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EFFECT OF ORGANIC (NEEM OIL) AND INORGANIC PESTICIDES (PROTRIN AND PHOSKILL) ON SOIL MICROFLORA AND EVALUATION OF THE GROWTH CHARACTERISTICS OF *AGROBACTERIUM* SP. AND *PSEUDOMONAS* SP. SOIL ISOLATES

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ABSTRACT

Pesticides are used to protect plants from harmful insects. This helps in increasing the crop yield. An unintended consequence of its application, is its effect on physicochemical properties of soil that could lead to changes in microbial populations and their activities. This influences the microbial ecological balance by altering the soil fertility and metabolic activity of soil microbial communities.

The study examines the response of microbial populations in soil after incorporation of pesticides [phoskill, protrin, neem oil]. The samples of soil were taken from an agricultural land from a depth of 10-15cm with no traces of pesticide application in the past 3years. The soil was fine sand with about 1.65% organic matter, pH of 6.53 and a water holding capacity of 10%. Enumeration of total bacteria, nitrogen fixing bacteria, fungi were done in control soil and pesticide applied soil at regular time intervals for a period of 21days. Growth characteristics of Agrobacterium and Pseudomonas isolated from the soil was studied in the presence and absence of the pesticides. Protein was isolated from their microbial cultures and quantified.

The results indicated that the use of pesticides affected the total number of heterotrophic bacteria, fungi and bacteria involved in nitrogen fixation. The observed effects strongly correlated with pesticide type and the duration of exposure. The stress induced by the presence of pesticides showed changes in the growth pattern of bacteria and the quantity (in g) of protein expression.

Keywords: Soil microflora, Pesticides, Enumeration, Growth curve, Protein.

1. INTRODUCTION

Microorganisms are very small forms of life found everywhere. Many live in colonies although single cells are found. Soil provides a good medium for their growth. Food sources are plentiful on the topsoil compared to subsoil. So, more organisms exist on the topsoil. They are also abundant in the area immediately next to plant roots (called the rhizosphere), where sloughed-off cells and chemicals released by roots provide ready food sources [1].

Many types of microorganisms like bacteria, actinomycetes, fungi, algae, protozoa and viruses are found in the soil. Each of these groups has different characteristics and functions in the soil they live in. Importantly, the interactions between these organisms influence soil fertility as much or more than the individual organism's activities [2].

They decompose organic matter, help plants to grow by nitrogen fixation, detoxify harmful chemicals (toxins), provide resistance against disease causing organisms, and stimulate plant growth. Soil microorganisms are the source of most antibiotics humans use to fight diseases [1].

Soil microorganisms contribute to soil fertility. A soil is said to be fertile based on its capacity to supply nutrients to plants; it is also described in terms of crop-producing power of the soil under given climatic conditions. The productivity is also affected by a combination of factors, like the climate, nature of crop and local agricultural practices adopted. Two forms of soil fertility are recognized. Active fertility, that is immediately available and potential fertility that becomes available by chemical or microbial action on minerals and organic matter.

But, microbes are the soil's primary crop, and they must be provided a balanced nutrition, particularly with respect to their major requirements for carbon and nitrogen. [3].

1.1. Soil composition

Soil composition is an important aspect of nutrient management. While soil minerals and organic matter hold and store nutrients, soil water is important for nutrient uptake. Soil air, too, plays a crucial role since many of the microorganisms that live in the soil need air to undergo the biological processes that release additional nutrients into the soil.

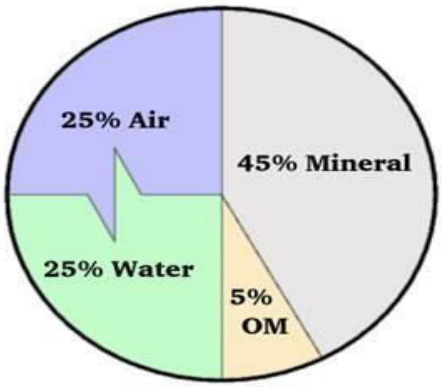


Fig. 1 Components in soil

The basic components of soil generally include minerals, organic matter, water and air. The typical soil consists of approximately 45% mineral, 5% organic matter, 20-30% water, and 20-30% air. The soil is very complex and dynamic. The composition of the soil can fluctuate on a daily basis, depending on numerous factors such as water supply cultivation practices, and/or soil type [4].

1.2. Pesticides

A pest is any organism that causes an economic loss or damage to the physical well being of human beings. It causes crop destruction by inflicting diseases in them, at times in human beings also. There are a number of chemicals used to destroy such pests. These chemicals are called as pesticides (*cides* means to kill). Pesticides are sprayed over crops, human dwellings etc.

1.2.1. Types of pesticides

Pesticides are of several types. Fungicides, Weedicides /Herbicicdes, Nematicides, Rodenticides, Insecticides, and Biopesticides are the various classes named based upon the type of pest to be controlled [5].

The use of pesticides in agriculture has rapidly increased during the last 40 years to increase crop yield. As a result of which we find that most pesticides have polluted water, soil, atmosphere and food.

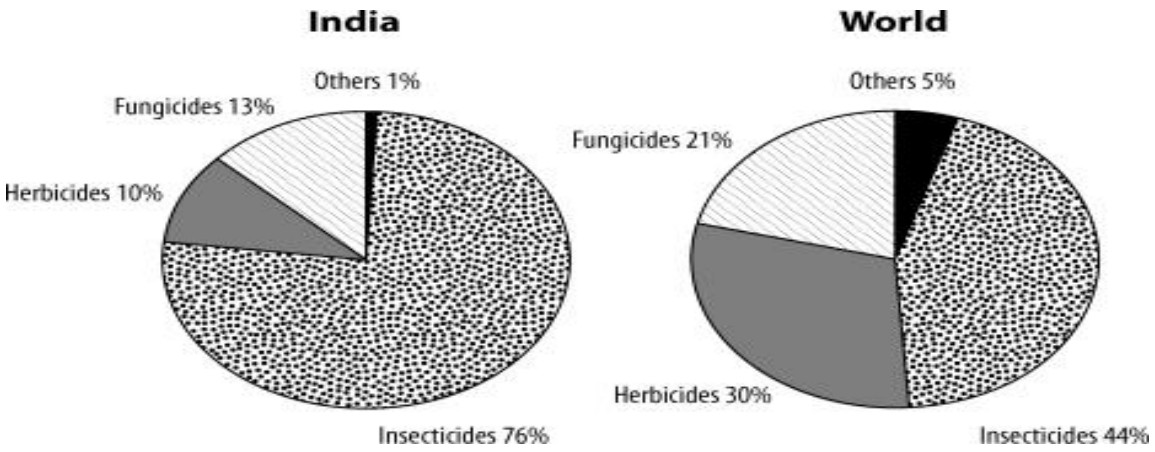


Fig. 2: Usage of Pesticides

Chemicals pose a potential risk to the ecosystem, in general and human beings in particular. It is estimated that with the onset of the green revolution around 800,000 people in developing countries have died due to pesticides. A report shows that nearly 20,000 people in developing countries die each year because of pesticide consumption through their food [6].

