DETECTING TOLERANCE OF *Bacillus Subtilis* TO THE COMMERCIALLY USED AGRICULTURAL PESTICIDES

A

Dissertation

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By

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This is to certify that **Ms. Saraswati Yonghang** has completed this project work entitled **"Detecting tolerance of *Bacillus subtilis* to the commercially used agricultural pesticides"** as a part of partial fulfillment of the requirements of Master's degree in Microbiology under my supervision. To my knowledge, this work has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

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ABSTRACT

Bacillus subtilis is an aerobic, spore forming, rod shaped, Gram positive soil bacterium. It is mostly found in soil and vegetation with an optimal growth temperature from 25-35℃. They produce endospores that allow the survival of extreme environmental conditions including heat and desiccation. Pesticides are substances that are meant to control pests. The term pesticide includes herbicides, insecticides, fungicides etc. Most pesticides are intended to serve as plant protection products which in general, protect plants from weeds, fungi, or insects. Because of extended persistence of fungicides, insecticides and herbicides in soil, slow rate of decomposition most of the pesticide residue remain in soil. It is desirable that actual or potential effect upon the soil microflora to be investigated. Hence, main motive of this research was isolation and biochemical characterization of *Bacillus subtilis* having Plant Growth Promoting Rhizobacterial (PGPR) characteristics from agricultural soil and detecting its tolerance on different pesticides. The bacterial strain was obtained with positive results of Gram staining, endospore staining, catalase degradation, citrate utilization, motility test, VP, starch hydrolysis, gelatin hydrolysis test. The growth promoting activity of bacterial strain was determined by Indole Acetic Acid production test, showed positive test. It was found that *Bacillus subtilis* was able to tolerate all the pesticide except Mancozeb where it did not show any growth in in-vitro examination. Enumeration of cfu/g soil by periodical interval by serial dilution method (10^8cfu/g) showed that all *Bacillus* isolates were able to grow first 45 day time interval. The number got increased and become higher at the interval of 90 day of inoculation then bacterial number got decreased at 135 day of inoculation. All soil inoculated bacterium with pesticide showed tolerance and gave positive growth in its number. The result of the study showed that the *Bacillus* spp are very good plant growth promoting agent with great potentiality to grow on pesticide polluted soil.

Key words: *Bacillus subtilis,* PGPR, pesticide, tolerance, DAI

LIST OF ABBREVIATIONS

NA	Nutrient Agar
NB	Nutrient Broth
PGPR	Plant Growth Promoting Rhizobacteria
CFU	Colony forming unit
HCN	Hydrogen Cyanide
MIC	Minimum Inhibitory Concentration
B. subtilis	*Bacillus subtilis*
IAA	Indole Acetic Acid
VOC	Volatile Organic Compound
g	gram
ml	milliliter
DAI	Day after inoculation
ANOVA	Analysis of Variance
Sig	Significant

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1 Background

Soil microorganisms have an important role in maintaining biogeochemical cycling, optimizing soil nutrient level for balancing soil fertility required for better plant diversity (Fitzsimons and Miller, 2010). Plant associated *Bacillus* species such as *B. subtilis* are crucial member of microbiome (Fierer, 2017). It is used as a biocontrol agent throughout the previous decade in the alternative to different chemically formulated pesticide (Fira et al, 2018; Köhl et al., 2019). These bacilli live in the rhizosphere of plant having special capacity to synthesize volatile organic substances and shorts of secondary metabolites which are responsible for plant protection as well as competing for ecological niches with that plant pathogens (Caulier et al, 2019; Kai, 2020). Plant phytopathogens are minimized with the biocontrol activity of *Bacillus* species by providing protection against plant diseases. Some of the antimicrobial secretion of *Bacillus* species includes; antibiotics, bacilysin, mycobacillin, bacillomycin, mycosubtilin, iturin, fengycin, and surfactin that has antifungal and antibacterial drug activity (Ntushelo et al, 2019).

Nowadays, concerned has shifted towards aerobic, Gram positive *Bacillus* species. One of them is *Bacillus subtilis* considering as a crucial biocontrol agent (Moszer, 1998). *Bacillus subtilis* keeps steady touch with the higher plants and aids in the development of those plants. In addition *Bacillus subtilis* has capacity to produce endospore and various antibiotics with broad spectrum activity. Hence it is unquestionably a valuable biocontrol agent. Biological management of plants by microbes might be more promising strategy than the extended use of pesticide which are costly and have harmful effect on plant as well as human being due to its prolonged accumulation. These formulations even are lethal to beneficial soil organisms (Leroux, 2003).

To maintain soil's fertility, Plant Growth Promoting Rhizobacteria takes part in various nutrients cycling process (Barea et al, 2004, Vikram and

Hamzeharghani, 2008). They facilitates plant growth by solubilizing insoluble phosphate, fixing atmospheric nitrogen and delivering it to plants and generating siderophore and phytohormones (Yadav et al, 2011, Zaidi et al, 2009). Due to the sensitivity of soil microflora to the changing surrounding, severe degradation of the microbial community could result from the perturbation of total microbial biomass and their unique composition (Lin et al, 2007).

Biopesticides currently account for 2.5% of the chemical market, which nevertheless represents a significant industry given that global chemical sales in 2005 were 267 billion bucks. Biopesticides have attached huge attention over the year with a market share of just o.2% in 2000 and anticipated 15% yearly increase. Additionally, since 2000, conventional pesticides have been steadily losing ground, with a predicted drop rate of 1.5% per year (Thakore, 2006).

Utilization of pesticides regarded as an essential component of modern agriculture for management of crop damaging pests that consequently results in a significant loses the amount of food production. However, prolonged pesticide usage with obstruction habits and failure to take fundamental precaution, can significantly increase the likelihood of accidental intoxication (Ntow et al., 2009, Paez et al, 2011). The greatest dangers of acute poisoning come from certain occupations like farming or pest control, whilst the populations as a whole faces a latent threat from their food chain (Ospina et al., 2009; Thundiyil et al, 2008). The estimated value of pesticide applied globally is 4 million tons (Elersek and Filipic, 2011). There are about 1800 million people working as farmer globally, and it has been estimated that twenty five million agricultural workers have non-intentional intoxication once a year (Alavanja, 2008). It results up to 20000 annual deaths and one million case of poisoning in underdeveloped nations (Duran-Nah & Colli-Quintal, 2000).

Optimum food supply of currently expanding global population at a rate of 1.05% per year is a significant issue for global agriculture (World Population Prospect, 2019). The development of chemical industry throughout the course

of 20th century results the number of exceedingly toxic molecules that misbalanced the ecosystem. Environmental contamination is inevitably exposed to human population through air, water, soil, and food (Gomez et al, 2011).

Soil microbes play an important role in soil fertility regulation, nitrogen cycling, plant diversity maintenance, and bioremediation of resistant contaminants (Fitzsimons and Miller, 2010). Plant-associated Bacillus species, such as B. subtilis, are important members of the microbiome (Fierer, 2017). Their potential use as biocontrol agents, which must be plant pathogens, has been highlighted in recent decades, making them a promising alternative to chemical pesticides (Fira et al, 2018; Köhl et al, 2019). Use of agricultural chemicals has harmed beneficial microorganisms and their physiological activities, which affect soil fertility (Wani et al., 2005; Sriniwas et al, 2008; Ahmad & Khan, 2010), which influences plant growth (Ahemad & Khan, 2010a).

According to FAO, about 66% people in Nepal are directly engaged in farming. The conventional farming pattern here includes use of chemical pesticide due to its rapid action. Nonetheless, it leads to a slew of issues relating to pest resistance to the active ingredients in such chemical protection products. As a result, more chemical control tools have been developed, which are neither environmentally nor economically favorable. It also disrupts the ecological equilibrium and has a negative influence on human health. These worrying elements prompted the hunt for novel, alternative pest management approaches for domesticated plants. Tolerance to pesticides of chosen biocontrol agents has been used as a fundamental component of integrated disease management (IDM). Combining pesticide-tolerant biocontrol agents with pesticides increased disease control while lowering the amount of fungicides needed for efficient disease management (Frances et al, 2002; Buck, 2004). When compared to separate components of disease management, the combined use of a biocontrol agent and a chemical fungicide proved successful against damping-off of tomato (Kondoh et al, 2001), Rhizoctonia root rot, and take-all of spring wheat (Duffy, 2000).

Because of its favorable impacts on soil and crop production, the use of naturally occurring plant growth promoting rhizobacteria (PGPR) improves plant growth while also assisting plants in coping with biotic and abiotic stresses. PGPR are types of biocontrol that can be used to substitute conventional pesticides and thereby reduce the amount of undesired chemicals residues in agriculture. Plant growth promotion, disease suppression and rhizosphere capabilities by PGPR, particularly bacilli, are all considered crucial needs for the creation of commercial products. Bacillus subtilis is a beneficial PGPR that promotes agricultural plant growth and is employed as a bioagent. It is a low-cost, environmentally friendly, and soil-dwelling organism that plays a significant role in integrated disease control. *Bacillus subtilis* is a good antagonist because it lacks inhibitory enzymes. It defends the plant against pathogens that live in the soil, such as *Fusarium*, *Phythium*, *Rhizoctonia. Bioresources* etc to replace artificial insecticides. Plant growth-promoting rhizobacteria are frequently innovative and potentially beneficial technologies in this area (Erhan & Nedim, 2006).

Today's agricultural scenario based up on the importance of integrated disease management and crop protection. Biocontrol agent may be an effective alternative for disease management instead of conventionally used chemical pesticides. Pesticides treatment including fungicides, insecticides, herbicides and antibiotics, by spraying, drenching and soil application may control these pathogen but these are not economical and cause hazardous effect on human health as well as increase environmental pollution. Prolong use of insecticides may lead to development of resistance in the target organism hence use of biocontrol agent may be an effective alternative for disease management. They are ecofriendly and do not induce resistance in pathogen as chemicals do (Sangeetha, 2009).

For instant due to extensive use of agricultural chemicals these beneficial microorganism and there physiological activities important to soil fertility adversely affected and also influenced on the plant growth. So it is important to study the tolerance of biocontrol agent on different concentration of commercially available pesticides in order to make them safe use for soil inhabiting beneficial microflora.

