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Plant-microbe interactive remediation of soil contaminated with Imidacloprid using Cowpea (*Vigna unguiculata* L. Walp) and Cowpea rhizospheric microorganism *Bacillus tequilensis*

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Abstract
Imidacloprid, one of the most used pesticides in agriculture protect crops from pest attacks but its high concentration in environment results in toxic effects on aquatic life, humans, and soil ecology. Therefore, in the present work, removal of excessive imidacloprid from soil using plants and their associated rhizosphere microflora was studied. Isolated Cowpea rhizosphere microorganism was inoculated in soil containing 1280 mg/kg Confidor pesticide (17.8% imidacloprid). This soil was then used for Cowpea cultivation. Individual and combined effects of Cowpea and *Bacillus tequilensis* to remove pesticide from soil were studied. Results revealed that *B. tequilensis* isolated from Cowpea rhizosphere removed 62.02% pesticide from bare soils while Cowpea and soil native microbes were able to remove 82.45% imidacloprid from soil on the last day of experiment. Approximately, 99% imidacloprid removal was observed from soil due to combined activity of isolated *Bacillus tequilensis*, soil microbes, and Cowpea. Effect of application of *B. tequilensis* culture was observed in terms of increase in plant growth as compared to control. Plant-microbe interactive remediation of imidacloprid contaminated soil was enhanced due to presence of *B. tequilensis,* Cowpea and native flora suggesting possible role of microbial population in the process of Phytoremediation.

Keywords: *B. tequilensis,* Confidor, Cowpea, Imidacloprid, Plant-microbe interactive remediation

Introduction
Pesticides like neonicotinoids are a group of synthetic chemicals that inhibit pests and insects, thus protecting the crops from getting damaged. Rapid uptake, broad-spectrum activity, and requirement of low dosage with strong insecticidal effects make neonicotinoids a favourite choice of farmers (Mamy *et al.* 2025) [16]. Despite such a versatile nature of neonicotinoids, about 5% of their active ingredient is taken up by crops, and remaining amount is transferred to soil (Aseperi *et al.* 2020) [2]. Soil ecology is thus disturbed by pesticide residues.
First commercial neonicotinoid pesticide, imidacloprid is commonly used in the form of spray near plant root areas and seed dressings on different crops like apple, cereals, cotton, maize, potatoes, rice, and sugarcane (Sarkar *et al.* 2001, Stoner & Eitzar, 2013) [22, 26]. It is known to act as an agonist on nicotinic acetylcholine receptors in the insect brains (Matsuda *et al.* 2011) [11]. Since it is a systemic pesticide, it is taken up by plants in the aboveground parts (Li *et al.* 2018). It persists in environment and can be taken up by non-target organisms which makes the environment unsafe (Uhl *et al.* 2015) [27]. Imidacloprid is toxic at 0.05 mg/L concentration to human beings (Pang *et al.* 2020) [20]. Although imidacloprid is an environmental contaminant, there is no routine monitoring of soil to check for its traces and this has raised concerns among the farmers (Sousa *et al.* 2018) [24].
Phytoremediation involves use of native plants to remove toxic chemicals from the environment. It is a cheaper, ecologically safer, socially acceptable, and aesthetically pleasing option (Futughe *et al.* 2020 [5], Velankar & Zunjarrao, 2025) [30]. Even though plants alone can remove many harmful agricultural contaminants from soil, some coordinated methodologies like plants along with microorganisms affiliated to them (rhizoremediation), can be considered as attractive choices for improving removal of agrarian xenobiotic compounds like pesticides from soil and help soil conservation (Kaur *et al.* 2022) [14]. The process of Phytoremediation is enhanced by activity of rhizospheric microflora. Variety of microorganism occurring in rhizosphere of the plant and native flora of the surrounding soil have ability to degrade different types of organic compounds including pesticides. They thus aid in improving the efficiency of phytoremediation of pesticides (Vaishampayan *et al.* 2007) [28, 29].
Ability of soil native microbes to degrade pesticides from soil strongly depends upon their long-term adaptation to the indigenous environment (Sharma *et al.* 2014) [23]. It is reported that microbes produce pesticide-degrading enzymes that convert pesticides in their respective metabolites (Hussain *et al.* 2009) [8]. Karn *et al.* (2017) [11] has shown removal of pentachlorophenol (PCP) in soil by simultaneous application of *Vibrio* sp. and biochar amendment. Thus, application of microbial activity helps in pesticides removal from soil.
In the light of imidacloprid toxicity on non-target species, its worldwide detection frequency, and high leaching potential in soil leading to soil pollution, present work was done to study the imidacloprid removal capability of Cowpea and its associated rhizosphere microorganisms from soil.