Guidelines for the approval of pesticides prescribe that the effects of pesticides on soil microorganisms and soil fertility should be determined before use. The fertility of soil depends not only on the texture of soil but also on the biological ability within it. The microbial diversity may have been changed following pesticide use, and such changes may affect soil fertility.

The use of pesticides to protect crops may alter the soil's biological ability either by direct or indirect action, but the knowledge of soil microbial ability to degrade pesticides and the influence of pesticides on microbial diversity in soil are still limited. To understand the effect of pesticides on soil microflora and their beneficial activities is an important part of the pesticide's risk assessment. It is not easy to predict the relationship between the chemical structure of pesticide and its effect on the various groups of soil microorganisms. Certain pesticides stimulate the growth of microorganisms, but other pesticides have depressive effects or no effects on microorganisms when applied at normal rates [7].

2. MATERIAL AND METHOD

2.1. Sample collection

Sandy soil samples were collected from an agricultural field at Cheyyar. The sampling land has not been used for agricultural purposes in the past five years.

Hence, no plant products or fertilizers have been used for a while. At the time of sampling, the site was covered by grass. Soil was collected from the middle layer and in a zig zag fashion to ensure homogeneity. The physical and chemical parameters of soil are measured.

Fig. 3: Sample collected Area

2.2. Pesticides used for the study

The pesticides were bought from commercial agricultural pesticides shop in Tiruvanamalai district, Tamilnadu.

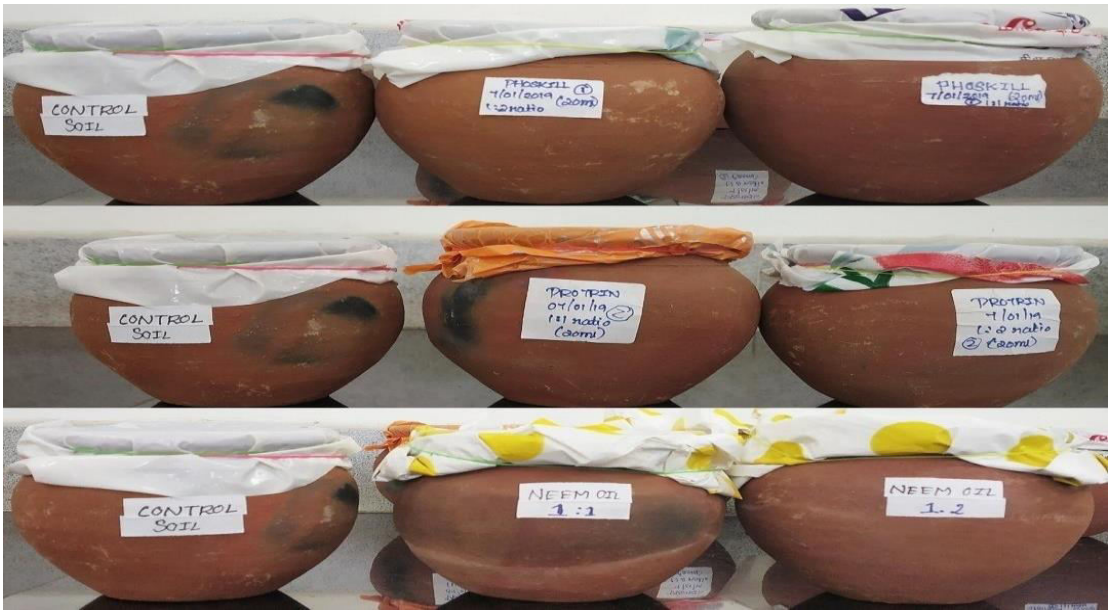
2.3. Preparation of soil for experiment

The soil was gently air-dried to make it suitable for sieving. Soil of particle size not exceeding 1mm was used for the experiment. Equal portions of soil weighing 500g was taken. The pesticides were diluted using distilled water in two different ratios of 1:1 and 1:2. They are added to the soil dropwise. The moisture content of the soil was maintained throughout the study by periodic addition of sterile distilled water. To avoid evaporation of water from soil and/or photo-degradation of pesticides, the pots were covered with polypropylene sheets and incubated in the dark, at room temperature for 28days. The control soil and soils treated with pesticides were incubated in triplicates [8].

2.4. Enumeration of soil samples

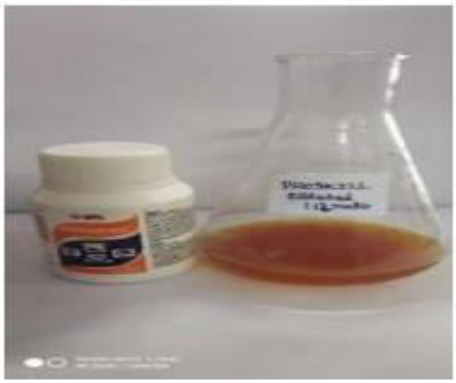
The total numbers of heterotropic bacteria was enumerated in the treated test soil samples and untreated control soil samples after 1, 14 and 21 days of pesticide application in Nutrient agar (peptic digest of animal tissue-5gm, sodium chloride-5g, beef extract-1.5g, yeast extract-1.5g, agar-5g, distilled water-1000ml, final pH-7.4). Total numbers of fungi was enumerated using Rose Bengal agar (glucose-10g, peptone-5g, K_2HPO_4-0.1g, $MgSO_4 \times 7H_2O$-0.5 gm, Rose Bengal- 0.033g, Agar-15g, distilled water-1000ml and final pH-7). Numbers of bacteria involved in nitrogen turnover were counted on YEMA agar

(Mannitol-10g, K_2HPO_4-0.5g, $MgSO_4.7H_2O$-0.2g, NaCl-0.1g, Yeast extract powder-0.4g, Agar-15g and distilled water-1000ml with the right calibration of pH-6.8-7). The number of colony forming units [CFU] in the selective media were determined by means of the serial dilution technique and spread plate method. Analysis was performed in triplicates [8].



