IMPACT OF COMBINATION OF HALOXYFOP R METHYL ESTER AND DICHLORVOS ON PLANT GROWTH PROMOTING RHIZOBACTERIA

BY

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DEPARTMENT OF MICROBIOLOGY

FACULTY OF LIFE SCIENCE

UNIVERSITY OF BENIN

BENIN CITY,

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, NIGERIA. IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF BACHELOR OF SCIENCE, B.Sc. (HONS.) IN MICROBIOLOGY

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CERTIFICATION

This is to certify that this project work was carried out by Gladys Onyinye NWOSU

(Miss) in the department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City under my supervision.

_____ _____

MR A. DUNKWU-OKAFOR DATE

(Project Supervisor)

APPROVAL

This project work was carried out by Gladys Onyinye NWOSU (Miss) in partial fulfillment of the award of a Bachelor of Science, B.Sc. (Hons) degree in the Department of Microbiology, University of Benin, Benin City.

Prof. (Mrs) F. I. AKINNIBOSUN **Date**
(Head of Department)

DEDICATION

This project is dedicated to Almighty God and to my parents Mr and Mrs NWOSU for their unending love and support.

ACKNOWLEGDEMENT

I want to express my deep gratitude to the Almighty God for his unending love and guidance. My sincere gratitude goes to my supervisor MR A. DUNKWU-OKAFOR for his help and assistance during my project work. Special thanks to the Head of Department PROF. (MRS). F.I. AKINNIBOSUN. Special appreciation also goes to DR. A. OGOFURE for his assistance during my project work and my parents Mr and Mrs NWOSU for their financial and emotional support all through this process.

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ABSTRACT

Pesticides are chemicals widely used in agriculture to control diseases and to help increase crop yield. Pesticides in particular insecticides can be very beneficial but have also been found to have harmful side effects on non-target insects. The effects of pesticides are not simply linear but complex through their interactions with a large variety of biotic and abiotic factors. Pesticides is a general word that describes numerous groups of insecticides, fungicides, herbicides, garden chemicals, household disinfectant sand rodents that are operated to both destroy and protect from pests. Rhizobacteria are root associated bacteria that can have detrimental, neutral or beneficial effect on plant grow. Nitrogen fixation is one of the beneficial processes performed by rhizobacteria. Plant growth promoting rhizobacteria were first identified by Kloepper and Schroth et al., (1978) to be soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth. Pesticides that persist in soil may have a long-lasting impact on Rhizobial survival and function. The soil was sprayed with the combination of the chemicals (Haloxyfop R Methyl Ester and Dichlovos) after they were diluted. Soil samples were obtained from the University of Benin (UNIBEN), life science land and were wrapped in a clean sterile polythene bag and labelled control and mixture of both chemicals. The materials for the experiment were sterilized, the differential agars were prepared and the soil samples were diluted. Distinct colonies were identified after growth were observed on the nutrient agar, these were restreaked on a freshly prepared agar to obtain the pure culture and incubated at 37°C for 24 hours. Growth on Bacillus cereus agar and Eosin methylene blue agar were observed by colony forms, colours and spore morphology. Using the nitrogen free medium, pure cultures were inoculated and incubated at 28°C for 1-7 days and growth on these plates confirms the ability to fix nitrogen. The results showed a gradual increase in the microbial count across 28 days (from $3.8\times10^4 \pm 707.11$ to $4.9\times10^4 \pm 4949.75$). Also there was little or no effect on plant growth promoting rhizobacterial activities of isolated microorganisms such as *Pseudomonas aeruginosa, Escherichia coli* and *Bacillus subtilis.* Chemicals that have little or no effects on the plant growth promoting rhizobacterial abilities of microorganisms should be used on farmlands.

CHAPTER ONE

INTRODUCTION

1.1 Background

Pesticides are plant protection products widely used in agriculture to control diseases and increase crop yield. Xenobiotic pesticides are a major cause for concern all over the world, given their persistence to their metabolite residues after degradation by artificial or natural means and adverse effects on the eco-biota (Chapman, 2002).

Most insecticides are produced as emulsifiable concentrates, suspension concentrates, wettable granules or wettable powders, depending on the properties of the active ingredient (AI) and the target market, and provide cost-effective pest control along with convenience of handling and compatibility with spray equipment. Past attempts to improve insecticide formulations have usually centered around greater rain fastness or photo stability, rather than reduced environmental risk or the complex issues related to uptake of dried deposits of a contact-acting compound from a plant surface, systemic movement of a stomach-acting compound or differential exposure to pests and natural enemies (Perrin, 2000).

Low-input farming is based on a reduction--but not necessarily elimination--of chemical fertilizers, insecticides, and herbicides. Farmers are adopting these practices primarily to reduce costs, but also because they want to minimize impact on the environment or because they perceive future pesticide regulations. In a search for information on how to farm with fewer

chemicals, it is helpful to examine alternative farming systems in existence that largely exclude chemicals in favor of biological farming practices (Diver, 1996).

Pesticides, in particular insecticides, can be very beneficial but have also been found to have harmful side effects on non-target insects. The effects of pesticides are not simply linear, but complex through their interactions with a large variety of biotic and abiotic factors. Furthermore, these effects manifest themselves at a variety of levels, from the molecular to metapopulation level (Bark *et al.,* 2018).

The multiplicity of beneficial effects of microbial inoculants, particularly plant growth promoters (PGP), emphasizes the need for further strengthening the research and their use in modern agriculture. PGP inhabit the rhizosphere for nutrients from plant root exudates.

In addition, the PGP microbes contain useful variation for tolerating abiotic stresses like extremes of temperature, pH, salinity and drought; heavy metal and pesticide pollution. Seeking such tolerant PGP microbes is expected to offer enhanced plant growth and yield even under a combination of stresses. This review summarizes the PGP related research and its benefits, and highlights the benefits of PGP rhizobia belonging to the family Rhizobiaceae, Phyllobacteriaceae and Bradyrhizobiaceae (Gopalakrishnan et al., 2015).One of the key elements of recent agricultural intensification is the increased use of pesticides (Morris *et al*., 2005).

The main thrust of this study is to investigate the effects of organic matter on the physical and the physicochemical properties of illitic soils. For this purpose, organic matter (peat) was added to inorganic illitic clayey soil at eight levels (0%, 5%, 10%, 12.5%, 15%, 17.5%, 20%, and 30% by weight). The physicochemical properties of the resulting soils were determined using a Grain

Size Analyzer (GSA) with specific surface area measurement, Scanning Electron Microscopy (SEM), and Infrared Spectroscopy (IR).

Aim

The aim of this study was to investigate the impact of Haloxyfop R methyl+DDVP ester on the activities of PGPR.

Objectives

The objectives of this study was to

1. identify the *PGPR* populations within sample farm land
2. determine the heterotrophic bacterial count before application of herbicide
3. compare *PGPR* counts upon application of haloxyfop R methyl ester for a 28 day period

CHAPTER TWO

Literature Review

2.1 PESTICIDES.

Pesticides are chemicals that are applied to private gardens, agricultural land, and other public areas to kill undesirable organisms. Pesticides in water resources adversely affect both the ecosystems and humans. Those materials have been thought as probable mutagens as they comprise constituents to trigger deviations in DNA. AccorGood Goodding to the world health organization (WHO), about 1000,000 human being are affected by acute poisoning by contact with pesticide. Each year, a death rate between 0.4 and 1.9% is recorded (Eddleston, 2020, Jia *et al*., 2020, Qiu *et al*., 2017, Thundiyil *et al.*, 2008). Work-related contact with pesticides could be behind 70% of these mortalities. Furthermore, constant contact to lower pesticides dosages was associated with a group of syndromes in the medium and long term, involving numerous tumors and nervous system disorders (Bertero *et a*l., 2020, Owens *et a*l., 2010). Likewise, WHO planned pesticides' taxonomy depending on their health hazard and their lethal performance in mice or other lab creatures administrated orally or dermally and approximating LD50 that creates mortality in 50% of the subjected animals (De Plaguicidas, 1998, Garcia *et a*l., 2012, Sjerps *et al*., 2019, WHO, 2004).

Pesticides are used in conjunction with the “industrial era” that changed the environment since the 1950s. In areas where monocular intensive cultivation takes place, pesticides have been applied as a typical pest control technique. Even with the advantages of chemistry, there are some drawbacks, many of which are so important to the point of disturbing the predator–prey interactions and damage biodiversity (El Nemr, 2011). Moreover, pesticides can have noteworthy health concerns.

The use of pesticides in agriculture is a subcategory of the largest group of manufactured compounds used in the present world. On the other hand, there is significant evidence that agricultural use of pesticides has a major impact on water quality and may cause severe environmental concerns (FAO, 1990, Warra and Prasad, 2020).

Although the amount of pesticides in practice is quite large almost 2 million metric tons of active ingredient, it is likely that greater use will be associated with a few pesticide products (Popp, Pető, & Nagy, 2013). These 2 million tons of pesticides can be divided into 47.5% herbicides, 29.5% insecticides, 17.5% fungicides and 5.5% other pesticides (De et al., 2014, El Nemr *et al*., 2004, El Nemr *et al*., 2003, Salem *et al*., 2014).

The present work is considered one of the first reviews that may explain briefly all aspects of using pesticides in environments starting from classification ending by degradation. Moreover, it describes the extraction and detection methods from the oldest to the modern techniques, as well as the disposal and treatment methods, while comparing the advantages and disadvantages of these methods and suggesting the optimized methods for complete removal of pesticides-containing wastewaters.

2.2 CLASSIFICATION OF PESTICIDES.

Pesticide is a general word that describes numerous groups of insecticides, fungicides, herbicides, garden chemicals, household disinfectants and rodenticides that are operated to both destroy and protect from pests (He, 1994, Eldridge, 2008, El Nemr *et al*., 2012, El Nemr *et a*l., 2012, El Nemr et al., 2012). These pesticides vary in their chemical and physical properties from one class to another. For that reason, it is praiseworthy to categorize them depending on their properties and study their particular groups. A synthetic pesticide is man-made chemicals, and does not exist in nature. They are classified into several groups based on their use. Currently, there are

three widely held method of pesticides' classification recommended by Drum (1980). These three common approaches of pesticides classes encompass: (i) the chemical structure of the pesticide, (ii) the entry mode, and (iii) the action of pesticide and the organisms they kill (Yadav et al., 2015). Chemical pesticides are classified into four types depending onto their sources: carbamate, organophosphate, organochlorine, and pyrethroid pesticides. On the other hand, there is another class of pesticides named biopesticides, which are naturally occurring or naturally derived materials especially from living organisms such as plants, fungi, bacteria, etc. Biopesticides are divided into three major groups: biochemical pesticides, microbial pesticides, and plant incorporated protectants. The methods that pesticides can use to interact with or reach the target pest are called *modes of entry* (Gerolt, 1969). Fig. 1 shows the sub-classification of insecticides.

2.2.1 MODE OF ENTRY

The routes of exposure involve ingested gastric toxins, contact exposure, expectorant and evaporators. Animals or plants when treated with systemic pesticides, the chemicals move to untreated parts of the organism. Systemic herbicides travel through plants and can reach untreated parts of roots, leaves, or stems. They are effective in killing weeds with an incomplete spray cap. They can successfully enter plant parts and travel through the plant's vascular system to destroy specific pests.

Some systemic insecticides can also be used in animals to reduce pests such as lice and fleas e.g. glyphosate and 2,4-dichlorophenoxyacetic acid (Fig. 2) (Buchel, 1983). Irregular pesticides act on target pests when they reach contact. Physical pesticides must come into contact with the lesion to be effective. The pesticide enters the lesion through the animal's epidermis after contact and causes poisoning leading to the death of the target pest. Examples of these pesticides

are diquat dibromide and paraquat (Fig. 2.0). (Seppo Saari et al., 2019, Yadav and Devi, 2017). The toxins may be transferred from the mouth to the stomach of the insects and then transferred to the rest of the digestive system, where they are absorbed into the body of the insect, which results in killing it.

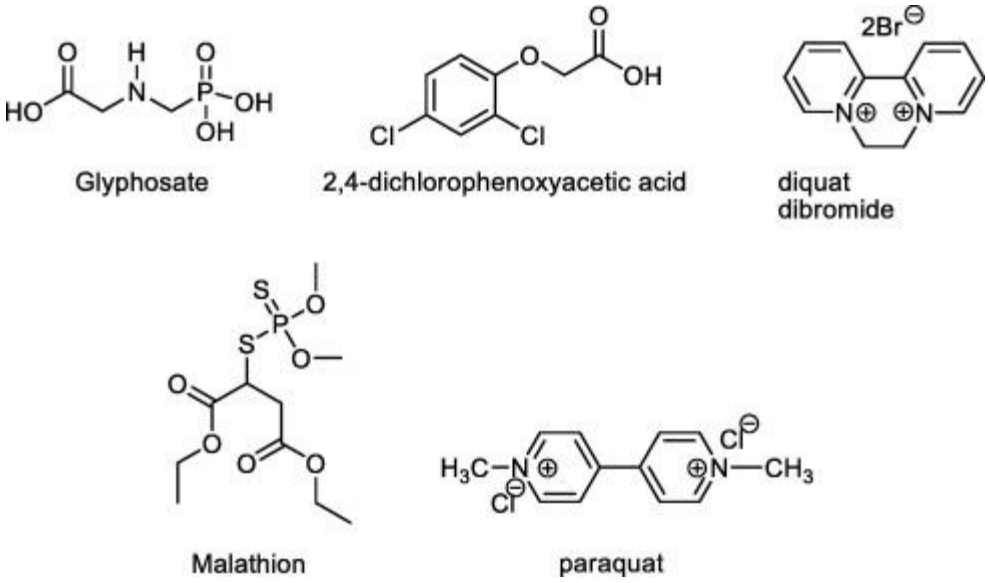

Fig. 2.0. Structure of Glyphosate, 2,4-dichlorophenoxyacetic acid, diquat dibromide, malathion and paraquat pesticides.