Materials and Methods

1. Chemicals, Reagents, Soil, and selection of Plant Seeds

Imidacloprid standard (99.9% purity) and 6-Chloronicotinic acid (98% purity) was purchased from Sigma Aldrich, USA, and Dr. Ehrenstorfer GmbH, Germany respectively. Confidor pesticide (Imidacloprid 17.8 SL, Bayer Crop Sciences) was purchased from local market. Mineral Salt Medium (MSM) with composition (per litre): 1.5g K_2HPO_4, 0.5g KH_2PO_4, 0.2g $MgSO_4.7H_2O$, 1.0g NaCl, pH-7 used for isolation of rhizosphere microflora and solvents required for High Performance Liquid Chromatography (HPLC) were procured from HiMedia. Research variety seeds of five leguminous plants *viz*., Cowpea, Black gram, Green Gram, Red Gram, and Soybean were purchased from Amar Seeds, Pune. Leguminous crops are usually fast growing hence the experimental demonstration becomes quick and easy. Black cotton soil was procured from local farms in Manjari, Pune.

2. Pesticide Concentrations used in the Study

A general survey carried out among the farmers confirmed that 40 mg/kg is the standard Confidor pesticide dose used in fields. Average pesticide concentration in surveyed soils was in the range of 45-110 mg/kg. Since the aim of the current study was to remediate pesticide contaminated soil, three imidacloprid concentrations of 320, 640 and 1280 mg/kg which are higher than the recommended dose were used. It was observed that up to 1280 mg/kg pesticide concentration, pesticide was easily soluble in water, hence this was the highest pesticide concentration that was chosen.

3. Selection of Plants Growing in the Highest Pesticide Concentration

In vitro seed selection was done by placing leguminous plant seeds on the germination paper in a petri plate. Tolerance of plants to the pesticide was studied by exposing the seeds to increasing concentration of pesticide (320, 640 and 1280 mg/kg) and thereby selecting the highest pesticide concentration for further studies. Ten seeds of each plant were placed in one petri plate containing pesticide treated germination paper. Control seeds were placed on distilled water treated germination paper. Seed germination was observed for a period of ten days.

From five types of seeds screened, only Cowpea seeds were able to germinate at the highest pesticide concentration of 1280 mg/kg. Thus, Cowpea plant and a pesticide concentration of 1280 mg/kg were selected. The final concentration of imidacloprid (active ingredient) in the dose of 1280 mg/kg pesticide was 228 mg/kg (17.8% of 1280 mg/kg pesticide).

4. Germination of Seeds and Isolation of Rhizosphere Microorganisms

Black cotton soil (10 kg) was spiked with 1280 mg/kg pesticide and used for the germination of Cowpea seeds. Seeds were allowed to germinate in natural conditions of humidity (56%) and temperature (22-28˚C). After one and half months of seed germination, plants were uprooted without cleaning the soil stuck to their roots and it was used for isolation of rhizospheric microorganisms.

5. Isolation of Cowpea Rhizosphere Microorganisms

Isolation of microbes from Cowpea rhizosphere was done as described by Gangola *et al*. (2021) [6] with minor modifications. Soil from Cowpea roots was homogenized in phosphate buffer with 1280 ppm pesticide. After centrifugation, the pellet was mixed with phosphate buffer and kept on shaker incubator for 48 hours at 37˚C. It was plated on MSM containing Confidor pesticide as a carbon source in three concentrations of 320, 640 and 1280 ppm. Total of seventeen bacterial colonies were obtained on the plates with different pesticide concentrations.