PREPARATION OF PESTICIDES FOR EXPERIMENT

PHOSKILL 1:1 RATIO



PHOSKILL 1:2 RATIO

PROTRIN 1:1 RATIO

PROTRIN 1:2 RATIO

Fig. 4: Preparation of soil for experiment

2.4.1. Isolation of Agrobacterium species

Agrobacterium was isolated from the rhizosphere soil, collected from the grass roots. One gram of soil was suspended in 100 ml of sterile distilled water and was inoculated in YEMA with congo red agar plate containing Mannitol (10g), K2HPO4 (0.5g), $MgSO_4$. $7H_2O$ (0.2g), NaCl (0.1g), Yeast extract powder (0.4g), Agar (15g) and distilled water (1000ml) with pH (6.8-7) and incubated at 37°C for 24 hrs. Bacterial culture were subsequently sub-cultured and used. Identification of the isolates were done by morphological and various biochemical methods [9].

Fig. 5: Soil sample from Root

2.4.2. Isolation of Pseudomonas species

Pseudomonas was collected from the root soil of plants. One gram of each soil sample was placed in 9ml of asparagine broth enrichment medium consisting of 2g per litre asparagine L-monohydrate, 1g per litre K_2HPO_4 and 0.5g per litre $MgSO_4.7H_20$ in order to enhance the *Pseudomonas* growth. The samples were incubated for 48 hours at 37°C until turbidity developed. A loopful of culture was transferred on to fresh nutrient agar plates and incubated for the development of bluish green colonies. These colonies were subsequently subcultured in nutrient broth. Identification of the isolates were done by morphological and biochemical methods [10].

Fig. 6: Soil sample in broth

2.5. Identification and characterization of isolated bacteria

2.5.1. Gram staining technique

Bacterial smear was prepared and fixed. The smear was stained with ammonium oxalate crystal violet for one minute. It was then washed with tap water and immersed in Gram's iodine for one minute. Again washed with tap water and blotted dry. The smear was flooded with 95% ethyl alcohol (decolorize) for 30 sec. It was again washed with water and blotted dry. Safranin was used as counter stain. Again the slide was washed with tap water, dried and examined under oil immersion objective on the microscope.

2.5.2. Biochemical test

2.5.2.1. Catalase test

This test was performed to study the presence of catalase enzyme in bacterial colonies. 24 Hr bacterial cultures were used for the test. A drop of the culture is taken on glass slides and one drop of H_2O_2 (1%) was added. Appearance of gas bubble indicated the presence of catalase enzyme.

2.5.2.2. Citrate utilization test

Carbon is provided in the form of citrate in this medium; however, if the bacteria can grow on the simmon's citrate agar, the colour changes from green to blue. To inoculate the slant, a loopful of bacterial culture was used; the slant was inoculated following stab and streak method and finally observed after incubation period of 24 h at 37°C [9].

2.5.2.3. Amylase test

10% soluble starch was prepared and sterilised. 20% of this solution is added to 100ml of melted nutrient agar. 0.1ml culture was spread over the plate and incubated at 37°C for 24 hours. After the incubation iodine was flooded over the plate.

2.5.2.4. Carbohydrate fermentation test

Peptone water bath (peptone-0.8g, Nacl-1.4g, NaHCo 3-0.02g, KCl-0.04g, $CaCl_2$- 0.04g, KH_2PO_4-0.24g, Na_2HPO_4-0.88g, distilled water-100ml) was prepared and autoclaved. 1% sugar solution and phenol red indicator was added. One loop full of culture was inoculated and incubated at 37°C for 24hours. Phenol red is yellow at pH 6.8 (acid production) and red at pH 8.4 (no acid production).

2.6. Pesticide susceptibility test

The performance of antimicrobial susceptibility testing

by the clinical microbiology laboratory is important to confirm susceptibility or to detect resistance in individual bacterial isolates. The bacterial sensitivity test was done by well diffusion method [11].

2.7. Bacterial growth curve

Spectrophotometer is a simple method used to analyze growth patterns by tracking changes in absorbance over time. The standard absorbance maxima is 660 nm for yellow to brown colour broth samples. Absorption maxima can be adjusted if the color is not in this range or if growth is expected to be lower or greater than average. This requires absorbance to be measured every 15 minutes for up to three hours. The readings are plotted on a graph with time on the X-axis and absorbance on the Y-axis [12].

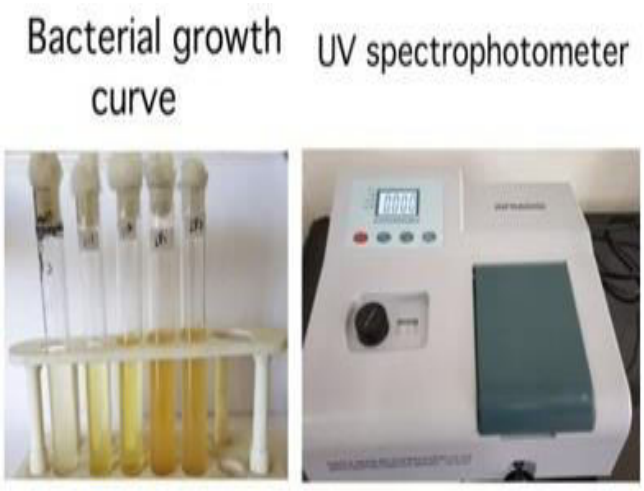


Fig. 7: Growth curve measurement

2.8. Isolation of protein from agrobacterium bacterial cells

Agrobacterium was grown at 37°C for 48hrs. Cells from 10ml culture were harvested by centrifugation (4,000×g), washed twice with phosphate buffered saline without Mg^{2+} and Ca^{2+}, and recentrifuged. The cells were then suspended in 10ml of ice-cold acetone (analytical grade), allowed to stand on ice for 5minutes, and collected by centrifugation (4,000×g). The pellet was subjected to acetone treatment from 5 to 30 minutes. Residual acetone was removed and the proteins were then extracted by incubating with 1.0 ml of 1% sodium dodecyl sulfate (SDS) for 2min and recentrifuged. Supernatant were discarded. The pellet was suspended phosphate buffer saline and used for protein estimation [13].