1.2 Objectives

General objective

To study tolerance of *Bacillus subtilis* to commercially used agricultural pesticides

Specific objectives

- To isolate and screen *Bacillus* spp from soil.
- To characterized *Bacillus subtilis* biochemically.
- To identify PGPR characteristics of *B. subtilis*
- To determine tolerance of *B. subtilis* towards different pesticides

CHAPTER II

LITERATURE REVIEW

2.1 Pesticides

Chemical known as pesticides are employed in agricultural, home, and institutional settings to eradicate plant pests such as, insects, and vermin. Herbicides, insecticides, fungicides, fumigants, and rodenticides are the primary categories of pesticides that are often utilized. Insecticides including organochlorines, organophosphates, and carbamates are of particular concern due to their toxicity and environmental durability. Most affluent nations have outlawed the use of organochlorine pesticides in agriculture and homes, while developing nations like Nepal continue to use them. Most pesticides have a broad range of activity and kill both species that are targets and those that are not. The majority of farmers are ignorant of the many types of pesticides, the degree of poisoning, safety measures, and possible risks to human health and the environment (Yassin et al, 2002).

Any agent intended to eradicate, deter, or regulate specific plant or animal life forms that are regarded as pests is known as a pesticide (NIEHS, 2019). Pesticide is the collaborative form of insecticide, herbicide, fungicide, rodenticide, molluscicides, nematicides, miticides, avicides, etc. The first known insecticide was sulfur, whose dust is said to have been utilized in ancient Mesopotamia 4500 years ago. Poisonous plants are used for pest management, according to the about 4,000-year-old Rig Veda (Rao et al, 2007).

Dichloro-Diphenyl-Trichloroethane (DDT), a commonly used chemical insecticide, had its first significant use in 1956. The 1950s saw the development of a wide range of further organochlorines, followed by the 1960s by organophosphates, the 1970s by carbamates, and the 1980s by synthetic pyrethroids. In recent, the nation did not make synthetic pesticides; instead, the active component was imported. India, China, Thailand, and Japan

produce the majority of the pesticides (Neupane, 2001; Winrock International, 2014).

Pesticides containing organochlorines have lengthy residual effects and can sustain in the environment for a very long time without losing their toxicity. Numerous organochlorine insecticides and their metabolites are extremely toxic and have been linked to a variety of harmful health outcomes, including cancer, neurological damage, abnormalities of the reproductive system, birth defects, and immune system damage (Agbeve et al, 2014 & Leena et al, 2012). These pesticides are volatile and can spread to far places (including aquatic bodies) where they have not been utilized via air drift and surface runoff (Kuranchie-Mensah et al, 2012). Many Organochlorine pesticides have been outlawed or had their usage restricted due to the negative effects connected with their use (Botwe et al., 2012)

Researchers have already identified a number of pesticide-related environmental concerns, including decreased biodiversity, nitrate leaching, decreased soil fertility, weed species that are resistant to common weedicides, increased costs for prevention and treatment that endanger human health, and acidification (Vema et al, 2013). The breakdown of xenobiotic substances can be accomplished by a variety of physical and chemical techniques; however these techniques are both expensive and ineffective. Numerous physical and chemical techniques are being looked at for xenobiotic compound degradation, however they are expensive and insufficient (Gangola et al, 2018). Therefore, the optimum option for environmental remediation would be biological approaches for degradation of xenobiotic chemicals from the contaminated locations by utilizing microbial metabolism. The benefits of employing this alternative remedial technique (bioremediation) are that it is extremely affordable, least dangerous, adaptable, and environmentally benign (Zang et al, 2020). Pesticides and other harmful pollutants are naturally broken down by bacteria without any expense or secondary contamination. Therefore, with a comprehensive grasp of their degradation mechanisms, researchers have carried out excellent investigations on the biodegradation of harmful pollutants. Researchers have isolated a variety of bacteria and fungi that are

effective at converting pesticides into simple and non-toxic forms (Akbar & Sultan, 2016).

2.1.1 Herbicides and its mode of action

The management of undesirable plants, or weeds, is done with the help of substances known as pesticides, also known as weed killers. Because they have the capacity to eradicate all plant material to which they are exposed, non-selective herbicides, sometimes referred to as broad spectrum herbicides in the market, can be used to clear waste ground, industrial and construction sites, railroads, and railway embankments. Herbicides that are selective in their control of weed species do so while causing little to no damage to the targeted crop. Additional key distinctions between selective and non-selective organisms are persistence, means of uptake (whether it is absorbed by only above-ground foliage, through the roots, or by other mechanisms), and mechanism of action (how it works). Herbicides were once made from substances like table salt and other metal salts. However, due to their adverse effects on human health, these products have steadily lost popularity and are now banned in certain countries. Herbicides have also been applied during hostilities and war (EPA, 2011).

Herbicides made up the largest share of the roughly $24.7 billion in global pesticide sales in 2012, accounting for over 44% of total sales, followed by insecticides, fungicides, and fumigants (Atwood et al, 2017). Herbicide is also used in forestry where it has been discovered that certain formulations can inhibit hardwood species in favor of conifers following clear-cutting pasture management, and the management of areas designated as wildlife habitat (Paul et al, 2007).

Herbicides are frequently categorized according to where they work. In general, plants that are vulnerable to the effects of a certain herbicide will exhibit comparable symptoms. Herbicide resistance management may be managed more efficiently if classification is based on the herbicide's site of action. If herbicide is neutralized sooner after its application, it is said as low residual activity. It might be due to effect of rainfall or microbial interaction

on soil. On the other hand strong residual activities are those having long persistency on soil (Vats, 2015).

Herbicides work by preventing, interfering with, disrupting, or reducing normal plant growth. A class of plant growth regulators known as synthetic auxins includes 2,4-D. It enters the plant through the leaves and is transported throughout. Stem curling, wilting leaves, and eventually plant death result from unchecked, unsustainable growth. A selective herbicide is pendimethaline. It acts as an inhibitor of plant cell division and cell elongation, which is how it works. This indicates that it stops the target weed's root and shoot from growing. The weed eventually perishes because it is unable to develop further (Green et al., 2011).

2.1.2 Fungicides and its mode of action

In areas having higher temperature than normal temperature of the environment, fungal crop loss has a particularly severe impact. The destruction caused by fungi happens in two stages: first, on the plant growing in the field, and then, postharvest loss, when they are being kept for onward transit. The growth of mold like tiny airborne infections on cooked food is the third form of contamination, which results in food degradation. Scientists have devised methods to reduce the loss of fungal food at every step. Nearly 25% of agricultural food products are useless owing to fungal infection, according to a gross yearly estimate (Pittet, 1998). The main problems with fugal crop loss include degradation brought on by a rise in fatty acid conditions, changes in the color and texture of food products, inadequate nutrition, and low germination of stored seeds (Dhingra et al, 2001).

Sometimes more than one host is required for a fungal pathogen to complete its life span process and cause illness (*Puccinia graminis var. tritici*, causative agent of black stem rust of wheat that requires *Berberis aristata* for completion of infection other than there main target wheat plant). Physical controls, such as the elimination of other than primary hosts and the incarceration of livestock and field remains, are thus the main strategies used by farmers to produce crops free from illness. Therefore, preserving sustainability and reducing pathogenic infection are key components of the

deep ecological movement for crop care. There have been instances of antibiotics used in agriculture becoming resistant to popular and extensively used ones (Dayan et al, 2009).

Inhibiting respiration and sterol biosynthesis are two common ways that fungicides work. Adenosine triphosphate synthesis, respiration, and lipid metabolism are all disrupted by mancozeb's interaction with sulfhydryl group amino acids and enzymes in fungal cells. Broad spectrum properties are present in another fungicide, carbendazim. It suppresses the assembly of fungi's microtubules and appears to bind to an unidentified location on tubulin (Tomlin, 2003).

1.2.3 Insecticides and its mode of action

The term "insecticide" refers to substances or techniques of chemical or biological origin that significantly manage or control insects. Control may be achieved by killing the insect or in some other way stopping it from acting in ways that are seen to be detrimental. Our range of insecticides before the start of World War II (1940) was constrained to a few arsenicals, petroleum oils, nicotine, pyrethrum, rotenone, sulfur, hydrogen cyanide gas, and cryolite. With the development of synthetic organic pesticides after World War II and Paul Muller's discovery of the insecticidal properties of DDT, a significant change in pest control happened during this time (1940). The impetus created by these inventories fueled more efforts to create synthetic pesticides throughout the course of the next 20 years. Following that, the age of organophosphates, carbamates, and pyrethroids began and were marketed in the pest control industry. Organochlorines' tenacity led to their widespread dispersion and aggregation in the biota, which had detrimental effects. In 1962, Rachel Carson's book "Silent Spring" sparked widespread awareness about the negative effects of pesticides (Gour et al, 2012).

Insecticides of the agricultural instruments more frequently linked to environmental hazards, even if other features of contemporary agriculture frequently have a bigger impact. They may have fatal or mild effects on non-target creatures (such as organisms that recycle soil nutrients, pollinate crops, and feed on pest species), as well as diminish and/or pollute food sources for

organisms at higher trophic levels. Their stated objective is to kill pests. Most pesticide poisoning incidents involving non-target creatures, especially those involving unfamiliar or uninteresting species in underdeveloped nations, are likely to go unreported. Direct effect or sublethal effects that appear as decreases in lifespan, rate of development, fertility, fecundity, sex ratio, and behavior may cause changes at the population level (e.g., feeding, foraging and reproduction). There is a plenty of resources that lists these impacts (Stark and Banks, 2003).

The majority of poisoning incidents are unintentional, but occasionally, pesticides are administered in ways that would unavoidably cause extensive harm to non-target species. The main method of eradicating the red-billed quelea (*Quelea quelea*), a serious pest of paddy in semi-arid sub-Saharan Africa, is to spray it with the organophosphate fenthion. As a consequence of being directly appied in the form of spay and from consuming damaged corpses, which may be discovered up to twenty kilometers or more from the applied locations, birds of prey, perching and songbirds (passerines) are recognized as common victims. Arthropods that live on land are also negatively impacted (McWilliam & Cheke, 2004).

Many insecticide act upon the insects nervous system, while other act as growth regulators or endotoxins. Emamactin benzoate is a brand-new pesticide with strong effectiveness against numerous Lepidoptera species that harm the fruit and leaves of agricultural crops. The active component, an avermectine-derived naturally occurring compound, paralyzes Lepidoptera larvae by activating the chloride channel in their neurological systems (Wunan et al, 2015). Another insecticide Imidachlorpid worked on the basis of reflecting the transmitted of stimulus of the insect nervous system. Imidachlorpid mimics the action of acetylcholine, but it is not degraded by enzyme acetylcholinesterase. It binds with acetylcholine receptor which results hyper excitation, convulsions, paralysis and death of the insect (Plumlee, 2004).