6. Screening of Isolates for the Growth on MSM with increasing Concentration of Pesticide thereby fixing the Highest Pesticide Concentration for Further Studies

Out of seventeen isolated colonies, only one culture colony was observed in the plate with highest pesticide concentration of 1280 ppm. Hence, this culture was selected for further studies. The selected bacterial culture (R2) was subjected to biochemical tests and identified using 16S rRNA gene sequencing. The contig of 16S rDNA was created and its percentage similarity was compared with the sequences present in National Centre for Biotechnology Information (NCBI) database using BLAST programme (Gangola *et al*. 2021) [6].

7. Flask Culture Study to demonstrate the ability of selected R2 Culture to remove Pesticide

The ability of selected culture to remove pesticide from medium was assessed *in vitro*, in MSM with 320, 640 and 1280 ppm pesticide. In 50 ml medium, 0.5 ml of the seed culture ($1.0X10^8$ cells/ml) was inoculated and incubated at 37°C for eighty days. Growth of the bacterial cells was recorded by measuring the absorbance at 600 nm using spectrophotometer (Chemito, Japan). Cell number was obtained by plating the cells on Luria Bertani agar and counting the Colony Forming Units per ml (CFU/ml).

8. Pesticide Residue Analysis from Bare Black Cotton Soil inoculated with R2 Culture

In vivo pesticide removal capability of R2 culture from natural soil was studied. Black cotton soil with no previous history of pesticide usage was selected for the present study. Soil was fortified with 1280 mg/kg pesticide and inoculated with R2 culture. Control soil with no culture but with pesticide was exposed to same natural conditions like the soil with R2 culture. These experiments were done in triplicate. Pesticide residues were extracted using QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) method and used for HPLC analysis (Gangola *et al*. 2015) [7].

9. QuEChERS Method for Extraction of Pesticide Residues from Soil Samples

Extracted pesticide residues were analyzed using HPLC at the time intervals of 5, 10, 20, 40, 80 and 118 days. Soil sample was mixed with acetonitrile and incubated on shaker incubator for 1 hr. The mixture was then allowed to settle down and liquid sample from top was separated and centrifuged. Sodium sulphate and hexane were added to supernatant and mixed to form two separate layers. Top layer was allowed to dry and resuspended in 2 ml acetonitrile, filtered, and subjected to HPLC analysis. When ten grams of soil was used for pesticide extraction, a final volume of 2 ml was made from it and 20 µl (equivalent to 100 mg sample) was injected in the HPLC instrument, which did not produce any background interference. From this, limit of Quantification (LOQ) of 0.01mg/kg and Limit of Detection (LOD) of 0.003 mg/kg were calculated (Sharma *et al*. 2014) [23].

10. Plant-Microbe interactive Phytoremediation: Growth of Cowpea in Black Cotton Soil spiked with Confidor Pesticide and amended with R2 Culture

R2 culture (~35 X 10^8 cells / 100 g soil) was inoculated in Black cotton soil spiked with 1280 mg/kg pesticide which was used for cultivation of Cowpea. This experiment was conducted to check the combined efforts of both, the Cowpea, and the culture to remove pesticide from soil. Before sowing the seeds, they were surface sterilized with 0.1% mercuric chloride. Twenty Cowpea seeds were sown in each pot containing 25 kg soil. A control set was made which had soil spiked with pesticide but without any culture. The experimental set up was done in triplicate.

11. QuEChERS Method for Extraction of Pesticide Residues from Cowpea

Pesticide was extracted from Cowpea as described by Zhang *et al*. (2022) [31] with some changes. Ten grams of plant sample was homogenized with acetonitrile, and the mixture was vortexed followed by addition of anhydrous magnesium sulphate and sodium chloride. The homogenate was incubated on a shaker incubator for 30 minutes after which it was centrifuged. Activated charcoal was added to the supernatant to remove the pigments. After 30 minutes of incubation, the solution was centrifuged. The supernatant was vacuum dried, resuspended in acetonitrile, filtered, and analysed using HPLC at time intervals of 5, 10, 20, 40, 80 and 118 days. The 118^{th} days experiment marked the completion of the life cycle of Cowpea.