Pesticides are classified based on the target pest object and are given special names to reflect their activities.The pesticide categories based on target pests can be summarized in Table 2.0

Table 2.0 Pesticide name and its type and target pests

Type of pests	Pesticides Example	Target pests/Function
Avicides	Avitrol (aminopyridine)	Kill birds
Acaricides	Bifenazate	Kill mites that feed on plants and animals
Attractant	Pheromones	Attracts wide range of pests
Algaecides	Copper sulfate	Control or kill growth of algae
Bactericides	Copper complexes	Kill bacteria or acts against bacteria
Biopesticide	Bacillus thuringiensis	Wide range of organisms
Bait	Anticoagulants	Wide range of organisms
Desiccants	Boric acid	Act on plants by drying their tissues
Defoliant	Tribufos	Removes plant foliage
Fungicides	Azoxystrobin, Chlorothalonil	Kill fungi (including blights, mildews, molds, and rusts)
Fumigant	Aluminum phosphide	Wide range of organisms

Type of pests	Pesticides Example	Target pests/Function
Herbicides	Atrazine, glyphosate, 2,4-D	Kill weeds and other plants that grow where they are not wanted
Insecticides	Aldicarb, Carbaryl, imidacloprid	Kill insects and other arthropods
Insect growth regulator	Diflubenzuron	Insects
Lampricides	Trifluromethyl	Target larvae of lampreys which are jawless fish latching on vertebrate fish in rivers
Larvicides	Methoprene	Inhibits growth of larvae
Molluscicides	Metaldehyde	Inhibit or kill molluscs i.e. snails usually disturbing growth of plants
Moth balls	Dichlorobenzene	Stop any damage to cloths by moth larvae or molds
Nematicides	Aldicarb, Ethoprop	Kill nematodes that act as parasites of plants
Ovicides	Benzoxazin	Inhibits the growth of eggs of insects and mites

Type of pests	Pesticides Example	Target pests/Function
Piscicides	Rotenone	Act against fishes
Plant growth regulator	Gibberellic acid, 2,4-D	Regulates plant growth
Predacide	Strychnine	Mammal predators
Repellents	Methiocarb	Repel pests by its taste or smell, vertebrates and invertebrates
Rodenticides	Warfarin	Control mice and other rodents
Silvicides	Tebuthiuron	Acts against woody vegetation
Termiticides	Fipronil	Kill termites
Virucides	Scytovirin	Act against viruses

(Fishel and Ferrell, 2013).

2.2.2 CHEMICAL STRUCTURE OF PESTICIDES.

The most widespread and appropriate method for classifying insecticides depends on their chemical composition and description of the active ingredients. It is a type of classification that provides evidence of efficacy, chemical and physical properties of special pesticides. Depending on the chemical composition, pesticides are classified into 4 main categories: organiochlorine, organic phosphorous, carbamate, pyrethrin and pyrethroid (Buchel, 1983).

2.2.2.1 Organochlorine pesticides (OCPs)

Organochlorines are stable chemicals that are very persistent in the environment and have the potential to accumulate in adipose tissue (Waliszewski et al., 2002, Waliszewski et al., 2003, Waliszewski et al., 2003, Waliszewski et al., 2004, Ragab et al., 2016). In humans, these compounds or their metabolites mostly work at the level of the central nervous system altering enzymatic nerve membranes and electrophysiological properties, which leads to changes in the kinetics of the flow of K^+ and Na^+ through the nerve cell membrane (Narahashi, Frey, Ginsburg, & Roy, 1992) and may cause symptoms such as acute poisoning death and seizures from apnea (Tordoir& van Sittert, 1994). Structurally, organochlorines fall into five classes (Blus, 2003): (1) DDT and its analogs including DDT and dichlorodiphenyldichloroethylene (DDE); Dichlorodiph enyldichloroethane (DDD) (2) hexachlorocyclohexane (HCH), such as lindane; (3) cyclodienes including aldrin, dieldrin, endrin (sometimes referred to as “drins” in the literature), heptachlor, c hlordane, and endosulfan; (4) toxaphene; and (5) mirex and chlordecone (Fig. 3). The field half-life time of some organochlorines such as DDT, DDE and DDD) is 15 years, while for aldrin and toxaphene is 365 and 9 days respectively (Sparling, 2016). The acute toxicity of most OCPs generally occurs at concentrations that are higher than those considered environmentally realistic

so death under natural conditions may be slow and often is seen as a general wasting away or chronic illness.

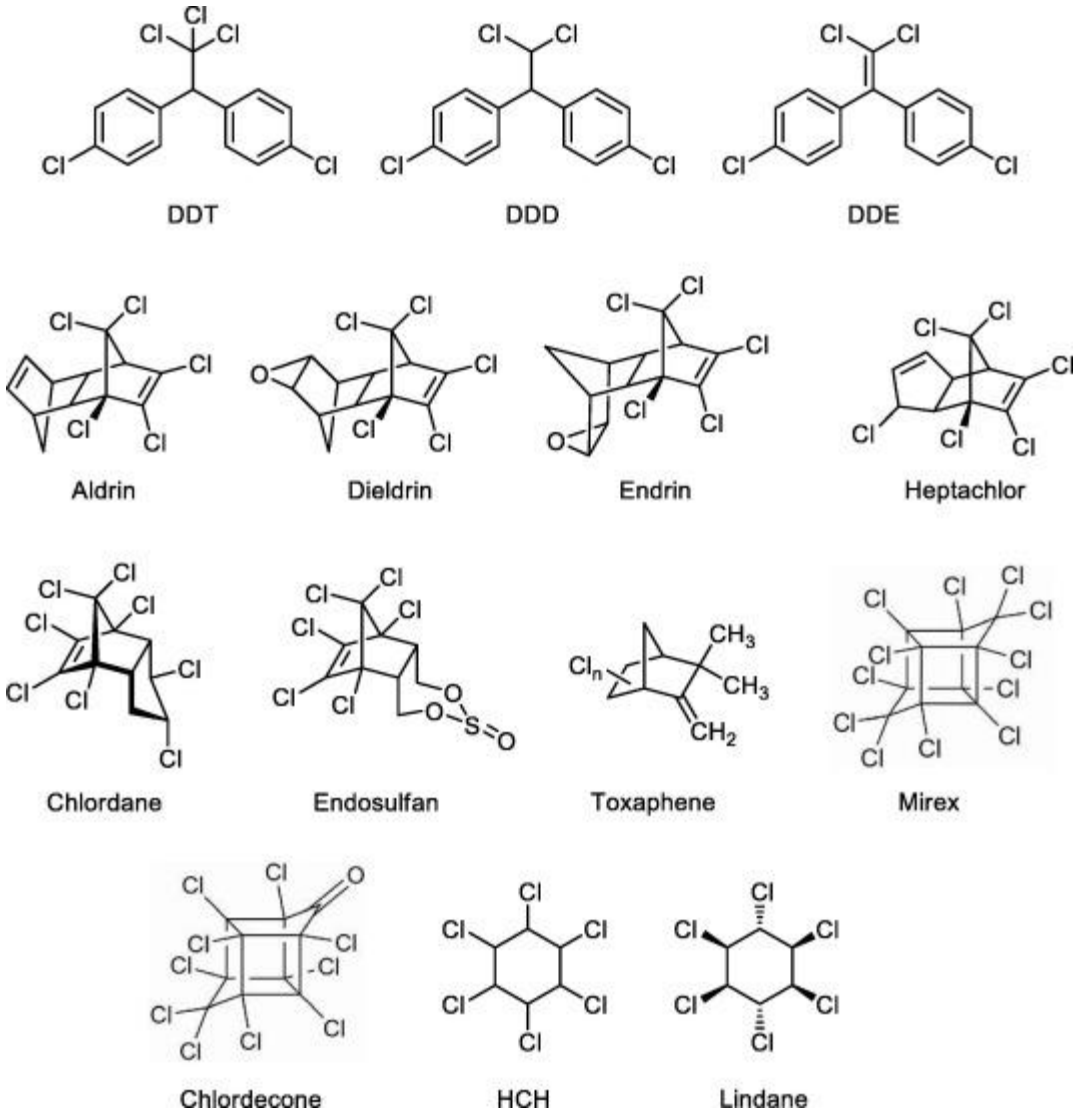

Fig. 2.1 Structure of some organochlorines.

2.2.2.2 Organophosphate pesticides

Some esters derived from phosphoric acid are known as organophosphate pesticides. These esters form are working in humans on the central nervous system by blocking the enzyme acetylcholine. This enzyme manages the amount and levels of the neurotransmitter acetyl cholinesterase, which disturbs the nerve impulse by the serene phosphorylation of the OH group in the active site of the enzyme (Fukuto, 1971, Krieger, 2001, Sogorb and Vilanova, 2002, Vale and Lotti, 2015). Intoxication symptoms are coma, dizziness, nausea, headache, cramps, convulsions, loss of reactions, and even death (El Nemr and El-Sadaawy, 2016, El Nemr et al., 2016, Perry et al., 2013, Sulbatos, 1994). Fig. 4 shows the chemical structure of some pesticides. The definitive treatment for organophosphate poisoning is atropine, which competes with acetylcholine at the muscarinic receptors. The initial dose for adults is 2 to 5 mg IV or 0.05 mg/kg IV for children until reaching the adult dose.

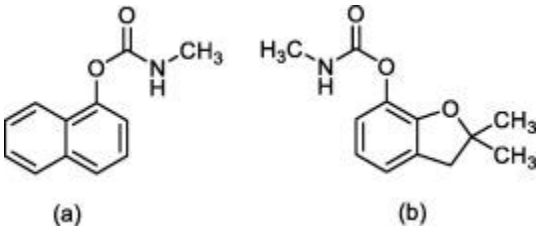

Fig. 2.2. Chemical structure of some pesticides (a) Carbaryl and (b) Carbofuran.

2.2.2.3 Carbamate pesticides

Some organic ester compounds derived from dimethyl *N*-methyl carbamic acid are used as herbicides, insecticides, nematicides and fungicides, and named as carbamates (Fig. 5). Carbamates such as thiobencarb, propoxur, molinate, disulfiram (Antabuse), pyridostigmine, methiocarb and carbaryl are widely used in both cats and dogs. The carbamate compounds toxicity varies according the molecular structure, but in general they have shorter duration than that of organophosphates and organochlorines, and the latter inhibits acetyl cholinesterase (Garcia et al., 2012, Winder, 2004). The treatment of acute carbamate toxicity is also similar to that of organophosphates. It should be noted that the carbamates are short-lived, therefore a higher level of caution is warranted in atropine administration (Eicher, 2009, Vale and Lotti, 2015). The acute poisoning symptoms occurring by organophosphate or carbamate insecticides is common and often severe. These poisoning symptoms appear in different organs and can be listed as follows: bronchial tree (wheezing, dyspnea, increased secretions, muscarinic (parasympathetic), cough, pulmonary edema, cyanosis, and bronchoconstriction), glandular stimulation (increased salivation, lacrimation, and sweating), cardiovascular effects (bradycardia, and hypotension), eye (miosis, and blurred vision), bladder dysfunction (incontinence, and frequency), Gastrointestinal manifestations (nausea, vomiting, abdominal cramps, diarrhea, and incontinence).

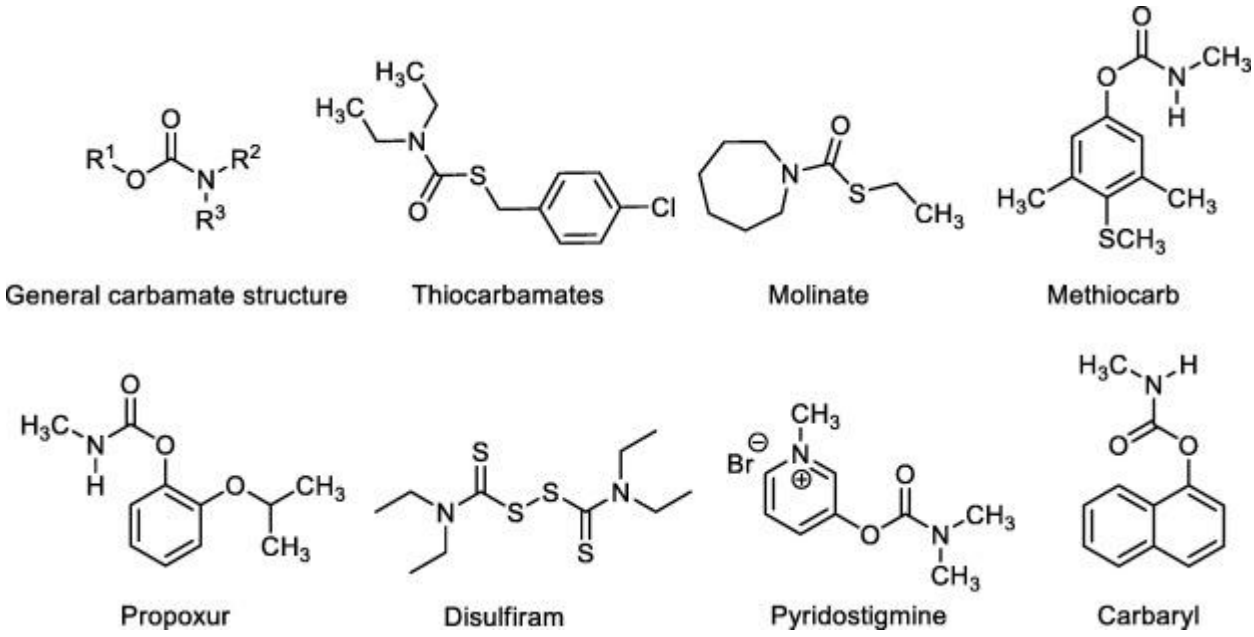

Fig. 2.3 Chemical structure of some carbamate and thiocarbamates pesticides.

