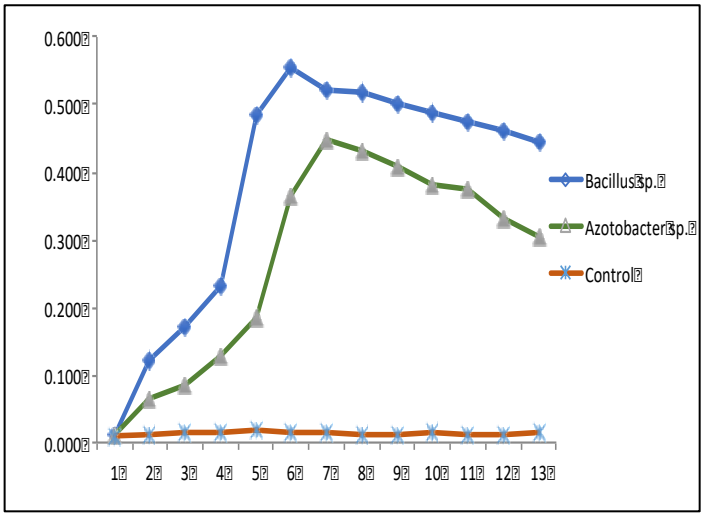


Figure13 Growth Curve of *Bacillus sp*. (blue), *Azotobacter sp*.(green) and control(red) isolated from Egg Plant sample in 10 ppm Cypermethrin enriched MSM. In the graph, x-axis represents the absorbance at 600 nm (OD) and y-axis represents the days of incubation.

CONCLUSION

The present study demarcates the phyllosphere microbiota on the selected plants, okra and eggplant, that are differentially assessed for their population distribution between controlled conditions of pesticide-sprayed plants and control plants. The diversity, composition, and biochemical / biomolecular profiling of the phyllosphere microbiome as a result of the conditions provided are indicative of the biochemical adaptations, metabolic potential that would be required to unravel the first impact on the plant / microbe biomass outcomes. Identification of the impact of phyllosphere on agriculture emanates from the fact that such first contact interface perceives the alterations of the environment or man-made alterations that directly affect the harboring microbiota, plant-microbe and microbe–microbe interactions overlaying the path towards the growth modulations, soil health, abiotic and biotic stress tolerance, entrenching the agriculture production and sustainability of the agro-ecosystems and overall environmental management (**Zhang *et al*., 2023**). An in-depth study using expression profiling of the microbes and their subsequent degradation or utilization of pesticides will be significant in assessing the potential role of phyllosphere in the biogeochemical cycling and further agroecosystems. The study design processed here can provide valuable details of the molecular events, microbial regulation of the plant host through the phyllosphere, putative process of bioremediation, and biomass production of the selected crops.

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