2.2 Pesticides used in Nepal

Nepal is an agricultural country having agriculture practices as a main economic recourse (Koirala et al, 2008; NHRC, 2010). In early 1950s reports, insecticides were used in Nepal to manage malaria, particularly to eliminate the sickness caused by mosquitoes for the Gandaki Hydropower Project (Dahal, 1995). Paris green, gramaxone, nicotine sulfates, and dichloro-diphenyl-trichloroethane (DDT) were the first chemicals introduced to Nepal and were all imported from the USA. Other organochlorines, organophosphates, carbamates, and synthetic pyrethroids were introduced after these compounds (Giri et al, 2014). Beginning in the early 1960s, pesticides were used in the agriculture sector. In the age of the green revolution farmers were advised to use larger varieties, such as better seeds, synthetic fertilizers, pest killer, etc., to produce the most yields possible from a crop. The pesticides and insecticides to control the many insect pests of crops weren't known to farmers prior to that time. Due of their current effectiveness in eliminating pests, farmers prefer using specific pesticides over broad-spectrum ones (Neupane, 1995).

Nepal only used but does not manufactured any kind of pesticides. It meets its pesticidal needs by importing pesticides from importing it from countries like China, India, Malaysia, Singapore, Italy, and Japan (GC, 2020). Till now, 54 types of insecticides were introduced to Nepal including 14 bio-pesticides. Some of highly toxic chemical pesticides such as Organochlorines, Benzene hexa chloride, lindane etc. were banned in different agricultural countries including Nepal. In Nepal, there are altogether 16,110 retailers, 5 pesticide formulators, 37 pesticide applicators, and 286 pesticide importers (PPQMC, 2021). Pesticide traders are mostly concentrated in the nation's commercial agricultural regions, including the plains, the valleys, and the areas in and around the nation's largest cities. However, the mid-hills, hills, and bigger rural portions of the nation are still unaffected by the pesticide industry. According to reports, 25% of farmers in Nepal's terai areas, 9% of those in the hilly region, and 7% of those in the mountains use pesticides on their farms (CBS, 2003). In comparison to other nations like India (0.481 kg/ha), China (2.0-2.5 kg/ha), Japan (10.8 kg/ha), Europe (1.9 kg/ha), and the United

States (1.5 kg/ha), pesticide inactive ingredient consumption is extremely low on average, at 0.396 kg/ha (Sharma, 2019).

Due to the increased demand, farmers are applying pesticides in their crops carelessly because they are heavily imported from other nations. In comparison to affluent nations, Nepal uses fewer insecticides and other pesticides, but the true issue is in the commercial pocket regions, where producers use far more than is necessary. Farmers generally believe that they are the only ones who can manage insect infestations chemically. One of the causes for Nepal's incorrect and excessive use of pesticides is a lack of farmer awareness and education, a lack of non-chemical methods for managing insect pests, as well as a lack of government supervision and oversight of pesticide usage regulations and practices (GC et al, 2021). Compared to cereal crops and other crops, vegetables are reported to use insecticides far more frequently. Commercial vegetable producers typically use pesticides more frequently. According to one study, farmers frequently apply pesticides even when the insects are not causing significant damage since more than 85% of imported insecticides are used to prevent different insect pests from destroying vegetable crops. According to reports, tomato and brinjal samples had pesticide residual concentrations of cypermethrin that above the allowable limit. The same study also revealed that cowpea has the highest content of deltamethrin, followed by cauliflower, tomato, and brinjal (Sharma, 2015).

Different legal rules apply to the usage of pesticides in Nepal. In order to reduce dangers to human health, animal welfare, and foreign enemies associated with this topic, the country's import, manufacturing, sales, distribution, and use of pesticides are governed by the Pesticide Act and Rule 1991 and 1994. To reduce environmental contamination and regulate agrochemicals, including pesticides, Nepal accepted the Stockholm, Basel, and Rotterdam Conventions. Currently, the Government of Nepal (GoN) has outlawed 14 chemicals (Chlorden, DDT, Dieldrin, Endrin, Aldrin, Heptachlor, Mirex, Toxaphen, B.H.C., Lindane, Phosphamidon, Organomercury fungicide, Methyl parathion, Monocrotophos) because of their toxicity, persistence, propensity for accumulation and biomagnification, and long-term serious threats (MOEST, 2007).

In the instance of the region where people practice conventional agriculture, Nepalese farmers are incredibly ignorant of the risks associated with pesticide use. According to a survey, over 73% of vegetable producers in the Gaidahawa Rural Municipality of the Rupandehi area reuse unused pesticides. Researchers have noted that farmers have reportedly abandoned pesticide packages and containers in their fields without considering the harm those materials provide (Bhandari et al, 2021). Chlorpyrifos had the highest value of all the pesticides identified in the area, measuring 177 g/kg in soil samples taken from three different soil depths (0–5 cm, 15-20 cm, and 35–40 cm). Despite being outlawed in Nepal since 2001, DDT remains were still present, demonstrating how tenacious it is in the environment (Boul, 1995)

2.2 Pesticides residues

In contrast, there haven't been many researches done in the past about the chemical residues in polluted soil. An extensive amount of chemical pollution in soil was revealed by a Pro-Public investigation conducted in 2005. The study also showed that while some pesticides, such as hexachlorocyclohexane, no longer left residues in the soil, others, such as heptachlor, cis, and transchlordane, did (MOEST, 2007).

Vegetable samples taken from Nepal's premier vegetable market, which is situated in the center of the nation capital city, Kathmandu, revealed the presence of carbamate and pesticides from the organophosphate group. In Nepal's commercial vegetable region, which includes the Sarlahi and Kavre districts, the tomato and cowpea were cultivated and had greater pesticide residues. The same study found that the samples that tested positive for pesticide residues utilizing the test kit approach, 21.38% of tomato samples and 18.75% of cowpea samples had subpar quality (Ghimire, 2020). Pesticide use is rising in Nepal by 10–20% year, which indicates that the country's agriculture is currently in danger due to both economic losses and other negative side effects (Diwaker et al, 2008).

A destination other than their intended target is reached by more than 98% and 85% of sprayed insecticides and herbicides respectively, including non-target species, air, water, bottom, sediments, and food as a pesticide residue (Miller,

2004). These residue flows off of fields, is dumped, is sprayed aerially, or is sprayed into water to kill algae, it contaminates both land and water. Due to the fact that airborne particles are dispersed by the wind to distant locations, pesticides can contribute to air pollution (Cornell University, 2007).

Pesticide residues in food have been the subject of various reports in Nepal. According to a recent assessment by the Department of Food Technology and Quality Control (DFTQC), the hazard of pesticides in Nepalese diets is miserable (Koirala et al, 2009/010). Data from the national pesticide surveillance program (1995–2005) showed that malathion (3.9%), BHC (3.1%), methyl parathion (2.8%), DDT (1.8%), and parathion (0.3%) were found in 12.1% of food samples. The greatest amount of pesticide residues, measured by commodity, were found in root vegetables (11.9%), and followed by leaf vegetables (10.9%) (Koirala et al, 2009).

The Government of Nepal has taken a number of actions to lessen the endanger to animal and atmosphere posed by pesticide usage. However, several studies have shown that farming peoples are improperly utilizing the herbicide and are not follow the application guidelines (Sharma, 2011; Shrestha et al, 2010). The majorities of them handle it without personal safety measure and improperly dispose of trash (Karmacharya et al, 2012).

2.3 *Bacillus subtilis*

A gram-positive bacterium known as soil-dwelling bacteria is *Bacillus subtilis*. A fast-growing, aerobic, gram-positive bacterium called B. subtilis has rod-shaped cells that are typically 2–6 m long and just under 1 m in diameter. With a doubling time of as short as 20 minutes, the ideal growing temperature is between 30 and 35 C. The cells have a propensity to organize into lengthy chains that are joined by unleaved septal wall material under certain growth conditions. Under situations of starvation, the cells can go through a complex two-cell differentiation process that results in the development of an endospore, which is expelled by lysis of the enclosing mother cell. Vegetative cells have the ability to move. As an alternative, they can create spore-containing biofilms and "fruiting bodies" (Bandow et al, 2002).

B. subtilis was initially identified in the nineteenth century, giving it a lengthy history. The typical lab strain, 168, has obscure beginnings, but investigations in the late 1950s revealed that it was naturally transformable using linear DNA, solidifying its place in genetics history (Zeigler, 2011). *B. subtilis*, originally extensively described by Ferdinand Cohn in 1872, is the "type strain" of the order Bacillales and the defining organism of the whole Firmicutes phylum (Cohn, 1872). There are 141 different *Bacillus* species listed in the most recent Bergey's Manual. Differentiating *B. subtilis* from other species in the genus is based on a wide variety of characteristics. The capacity to hydrolyze and use multiple carbon sources, colony, cell, and spore shape, as well as tolerance of salt, pH, and temperature change, stand out among them. (De Vos & Logan, 2009)

B. subtilis is frequently discovered in close proximity to the roots of many plants (Cazorla et al, 2007). *B. subtilis* is frequently utilized as a biofertilizer since it has several advantageous properties for the plant (Lucy et al, 2007). *B. subtilis* stimulates plant growth in artificial media by secreting cytokinin hormones and volatiles that alter the homeostasis of plant hormones (Zhang H, et al, 2007). In addition, *B. subtilis* can directly stop bacterial pathogens from infecting the plant by releasing the AiiA enzyme. AiiA is a lactonase that inhibits acylhomoserine-lactone compounds that control how various plant pathogens express their virulence genes. Surfactin, a substance secreted by *B. subtilis*, functions as an antibiotic against diseases like *Pseudomonas syringae*. (Bais et al, 2004).

2.3.1 *Bacillus* as Plant Growth Promoting Rhizobacteria (PGPR)

Over a century ago, the area of soil that surrounds plant roots known as the rhizosphere was characterized as "a nesting location for a rich and active variety of microorganisms." Some of these microbes live alongside plants as mutualists, while others are plant pathogens. Plant Development-Promoting Rhizobacteria (PGPR) is a term coined by Kloepper in 1980 to describe bacteria that flourish in the rhizosphere, colonize the roots, and encourage plant growth. *Bacillus subtilis* is one such rhizobacterium that encourages plant development (Barea et al, 2005). Nonpathogenic soil bacterium living

in association with roots of higher plants enhance the accommodative potential of the hosts and increase their growth. PGPR have varied traits, that permit them to act as bio-control agents: suppression of diseases caused by phytopathogens with the assembly of a wide vary of antimicrobial compounds (Ongena et al, 2005b).

Within the field of biopesticides, the *Bacillus* species and different PGPR play a very important role as a result of they turn out a range of antimicrobial agents together with lipopeptides, antibiotics, and enzymes that promote the expansion of plants and inhibit the pathogenic microorganisms (Teixeira et al, 2010). Inoculations of soils with biocontrol agent involve application of high densities of viable microbes for speedy formation of the host rhizosphere and is so expected to a minimum of transiently perturb the composition and performance of soil microorganism communities (Trabelsi and Mhamdi, 2013).