12. HPLC Conditions used for analysing Pesticide Residues from Cowpea and Soil Samples

HPLC conditions used for the experiment were adopted from Jawad & Hermize (2020) [10] and standardized. HPLC instrument (Shimadzu, Kyoto, Japan) with a C18 column and UV detector at 270 nm was used. Mobile phase comprised of Acetonitrile: Water (10:90 v/v). Oven temperature of the instrument was set at 40˚C, and pressure exerted was 100 bars with a run time of 15 minutes. Samples were injected in the column with a flow rate of 1 ml/min. Along with the samples, 500 ppm of standard solutions of pure Imidacloprid, 6-Chloronicotinic acid and pesticide were run on HPLC using same conditions. Quantitation of pesticide and its metabolites in environmental samples is directly related to the data interpreted using HPLC chromatograms. Retention times of highest peaks in Cowpea and soil samples were compared to the peaks of standard solutions and residues of pesticide (imidacloprid) present in them were calculated using the formula given by Farouk *et al*. (2014) [4]:

$$\text{Concentration of imidacloprid residue } (mg/kg) = \frac{\begin{pmatrix}\text{Peak Area of the sample} \\ X \text{ Volume of cleaned extract } (L) \\ X \text{ Dilution factor} \\ X \text{ Concentration of standard } (mg/L)\end{pmatrix}}{\text{Peak Area of the standard } X \text{ Weight of sample } (kg)}$$

Residue values were converted to percentage values and used in the results.

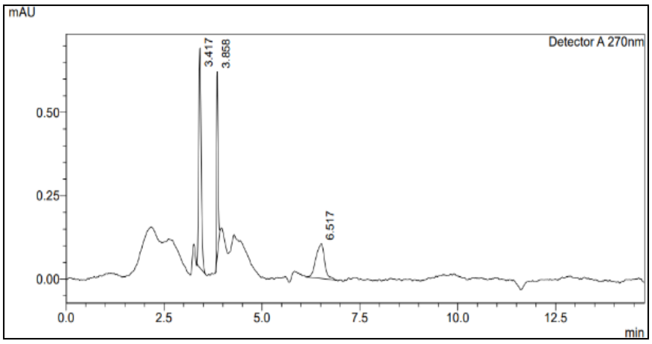

Fig 1: Pure Imidacloprid solution (500 ppm) (Highest peak is visible at 3.417 minutes with an area of 2824)

Peak#	Ret. Time	Area	Height
1	3.417	2824	660
2	3.858	1427	553
3	6.517	1588	104
Total		5839	1318

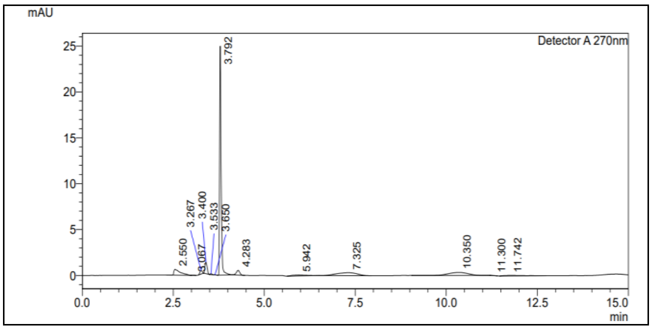

Fig 2: Soil spiked with 1280 mg/kg pesticide and amended with *B. tequilensis* (Highest peak is visible at 3.400 minutes with an area of 5140)

Peak#	Ret. Time	Area	Height
1	2.550	7729	635
2	3.067	91	26
3	3.267	1744	392
4	3.400	5140	1276
5	3.533	343	111
6	3.650	63	23
7	3.792	72595	24945
8	4.283	3406	513
9	5.942	2797	106
10	7.325	12560	319
11	10.350	12709	329
12	11.300	114	15
13	11.742	2005	58
Total		121295	28748

Under the standardized HPLC conditions, pure imidacloprid chromatogram exhibited a peak at 3.417 minutes with an area of 2824 (Fig. 1). Pesticide chromatogram exhibited three peaks at 3.442, 4.283 and 6.533 minutes. Peak at 3.442 minutes had similar retention time as that seen in pure imidacloprid chromatogram (3.417 minutes). Hence, this peak in pesticide chromatogram can be of imidacloprid. The chromatograms of soil samples had many unidentified peaks apart from peaks at similar retention times like standard solutions (Fig. 2) (Oke & Zunjarrao, 2022) [19].