2.2.2.4 Pyrethroid pesticides

Pyrethroids are natural insecticides derived from the pyrethrum extracts of chrysanthemum flowers known as pyrethrin found in Kenya. It works on the central nervous system, which causes fluctuations in the dynamics of sodium cation channels in the membrane of the nerve cell, which leads to an increase in the time of opening of the sodium channels. Most pyrethroid insecticides (2.4) share several characteristics such as low toxicity to birds and mammals; high toxicity to arthropods since it requires very low doses to kill insects; highly toxic to fish if applied directly to water; and fast-acting especially against chewing insects.. In addition, they are poorly soluble in water (Gupta and Crissman, 2013, Seppo Saari et al., 2019). There are over 1000 different pyrethroids used today, although fewer than a dozen are available in the United States.

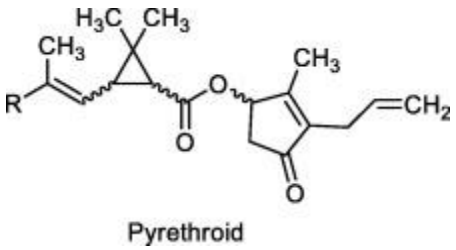

Fig. 2.4 General structure of pyrethroids pesticides.

2.3 Pesticides in water resources

Pesticides enter the water by flowing (run-down) or filtering (leaching). Both are related to the Earth's hydrological cycle. When considering the use of urban water for surface flow, pesticides in municipal wastewater are appropriate for the hydrological model (Gago-Ferrero et al., 2017, Khalid et al., 2020, Kuster et al., 2010, Majewski and Capel, 1996, Peña et al., 2020).

Factors that regulate the pesticides impact on water quality (FAO, 1990)

- Contaminants that occur as impurities inside the active ingredient.

- The active ingredients in the pesticide formulation.

- Degradable compounds that resulted after or during microbial, or chemical and photochemical degradation of the active components.

- Additives mixture with active ingredients (preservatives, emulsifier's wetting agents, solvents or diluents, adhesives and buffers).

2.4 Pesticides impact on human health and hazardous levels

The degradation of pesticide runoff in water has two major impacts on human health (Ferrer and Cabral, 1995, Ferrer, 2003; El Nemr et al., 2012, El Nemr et al., 2012, El Nemr et al., 2012, Bergmann, 2019). The first is the ingesting of shellfish and fish that are contaminated by pesticides. This may jeopardize economies depending on the survival of fish that live at the bottom of the main agricultural submerged parts. The second is the direct ingesting of water contaminated with pesticide (Bergmann, 2019, Bhandari et al., 2020, Łozowicka et al., 2020, Margni et al., 2002; Kamrin, 1997, Leite et al., 2009).

2.4.1 Pesticides ecological effects

Pesticides are involved in a wide range of organic micro pollutants that have negative environmental effects. Several groups of pesticides have specific mechanism of contamination of living organisms, which is why generalization is difficult. The two main mechanisms are biomagnification and bioconcentration (Tian et al., 2018, Zhen et al., 2019).

2.4.1.1 Biomagnification

The term determines the increasing level of a chemical for food energy that is converted within the food chain. Going up the food chain, less living organisms are eaten by large organisms, thus the level of pesticides and other chemicals is gradually amplified in tissues and other organs. A very high level can be observed in higher predators, including humans (Guan et al., 2020, Liu et al., 2020, Wang et al., 2019, Zhou et al., 2018).

2.4.1.2 Bioconcentration

The term defines the transmission of a chemical into an organism from the surrounding medium. The ecological impacts of pesticides are different and are frequently inter-related. Several pesticides have noticeably diverse impacts on aquatic ecosystems, this is why it is very hard to create a broad view about this problem. Many of these effects are chronic and often not observed by regular observers but they have many concerns about the entire food chain. Examples for some physiological effects can be summarized as the following: 1. Reproductive failure or inhibition, 2. Disturbance of endocrine (hormonal) system, 3. Death, 4. Immune system suppression, 5. Tumors, cancers and lesions on animals and fishes.

2.5 Pesticides degradation in water, soil and their metabolism process

Besides photochemical and chemical processes, there are 2 major biological mechanisms that lead to pesticides degradation. The first is microbiological reactions in water and soil and the second is the pesticide metabolism that organisms consume as part of their absorption of food. Although both mechanisms are beneficial in the sense that the toxicity of pesticides changes, metabolic processes lead to undesirable effects. The energy consumed in the metabolism of pesticides and other foreign organisms is not provided for other basic body functions and can severely disrupt the reproduction and growth of the organism (e.g. fish) (Khan and Pathak, 2020, Ouyang et al., 2020, Yang and Zhang, 2019).

Various pesticides rapidly degrade in the soil in a process called mineralization that turns pesticides into smaller compounds such as NH_3, H_2O, and CO_2. Chemical reactions such as

photolysis and hydrolysis lead to this degradation process. The main pathway for mineralization is usually the microbial metabolism and demolition. Soil microorganisms consume pesticides as a source of carbon or other nutrients. Some chemicals, for example [2,4-dichlorophenoxyacetic acid], degrade fairly quickly in the soil, while others are degrade less easily [2,4,5-trichlorophenoxyacetic acid].

2.6 INSECTICIDE

Neonicotinoids are a class of neuro-active insecticides chemically similar to nicotine. Imidacloprid, of the neonicotinoid family, is the most widely used insecticide in the world[Yamamoto, 1999]. In the late 1990s neonicotinoids came under increasing scrutiny over their environmental impact and were linked in a range of studies to adverse ecological effects, including honey-bee colony collapse disorder (CCD) and loss of birds due to a reduction in insect populations. In 2013, the European Union and a few non EU countries restricted the use of certain neonicotinoids [Cressy, 2013, Gill et al., 2012, Dicks, 2013, Stoddart, 2012, Osborne, 2012, Cressy, 2013, Bees and Pesticides, 2013]. Organophosphate and carbamate insecticides have a similar mode of action. They affect the nervous system of target pests (and non-target organisms) by disrupting acetylcholinesterase activity, the enzyme that regulates acetylcholine, at nerve synapses. This inhibition causes an increase in synaptic acetylcholine and overstimulation of the parasympathetic nervous system.[Colović, 2013] Many of these insecticides, first developed in the mid 20th century, are very poisonous. Although commonly used in the past, many older chemicals have been removed from the market due to their health and environmental effects (*e.g.* DDT, chlordane, and toxaphene).[Public Health Statement for DDT, DDE and DDD, 2018, Medical Management Guidelines(MMGs), 2018, Toxicological Profile for Toxaphene, 2018]. Many organophosphates do not persist in the environment.

Pyrethroid insecticides were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemums. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system[Soderlund, 2010].

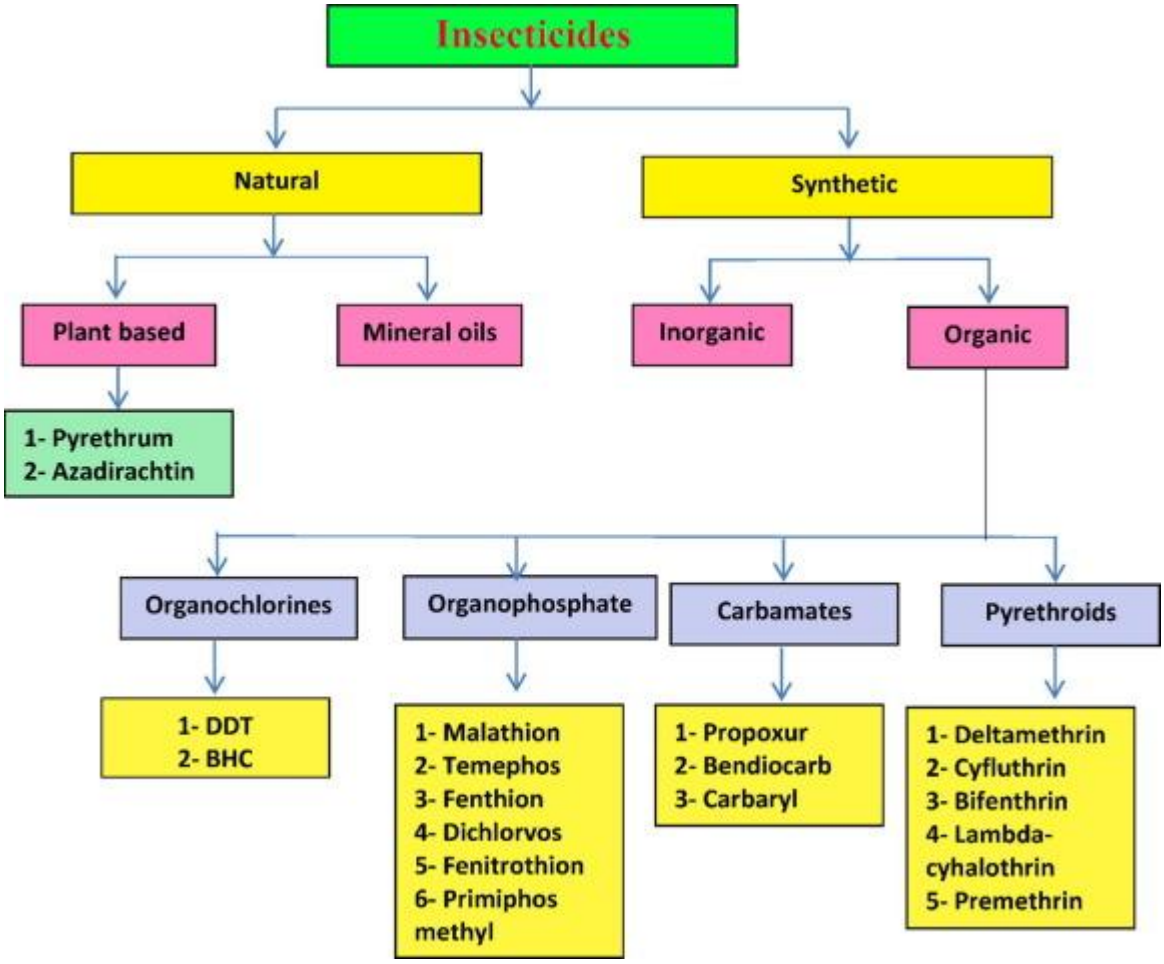

Fig 2.5 Classification of Insecticides (Yadav & Devi, 2017).

2.7 Herbicides

A number of sulfonylureas have been commercialized for weed control, including: amidosulfuron, flazasulfuron, metsulfuron-methyl, rimsulfuron, sulfometuron-methyl, terbacil,[Appleby et al, 2002] nicosulfuron,[Nicosulfuron, 2013] and triflusulfuronmethyl.[EFSA,

2009] These are broad-spectrum herbicides that kill plants weeds or pests by inhibiting the enzyme acetolactate synthase. In the 1960s, more than 1 kg/ha (0.89 lb/acre) crop protection chemical was typically applied, while sulfonylureates allow as little as 1% as much material to achieve the same effect.[Lamberth et al, 2013]

2.8 Biopesticides

Biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals. For example, canola oil and baking soda have pesticidal applications and are considered biopesticides. Biopesticides fall into three major classes:

- Microbial pesticides which consist of bacteria, entomopathogenic fungi or viruses (and sometimes includes the metabolites that bacteria or fungi produce). Entomopathogenic nematodes are also often classed as microbial pesticides, even though they are multi-cellular.[Coombs, 2013, Borgio et al, 2011]
- Biochemical pesticides or herbal pesticides[Pal et al, 2013] are naturally occurring substances that control (or monitor in the case of pheromones) pests and microbial diseases.
- Plant-incorporated protectants (PIPs) have genetic material from other species incorporated into their genetic material (*i.e.* GM crops). Their use is controversial, especially in many European countries.[Plant Incorporated Protectants (PIPs), 2017]

By pest type

Pesticides that are related to the type of pests are:

Type	Action
Algicides	Control algae in lakes, canals, swimming pools, water tanks, and other sites
Antifouling agents	Kill or repel organisms that attach to underwater surfaces, such as boat bottoms
Antimicrobials	Kill microorganisms (such as bacteria and viruses)
Attractants	Attract pests (for example, to lure an insect or rodent to a trap).
Biopesticides	Biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals
Biocides	Kill microorganisms
Disinfectants and sanitizers	Kill or inactivate disease-producing microorganisms on inanimate objects
Fungicides	Kill fungi (including blights, mildews, molds, and rusts)
Fumigants	Produce gas or vapor intended to destroy pests in buildings or soil
Herbicides	Kill weeds and other plants that grow where they are not wanted
Insecticides	Kill insects and other arthropods
Miticides	Kill mites that feed on plants and animals
Microbial pesticides	Microorganisms that kill, inhibit, or out compete pests, including insects or other microorganisms
Molluscicides	Kill snails and slugs
Nematicides	Kill nematodes (microscopic, worm-like organisms that feed on plant roots)
Ovicides	Kill eggs of insects and mites
Pheromones	Biochemicals used to disrupt the mating behavior of insects
Repellents	Repel pests, including insects (such as mosquitoes) and birds
Rodenticides	Control mice and other rodents
Slimicides	Kill slime-producing microorganisms such as algae, bacteria, fungi, and slime molds