In soil system, PGPR participates in myriad of utilization processes of nutrients in order to sustain the soil fertility (Barea et al, 2004, Vikram and Hamzeharghani, 2008). PGPR facilitates plant growth by solubilizing insoluble phosphate, fixing atmospheric nitrogen and transporting it to plant, facilitating uptake of alternative plant nutrients, synthesizing siderophore and phytohormones (Yadav et al, 2011; Zaidi et al, 2009). Since soil organisms are sensitive to environmental change, a significant degradation of the microbial community might occur following disturbance of total microbial biomass and their specific composition (Lin et al, 2007).

2.3.2 *Bacillus subtilis* as biocontrol agent

Bacillus has a significant capacity to generate a wide variety of volatile organic compounds (VOCs) and soluble bioactive secondary metabolites due to its effectiveness in plant protection and ongoing presence in the competitive rhizosphere niche (BSMs). Within the patterns of VOCs generated by *Bacillus*, a high structural variety is confirmed (Caulier et al, 2019; Kai, 2020). Plant pathogens are managed by the biocontrol activities of the *Bacillus* species, which in turn protects against plant diseases. A few of the antimicrobial compounds produced by *Bacillus* species are antibiotics,

bacilysin, mycobacillin, bacillomycin, mycosubtilin, iturin, fengycin, and surfactin, which has both antifungal and antibacterial pharmacological activity (Ntushelo et al, 2019).

Now, the focus has shifted to the gram-positive *Bacillus* species, which generate aerobic reproductive structures. *B. subtilis*, a Gram-positive model organism (Moszer, 1998), is one of them and is now understood to be a potent biocontrol agent. As a soil rhizobacterium and immediate neighborhood of plant roots, *B. subtilis* maintains stable contact with higher plants and promote their growth. Additionally, its ability to make endospores and different antibiotics with a broad spectrum activity is undoubtedly helpful biocontrol agent. Biological management of plants by microorganisms may have promising approach as compared to the extended use of pesticides which is usually expensive and accumulate in plants resulting adverse effects on humans. Such chemicals can even be fatal to beneficial soil organisms (Leroux, 2003).

2.4 Tolerance of *Bacillus spp* to pesticides

Any chemical employed by humans to support the management of an agricultural ecosystem is referred to as an agrochemical. Typically, the term "agrochemicals" is used to describe a wide variety of chemical pesticides (including herbicides, fungicides, and insecticides) that are frequently employed to mitigate the damage caused by various pests. Though it has been determined that using pesticides is crucial for increasing agricultural output, it has also been observed that using these substances excessively or carelessly might harm the environment and cause microorganisms to grow poorly (Cederberg et al, 2019). Agrochemicals can be applied and then used by soil microorganisms as a source of nutrients. After being consumed, these substances are broken down by bacteria, which lead to the creation of new metabolites that may be significantly more toxic to plants than the original molecules (Magnoli et al., 2020). As a result, numerous researchers have recovered pesticide-resistant bacteria that could be used as microbiological agents to increase agricultural yield in polluted soil in order to overcome these difficulties. As an illustration, many varieties of *Bacillus* have been found to

increase crop output and productivity while tolerating higher amounts of pesticides (Radhakrishnan and Lee, 2016). Yet, a special characteristic of microorganisms such as N2-fixers and phosphate solubilizers, which may be caused by constitutive or induced mechanisms, is their ability to endure even greater rates of pesticides (Kirubakaran et al, 2019).

For increasing crop output under sustainable agriculture, only efficient microorganisms can replace chemical fertilizers and pesticides. These microorganisms can also adapt their physiological and genetic characteristics to the environment, which gives them the ability to thrive in pesticide- and environmentally-unfavorable-rich environments. Chemical breakdown in soil is accomplished by bioremediation and microbiological processes. Bacillus-related bacteria, which have an amazing ability to break down xenobiotic pollutants, have been found in many different parts of the world. Local microorganisms such as bacteria, which have the remarkable ability to use a wide variety of xenobiotics as their only source of energy and carbon, remove these pesticide contaminants from the soil (Siddique et al, 2003).

2.5 Biodegradation of pesticide by *Bacillus* spp

Pesticides can be converted into environmentally benign materials through a process known as biodegradation. Pesticides can degrade in soil, water, and plants. Pesticide-degrading microorganisms could be found naturally or could be deliberately introduced into the environment. Microorganisms, particularly fungus, bacteria, and yeasts, which consume pesticides as a source of food and energy, carry out the most prevalent type of degradation in the soil. According to Wróblewska-Krepsztul et al. (2017), *Bacillus* spp. is one of the organisms with the highest pesticide degradation activity.

Pesticides, fungicides, herbicides and antibiotics used carelessly to control phytopathogens that harm crop productivity remain in the environmental for longer periods of time and destabilize soil ecosystems, harming PGPR (Ahemad et al, 2009). Pesticides make up 10 of the 12 most harmful and persistent organic compounds, according to the Stockholm Convention on Persistent Organic Pollutants (Gilden et al, 2010).

The studies focused on the degradation of pesticides by four *B. subtilis* strains: DR-39, CS-126, TL-171 and TS-204, which were isolated from grapevines or the grape rhizosphere and examined in a liquid culture, on grape berries, and in vineyard soil. Each of the four *B. subtilis* strains improved profenofos degradation in each of the three matrices. The findings suggest that all four *B. subtilis* strains were capable of degrading profenofos even when other carbon sources were present in the medium, at a level of 90% (TS-204, TL-171, CS-126) or 79% (DR-39), as opposed to the 52% degradation seen in the uninoculated control. According to the in vitro profenofos degradation kinetics, the half-life was shorter in the presence of the B. subtilis strains, dropping from 12.90 days in the uninoculated spiked control to 4.03 days (Salunkhe et al, 2013).

With the use of an organic fertilizer enriched with strains of the antagonistic bacteria *Bacillus* spp., *Trichoderma* spp., and mycorrhizal fungi *Glomus* spp., the removal of boscalid and pyraclostrobin residue in apple fruit was examined. In comparison to the control, there were 52% and 41% lower residue levels of boscalid and pyraclostrobin, respectively. The soil fertility is increased, the roots and above-ground sections of plants are encouraged to grow healthily, and plant growth and development are simulated by this organic fertilizer. It boosts a plant's natural defense mechanism, enhances the quality of the fruit while it's being stored, and lowers the amounts of chemical plant protection product residue in orchards (Podbielska et al, 2018)

CHAPTER III

MATERIALS AND METHODS

This study was carried out in Microbiology Laboratory of Central Campus of Technology, Dharan. The laboratory techniques were according to standard methods.

3.1 Materials required

The materials equipment, media and reagent used and their application in this study are systematically listed in appendix-I

3.2 Methods

3.2.1 Study design

The study was conducted from January to July 2022. This study was the laboratory based cross-sectional study. All the work concerning to this research was carried out in Microbiology Laboratory of Central Campus of Technology, Hattisar, Dharan.

3.2.2 Sample collection

Five soil samples were collected from Jute Research Center, Itahari, Morang following proper sampling parameters for soil collection, handling and analysis. Samples were collected from 10-20 cm depth soil and were aseptically placed in the soil sample bag/plastics bag and bag was zip locked. Sterile gloves were used during sample collection and proper tags will be provided for each one (Ubalua, 2014). The soil samples were brought to the Microbiology Department of Central Campus of Technology campus for further analyses.

3.2.3 Laboratory set up

Laboratory setting was done in microbiology laboratory, Central Campus of Technology, Hattisar, Dharan.

3.2.4 Cleaning and sterilization of glass wares

The test tubes, pipettes, conical flasks, beakers, and other items used in the experiment were carefully cleaned and dried before use. The pipettes and petri plates were sterilized in a hot air oven at 160°C for two hours after being covered in silver foil (Aneja, 2004).

3.3 Isolation and characterization of *Bacillus subtilis*

With 9 ml of sterile, distilled water, one gram (1g) of each soil sample was suspended. After that, the soil suspension was heated to 60 degrees Celsius for one hour in a water bath to destroy non-spore producing organisms (Ubalua, 2014). On nutrient agar medium, a streak of soil suspension was used to inoculate the area. The inoculation plate was kept in an aerobic environment at 37 °C for 24 hours, after which colonies were spotted and observed. In order to identify the colonies later, they were subcultured onto nutrient agar slants and showed the cultural traits typical of the Bacillus species, such as being round or irregular, thick and opaque, and cream-colored colonies.

3.4 Identification of *Bacillus subtilis*

Taxonomic characteristics were largely used to identify Bacillus subtilis isolates. The distinctive physical, cultural, and biological traits were seen (Bergey, 2004; Cowan and Steel, 2003)

3.4.1. Cultural characterization

On nutrient agar plates, colonies from isolates were studied for their size, colour, shape, margin, and elevation.

3.4.2 Morphological characterization

The Gram staining technique was used to identify morphological traits such as cell shape, cell organization, and the organism's response to Gram's staining. The isolates were also morphologically characterized using the endospore-staining technique.

3.4.3 Biochemical characterization

Biochemical tests such as catalase degradation, citrate utilization, motility, indole, MR, VP, starch hydrolysis, gelatin hydrolysis test were carried out according to standard procedures.

3.5 PGPR characteristics

3.5.1 Indole acetic acid production

The qualitative analysis of Indole acetic acid was done by inoculating *Bacillus subtilis* in nutrient broth containing 5µg/ml L-tryptophan for 48 hours at 28℃ (Zakry et al, 2010). After incubation cell free supernatants were then prepared by centrifuging the broth at 5000 rpm for 20 minutes. 1ml of supernatants was mixed with 2 ml of Salkowski reagent and kept in the dark room for 20 minutes then pink color was noted for IAA positive test. Isolate having highest pink color was used for tolerance of pesticides.

3.5.2 Hydrogen cyanide production

HCN production was detected by inoculating the bacteria on modified nutrient agar media amended with 4% glycine according to Lorck (cited by Agbodjato et al., 2015). Whatman filter paper no.1 previously soaked in 2% sodium carbonate in 0.5% of picric acid was placed in the lid of the petri dish and seal with the paraffin to air tight. HCN production was detected by color orange to brownish red. The isolates producing highest amounts of color change was selected to use for tolerance of pesticides.

3.5.3 Determination of ammonia production

The selected isolates were tested for the production of ammonia using the qualitative method of Ahmad et al (2008). Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 hrs at 28±2℃. Nessler's reagent (0.5 ml) was added in each tube. The development of brown to yellow color was indicative for ammonia production.