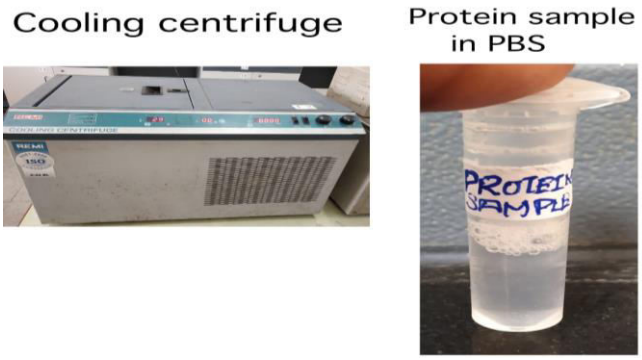


Fig. 8: Isolation of protein

2.9. Estimation of protein by biuret method

Copper sulphate reacts with the peptide bond in an alkaline medium to give a purple colour which can be measured at 540 nm.

The standard protein BSA was prepared (2mg/ml). The Biuret reagent was prepared by adding 3 g of $CuSO_4 \cdot 5H_2O$ and 9 g of sodium potassium citrate to 500 mL of 0.2 N NaOH solution, followed by the addition of 5 g of KI. The resulting solution was then brought to a total volume of 1 L with 0.2 N NaOH [14].

3. RESULTS AND DISCUSSIONS

Soil is a natural habitat for million of microorganisms which are a very important part of the ecosystem. The present study shows the results of the use of pesticides on agricultural soil and its varied effects on micro-organisms dwelling in it [8]. Physical and chemical properties of the soil used for the study were assessed and are given below.

Table 1: Analysis of soil

PARAMETERS	UNITS	VALUE
Ph	-	6.53
Electrical conductivity	µc/cm	132
Soil texture (sand)	%	80
Soil texture (silt)	%	2
Soil texture (clay)	%	18
Calcium as Ca	Meq/100g	5.19
Magnesium as Mg	Meq/100g	3.2
Sodium as Na	Kg/ha	40
Potassium as K	Kg/ha	50
Total organic matter	%	1.65
Total nitrogen	%	4.18
Available phosphorus	Kg/ha	44.6
Available Sulphate	Mg/ha	60.0
Cation exchange capacity	Meq/100g	945.4
Biomass C	µg/100g	620
Water holding capacity	%	10

Soil samples are prepared for the study by the addition of pesticides, namely, Protrin and Phoskill (inorganic) and Neem oil (organic). Each of these pesticides were diluted with water to give two different concentrations. The soil samples will be referred to as below.

Table 2: Preparation of soil for pot experiment

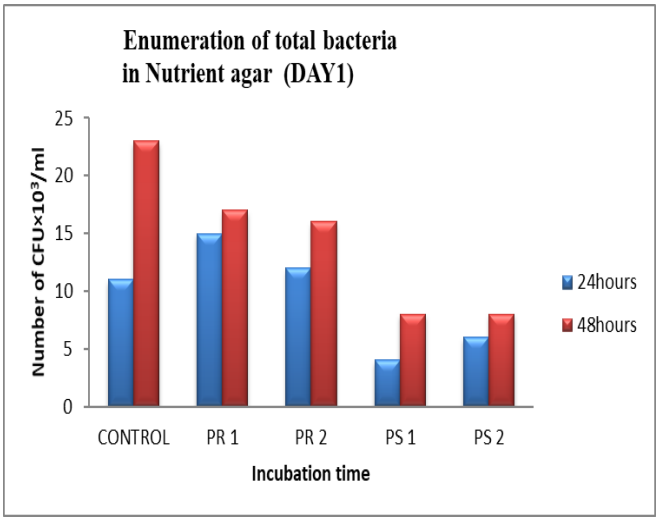
Soil Samples	Referred names
Soil + Protrin (1:1)	PR 1
Soil + Protrin (1:2)	PR 2
Soil + Phoskill (1:1)	PS 1
Soil + Phoskill (1:2)	PS 2
Soil + Neem oil (1:1)	NM 1
Soil + Neem oil (1:2)	NM 2

3.1. Effect of inorganic and organic pesticides on soil microflora

3.1.1. Enumeration studies

The bacterial and fungal count in test soil is compared with the control soil on the day following pesticide addition. Soil samples are enumerated in nutrient agar for the total bacterial count and on YEMA agar, which is a special media for nitrogen fixing bacteria. Fungal counts were measured on Rose Bengal agar. All the plates were prepared in triplicates.

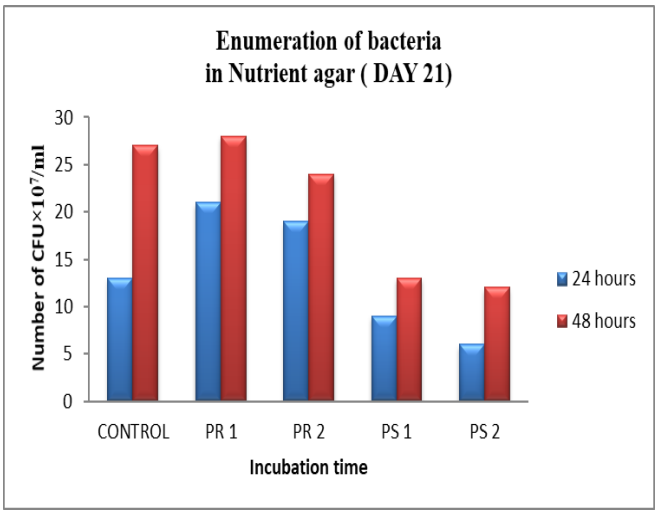
Graph 1 shows the bacterial count in the soil samples treated with inorganic pesticides. There is a significant reduction in the bacterial count with application of Phoskill and moderate reduction in bacterial count with the application of Protrin.