Table 2.1 Types of pesticides and their actions

2.9 PLANT GROWTH PROMOTING RHIZOBACTERIUM

2.9.1 Rhizobacteria

Rhizobacteria are root-associated bacteria that can have a detrimental (parasitic varieties), neutral or beneficial effect on plant growth. The name comes from the Greek *rhiza*, meaning root. The

term usually refers to bacteria that form symbiotic relationships with many plants (mutualism). Rhizobacteria are often referred to as plant growth-promoting rhizobacteria, or PGPRs. The term PGPRs was first used by Joseph W. Kloepper in the late 1970s and has become commonly used in scientific literature.[Vessy et al., 2003]

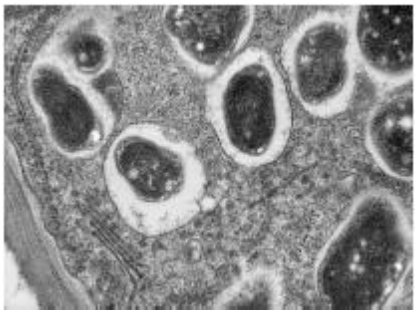

Fig: Cross section though a soybean (*Glycine max* 'Essex') root nodule: The rhizobacteria, *Bradyrhizobium japonicum*, colonizes the roots andestablishes a nitrogen-fixing symbiosis. This high magnificationimage shows part of a cell withsingle bacteroids

Generally, about 2–5% of rhizosphere bacteria are PGPR.[Antoun et al., 2005] They are an important group of microorganisms used in biofertilizer. Biofertilization accounts for about 65% of the nitrogen supply to crops worldwide. PGPRs have different relationships with different species of host plants. The two major classes of relationships are rhizospheric and endophytic. Rhizospheric relationships consist of the PGPRs that colonize the surface of the root, or superficial intercellular spaces of the host plant, often forming root nodules. The dominant species found in the rhizosphere is a microbe from the genus *Azospirillum*. [Bloemberg et al., 2001] Endophytic relationships involve the PGPRs residing and growing within the host plant in the apoplastic space.[Vessy, 2003]

2.9.2 Nitrogen Fixation

Nitrogen fixation is one of the most beneficial processes performed by rhizobacteria. Nitrogen is a vital nutrient to plants and gaseous nitrogen (N2) is not available to them due to the high energy required to break the triple bonds between the two atoms. [Cain et al., 2011] Rhizobacteria, through nitrogen fixation, are able to convert gaseous nitrogen (N2) to ammonia (NH3) making it an available nutrient to the host plant which can support and enhance plant growth. The host plant provides the bacteria with amino acids so they do not need to assimilate ammonia. [Willey et al., 2011] The amino acids are then shuttled back to the plant with newly fixed nitrogen. Nitrogenase is an enzyme involved in nitrogen fixation and requires anaerobic conditions. Membranes within root nodules are able to provide these conditions. The rhizobacteria require oxygen to metabolize, so oxygen is provided by a hemoglobin protein called leg hemoglobin which is produced within the nodules. [Cain et al., 2011] Legumes are well-known nitrogen-fixing crops and have been used for centuries in crop rotation to maintain the health of the soil acids are then shuttled back to the plant with newly fixed nitrogen. Nitrogenase is an enzyme involved in nitrogen fixation and requires anaerobic conditions. Membranes within root nodules are able to provide these conditions. The rhizobacteria require oxygen to metabolize, so oxygen is provided by a hemoglobin protein called leghemoglobin which is produced within the nodules. [Cain et al., 2011] Legumes are well-known nitrogen-fixing crops and have been used for centuries in crop rotation to maintain the health of the soil.

2.9.3 Symbiotic relationship

The symbiotic relationship between rhizobacteria and their host plants is not without costs. For the plant to be able to benefit from the added available nutrients provided by the rhizobacteria, it needs to provide a place and the proper conditions for the rhizobacteria to live. Creating and maintaining root nodules for rhizobacteria can cost between 12–25% of the plant's total photosynthetic output. Legumes are often able to colonize early successional environments due to the unavailability of nutrients. Once colonized, though, the rhizobacteria make the soil surrounding Symbiotic relationships the plant more nutrient rich, which in turn can lead to competition with other plants. The symbiotic relationship, in short, can lead to increased competition. [Cain et al., 2011] PGPRs increase the availability of nutrients through the solubilization of unavailable forms of nutrients and by the production of siderophores which aids in the facilitating of nutrient transport. Phosphorus, a limiting nutrient for plant growth, can be plentiful in soil, but is most commonly found in insoluble forms. Organic acids and phosphotases released by rhizobacteria found in plant rhizospheres facilitate the conversion of insoluble forms of phosphorus to plantavailable forms such as $H2PO4^-$. PGPR bacteria include Pseudomonas putida, Azospirillum fluorescens, and Azospirillum lipoferum and notable nitrogen-fixing bacteria associated with legumes includes Allorhizobium, Azorhizobium, Bradyrhizobium, and Rhizobium. [Willey et al., 2011] Though microbial inoculants can be beneficial for crops, they are not widely used in industrial agriculture, as large scale application techniques have yet to become economically viable. A notable exception is the use of rhizobial inoculants for legumes such as peas. Inoculation with PGPRs ensures efficient nitrogen fixation, and they have been employed in North American agriculture for over 100 years.

2.9.4 Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) were first defined by Kloepper and Schroth[Kloepper et al., 1978] to be soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth.[Aziz et al., 2012] The following are implicit in the colonization process: ability to survive inoculation onto seed, to multiply in the Plant growth-promoting rhizobacteria spermosphere (region surrounding the seed) in response to seed exudates, to attach to the root surface, and to colonize the developing root system.[Kloepper et al., 1993] The ineffectiveness of PGPR in the field has often been attributed to their inability to colonize plant roots.[Bloemberg et al., 2001, Benizri et al., 2001] A variety of bacterial traits and specific genes contribute to this process, but only a few have been identified. These include motility, chemotaxis to seed and root exudates, production of pili or fimbriae, production of specific cell surface components, ability to use specific components of root exudates, protein secretion, and quorum sensing. The generation of mutants altered in expression of these traits is aiding our understanding of the precise role each one plays in the colonization process.[Lugtenberg et al., 2001, Persello-Cartieaux et al., 2001] Progress in the identification of new, previously uncharacterized genes is being made using nonbiased screening strategies that rely on gene fusion technologies. These strategies employ reporter transposons. [Roberts et al., 1998] and in vitro expression technology (IVET)[Rainey et al., 1999] to detect genes expressed during colonization. Using molecular markers such as green fluorescent protein or fluorescent antibodies, it is possible to monitor the location of individual rhizobacteria on the root using confocal laser scanning microscopy.[Bloemberg et al., 2001, Bloemberg et al., 2000, Sørensen et al., 2001] This approach has also been combined with an rRNAtargeting probe to monitor the

metabolic activity of a rhizobacterial strain in the rhizosphere and showed that bacteria located at the root tip were most active.

2.9.4 Mechanisms of action

PGPRs enhance plant growth by direct and indirect means, but the specific mechanisms involved have not all been well characterized.[Kloepper *et al.,* 1993] Direct mechanisms of plant growth promotion by PGPRs can be demonstrated in the absence of plant pathogens or other rhizosphere microorganisms, while indirect mechanisms involve the ability of PGPRs to reduce the harmful effects of plant pathogens on crop yield. PGPRs have been reported to directly enhance plant growth by a variety of mechanisms: fixation of atmospheric nitrogen transferred to the plant,[Zakry et al., 2012] production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones.[Riaz et al., 2021] Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPRs has also been

reported. PGPR strains may use one or more of these mechanisms in the rhizosphere. Molecular approaches using microbial and plant mutants altered in their ability to synthesize or respond to specific phytohormones have increased understanding of the role of phytohormone synthesis as a direct mechanism of plant growth enhancement by PGPRs.[Glick et al., 1995] PGPR that synthesize auxins, gibberellins and kinetins or that interfere with plant ethylene synthesis have been identified.[Chaitanya et al., 2015]

Development of PGPRs into biofertilisers and biopesticides could be a novel way of increasing crop yield and decreasing disease incidence, [Vessy, 2003] whilst decreasing dependency on chemical pesticides and fertilisers which can often have harmful effects on the local ecology and environment.[Muth et al., 2019]

2.9.6 Pathogenic roles

Studies conducted on sugar beet crops found that some root-colonizing bacteria were deleterious rhizobacteria (DRB). Sugar beet seeds inoculated with DRB had reduced germination rates, root lesions, reduced root elongation, root distortions, increased fungi infection, and decreased plant growth. In one trial the sugar beet yield was reduced by 48%.[Suslow et al., 1982] Six strains of rhizobacteria have been identified as being DRB. The strains are in the genera *Enterobacter*, *Klebsiella*, *Citrobacter*, *Flavobacterium*, *Achromobacter*, and *Arthrobacter*. Due to a large number of taxonomic species yet to be described, complete characterization has not been possible as DRB are highly variable.[Suslow *et al.,* 1982] The presence of PGPRs has proven to reduce and inhibit the colonization of DRB on sugar beet roots. Plots inoculated with PGPRs and DRBs had an increase in production of 39% while plots only treated with DRBs had a reduction in production of 30%.[Suslow *et al.,* 1982]

2.9.7 Biocontrol

Rhizobacteria are also able to control plant diseases that are caused by other bacteria and fungi. Disease is suppressed through induced systemic resistance and through the production of antifungal metabolites. Pseudomonas biocontrol strains have been genetically modified to improve plant growth and improve the disease resistance of agricultural crops. In Biocontrol agriculture, inoculant bacteria are often applied to the seed coat of seeds prior to being sown. Inoculated seeds are more likely to establish large enough rhizobacterial populations within the rhizosphere to produce notable beneficial effects on the crop. [Vessy, 2003].

2.9.8 Effect of pesticides and insecticides on PGPR

Of the different legumes grown around the world, chickpea [Cicer arietinum (L.)] is one of the most widely grown legumes. In India, chickpea occupies 7.1 million ha with a production of 5.75 million tones, accounting for 31% and 31% of total pulse area and production, respectively (ICAR, 2006). Chickpea replenish nitrogen in soils by forming specific symbiosis with its cognate N2 fixing bacterium, Mesorhizobium that convert atmospheric N2 to ammonia and other compounds and transport it to the growing plants (Wani et al., 2008). The efficiency of this approach, however, depends on principally maximizing symbiotic N2 fixation (SNF) and plant yield to resupply organic and inorganic nitrogen and other nutrients to soils. Rhizobial inoculants as bio-fertilizers are therefore, applied to soils/seeds of legumes to ensure effective nodulation and subsequent N2 fixation and consecutively, to increase the nitrogen pool of soils (Dudeja and Singh, 2008). The inoculants are often used together with agrochemicals,which besides containing essential nutrients also contain contaminants and toxic elements. The exposure of these chemicals to field-grown plants could be either intentional (e.g. by spraying the legumes with pesticides) or through residues remaining from previous applications (Khan et al., 2004). Of these chemicals, pesticides and their microbially degraded products interact with soils and rhizosphere microorganisms including rhizobia and cause DNA, protein, oxidative or membrane damage (Pham et al.,2004). In addition, the common use of pesticides in agricultura practices has been shown to affect N2 fixation adversely, either directly by affecting the rhizobia (Mallik and Tesfai, 1985; Anderson et al., 2004) or disrupting the signaling between legumederived phytochemicals (luteolin, apigenin) and Rhizobium NodD receptors (Fox et al., 2007) or indirectly by reducing photosynthate allocation to the nodules for N2 fixation (Sprout et al., 1992;Koopman et al., 1995; Datta et al., 2009) or by restricting root growth and hence reduce the

number of sites available for infection (Eberbach and Douglas, 1991). Additionally, pesticides that persist in soils may have a long-lasting impact on rhizobial survival and function (Eberbach and Douglas, 1989; Martenssonand Nilsson, 1989; Eliason et al., 2004).

CHAPTER THREE

3.0 Materials and methods

3.1 Spraying of soil

The soil was sprayed with the combination of the chemicals (haloxyfop R methyl ester + DDVP). The soil area was partitioned into four and one out of this was used as the control portion. 0.25L of the pesticide was mixed in 7.5L of water, 2L was taken and sprayed on the pesticide portion of the soil. For the insecticide portion, 25ml of the insecticide was taken and mixed in 7.5L of water. From this, 2L of the mixed insecticide and water was later taken and sprayed on the soil with the use of the knapsack device. For the mixture portion of the soil which is the part were a mixture of the insecticide and herbicide was to be sprayed, 1L of mixed herbicide and 1L of mixed insecticide were mixed together and sprayed with the use of the knapsack device.

3.2 RESEARCH MATERIALS

3.2.1 Sources of samples

Soil samples were obtained from the University of Benin (UNIBEN), Faculty of Life Science land but under three different soil condition in the same geographical area. The soil were wrapped in a clean, sterile Polythene bag and labelled Control (C), and Mixture of pesticide and herbicide (M) according to the different soil conditions they were obtained.