3.5 Study of tolerance and sensitivity for *Bacillus subtilis* towards pesticides

A Biocontrol bacterium was tested in vitro for their compatibility to the fungicides, insecticides and herbicides. 1 ml bacterial suspensions having concentration of 10^8cfu/ml, were pipetted out in Petri dishes containing nutrient agar media amended with the Fungicides (Mancozeb, Carbendazim), Insecticides (Imidachlorpid, Emamactin benzoate) and Herbicides (2,4-D, Pendimethaline) with their respective conmcentrations. The suspensions were dispersed over the medium. Then Petri plates were incubated at 25°C for 48 hr. After 48 hr. number of colonies were counted and compared with control.

Step-by-step regression analysis, where the dependent variable was the number of colonies and the independent variable was the concentration of pesticides, was carried out to assess the tolerance and sensitivity of Bacillus subtilis to various pesticides. With the help of this investigation, the ideal pesticide concentration and highest bacterial count were discovered.

3.6 Enumeration of cfu/g soil by periodical interval by serial dilution method (10^8cfu/g)

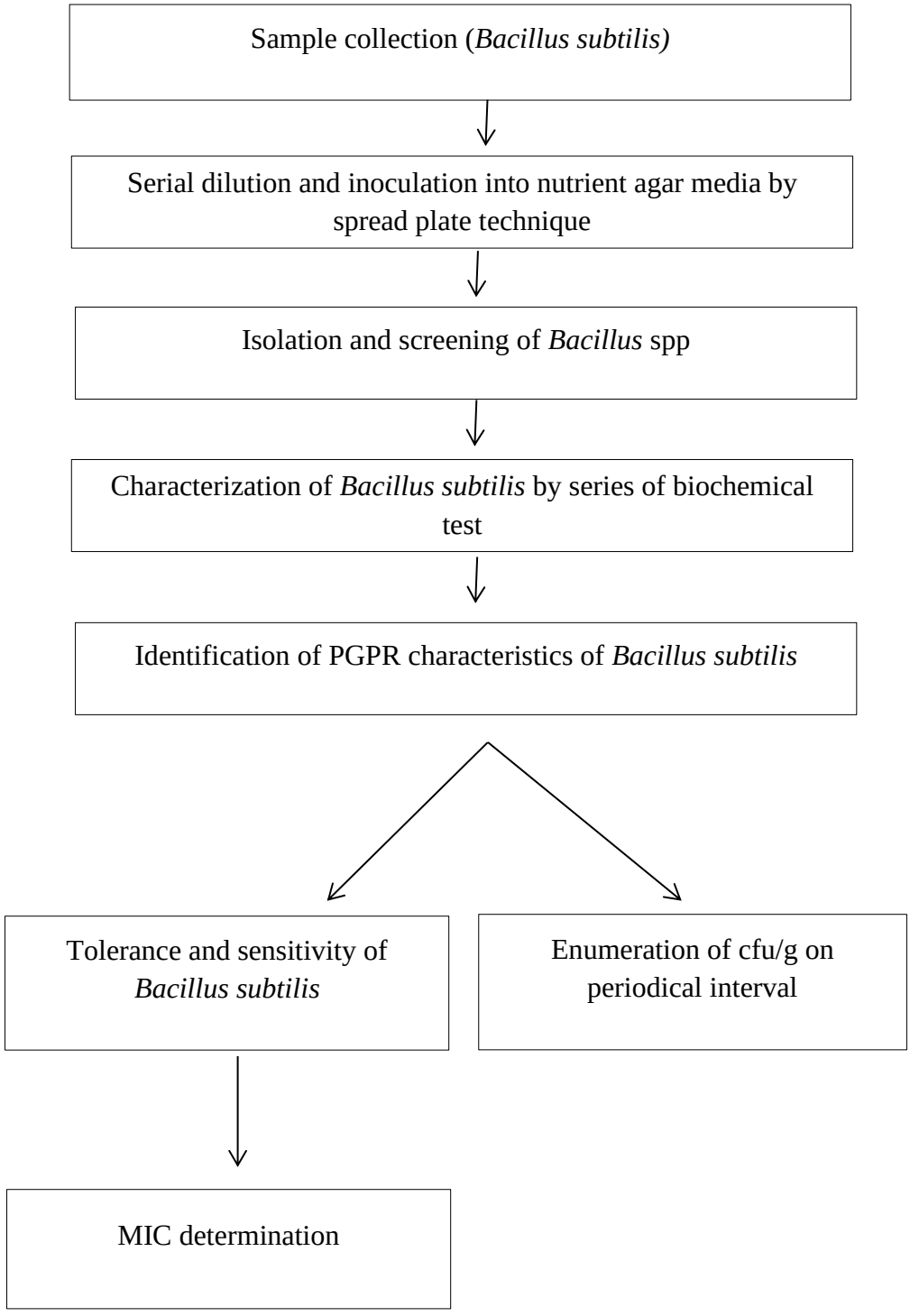
Carrier based culture of *B subtilis* was prepared. It was inoculated in nutrient broth. Eight days old broth cultures used for preparing carrier base culture of bioagent in 1:2 proportions i.e. 100 ml broth culture of *B subtilis* will be mixed in 200 gm of sterilized talcum powder and air dried for a day. Plastic pots having capacity 1kg soil were disinfected by autoclaving. Sterilized soils were inoculated with carrier based *B subtilis*. Fungicides, Insecticides and Herbicides individually at recommended concentration were added in the soil. Carrier based *B subtilis* was added at 10gm/kg soil. Bacterium suspension with soil was used as a control. Enumeration of *B subtilis* was performed at the concentration of 10^8cfu/g with the interval of 45 days, 90 days and 145 days.

3.7 Minimum inhibitory concentration (MIC)

MIC was determined using well diffusion method (Mazolla et al., 2009). The prepared MHA plates were inoculated with the *Bacillus subtilis.* Three wells of 5 mm diameter were made at equidistant to each other. Each well was

labeled for the amount of extract to keep on. The fungicides (Mancozeb, Carbendazim), insecticides (Imidachlorpid, Emamactin benzoate) and herbicides (2,4-D, Pendimethaline) with their specific concentrations were added in those of three wells. The entire system was left undisturbed and given some time to dry. For the purpose of determining the minimal inhibitory concentration, the plates were infected at 37°C for 24 hours.

Outline of the Study



CHAPTER IV

RESULTS

4.1 Population of *B. subtilis*

Out of 5 soil samples collected from different rhizosheric locations of Jute Research Center, Itahari, *Bacillus* spp were isolated from 3 samples by serial dilution technique using Nutrient agar media. 60% samples were found positive for *B. subtilis* (fig. 1).

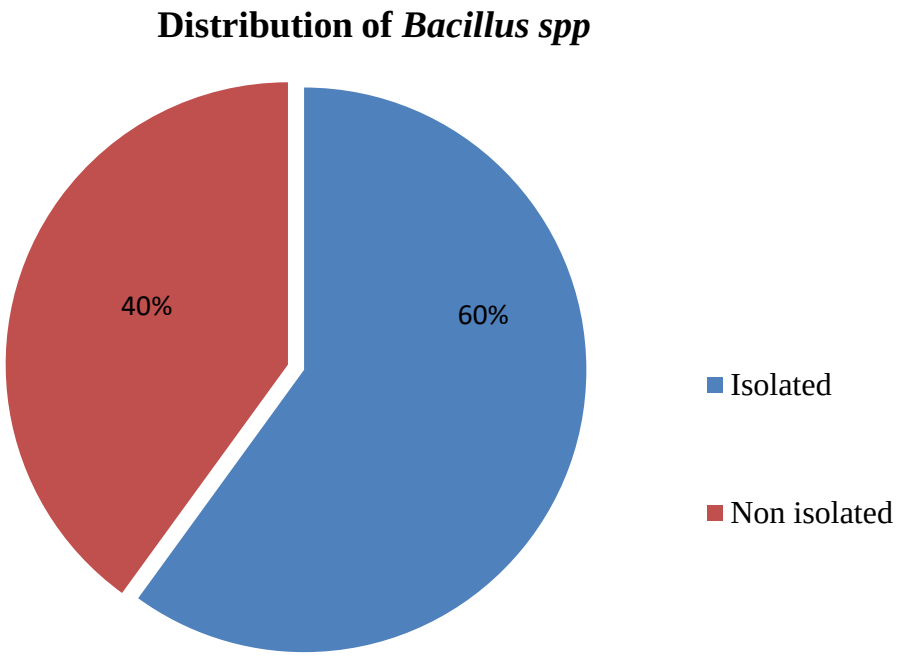


Figure 1 Distribution of *Bacillus* spp from 5 soil samples collected

4.2 Morphology and biochemical characteristics of *B. subtilis*

All 5 samples were purified and characterized through microscopically as well as using set of biochemical tests. The test results are shown in table 1

Table 1 Morphology and biochemical characteristics of *B. subtilis*

Test performed	**S1**	**S2**	**S3**	**S4**	**S5**
Pigmentation	Creamy	Creamy	Creamy	Creamy	Creamy
Gram	Positive	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod
Endospore	+ ve	+ ve	+ ve	+ ve	+ ve
Catalase	+ ve	_ ve	+ ve	+ ve	- ve
Citrate	+ ve	+ ve	+ ve	+ ve	_ ve
Motility	+ ve	+ ve	+ ve	+ ve	+ ve
Indole	- ve	- ve	- ve	- ve	+ ve
MR	- ve	+ ve	- ve	- ve	- ve
VP	+ ve	+ ve	+ ve	+ ve	+ ve
Gelatin	+ ve	- ve	+ ve	+ ve	+ ve
Starch hydrolysis	+ve	+ ve	+ ve	+ ve	- ve

Among the tested isolates, sample 1, 2 and 4 showed exact morphological and biochemical properties to the *B. subtilis.*

4.3 Growth promoting characteristics

Growth promoting activities of *B. subtilis* were detected on the basis of IAA production, HCN production and ammonia production test. Isolates were tested for indole acetic acid production qualitatively on the basis of pink color production by cell free supernatant after addition Salkowski reagent. Out of 5 isolates, 2 isolates were positive for IAA production.

Similarly, hydrogen cyanide production test was quantified on the basis of conversion of yellow colored whatman filter paper soaked in 2% sodium carbonate in 0.5% picric acid into brownish red. Out of 5 isolates, 3 were positive for HCN production.

Ammonia production test was carried out in peptone water. Test was positive for the ammonia production by development of brown to yellow color. Out of 5 samples, 4 isolates were positive for ammonia production (Table 2).

Table 2 Growth promoting characteristics

Samples	IAA Production	HCN Production	Ammonia Production
S1	+ve	+ve	+ve
S2	-ve	-ve	+ve
S3	-ve	+ve	+ve
S4	-ve	-ve	-ve
S5	+ve	+ve	+ve

4.3 Tolerance and sensitivity of *B. subtilis* to different pesticide

This experiment was carried out to study the tolerance and sensitivity of *Bacillus subtilis* to different pesticides. The step wise Regression analysis was done at different concentration of pesticides in which dependent variable was no of colonies and Independent variable was pesticides concentration.