Graph 1: Total bacterial in soil sample in Day 1

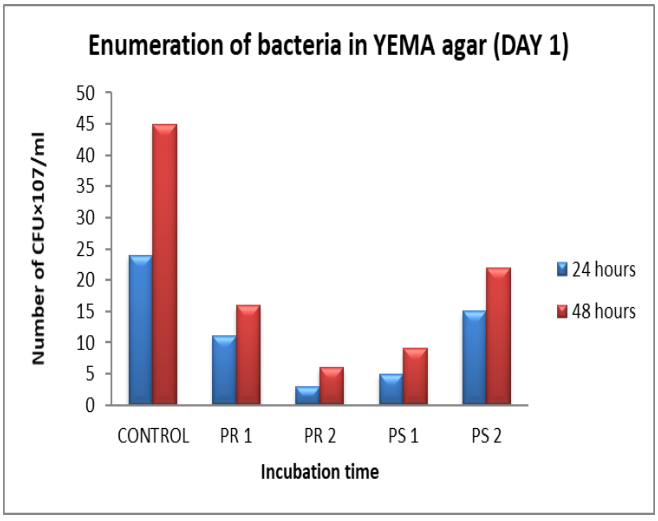
Graph 2 shows that, after three weeks, notable elevation in the bacterial count is observed in the test soils showing that the effect of the pesticide reduces with the time. On the other hand, the bacterial count is not very much affected by the concentration of the pesticide. Only presence or absence of the pesticide is seen to affect bacterial populations.

The number of nitrogen fixing bacteria were enumerated on a special medium (YEMA). The results are shown below.



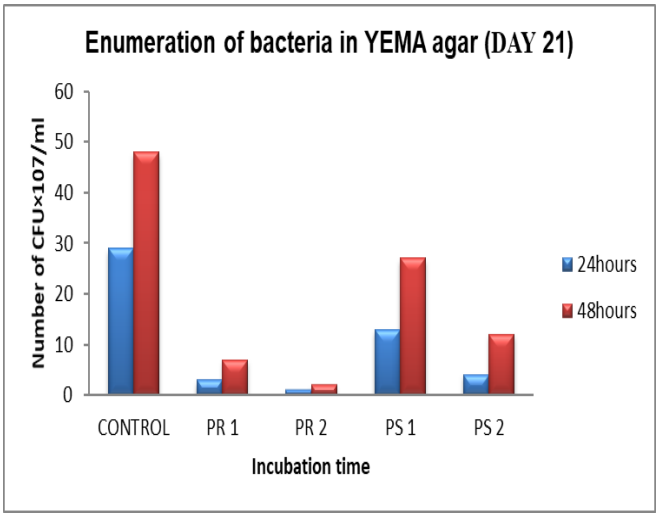
Graph 2: Total bacterial in soil sample after 21 days

From the graph 3, it is clearly seen that Protrin which diminished the total bacterial count to lesser extent compared to Phoskill, has a stronger negative impact on the nitrogen fixers. Use of such pesticides may have a gross impact on soil fertility over prolonged use.



Graph 3: Total number of nitrogen fixing bacteria (day 1)

Graph 4 shows that there is a natural increase in the number of nitrogen fixers in the control soil. However, the effect of the pesticide - Protrin persists even after 21 days and keeps the level of nitrogen fixing bacteria in test soil evidently lower. While the effect of phoskill declines with time to allow a marginal rise in the number of nitrogen fixers



Graph 4: Total number of nitrogen fixing bacteria (day 21)

Table 3: Enumeration of fungi in control and the test soil samples

SOIL SAMPLES	1st day	21 st day
CONTROL	22	1
PR 1	4	2
PR 2	4	1
PS 1	4	1
PS 2	3	1

The number of fungi in the control soil is much higher than the test samples on day 1. But on the day 21, the difference becomes narrow as there has been a natural decline in the fungi, may be due to lack of moisture. However, the drop in fungal count immediately following addition of pesticides is significant. Fungal colonies were stained using Lacto phenol blue staining.

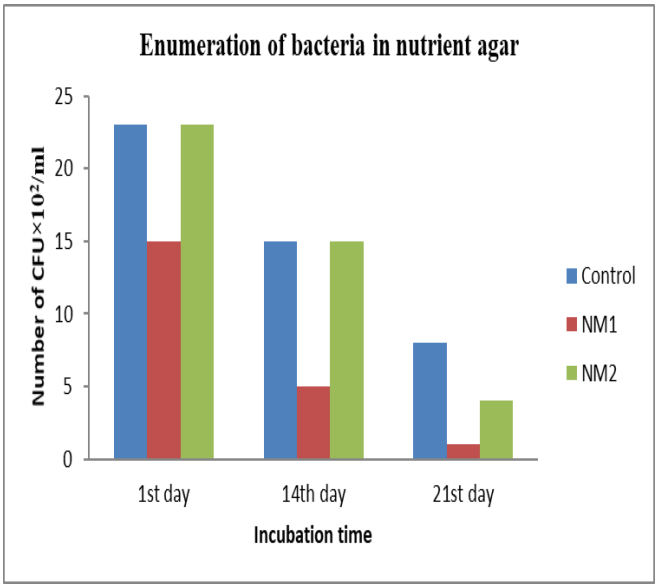
Collectively analyzing the data, it can be concluded that though the impact of the pesticides on the total bacterial and fungal count is not alarming, its impact on the count of nitrogen fixing bacteria is of concern.

Several environmental factors also play an important role in pesticide persistence, namely, sunlight, plant and animal metabolism, water, dissociation, sorption and bioaccumulation [15].

For thousands of years, Indian farmers are conscious of the insecticidal properties of neem. Neem oil isolated from the seeds neem may be a better pesticide. Azadirachtin is the most active component of neem, active in repelling and killing pests. Microbes and light can breakdown the pesticide in soil, water and on plants.