3.3 EXPERIMENT METHODOLOGY

The experimental methodologies were as follows: sterilization of materials, preparation of agar; serial dilution of soil samples; characterization and identification of bacteria isolates and determination of their plant growth promoting rhizobacterium potentials.

3.3.1 Sterilization of Materials

Materials such as Petri dishes, pipettes, glass containers (conical flask, round bottom flask), and bottles were drained and dried. They were wrapped with aluminum foil and sterilized in a hot-air oven at 160oC for an hour. They were allowed to cool after sterilization before usage. An aseptic working environment was achieved using a Bunsen burner flame and the disinfection of work surfaces with alcohol.

All media used were obtained from Oxoid and were prepared according to manufacturers' instructions. The media used in this study include Plate count agar, Bacillus cereus agar (BCA), Eosin methylene blue agar (EMB), Mannitol salt agar (MSA), Pseudomonas cetrimide agar (PCA), Triple sugar iron agar (TSI), Simmons citrate agar (SCA) and Mueller Hinton agar (MHA) (Willey et al., 2008).

Following pour plate culture of bacterial isolates from the samples, single colonies were subcultured on tryptone soya agar and incubated for 24 h. at environmental temperature (28±20C). The colonies were Gram stained and identified using standardized cultural and biochemical techniques as stipulated by Bridson (2006) in Oxoid manual. Differential Media (Oxoid) such as Chromogenic Bacillus cereus agar with chromogenic Bacillus cereus selective supplement, Sorbitol MacConkey agar with Cefixime-Tellurite Supplement, Eosine methylene blue agar, Pseudomonas cetrimide agar (supplemented with glycerol), Salmonella Shigella agar, Mannitol salt agar, and triple sugar iron agar slants were used for successful isolation and culture of bacterial isolates from samples. Further

confirmation of bacteria identity were carried out using biochemical tests such as citrate (simon citrate agar {Micromaster}), indole, Oxidase, Urease, sugar fermentation, catalase, 3% KOH, Gas formation, and H2S formation etcetera.

3.3.2 Serial dilution of soil samples

Serial dilution was done on the soil samples. 10g of each sample was added to 90ml of already sterilized water in 7 conical flask. From this 1ml of solution was introduced into 9ml of test tube. From this test tube, 1 ml of solution was introduced into another test tube containing 9ml of sterilized water. From this solution, 0.5 ml is taken and inoculated on a petri dish. 15 ml of EMB agar is added to the petri dish. For the nutrient agar plates, 0.1 ml of the solution is added to a petri dish of which 15 ml of nutrient agar is added**.**

3.3.3 Pure culture

One single distinct colony was identified and re-streaked as a primary inoculant on the surface of a nutrient agar plate medium. Pure cultures were checked from nutrient agar plates. After achieving a pure culture, the same colony was streaked onto a nutrient agar slant. These cultures were incubated at 37°C for 24h hours.

3.3.4 Sub-culturing of bacteria isolates

A single bacterial colony was picked up using a sterilized wire loop and placed on a fresh nutrient agar medium. The cultures were incubated at room temperature for 48 hours to obtain a pure culture.

3.3.5 Phenotypic Identification of Bacteria from Samples

Pure cultures of the bacterial isolates were obtained from the subculture of a single colony from the successful pour plate technique and were characterized using cultural, morphological and biochemical methods. Several tests, such as Gram reaction, catalase, urease, indole, oxidase, citrate utilization and respective reactions of bacteria on triple sugar iron agar, were carried out to identify bacterial isolates presumptively

3.3.6 Gram Staining Test

A Gram staining test was carried out to determine the presence of Gram-positive and Gram-negative isolates. Neat, grease-free and sterile-dried microscope slides with labels were smeared using a sterilized loop, and the organism was air-dried and heat-fixed over a flaming Bunsen burner. The fixed smear was saturated with drops of crystal violet, left for one minute to react and washed off with distilled water. Lugol's iodine, which serves as a mordant, was added, left for one minute, and washed off with distilled water. The smear was decolourized with 95% ethyl alcohol, went for 30 seconds and washed off with distilled water. Then, the streak underwent counter staining using safranin solution for one minute and was rinsed with distilled water. Lastly, the smear was allowed to air dry and immersion oil was added for a microscopic view on an immersion objective lens light microscope. Colours, shapes and arrangements were used in identifying the organisms. Gram-positive organisms maintained the crystal's purple colour, while Gram-negative retained the pink of safranin.

3.3.7 Potassium Hydroxide (KOH) test

The potassium hydroxide (KOH) test was used to determine or confirm Gram-negative bacteria, to quickly differentiate between Gram-negative and Gram-positive bacteria as a complement to Gram staining. KOH breaks down the thin peptidoglycan bacterial cell walls of Gram-negative bacteria but does not affect the thick layer of Gram-positive cell walls. Disruption of bacterial cell walls lyses the cell and releases its contents, including the genetic material. A drop of 3% KOH solution was applied on a labelled clean microscope slide and smeared with pure isolated culture using a loop. It was stirred carefully and observed that the solution turned to be a viscous or dense suspension, which formed a slimy or mucoid string within 60 seconds, and the appearance of that indicated a positive result as the presence of Gram-negative isolates. While non-slimy viscous suspensions remained negative results.

3.4. Biochemical Test

To better characterize these isolates, biochemical tests were conducted, which included:

3.4.1. Indole Test

An indole test was carried out to demonstrate the ability of certain bacteria that can decompose amino acid tryptophane to indole. The indole production test is essential in identifying the Enterobacteriaceae family that breaks down the amino acid tryptophan by releasing indole in the presence of intracellular enzymes called "tryptophanase." Several drops of Kovac's indole reagent were placed on a filter paper. A portion of a pure isolated colony picked from the TSA pure culture with an inoculating loop was smeared onto the reagent-saturated area of the filter paper. It was allowed and examined to observe for colour development within 2 - 3 minutes. In this spot test,

indole combined with the reagents in the filter paper matrix to produce a blue-to-blue-green colour change on the bacterial smear and adverse reactions remained colourless or light pink.

3.4.2 Oxidase Test

The oxidase test was carried out to detect the presence of a cytochrome oxidase or indophenol oxidase that will catalyze electrons between electron donors in the bacteria and a redox dye known as tetramethyl-p-phenylene-diamine. The dye would be reduced to deep purple colour if yielded to positive reactions.

Several reagents can be used for this study, but Kovacs oxidase reagent: 1% tetra-methyl-p-phenylenediamine dihydrochloride in water, was used. The filter paper was saturated with a Kovacs oxidase reagent solution, and a speck of the pure culture was smeared on it with a platinum loop. It was allowed and observed for colour development within 10 - 60 seconds. The appearance of a deep purple-blue/blue colour indicated oxidase production and the negative result was when no colour changed.

3.4.3 Catalase Test

This test was used to distinguish between bacteria that produce the catalase enzyme, such as Staphylococci, and bacteria that do not, such as Streptococci. Catalase catalyzes the breakdown of hydrogen peroxide (H2O2) to oxygen (O2) and water (H2O). In this test, 2mL of hydrogen peroxide solution was poured into a test tube, and some colonies of the test organism were picked and immersed into the H2O2 solution using a sterile glass rod. The bacteria that generated catalase

(positive result) produced gas bubbles (oxygen), but those that did not possess catalase enzyme had none (negative result).

3.4.4 Citrate Utilization Tests

The citrate utilization test is a part of the test used to differentiate organisms on their ability to utilize citrate as the primary energy source. A citrate test was performed to differentiate members of Enterobacteriaceae capable of fermenting citrate in the presence of the enzyme citrate. Simon's citrate agar contained citrate as significant energy and was prepared for inoculation on Petri dishes. Well-prepared and sterilized citrate agar plates were inoculated from the pure isolated culture by streaking the surface with a sterilized loop. The plates were then incubated at 37°C for 24 hours. There were changes in colour due to bacterial growth of the organisms on the medium due to citrate metabolism, which gave a positive citrate test. The shift in pH turns the bromothymol blue indicator in the medium from green to blue (positive result). A negative test was demonstrated with no growth, no colour change, or the colour of the medium remains green.

3.4.5 Urease test

The urease test is used to identify bacteria capable of producing the urease enzyme. The organisms that secrete urease can hydrolyze urea to ammonia and carbon dioxide. This test was used to distinguish urease-positive bacteria from other Enterobacteriaceae. The isolated pure bacteria were inoculated into well-prepared and autoclaved Christensen-modified urea broth and incubated for 24 hours at 37°C. Urease-positive cultures produced a pink colour due to a change in the indicator's

colour in the presence of ammonia, while the negative result remains no colour change or yellow-orange colour.

3.4.6 Mannitol Test

The mannitol test is selective (the presence of high salt concentration; sodium chloride inhibits most Gram-negative and Gram-positive bacteria) and differential test (the ability of the organism to ferment or not the mannitol). The ability to ferment mannitol induces acidification, changing the medium's coloration from red to yellow. A well-prepared mannitol salt agar was autoclaved at 121oC for 15 minutes, cooled and plated. Inoculated with pure isolates and incubated for 24 hours at 37oC. Growths were observed with yellow zones and yellow colonies as positive results. The negative result remained red-pink colour with growths.

3.4.7 Triple Sugar Iron (TSI) test

The Triple Sugar Iron (TSI) test is an ability to test an organism's capability to ferment sugars and to produce hydrogen sulphide (H2S) or gas (O2), or both. The test was used primarily to differentiate members of the Enterobacteriaceae family based on their sugar fermentation patterns and from other Gram-negative rods. An agar slant prepared of a TSI agar was used in carrying out this test in a sterile test tube at a slanted angle. The slanted medium was inoculated with TSA pure culture using a straight inoculation needle by stabbing first through the center to the bottom of the tube and streaking the agar slant's surface. After inoculations, the test tubes were covered with foil paper and left at an ambient temperature of 36°C to incubate for 24 hours. Reactions on test tubes were examined, and sugar fermentations were indicated by the production of H2S, gas and a change in colours from red (alkaline) to yellow (acid). When an alkaline/acid (red top/yellow bottom) slant reaction appeared, it only indicated dextrose (glucose) fermentation. When an acid/acid (yellow top/yellow bottom) slant

reaction appeared, it showed the fermentation of dextrose, lactose and/or sucrose. The appearance of an alkaline/alkaline (red top/red bottom) slant reaction represented the absence of sugar fermentation. The blackening of the medium in the slant indicated H2S production. Bubbles, cracks, or bottom-raised space in the slanted agar indicated gas production (formation of CO2 and H2).

3.5 Growth on Differential Media

3.5.1 Bacillus Cereus Agar Base

Bacillus cereus agar was used to identify and isolate Bacillus species and pathogenic Staphylococci species. Bacillus cereus agar helps to restrict the growth of Gram-negative bacteria, and this differentiating media allows the differentiation of Gram-positive Bacillus species. Autoclaved dissolved Bacillus cereus agar at 121oC for 15 minutes; then was allowed to cool and poured into Petri dishes. Isolated pure cultures were inoculated by streaking on the medium and incubated the plates at 37°C for 24 hours. Plates were examined and observed for typical growths by colony forms, colours and spore morphology.

3.5.2 Eosin Methylene Blue (EMB) agar

Eosin Methylene Blue (EMB) agar is a differential medium that inhibits the growth of Gram-positive bacteria and is used to indicate Gram-negative pathogenic enteric bacteria by distinguishing between organisms that ferment lactose and those that cannot cope with a colour indication. A sterile petri plate was prepared with EMB, which was autoclaved at 121°C for 15 minutes, allowed to cool and inoculated with pure inoculums by streaking. Inoculated plates were incubated at 37°C for 24 hours and examined plates for colonial morphological changes. Lactose fermenting bacteria produced dark

colonies with green metallic sheen or pink mucoid colonies (positive result), and lactose non-fermenters were colourless (negative result).

3.5.3 Bile Esculin Azide (BEA) agar

This is a selective and differential agar used to differentiate bacteria based on their ability to hydrolyze esculine in the presence of bile and azide. A sterile petri plate was prepared with BEA and autoclaved at 121degree Celsius for 15 minutes, allowed to cool and inoculated with pure inoculums by streaking. Inoculated plates were incubated at 37 degrees Celsius for 24 hours and plates were examined for colour changes. The enzyme esculinase produced by some bacteria can hydrolyze esculin into esculetin and glucose. Esculentin reacts with iron ions in the medium to produce a black precipitate which suggest the presence of esculinase-producing bacteria. This is used to identify members of the enterococcus genus which are capable of esculin hydrolysis.

3.5.4 MacConkey agar

This is also a selective and differential agar used to differentiate between lactose-fermenting and non-lactose fermenting bacteria. The MacConkey agar contains lactose and pH indicators. A sterile petri plate was prepared with MacConkey agar and autoclavedat 121 degree Celsius for 15 minutes, allowed to cool and inoculated with pure inoculums by streaking. Inoculated plates were incubated at 37 degrees Celsius for 24 hour and the plates were examined. Lactose-fermenting bacteria will produce acid during lactose fermentation which lowers the pH of the medium and causes colonies to appear pink or red indicating the presence of bacteria from the Enterobacteraceae family. Non-lactose

fermenting bacteria will not change the pH resulting in colourless or pale colonies suggesting the presence of many pseudomonas species.

3.6 Preparation of medium for for rhizobacterium potential of bacterial isolates

3.6.1 Preparation of Pikovskya's Agar for Phosphate Solubilization test

This was done by mixing 0.0057g of Pikovskya's agar in 160ml of water. This was sterilized using an autoclave after which it was allowed to solidify before been distributed on petri dish.