4.3.1 Tolerance and sensitivity of *B. subtilis* to herbicides

Data presented in table 3 indicate that *B. subtilis* tolerated the effect of herbicides. Regression coefficient (R^2) having positive value for 2,4-D (0.998) and Pendimethaline (1.00) suggest that bacterial count (dependent variable) is strongly rely on the applied pesticide concentration (independent variable). Incorporation of insecticides in growth medium with different concentrations recorded minimum to maximum count of *B. subtilis*. Among this 2,4-D at the rate 0.03% (6×10^8 cfu/ml) gave minimum count and at the rate 0.01% (30×10^8 cfu/ml) gave maximum count of *B. subtilis*. On the other hand Pendimethaline at the rate 0.4% (55×10^8 cfu/ml) gave minimum count and at the rate 0.2% (104×10^8 cfu/ml) gave maximum count of *B. subtilis*.

Table 3 Tolerance and sensitivity of *B. subtilis* to herbicides

Coefficient	**Herbicides**	
	2,4-D	Pendimethaline
A	42.333	5.2830
B	-120.000	-3.1391
R^2	0.998	1.000

Coefficient of A indicates pesticidal treatment concentration and coefficient B indicates increased or decreased in number of colonies in correspondence with pesticidal concentrations.

4.3.2 Tolerance and sensitivity of *B. subtilis* to fungicides

Data presented in table 4.4 indicate that *B. subtilis* tolerated the effect of fungicides. Regression coefficient (R^2) having positive value for Carbendazim (1.00) suggest that bacterial count (dependent variable) is strongly rely on the applied pesticide concentration (independent variable) whereas, Mancozeb treatment did not show any microbial growth. Incorporation of Carbendazim in growth medium with different concentrations recorded minimum to maximum count of *B. subtilis*. Among this Carbendazim at the rate 0.3% (46×10^8 cfu/ml) gave minimum count and at the rate 0.1% (131×10^8 cfu/ml) gave maximum count of *B. subtilis*.

Table 4 Tolerance and sensitivity of *B. subtilis* to fungicides

Coefficient	**Fungicides**	
	Mancozeb	Carbendazim
A	-	159.000
B	-	-565.714
R^2	-	1

Coefficient of A indicates pesticidal treatment concentration and coefficient B indicates increased or decreased in number of colonies in correspondence with pesticidal concentrations.

4.3.3 Tolerance and sensitivity of *B. subtilis* to insecticides

Data presented in table 5 indicate that *B. subtilis* tolerated the effect of insecticides. Regression coefficient (R^2) having positive value for Emamactin benzoate (0.997) and Imidachlorpid (0.981) suggest that bacterial count (dependent variable) is strongly rely on the applied pesticide concentration (independent variable). Incorporation of insecticides in growth medium with different concentrations recorded minimum to maximum count of *B. subtilis*. Among this Imidacloprid at the rate 0.03% (82×10^8 cfu/ml) gave minimum count and at the rate 0.01% (203×10^8 cfu/ml) gave maximum count of *B. subtilis*. On the other hand Emamactin benzoate at the rate 0.05% (58×10^8 cfu/ml) gave minimum count and at the rate 0.03% (144×10^8 cfu/ml) gave maximum count of *B. subtilis*.

Table 5 Tolerance and sensitivity of *B. subtilis* to insecticides

Coefficient	**Insecticides**	
	Emamactin benzoate	Imidachlorpid
A	221.667	268.333
B	-4300	-6050.000
R^2	0.997	0.981

Coefficient of A indicates pesticidal treatment concentration and coefficient B indicates increased or decreased in number of colonies in correspondence with pesticidal concentrations.

4.4 Enumeration of *B. subtilis* (10g/kg) soil population at different intervals from herbicide amended soil (10^8 cfu/g soil)

Data recorded in Table 6 indicated the incorporation of herbicides in soil with the addition of *B. subtilis* at the rate 10g/kg soil showed that the population of *B. subtilis* was lower at 45 days of inoculation. Initial count of *B. subtilis* was lower at 30 days inoculation. The load was increased at 90 days and declined at 135 days of inoculation. All the treatments were compared to control having no pesticidal treatment.

Table 6 Load of *B. subtilis* (10g/kg soil) at different time intervals

SN	Treatment	45 DAI (cfu/g)	90 DAI (cfu/g)	135 DAI (cfu/g)
1	2,4-D	27.67	71.67	23
2	Pendimethaline	64.67	102.33	80
3	Control	109	127	95
p-value		0.000	0.002	0.000
F-test		sig	sig	sig

Results: $p<0.05$. There was significant difference in number of colonies within herbicidal treatment groups in different time intervals.

Significant F-test showed that the numbers of bacterial count were varied with the different interval of time in pesticide amended soil.

Note: Mean of three replications was taken.

4.5 Enumeration of *B. subtilis* (10g/kg) soil population at different intervals from fungicides amended soil (10^8 cfu/g soil)

Data recorded in Table 7 indicated the incorporation of fungicides in soil with the addition of *B. subtilis* at the rate 10g/kg soil showed that the population of *B. subtilis* was lower at 45 days of inoculation. Initial count of *B. subtilis* was lower at 45 days inoculation. The load was increased at 90 days and declined at 135 days of inoculation. All the treatments were compared to control having no pesticidal treatment.

Table 7 Load of *B. subtilis* (10g/kg soil) at different time intervals

SN	Treatment	45 DAI (cfu/g)	90 DAI (cfu/g)	135 DAI (cfu/g)
1	Mancozeb	63.33	95.33	55
2	Carbendazim	15	75.33	55
3	Control	109	127	95
p-value		0.000	0.001	0.000
F-test		sig	sig	sig

Results: $p<0.05$. There was significant difference in number of colonies within fungicidal treatment groups in different time intervals.

Significant F-test showed that the numbers of bacterial count were varied with the different interval of time in pesticide amended soil.

Note: Mean of three replications was taken.

4.6 Enumeration of *B. subtilis* (10g/kg) soil population at different intervals from insecticide amended soil (10^8 cfu/g soil)

Data recorded in Table 8 indicated the incorporation of insecticides in soil with the addition of *B. subtilis* at the rate 10g/kg soil showed that the population of *B. subtilis* was lower at 30 days of inoculation. Initial count of *B. subtilis* was lower at 30 days inoculation. The load was increased at 60 days and declined at 90 days of inoculation. All the treatments were compared to control.

Table 8 Load of *B. subtilis* (10g/kg soil) at different time intervals

SN	Treatment	45 DAI	90 DAI	135 DAI
1	Imidachlorpid	42	72.67	33.33
2	Emamactin benzoate	34.33	104.33	53.67
3	Control	109	127	95
p-value		0.001	0.002	0.000
F-test		sig	sig	sig

Results: $p<0.05$. There was significant difference in number of colonies within insecticidal treatment groups in different time intervals.

Significant F-test showed that the numbers of bacterial count were varied with the different interval of time in pesticide amended soil.

Note: Mean of three replications was taken.

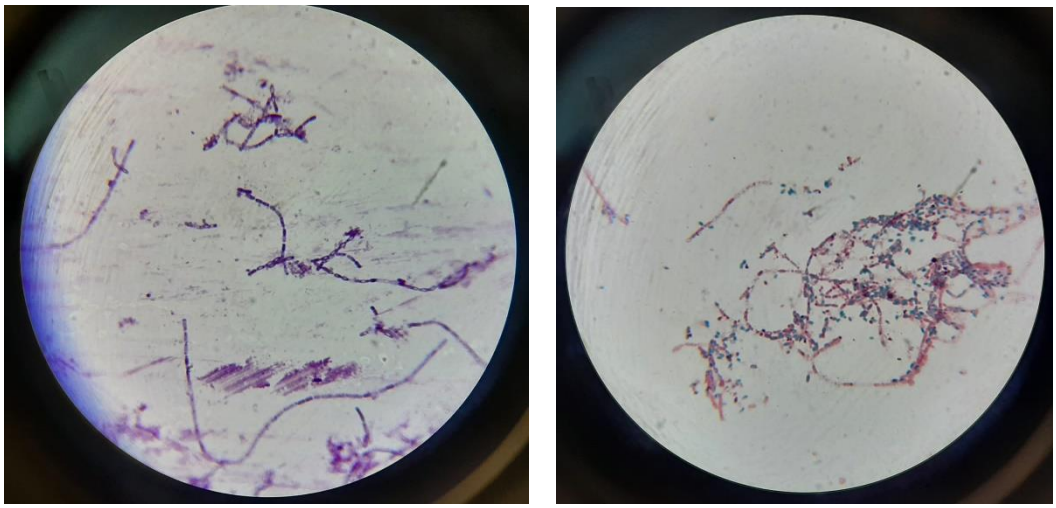
4.7 Minimum inhibitory concentration determination

MIC values of different pesticide were determined by Agar diffusion method. The values obtained were given in the table 9. Zone of inhibition of 2,4-D was increased from 1.7 cm to 2.2 cm when the concentrations of the pesticides were increased from 0.1% to 0.3%. Pendimethaline, Emamactin benzoate, Mancozeb and Carbendazim followed the similar manner to that of 2,4-D. It indicated that *B. subtilis* had certain level of resistance against recommended concentration of pesticides. From the data below, it can be assured that *B. subtilis* were able to tolerate particular concentration of the different chemical pesticide.