Table 4: Enumeration total bacteria in Nutrient agar

	1st day	14th day	21st day
Control	23	15	8
NM1	15	5	1
NM2	23	15	4

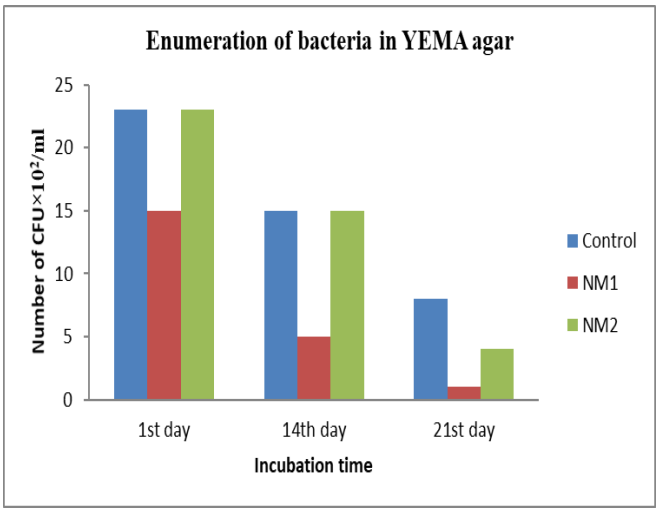


Graph 5: Total number of bacteria in soil sample (NM samples)

Table 5: Enumeration of nitrogen fixing bacteria in YEMA agar (NM samples)

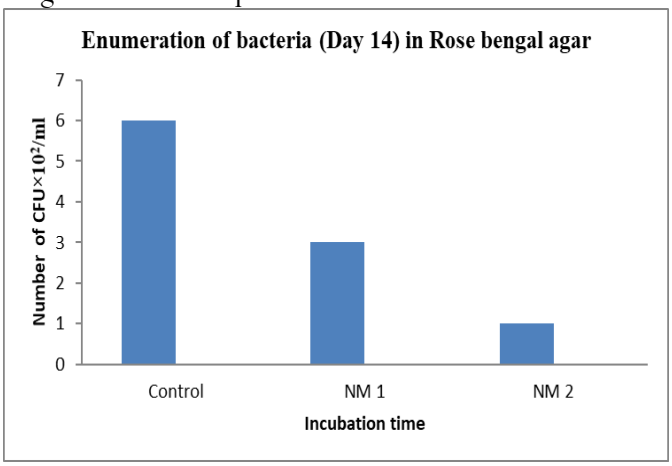
	1st day	14th day	21st day
Control	23	15	8
NM1	15	5	1
NM2	23	15	4

The Graph 6 shows a very significant decline in the counts of total bacteria as well as in nitrogen fixers. This could be due to the persistence of Neem oil. The half life of azadirachtin in soil ranges from 3-44 days.It is only 48 hours to 4 days in water. [15].



Graph 6: Total number of Nitrogen fixing bacteria in soil sample (NM samples)

Graph 7 shows the markable decline in the count of fungi in the test samples



Graph 7: Total number of fungal in soil sample

3.2. Isolation of agrobacterium

The organism was isolated from red colonies on the CRYEMA plates. The organism was identified with the help of gram staining, motility and some biochemical test namely catalase test, amylase test, carbohydrate digestion test and citrate utilization test.

3.3. Isolation of pseudomonas

Rhizosphere soil was inoculated in a special Asparagine medium. 48 hour culture was streaked on the surface of the nutrient agar. Bluish green colonies were picked and subcultured in nutrient broths and characterised by gram staining, motility and some biochemical test namely catalase test, amylase test, carbohydrate digestion test and citrate utilization test. The results shown in following pictures.

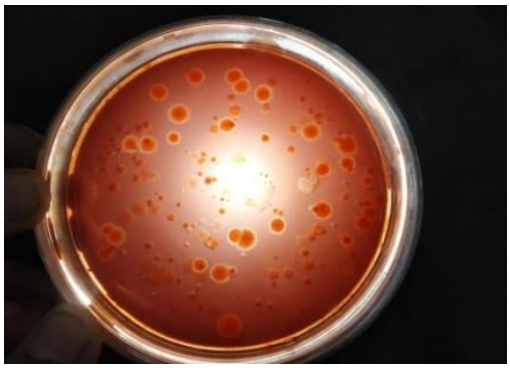


Fig. 9: Agrobacterium colonies in CRYEMA agar

Fig. 10 Pseudomonas in Nutrient agar

Table 6: Characteristics of isolated bacteria

Test	Agro-bacterium spp	Pseu-domonas spp
Staining	+	+
Motility	+	+
Catalse test	+	+
Amylase test	+	+
Carbohydrate digestion test	+	+
Citrate utilization test	+	+

3.4. Pesticide sensitivity by well diffusion method

The figure 13 shows the results of the sensitivity of Agrobacterium to the three pesticides. The organisms show sensitivity only towards Phoskill.

Fig. 14 shows that the Pseudomonas also shows similar results. Phoskill is a more harmful pesticide compare to others.