13. Data Analysis

Two-way analysis of variance (ANOVA) was used for data analysis using GraphPad Prism software (version 9). The mean values of experiments were compared using Tukey tests for Multiple Comparisons at $p \leq 0.05$ level of significance. Data was represented as Mean ± Standard Deviation (SD) and converted to percentage values. The difference was considered significant if p was smaller than or equal to 0.05 ($p \leq 0.05$) (Islam & Kato-Noguchi, 2013) [9].

Results and Discussion

1. Identification of Isolated Rhizosphere Microorganisms

Based on 16S rRNA gene sequence comparison with the data present in GenBank Database, the isolated culture was identified as *Bacillus tequilensis* with 99.80% similarity. The organism was Gram positive with thick purple rods. It was motile, non-capsulated and endospore producing without any pigment formation. The sequence of the identified organisms was deposited to NCBI with GenBank Accession number MW009674.

2. Growth of Cowpea in Soil amended with *B. tequilensis* and spiked with Pesticide

Cowpea grown in pesticide spiked soil inoculated with *B. tequilensis* had good height as compared to the plants grown in soil without *B. tequilensis* and with pesticide (Fig. 3 and Fig. 4). The numerical data supporting growth of plants in terms of height is provided in Table 1. This morphological change in Cowpea can be attributed to the presence of *B. tequilensis* in soil along with other soil native microbes. Similar observations were reported by Spence & Bais (2015) [25] who claimed that soil microbes are known to promote plant growth and development during stress conditions using some biochemical processes. They stimulate the growth of plants by making more nutrients available to them, increasing the production of growth hormones, and helping them in disease suppression (Narwal *et al*. 2023) [18].

Table 1: Height of Cowpea plants grown in soil with different treatments of pesticide and *B. tequilensis*

Height of plants grown in soil amended with pesticide in different concentrations and B. tequilensis (cm)				Height of plants grown in soil amended with pesticide in different concentrations and without B. tequilensis (cm)			
Control	320 mg/kg	640mg/kg	1280 mg/kg	Control	320 mg/kg	640 mg/kg	1280 mg/kg
42±0.05	31.07±0.09	26.8±0.19	35.65±0.65	35.1±0.07	29.98±0.04	23.77±0.61	26.15±0.01

Average height of 20 plants in Centimetres ± Standard Deviation is considered.

Fig 3: Cowpea plants grown in soil spiked with different pesticide concentrations and inoculated with *B. tequilensis*

Fig 4: Cowpea plants grown in soil spiked with different pesticide concentrations without *B. tequilensis*

3. Removal of Pesticide from Bare Black Cotton Soil amended with *B. tequilensis* and spiked with Pesticide

Studies related to pesticide removal are crucial in understanding the fate of pesticide in nature. They are indicators of the potential risk of use of any pesticide (Cheah *et al*. 1998) [3]. It was observed that bare soil spiked with pesticide and supplemented with *B. tequilensis* retained less pesticide residues as compared to the control bare soil on all the days. On 5^{th} and 10^{th} day, residues in control soil were in the range of 90-98.8%; which further decreased to 60.87-63% on the last two days. In soil supplemented with *B. tequilensis,* 72.12-83.21% pesticide residues were detected on the first two days which reduced to ~38% on the last day. The difference in decrease of imidacloprid residues in control and *B. tequilensis* supplemented, pesticide spiked soil was statistically significant on 5^{th} and 40^{th} day at $p^{**} \leq 0.01$ level while on all the remaining days the difference was significant at $p^{****} \leq 0.0001$ level. Thus, coordinated action of native soil microbes and isolated *Bacillus tequilensis* removed more pesticide from soil than the sole native organism's imidacloprid removal capacity from control bare soil.