Table 9 Zone of inhibition of pesticides for MIC determination

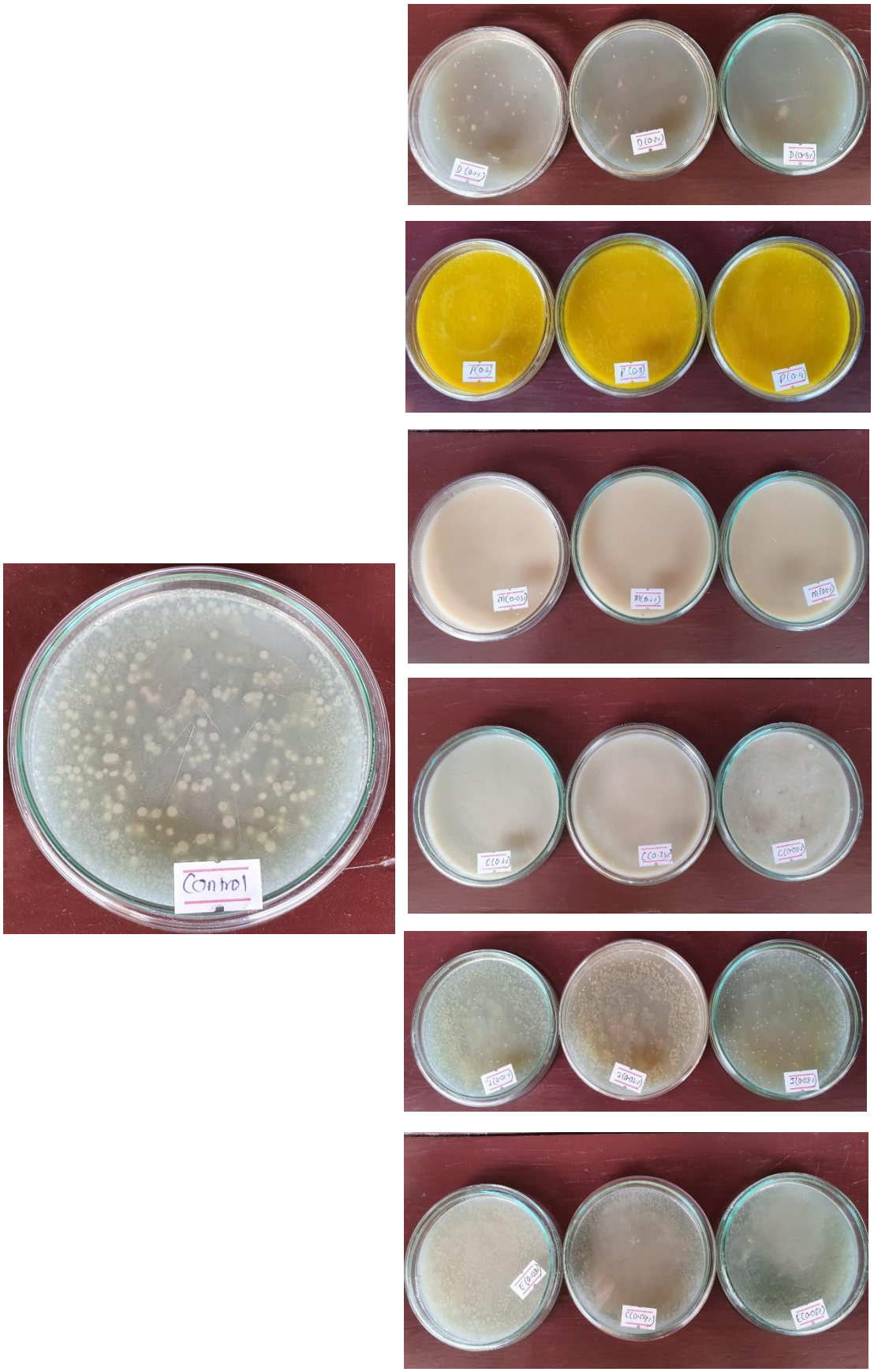
Treatment	Concentrations (%)	Diameter (cm)
2,4-D	0.1	1.7
	0.2	2
	0.3	2.2
Pendimethaline	0.2	2.1
	0.3	2.5
	0.4	2.9
Emamactin benzoate	0.03	1.3
	0.04	2
	0.05	2.3
Mancozeb	0.1	2
	0.25	2.3
	0.3	2.5
Carbendazim	0.05	1
	0.1	1.5
	0.2	1.7
Imidachlorpid	0.01	1.5
	0.02	1.6
	0.03	1.9

PHOTOGHAPHS

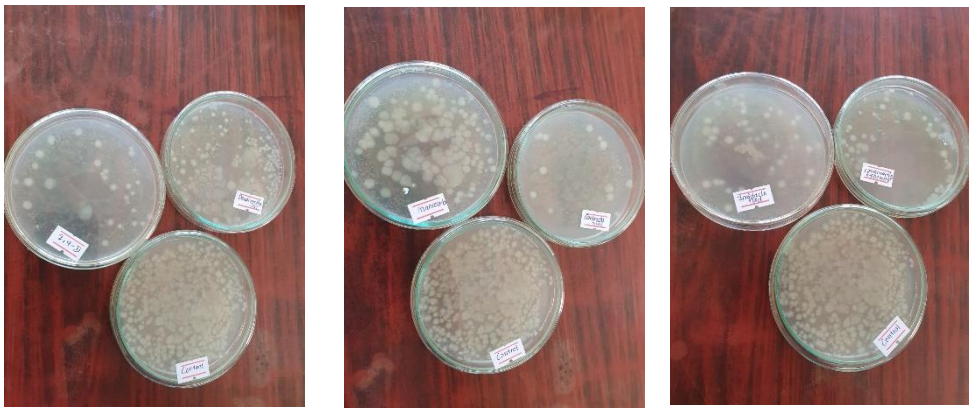


Photograph 1: Microscopic view of Gram staining and spore staining of *B. subtilis*

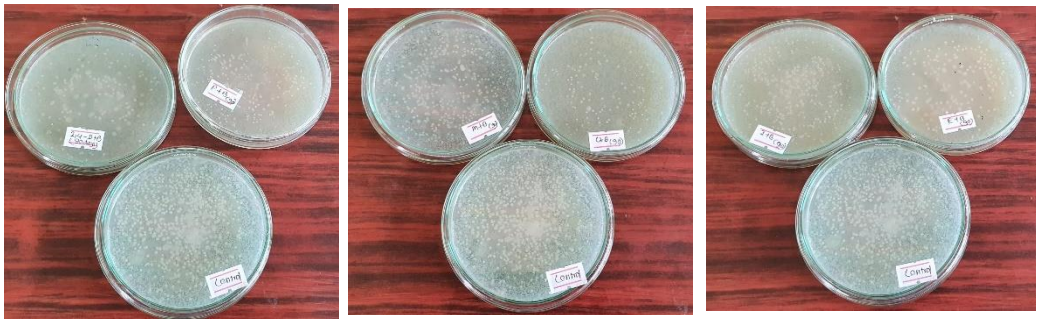
Photograph 2: In vitro screening of HCN production and IAA production of *B. subtilis*



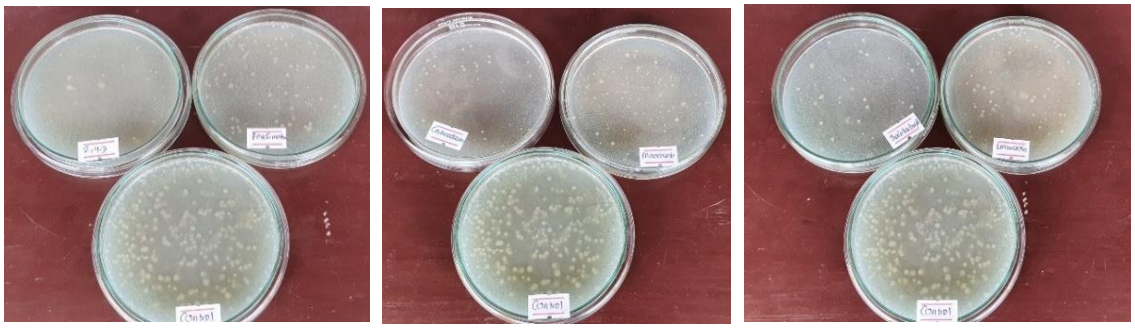
Photograph 3: Tolerance and sensitivity of *B. subtilis* to herbicide, fungicide and insecticide



Photograph 4: Enumeration of *B. subtilis* population at 45 days from pesticide amended soil



Photograph 5: Enumeration of *B. subtilis* population at 90 days from pesticide amended soil



Photograph 6: Enumeration of *B. subtilis* population at 135 days from pesticide amended soil

CHAPTER V

DISCUSSION

The present investigation deals with the PGPR activity of *Bacillus subtilis* and tolerance or sensitivity of *B subtilis* towards different concentration of pesticides in soil. In this study five bacterial isolates were screened from five soil samples and among them bacteria with PGPR activity was selected. This isolate was subjected to pesticide amended cultural media. It also aimed to determine the effect of different concentration of pesticide on load of selected strain.

In agricultural fields, diverse populations of *Bacillus* species, which create aerobic endospores, can be found and either directly or indirectly influences crop productivity. These *Bacilli* exhibit a variety of physiological characteristics that enable them to endure challenging environmental conditions for extended periods of time, including multilayered cell walls, stress-resistant endospore formation, secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes. The growth of plants is known to be aided by numerous *Bacillus* species. The main growth-promoting mechanisms include the production of growth-stimulating phytohormones, solubilization and mobilization of phosphate, siderophore production, antibiosis, or the production of antibiotics, inhibition of ethylene synthesis in plants, and induction of systemic pathogen resistance in plants (Richardson et al., 2009). The *Bacilli* make up a sizeable portion of the soil's microbial ecosystem. This bacterial genus's ability to sporulate makes it easier for them to survive in a wide range of settings.

Five isolates were taken from jute agricultural soil and assumed to be B. subtilis based on cultural, morphological, and Gram's reaction. However, only three of the isolates were confirmed to be B. subtilis based on a series of biochemical tests. The biochemical system of identifying Ubalua's sensitivity may be to blame for the drop in the number of presumed isolates (2014).

Percentage occurrence of *B. subtilis* on soil was 60% obtained with biochemical tests. This might be due to the lack of humic soil which has more organic content resulting more bacterial mass. This supports Bello's (2016) research, which showed that isolation rates were higher in organically rich soil (50%) than in nutrient-poor soil (20%). This concurs with Ubalua's (2014) findings, which showed that *B. subtilis* is typically found in agricultural soil. The outcome of diversity is also related to numerous studies on the diversity of Bacilli in the soil's rhizosphere, which have shown that rhizosphere competence is a trait shared by all strains, not only those belonging to a particular species or genus. These studies have revealed that the rhizosphere of soil is dominated by *Bacillus* species (Maplestone and Campbell 1989).

The generation of ammonia is a unique trait of bacteria that promote plant growth since it directly enhances plant growth and yield. Ammonia-positive strains, also known as diazotrophic or nitrogen-fixing microorganisms, are a key strain that promotes plant growth since it is the main source of nitrogen for plant growth and yield. According to Verma et al. (2013), isolated from rhizosphere soils, plant growth-promoting microorganisms have a higher capacity for ammonia synthesis. Recently, Beneduzi et al. (2008) argued in favor of the *Bacillus* having the capacity to fix nitrogen, synthesis IAA, HCN, and solubilize phosphates, all of which support crop growth. Hence, this study only has taken the *B subtilis* having all the above characteristics for further study.