3.6.2 Preparation of Nitrogen free medium for Nitrogen Fixation test

The following salts were used for the preparation of the media for nitrogen fixation; sucrose 5g, K2 HPO4 0.25g, MgSO4 0.125g, NaCl 0.125g, FeCl3 0.025g, Na2MoO4.2H2O 0.00125g, CaCO3 0.5g, Agar 3.75g. This were all mixed in 250ml of distilled water and allowed to sterilize in an autoclave at 121 degree Celsius for 15 minutes after which it was allowed to cool before been poured in a petri dish.

3.7 Rhizobacterial potential of bacteria isolates

3.7.1 Screening for Nitrogen fixation activity: A day old culture of bacterial isolates grown on nutrient agar was streaked on a Jensen's Nitrogen free medium otherwise knows as NFM (formulated via the addition of: 20g/L sucrose, 1g/L K2HPO4, 0.5 g/L MgSO4.7H2O, 0.5 g/L NaCl, 0.1 g/L FeCl3, 0.005g/L Na2MoO4.2H2O, 2g/L CaCO3, 15g/L agar). Plates were incubated at 28°C for 1- 7 days. Growth on nitrogen deficient medium confirms the ability to fix nitrogen (Weselowski et al., 2016).

3.7.2 Screening for Phosphate Solubilization activity: The bacteria was cultured in replicates on prepared Pikovskya's agar (Micromaster) plates and incubated at 30°C for 3 days. A zone of clearing around the colonies after 1-3 days was scored as positive for phosphate solubilization. The diameter of the halozone and its bacterial colony from individual isolates was measured. The data obtained was used to calculate solubilization index (SI) using the formula below (Doilom et al., 2020).

3.7.2 Screening for Ammonia production: Freshly grown bacterial cultures were inoculated in 10 ml nutrient broth and incubated at 30°C for 48h in a rotator shaker. After incubation, 0.5 ml of Nessler's reagent was added to each tube. The development of a yellow to brown colour indicated a positive reaction for ammonia production (Kumar et al., 2012)

CHAPTER FOUR

4.0 RESULT

To obtain the heterotrophic bacteria cont, the volume of inoculum used was 0.1ml after a 100 fold dilution. The Agar used for this purpose was Nutrient Agar .

To obtain the coliform bacteria cont, the volume of inoculum used was 1ml after a 100 fold dilution was conducted. The Agar used for this purpose was Eosin Methylene blue Agar.

On Day 0, bacterial isolates with plant growth promoting rhizobacterium characteristics included *Pseudomonas aeruginosa*, *E. coli, Bacillus cereus*, *Bacillus subtilis*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Aeromonas hydrophila* and *Proteus vulgaris*.

After 14 days, the number of bacterial isolates with plant growth promoting rhizobacterium characteristics included *Pseudomonas aeruginosa*, *E. coli* and *Bacillus subtilis.*

Day 0 CONTROL

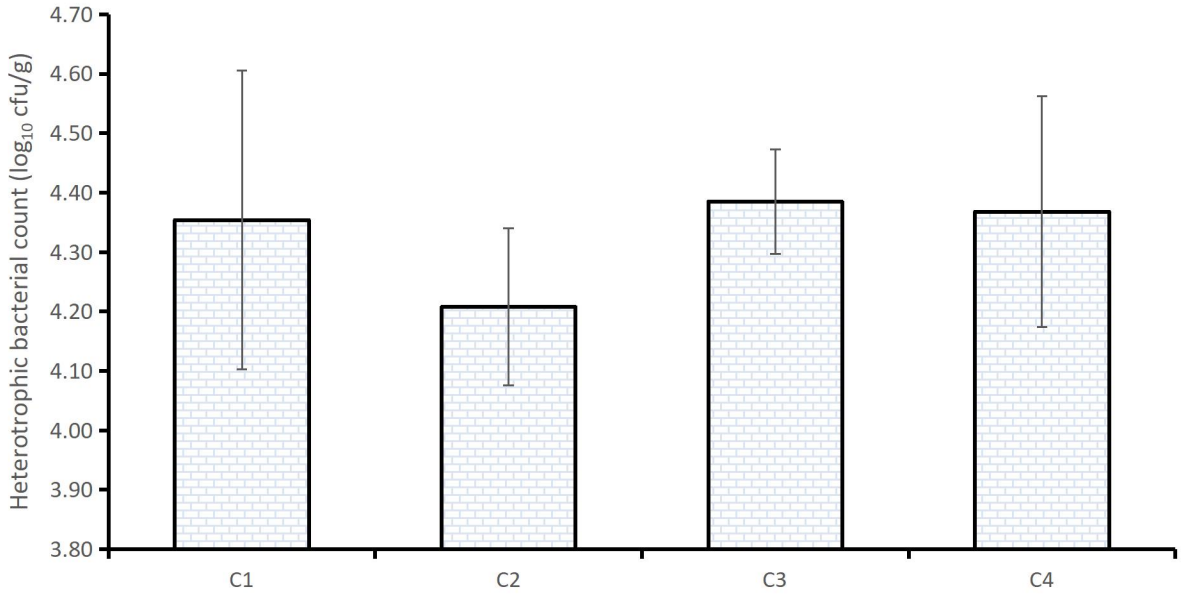

FIGURE 4.1 Heterotrophic bacterial count of isolates on control (Day 0)

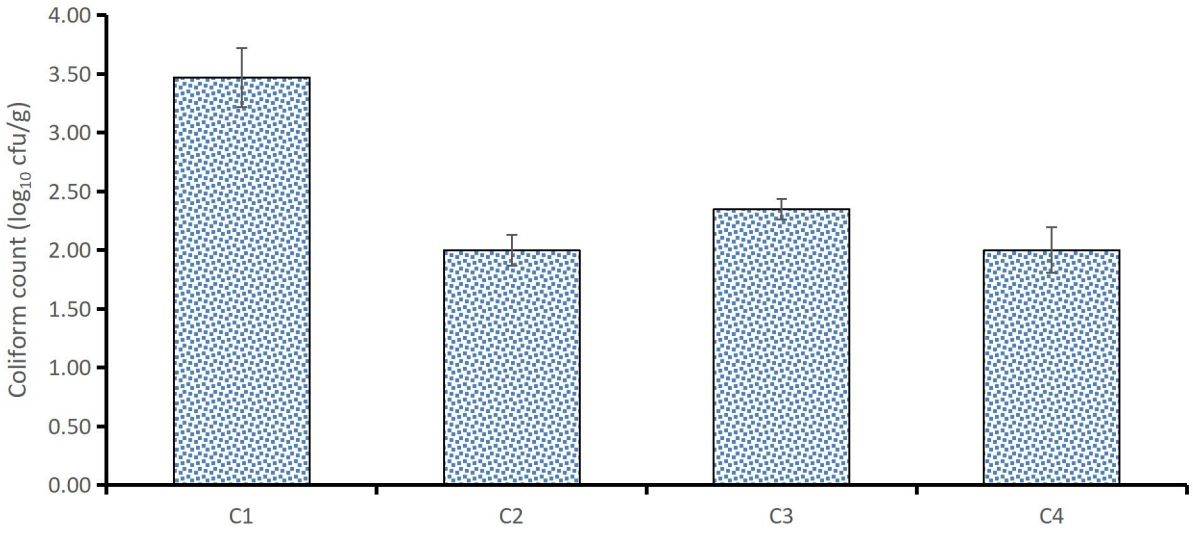

FIGURE 4.2 Coliform bacterial count of isolates on control (Day 0)

Table 4.1: Morphological and Biochemical Characteristics of Bacterial Isolates (Day 0)

Morphological								
Elevation	Raised	Flat	Flat	Flat	Flat	Flat	Raised	Flat
Margin	Entire	Undulate	Undulate	Undulate	Entire	Entire	smooth	Entire
Color	lemon	Cream	Cream	Cream	Cream	Cream	Cream	Cream
Shape	Circular	Irregular	Irregular	Irregular	Circular	Circular	concave	Circular
Size	Medium	Large	large	large	Small	Medium	small	Medium
Gr. diff. agar	PCA	EMB	BCA	BCA	EMB	SSA	MCC	SSA
Colour	green	green	Straw	Blue	Pink	black	cream	black
Staining								
Gram stain	-	-	+	+	-	-	-	-
cell type	rod	Rod	Rod	Rod	Rod	rod	coccobacilli	rod
Arrangement	disperse	disperse	disperse	disperse	disperse	disperse	single	disperse
Color	pink	pink	purple	purple	pink	pink	cream	pink
Spore staining	-	-	+	+	-	-	-	-
Biochemical								
KOH String Test	+	+	-	-	+	+	+	+

Catalase	+	+	+	+	+	+	+	+
Indole	-	+	-	-	-	-	+	+
Citrate	+	-	+	+	+	+	+	+
Oxidase	+	-	-	-	-	-	+	-
Motility	+	+	+	+	-	+	+	+
Urease	+	-	-	-	+	+	-	+
Glucose	-	+	+	+	+	+	+	+
Sucrose	-	-	+	+	+	-	-	-
Lactose	-	+	+	+	+	-	-	-
Mannitol	-	-	+	-	-	-	+	-
Gas formation	-	+	-	-	+	+	(-/+)	-
H_2S formation	-	-	-	-	-	+	-	+
TSI (Slant/Butt) reaction	K/K	A/AG	A/A	**A/AG**	**A/AG**	**K/AG H_2S**	K/A (G*)	**K/A H_2S**
Esculin Hydrolysis	-	-	-	-	+	+	+	+
Identity	*Pseudomonas aeruginosa*	*E. coli*	*Bacillus subtilis*	*Bacillus cereus*	*Klebsiella oxytoca*	*Proteus mirabilis*	*Aeromonas hydrophila*	*Proteus vulgaris*

TABLE 4.2: Plant Growth Promoting Rhizobium Characteristics of Bacterial Isolates (Day 0)

Isolates	NFM	Ammonia production	Phospate Solubilization
Pseudomonas aeruginosa	+	+	+
E. coli	+	-	+
Bacillus subtilis	+	+	+
Bacillus cereus	+	+	+
Klebsiella oxytoca	+	-	+
Proteus mirabilis	+	+	+

Aeromonas hydrophila	+	+	-
Proteus vulgaris	+	+	+

Day 14 CONTROL

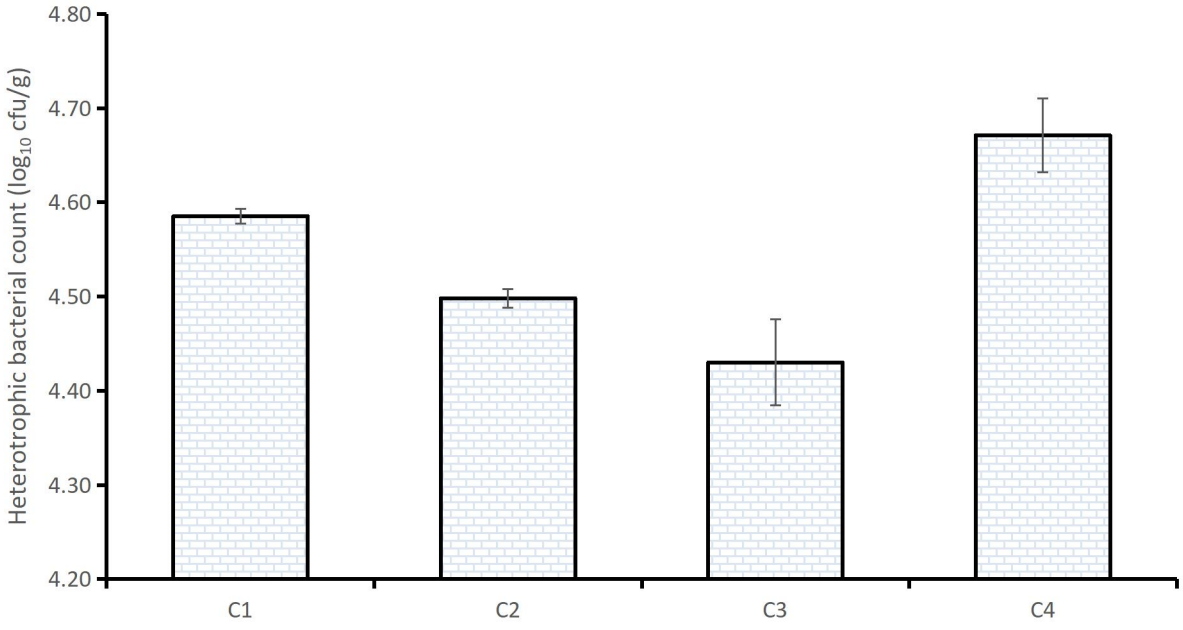

FIGURE 4.2 Heterotrophic bacterial count of isolates on control (Day 14)