4. Removal of Pesticide from Black Cotton Soil under Cowpea cultivation amended with *B. tequilensis* and spiked with Pesticide

The pesticide residues in control soil and in soil inoculated with *B. tequilensis* under Cowpea cultivation were compared. On last two days, the concentration of imidacloprid went below the detection limit of the HPLC instrument. Hence, it was observed that Cowpea removed maximum pesticide (~99%) from soil along with *B. tequilensis*. On the last day in control soil containing indigenous microbes without *B. tequilensis,* 17.55% pesticide was present and 82.45% pesticide was removed by Cowpea. Pesticide residues present in soil on the last day of experiment were considered as after this day the whole plant was uprooted from soil and the remediation process by plants was stopped.

Overall results suggest that the presence of both, *B. tequilensis* and native microbes in soil helped Cowpea to remove pesticide more efficiently as compared to the Cowpea plant's pesticide removal capability from control soil which had only the soil native microbes. On 80^{th} and 118^{th} day, no pesticide was detected from soil inoculated with *B. tequilensis* suggesting its extremely efficient removal from soil.

Results presented here proved the concept of Phytoremediation, which is the remediation of polluted soil using plants and their associated microorganisms. Microorganism isolated in the study was effectively reducing pesticide from soil.

Indiscriminate use of imidacloprid in agricultural fields can negatively influence non-target organisms and overall soil ecology. This calls for an urgent need to remove it from soil using method like Phytoremediation. In the present work, an attempt was made to isolate plant associated rhizosphere microflora, *B. tequilensis* from Cowpea and check its pesticide removal capability from bare soil and soil under Cowpea cultivation. Pesticide removal capability of Cowpea plant was also assessed.

There are a couple of reports on Plant-microbe interactive removal of different pesticides from soil. Romeh (2020) [21] analysed the synergistic effect of *Plantago major* and microbes to remove 0.05 g/kg imidacloprid from polluted soils. Results suggested that high imidacloprid removal was achieved in soil under *P. major* cultivation, with biofertilizer and inoculated with useful microbes.

Akbar and Sultan (2016) [1] studied the Phytoremediation capacity of Cowpea using plant-microbe interactive approach. They isolated two bacteria, *Achromobacter xylosoxidans* and *Ochrobactrum* sp., from chlorpyrifos contaminated agricultural soil. Initial chlorpyrifos concentration of 100 mg/L was reduced by 85% within 10 days. Cowpea was grown in presence of isolated organisms in sterile and non-sterile soil. These organisms were able to remediate chlorpyrifos containing soil at a concentration of 200 mg/kg. Organisms also had some plant growth promotion traits like nitrogen fixation and enhanced cell growth.

Isolation and characterization of *Bacillus* sp. degrading pentachlorophenol from secondary sludge of paper and pulp industry was performed by Karn *et al*. (2010) [13]. Based on 16S rRNA sequencing, the organisms were identified as *Bacillus megaterium, Bacillus pumilus*, and *Bacillus thuringensis.* The isolated bacterial strains were able to remove 90% pentachlorophenol from sludge. It was thus concluded that bacteria can be effective degraders of pentachlorophenol and can be successfully used in treatment of pentachlorophenol polluted sites. Karn *et al*. (2014) [12] also studied mineralization of 2,3,4,6 tetra chlorophenol by two microorganisms, *Bacillus* sp. and *Staphylococcus* sp. isolated from the secondary sludge of paper and pulp mill. They reported that a consortium of these organisms was able to remove almost entire compound within fifteen days of the treatment highlighting the importance of microbes isolated from environmental sources in the process of bioremediation.

In another work by Vaishampayan & Kanekar (2007) [28, 29], effect of atrazine on the growth of Cowpea and removal of atrazine residues with different inoculum sizes of *Arthrobacter* sp. from soil were studied. It was observed that, with increased inoculum size in soil, plant had more height and less biomass. This study concluded that *Arthrobacter* sp. can be used for bioremediation of atrazine-polluted soils efficiently.

Conclusion

Phytoremediation is considered as an interesting technology to treat soils polluted at the top level. Effective role of soil microbial population in phytoremediation of pesticide polluted soils is proven in the present work which highlights the fact that plants can remediate contaminated soil better in the presence of microbes. However, more detailed research work in this area needs to be undertaken to make this technology more successful.

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