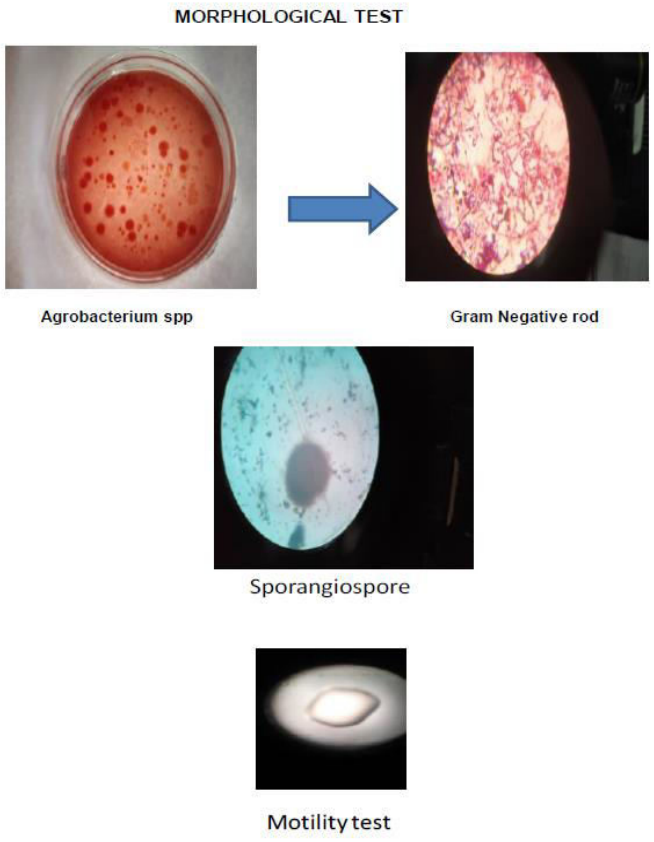


Fig. 11: Morphological test of isolated bacteria

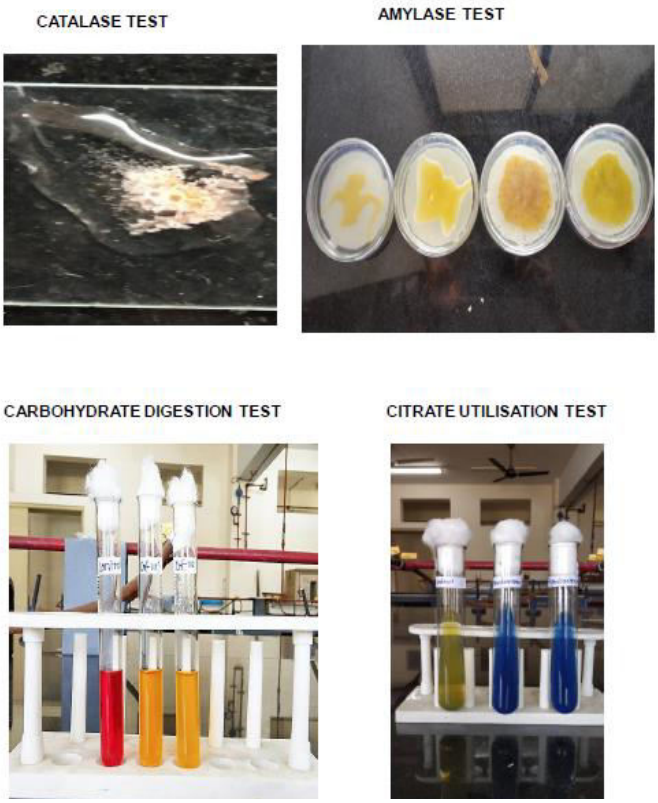


Fig. 12: Biochemical tests of isolated bacteria

Table 7: Measurement of sensitivity test (cm)

Pesticides	Agrobacterium	Pseudomonas
Phoskill	1.3	0.5
Phoskill 1:1	1	0.3
Phoskill 1:2	1	0.3

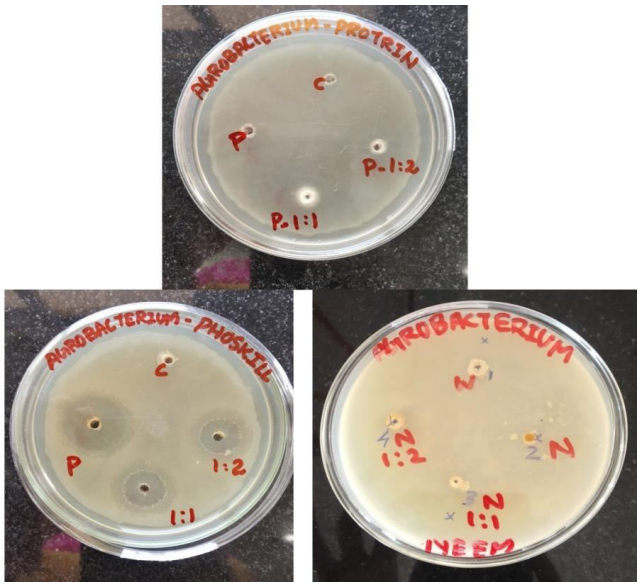


Fig. 13: Agrobacterium sensitivity test

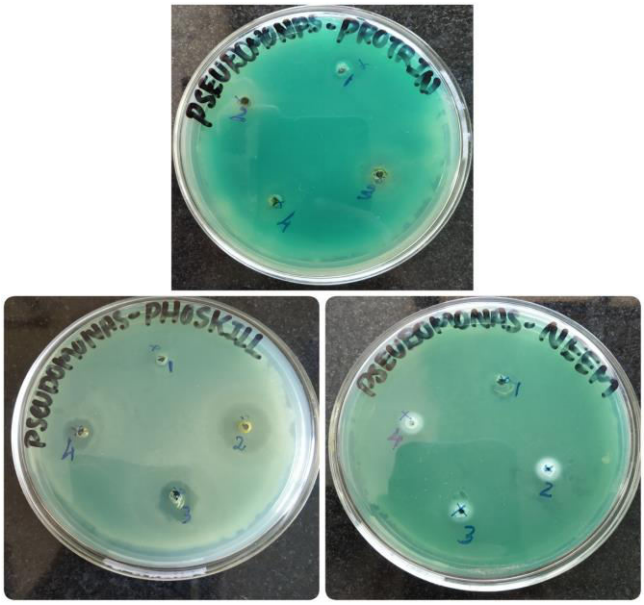


Fig. 14: Pseudomonas sensitivity test

3.5. Bacterial growth curve

The growth of both Agrobacterium and Pseudomonas in nutrient broth was studied spectrophotometrically at 660nm in visible range. The absorbance of the culture is measured at regular intervals of time for maximum

period upto 62hrs. The results are represented graphically as below.

The growth curve shows that both have a lag phase for a duration of 90mins after which the organisms start multiplying, The rate of growth of Agrobacterium is much higher compare to Pseudomonas.

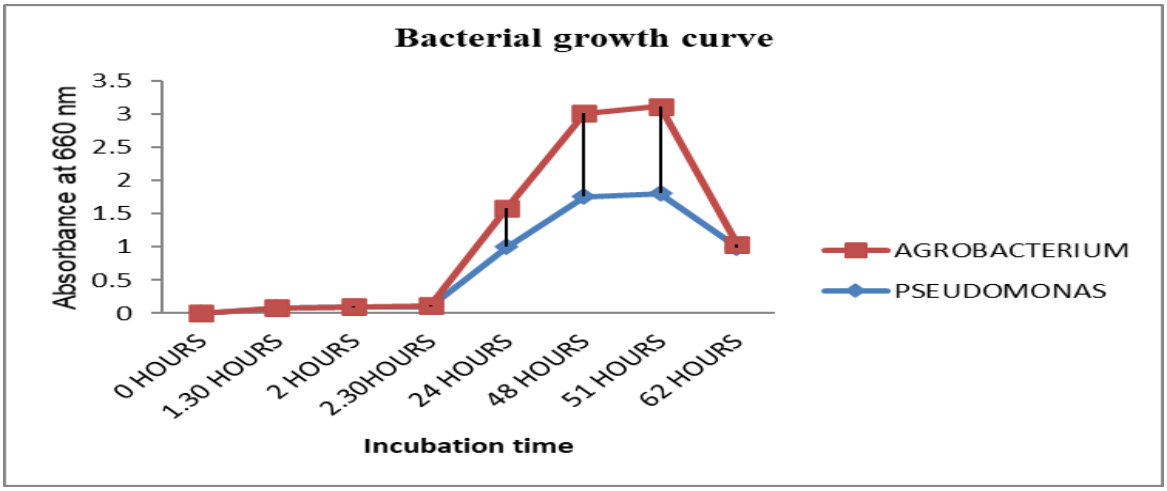
The growth of the bacteria was assessed in media to which the pesticide phoskill was added. To one set of tubes the organism was inoculated to the media containing phoskill, to a second set of tubes phoskill was introduced 24 hours after inoculation of the respective organisms. The growth of the organisms under different conditions was monitored.