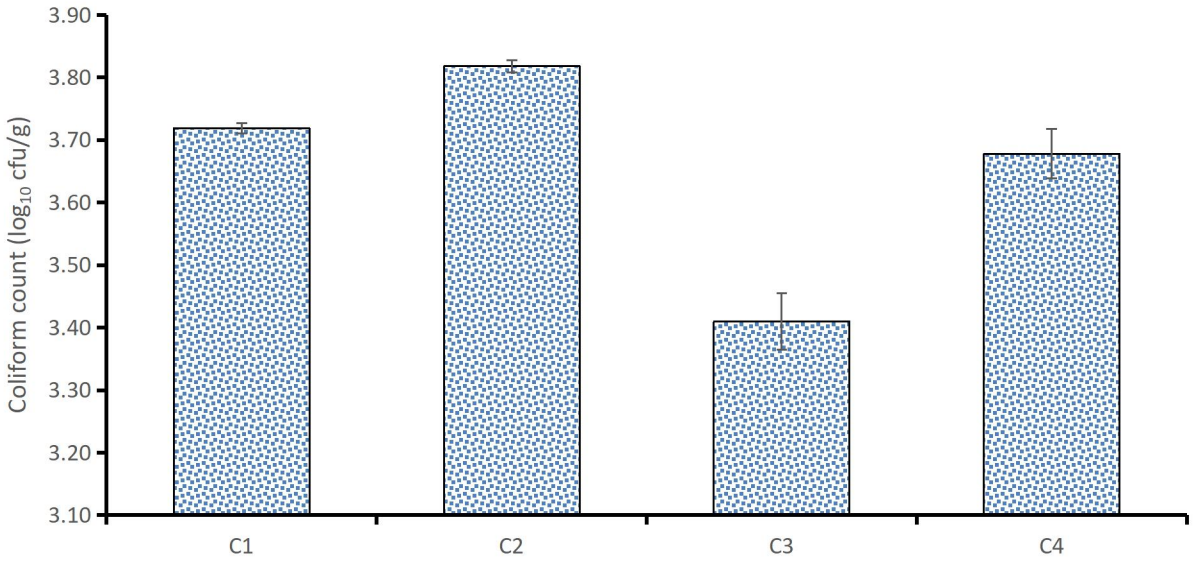

FIGURE 4.4 Coliform bacterial count of isolates on control (Day 14)

Day 14 Mixed

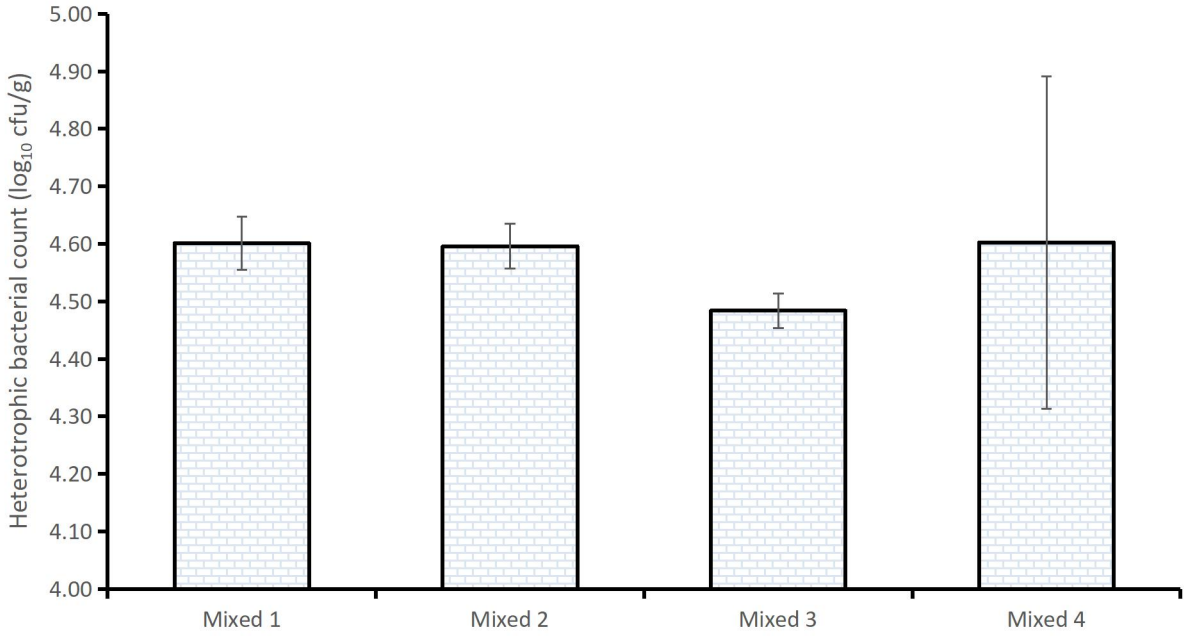

FIGURE 4.5 Heterotrophic bacterial count of isolates on mixed (Day 14)

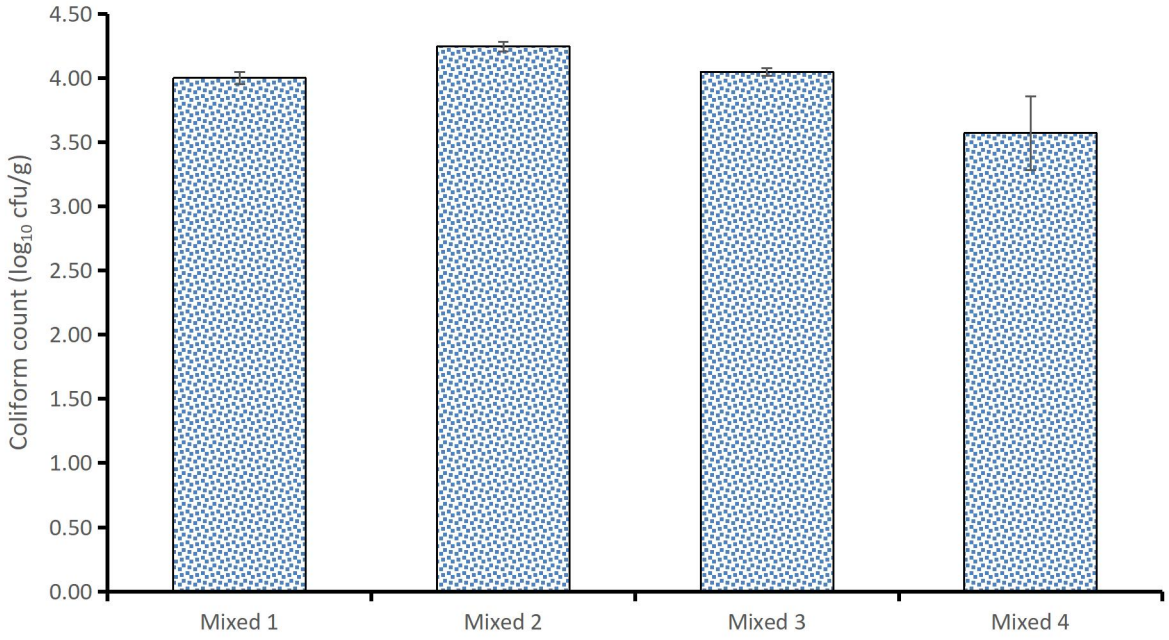

FIGURE 4.6 Coliform bacterial count on isolates on mixed (Day 14)

TABLE 4.3: Plant Growth Promoting Rhizobacterium Characteristics of Bacterial Isolates (after 14 days)

Isolates	NFM	Ammonia production	Phospate Solubilization
Pseudomonas aeruginosa	+	+	+
E. coli	+	-	+
Bacillus subtilis	+	+	+

Day 28 control

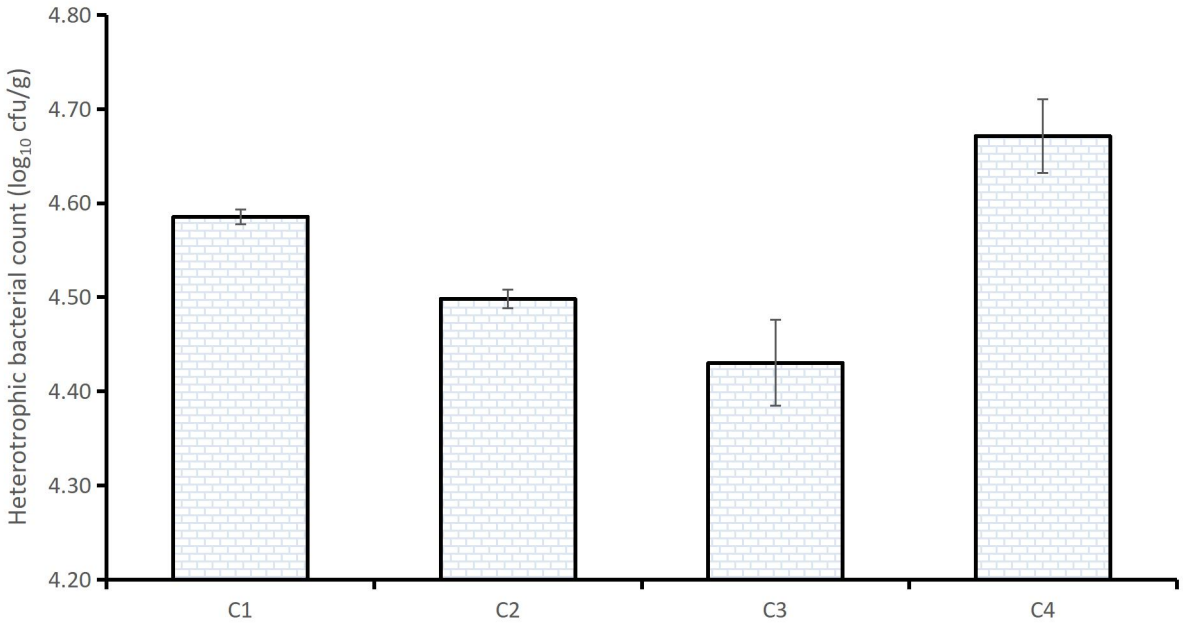

FIGURE 4.7 Heterotrophic bacterial count on isolates on control (Day 28)

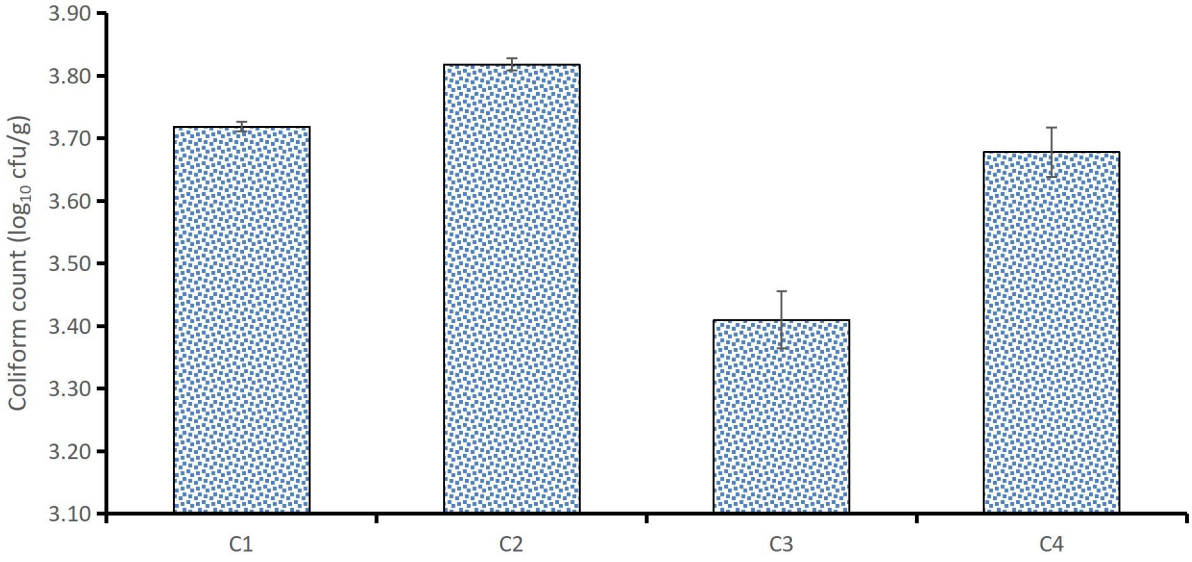

FIGURE 4.8 Coliform bacterial count on isolates on control (Day 28)

Points

CFU meaning Colony forming unit

g meaning grams

Day 28 mixed

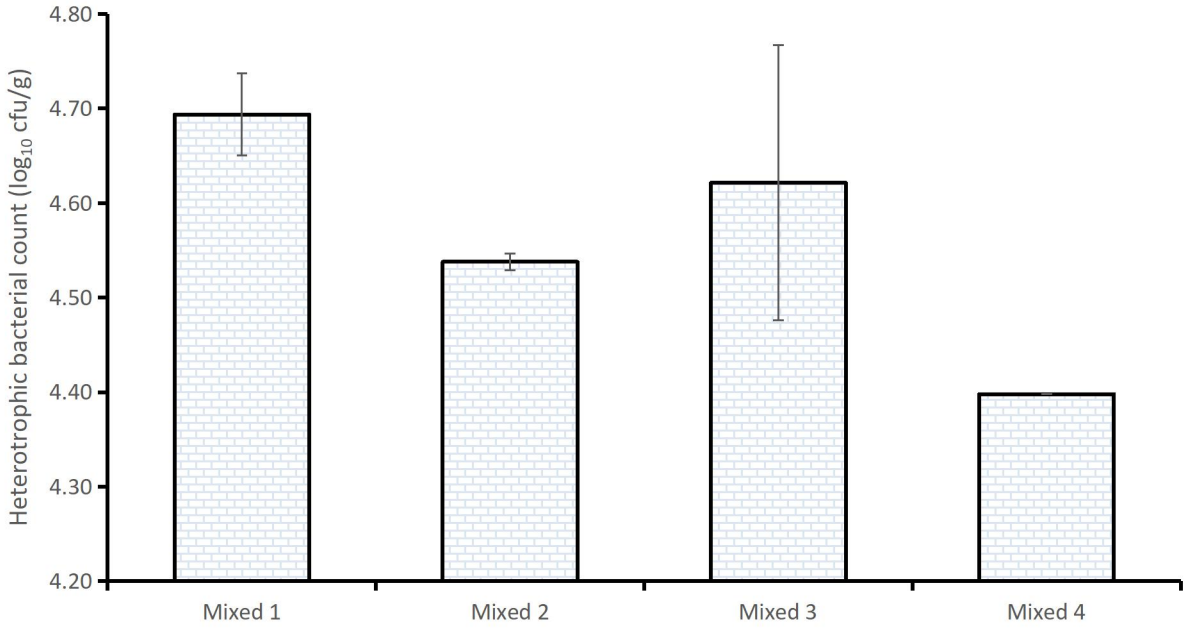

FIGURE 4.9 Heterotrophic bacterial count on isolates on mixed (Day 28)

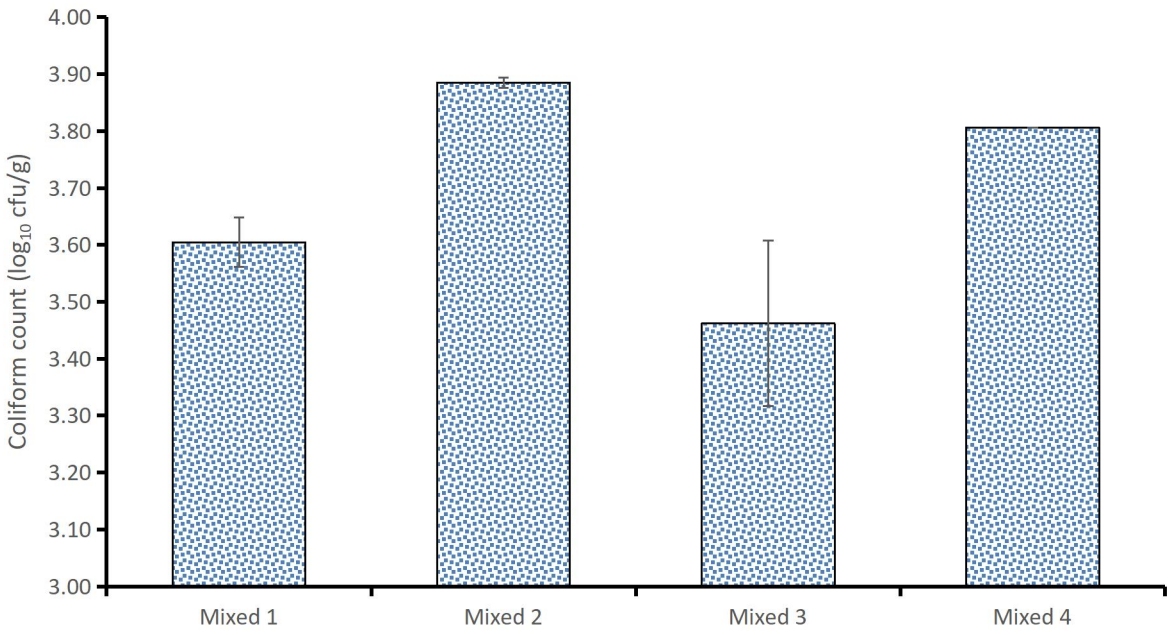

FIGURE 5.0 Coliform bacterial count on isolates on mixed (Day 28)

Isolates	NFM	Ammonia production	Phospate Solubilization
Pseudomonas aeruginosa	+	+	+
E. coli	+	-	+
Bacillus subtilis	+	+	+

TABLE 4.4 Plant growth promoting rhizobacterium characteristics of bacterial isolates

CHAPTER FIVE

5.1 DISCUSSION

PGPR colonize plant roots and exert beneficial effects on plant growth and development by a wide variety of mechanisms. To be an effective PGPR, bacteria must be able to colonize roots because bacteria need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects. The exact mechanism by which PGPR stimulate plant growth is not clearly known, although, several hypothesis such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved.

Soil samples were serially diluted and were cultured on general purpose agar and Eosin Methylene Blue. Gram staining was done to differentiate between Gram positive and Gram negative bacteria. Pottasium hydroxide string test was conducted. Biochemical tests such as citrate, Triple sugar iron test, Oxidase test and indole test were performed. Nitrogen fixation test was performed using Nitrogen free medium and plates with no growth indicated the inability of the bacterium to fix Nitrogen. Phosphate solubilization test was performed using Pikoskya agar. Tests for ammonia production was also conducted on the bacteria isolates. The total heterotrophic count showed an increase from 3.8×10^4+-707.11 to 4.9×10+-4949.75 and little or no effect on the PGPR ability of the isolated microorganisms such as nitrogen fixation and phosphate solubilization.

Phosphorus is one of the major nutrients, second only to nitrogen which is required by plants. Most of the phosphorus in the soil is present in the form of insoluble phosphates and cannot be utilized by the plants (Nautiyal, 1999). The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists as it can enhance the availability of phosphorus and iron for plant growth. In our experiment, six bacteria were found to be efficient phosphate solubilizers, and their phosphate solubilizing efficiency has been calculated and tested in plant growth. The exact mechanism by which PGPR stimulate plant growth is not clearly established, although several hypotheses such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization, and promotion of the mineral nutrient uptake are usually believed to be involved (Lalande et al., 1989; Liu et al., 1992; Glick, 1995; Bowen and Rovira, 1999). Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants (Pradhan and Sukla, 2006). The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists as it can enhance the availability of phosphorus and iron for plant growth. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to rice that represent a possible mechanism of plant growth promotion under field conditions (Verma et al., 2001). In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate-solubilizing bacteria is commonly found in the rhizosphere (Raghu and MacRae, 1966).

It is important to note that several phosphate-solubilizing bacilli occur in soil (Skrary and Cameron, 1998) but their numbers are not usually high enough to compete with other bacteria commonly established in the rhizosphere (Lifshitz et al., 1987). A large body of evidence suggests that PGPR enhance the growth, seed emergence and crop yield, and contribute to the

protection of plants against certain pathogens and pests (Dey et al., 2004; Kloepper et al., 2004; Kokalis-Burelle et al., 2006; Herman et al., 2008; Minorsky, 2008).

Conclusion

This study showed that there was an increase in the heterotrophic count of the isolated microorganisms due to the combination of haloxyfop R mehyl and Dichlorvos. The combination of these chemicals also had little or no effect on the ammonia production and phosphate solubilization potential of the bacteria isolates. They also resulted in the reduction of bacteria isolates with plant growth promoting rhizobacterium characteristics. Chemicals that have little or no effect on the PGPR ability of microorganisms should be used on farmlands.

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APPENDIX

Sample 1

Day 0

TABLE 1: Colony count

ISOLATES	NA	EMB
	A	B
C1 A	66	95
B	46	95
C2 A	26	29
B	36	_
C3 A	18	43
B	35	_
C4 A	89	13
B	96	_

TABLE 2: Differentials

Isolate	**MAC**	**BCA**	**BAA**
C1A1	Cream	Cream	+
C1A2	Cream	Cream	+
C1A3	Cream	White	+

C1A4	Cream	Yellow	+
C2A1	Pale pink	Yellow	+
C2A2	Cream	Cream	-
C3A1	Cream	Milky cream	+
C3A2	Cream	Cream	-
C3A3	Pink	Cream	-
C1B1	Red	Red	+
C1B2	Red	Red	+
C1B3	Pale pink	Milky white	-
C4B1	Cream	Cream	-
C4B2	Cream	Opaque	-
C4B3	Cream	Cream	

TABLE 3: TSI RESULTS

Isolate	**MAC**	**BAA**	**Citrate**	**PKA**	**Slant**	**Butt**	**Gas**	**H2S**
C1A1	Pale pink	+	+	2.5mm	K	A	-	+

C1A2	Pale pink	+	+	2.5mm	A	A	+	-
C1A3	Pale pink	+	+	6mm	A	A	+	-
C1A4	Pale pink	-	-	4.67mm	A	A	+	-
C2A2	Pale pink	-	-	No zone	A	A	-	+
C2A1	Pale pink	+	+	4mm	A	A	+	+
C3A2	Pale pink	-	-	No zone	K	A	-	+
C3A3	Pink	+	-	No zone	K	A	-	+
C4B1	Pink	-	-	6mm	K	A	-	+
C4B3	Pink	+	+	2.5mm	K	A	-	+

TABLE 4: NFM RESULTS

Isolates	**NFM**
C1A1	+
C1A2	+
C1A3	+
C2A1	+
C2A2	+
C1A4	+
C3A2	+
C3A3	+

C4B1	+
C4B3	+
S2B1	+
M3A3	-
M4A1	-
EMB	
C1B	+
C2B2	+
H2A1	+

TABLE: COLONY COUNT Day 14 Control

NA	34	15	13	20	21	28	32	17
EMB	25	35	1	1	5	1	1	1

A. Morphological Characteristics

	HSM	**CHSM**	**CSHM**	**HSM**	**CHSM**	**C**	**C**
Elevation	Raised	Flat	Flat	Flat	Flat	Flat	Raised
Margin	Entire	Undulate	Undulate	Undulate	Entire	Entire	smooth
Color	lemon	Cream	Cream	Cream	Cream	Cream	Cream
Shape	Circular	Irregular	Irregular	Irregular	Circular	Circular	concave

Size	Medium	Large	large	large	Small	Medium	small
Gr. diff. agar	PCA	EMB	BCA	BCA	EMB	SSA	MCC
Colour	green	green	Straw	Blue	Pink	black	cream
Staining							
Gram stain	-	-	+	+	-	-	-
cell type	rod	Rod	Rod	Rod	Rod	rod	coccobacilli
Arrangement	disperse	disperse	disperse	disperse	disperse	disperse	single
Color	pink	pink	purple	purple	pink	pink	cream
Spore staining	-	-	+	+	-	-	-
KOH String Test	+	+	-	-	+	+	+
Catalase	+	+	+	+	+	+	+
Indole	-	+	-	-	-	-	+
Citrate	+	-	+	+	+	+	+
Oxidase	+	-	-	-	-	-	+
Motility	+	+	+	+	-	+	+
Urease	+	-	-	-	+	+	-
Glucose	-	+	+	+	+	+	+
Sucrose	-	-	+	+	+	-	-
Lactose	-	+	+	+	+	-	-
Mannitol	-	-	+	-	-	-	+
Gas formation	-	+	-	-	+	+	(-/+)
H2S formation	-	-	-	-	-	+	-
TSI (Slant/Butt) reaction	K/K	A/AG	A/A	A/AG	A/AG	K/AG H2S	K/A (G*)
Esculin Hydrolysis	-	-	-	-	+	+	+
Identity	Pseudomonas aeruginosa	E. coli	Bacillus subtilis	Bacillus cereus	Klebsiella oxytoca	Proteus mirabilis	Aeromonas hydrophila

TABLE: COLONY COUNT Day 14 Mixed

NA	37	43	42	37	32	29	64	25
EMB	120	84	172	181	88	142	34	41

MORPHOLOGICAL CHARACTERISTICS

Elevation	Raised	Flat	Flat
Margin	Entire	Undulate	Undulate
Color	lemon	Cream	Cream
Shape	Circular	Irregular	Irregular
Size	Medium	Large	large
Gr. diff. agar	PCA	EMB	BCA
Colour	green	green	Straw
Staining			
Gram stain	-	-	+
cell type	rod	Rod	Rod
Arrangement	disperse	disperse	disperse
Color	pink	pink	purple
Spore staining	-	-	+
KOH String Test	+	+	-
Catalase	+	+	+
Indole	-	+	-
Citrate	+	-	+
Oxidase	+	-	-
Motility	+	+	+
Urease	+	-	-
Glucose	-	+	+
Sucrose	-	-	+
Lactose	-	+	+
Mannitol	-	-	+
Gas formation	-	+	-
H2S formation	-	-	-
TSI (Slant/Butt) reaction	*K/K*	*A/AG*	*A/A*
Esculin Hydrolysis	-	-	-
Identity	*Pseudomonas aeruginosa*	*E. coli*	*Bacillus subtilis*

TABLE: COLONY COUNT Day 28 Mixed

NA	25	53	42	44	54	36	33	43
EMB	105	83	81	79	52	103	31	27

TABLE: Identity of Bacteria Isolates

Morphological			
Elevation	Raised	Flat	Flat
Margin	Entire	Undulate	Undulate
Color	lemon	Cream	Cream
Shape	Circular	Irregular	Irregular
Size	Medium	Large	large
Gr. diff. agar	PCA	EMB	BCA
Colour	green	green	Straw
Staining			
Gram stain	-	-	+
cell type	rod	Rod	Rod
Arrangement	disperse	disperse	disperse
Color	pink	pink	purple
Spore staining	-	-	+
Biochemical			
KOH String Test	+	+	-
Catalase	+	+	+
Indole	-	+	-
Citrate	+	-	+
Oxidase	+	-	-
Motility	+	+	+
Urease	+	-	-
Glucose	-	+	+
Sucrose	-	-	+
Lactose	-	+	+
Mannitol	-	-	+
Gas formation	-	+	-
H_2S formation	-	-	-
TSI (Slant/Butt) reaction	K/K	A/AG	A/A

Esculin Hydrolysis	-	-	-
Identity	*Pseudomonas aeruginosa*	*E. coli*	*Bacillus subtilis*