The growth of bacteria, both Agrobacterium and Pseudomonas, in first set of tubes are severely inhibited in the presence of pesticide, however the organism in other set of tubes , that have grown in absence of pesticide for the first 24 hours are able to tolerate the stress caused by the introduction of pesticides.

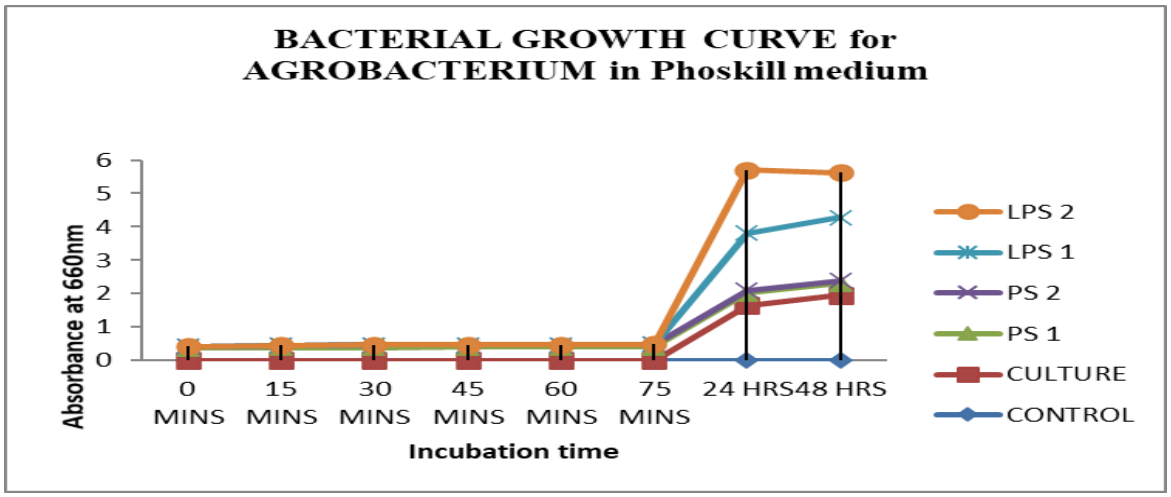
Stress in environment generally results in increase of ROS in living systems. Antioxidant enzymes offer natural defense against cell damage caused by ROS. Therefore, it is anticipated that there will be an elevation in antioxidant enzyme levels, hence in total protein.

3.6. Isolation of protein

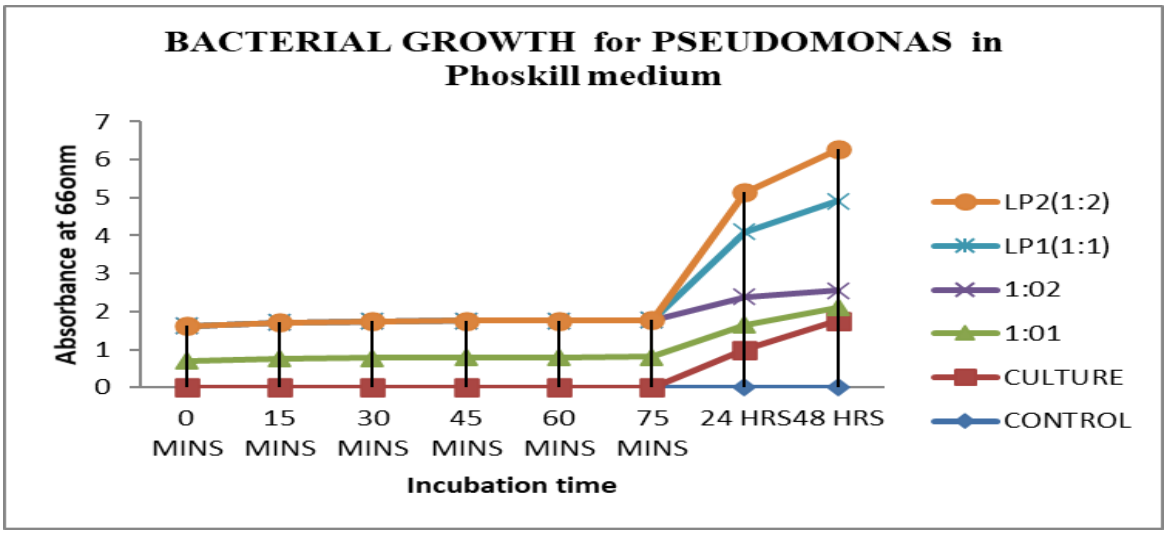
Protein in control and pesticides treated cultures were isolated. The isolated protein is suspended in PBS and estimated. The amount of protein isolated from the different cultures was quantified by biuret method. 53 µg of protein was isolated from the Agrobacaterium grown in the presence of Neem oil. The control culture yielded only 40µg.



Graph 8: Growth curve for isolated bacteria



Graph 9: Growth curve for Agrobacterium



Graph 10: Growth curve for Pseudomonas

4. CONCLUSION

Plants and microbes have a very cooperative relationship in natural environments like soil. Plants and trees, grass and food crops, all depend upon microorganisms in the soil to get water, absorb nutrients, to protect from pathogens and pest, prevent nutrient loss and breakdown compound that could inhibit growth. These soil microbes, in return, benefit from the health of plants growing within the soil and substances secreted from the plants rootage. This relationship creates a dynamic living system that is easily broken by conventional systems that use pesticides, herbicides and fertilizers. The chemicals that are intended to reinforce plant growth can in reality destroy soil system, killing or causing mutation on the soil microbes in order to survive in this ecosystem.

The results of the present study confirms that gross picture of the situation is not alarming as the total count of heterotrophic bacteria has been lowered very marginally. But the response of individual organisms may be a cause of concern. There are some pesticides that trigger the multiplication of bacteria. Hence the choice of pesticides is the key to maintain the fertility of the soil.

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Conflict of Interest

The authors declare no conflict of interest.

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