In vitro study clearly established that bacterium was able to tolerate the recommended fungicidal dose of Carbendazim at the rate 0.1%, 0.25% and o.3% and gave bacterial growth 131×10^8 cfu/ml, 102×10^8 cfu/ml and 46×10^8 cfu/ml respectively. Comparable research has been done on *Bacillus* spp., which is known for their strong tolerance activity, by Goswami (2014) and this paper. In addition to hydrolytic enzymes, they produce a wide range of potent antifungal metabolites. In the breakdown of pesticides, they play a vital role. The secretion of hydrolytic enzymes, the production of antimicrobial substances, the competition for nutrients, or a combination of these mechanisms have all been put forth as possible explanations for why Bacillus

spp. inhibit pathogenic fungi. The most well-known and crucial technique to prevent the pathogen from invading the tissues of the host plant is antagonist activity, also known as antibiosis. Whereas, Mancozeb did not shows any bacterial growth which might be due to sensitivity of *B subtilis* to the Mancozeb treatment. According to Magnoli et al., 2020, after consumption, these chemicals were not degraded by bacterium. In this case such metabolite is more harm to the soil microflora.

In vitro studies clearly established that bacterial antagonist found able to tolerate the recommended dose of insecticides. Ahemad and Khan (2011a) tested rhizobacterial strains for tolerance to insecticides. Rhizobacteria was found to tolerate almost all insecticides and to overcome the toxic effect of pesticides including insecticides rhizobacteria may either biodegrade or hydrolyze pesticides enzymatically. Similarly bacterial species was able to tolerate the recommended dose of herbicides. This result is similar with Ahmad et al (1995), who reported the influence of herbicide in *Bacillus sp*. This means it is possible that these bacteria can metabolize the pesticide or require a much higher concentration of pesticide in order to be affected.

The result obtained from enumeration of *B. subtilis* (10g/kg) soil population at different intervals from fungicides amended soil (10^8 cfu/g soil) showed that bacterial species able to resist the recommended concentration of insecticides, herbicides and fungicides. They show minimum growth at the interval of first 45 days. Soil incorporated with herbicidal treatment of 2,4-D and Pendimethaline showed 27.67×10^8 cfu/g and 64.67×10^8 cfu/g respectively. In this soil with fungicidal treatment of Mancozeb and Carbendazim showed 63.33×10^8 cfu/g and 15×10^8 cfu/g respectively. Comparable results were seen in soil treated with imidachlorpid and emamactin benzoate for insecticidal purposes, which exhibited 42×10^8 and 34.33×10^8 CFU/g, respectively. Wesley et al. (2017) claim that changes in growing conditions, such as pH changes brought on by the formation of acid or alkali, variations in test methodology, and particular factors, may have affected an organism's tolerance to or susceptibility to pesticides.They have to adapt in such environment at first.

Bacteria afterwards displayed its maximum growth within a 90-day period. This could be as a result of bacteria using the pesticides as a source of energy during the biodegradation process. *Bacillus* isolates are likely using agrochemicals as carbon and energy sources through partial transformation events that can happen with a variety of pesticide chemical families (Briceno et al., 2020). As a result of pesticide use and persistence, additional causes could include the establishment of novel pesticide breakdown routes and genetic mutation, which could increase soil bacteria's multi-drug resistance (MDR) (Al-Waili et al., 2012). Continuous pesticide exposure would exert constant pressure on the genes, leading to drug resistance, according to Pan Hau et al. (1981). The basic mechanism of resistance is the creation of slime materials (glycocalyx) or biofilms by microorganisms. The decrease in porin proteins, which can make it easier for molecules to flow through cell membranes, seems to be a factor in the resistance. Following 135 days of soil injection, the bacterial species showed signs of population reduction. The outcome was consistent with the earlier discovery made by Prescott et al. (2002) that a lack of nutrients in the growth medium caused a drop in the microbial population's size.

Various pesticides used in the study displayed in table 9 shows variation in their toxicity level. One thing was sure that *Bacillus* strain able to give clear zone of inhibition in the recommended dose of pesticide. It suggests that recommended doses of pesticides were tolerable for *Bacillus* for its growth. The result was comparable with the study carried out by Khan et al, (2006), where he found that the MIC values for *Bradyrhizobium* towards pesticide ranged from 3200 to 6400 µg/ml showed relative amount of tolerance to the applied dose of pesticide.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Bacillus can readily be isolated from soil sample with frequency 60%. This is the average concentration of bacterium in soil. The strain was able to show series of biochemical requirement given by standard protocols.

From the study we can conclude that *Bacillus* spp have potentiality to perform PGPR activity. The rhizospheric community is extremely complex and is made up of a wide range of creatures that interact with one another, affect one another, and respond to their surroundings. Numerous Bacillus spp. isolates have been transformed into PGPR.

The result of the study support *Bacillus* spp. is a good pesticide resistant soil microflora. By enhancing bacterial isolates' ability to survive in contaminated soil environments, pesticide tolerance may help retain pesticide resistance genes by boosting environmental selection pressure.

In this study, a few rhizobacterial isolates were chosen because they had the inherent traits of pesticide tolerance, numerous antibiotic resistances, and the ability to synthesis a wide variety of PGP compounds. Because of these intriguing characteristics, rhizobacteria are an appealing, agronomically viable, and long-term prospective alternative for crop production. Microorganisms constantly change as a result of their environment.

However, the findings in this work are based on laboratory experiments, and additional study is needed to verify this information in a practical setting (field experiments). Further study is required to determine the molecular mechanisms behind the emergence of antibiotic resistance and pesticide tolerance in rhizobacteria..

6.2 Recommendations

Based on the result and findings of the experiment, the recommendations made are as follows:

1. *Bacillus* strain screened can be potentially used as biofertilizer.
2. It can be used in soil contaminated with commercial pesticide.
3. Reduction of pesticide concentrations in soil by *Bacillus* spp need to be measured to support degradation of pesticide by selected strain.
4. Tolerance level of *Bacillus* spp. recommended that the mixed formulation of *Bacillus* spp. with chemical pesticide have better performance in controlling pest with increasing soil fertility.
5. In present, people are looking for organically grown products, *Bacillus* spp can be recommended for mass production and awareness for use as bio pesticide and biofertilizer.

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APPENDICES

APPENDIX-I

Materials and equipment

List of materials

1.Glasswares

Beaker	Conical flask
Petri plates	Measuring cylinder
Test tubes	Glass rod
Micropipette	Micropipette tips
Glass slides	Dolly rods

2. Miscellaneous

Bunsen burner	Gloves
Hi-media sterile cotton swabs	Bacteriological loop
Forceps	Permanent marker
Soaps	Labeling tags
Test tube rack	Ice box

3. Equipments

Autoclave	Hot air oven
Incubator	Refrigerator
Compound Microscope	Weight Balance

4. Chemical and Reagents

Crystal Violet (CV) solution 0.1%	30% acetic acid
Methylene blue	Nessler's reagent
Safranin	Lysol

5. Cultural media

Nutrient agar	Tryptone
Peptone	Nutrient broth

6. Biochemical media

Glucose	Sucrose
Fructose	Simon's Citrate agar

APPENDIX-II

Culture and media used in research

1. Nutrient Agar

Ingredients	gm/litre
Beef extract	0.5 g
Yeast extracts	1 g
Peptone	2.5 g
Distilled water	500 ml

2. Nutrient Broth

Ingredients	gm/litre
Peptone	5.0g
Sodium chloride	5.0g
Beef Extract	1.5g
Yeast Extract	1.5g
Final p^H	7.4±0.2

1.3 gm of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121°C for 15 minutes.

3. Peptone broth

Ingredients	gm/litre
Peptone	10.0
Sodium Chloride	5.0
Final p^H (at 25°C)	7.4±0.2

4. Muller Hinton Agar (MHA)

Ingredients	**gm/litre**
Beef infusion Broth	300.0g
Casein Acid Hydrolysate	17.0g
Starch	1.0g
Agar	17.0g
Final P^H	7.0±0.2

3.8 gm of media was suspended in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121^{o}C for 15 minutes. It was then poured while at 45-48oC into sterile petriplates in 25 ml quantity.

5. Starch Agar

Ingredients	**gm/litre**
Beef extract	3
Soluble starch	10
Agar	12
p^H	7.0±0.2

Suspend 25gm of powder in 1 liter purified water and mix thoroughly. Heat and boil for 1 min, autoclave 121^{o}C for 15 minutes.

APPENDIX III

Composition and preparation of different reagents

1. Gram staining reagents

i. Crystal violet Gram stain

Crystal violet	20g
Ammonium oxalate	9g
Ethanol or methanol, absolute	95ml
Distilled water	1 litre

Preparation:

Crystal violet is weighed and transferred to a clean bottle and absolute ethanol is added and mixed until dye is completely dissolved.

Ammonium oxalate is weighed and dissolved in about 200 ml of distilled water. Then it was added to the stain and total volume is made 1 litre by adding distilled water and mixed well.

ii. Iodine Solution

Potassium iodide	1.5g
Iodine	1.0g
Distilled water	150ml

Preparation:

Potassium iodide is weighed and transferred to a clean bottle 30-40 ml of distilled water is added to Potassium iodide and mixed until it is fully dissolved.

Iodine is weighed and added to potassium iodide solution and mixed well. Final volume is made 150ml by adding distilled water and mixed well.

iii. Acetone-alcohol decolorizer

Acetone	500ml
Ethanol (absolute)	475ml
Distilled water	25ml

To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then 500 ml acetone was added and mixed well.

iv. Counter stain solution

Safranin	10gm
Distilled water	1 lit

In a piece of clean paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then after, 1 liter distilled water was added to the bottle and mixed well until safranine dissolves completely.

v. Catalase reagent (To make 100ml)

Hydrogen peroxide solution	3ml
Distilled water	97ml

Preparation:

To 97 ml distilled water, 3 ml of hydrogen peroxide solution was added and mixed well.

APPENDIX-IV

Statistical analysis output

ANOVA

Bacterial colonies (45 DAI) of 2,4-D and Pendimethaline herbicides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5437.524	2	2718.762	119.070	.000
Within Groups	91.333	4	22.833		
Total	5528.857	6			

H_0: There is no statistical difference in number of colonies within herbicidal treatment group in day 45.

H_1: There is statistical difference in number of colonies within herbicidal treatment group in day 45.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Herbicidal treatment affects the number of colonies of *B. subtilis* on soil in day 45.

ANOVA

Bacterial colonies (90 DAI) of 2,4-D and Pendimethaline herbicides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2782.095	2	1391.048	39.369	.002
Within Groups	141.333	4	35.333		
Total	2923.429	6			

H_0: There is no statistical difference in number of colonies within herbicidal treatment group in day 90.

H_1: There is statistical difference in number of colonies within herbicidal treatment group in day 90.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Herbicidal treatment affects the number of colonies of *B. subtilis* on soil in day 90.

ANOVA

Bacterial colonies (135 DAI) of 2,4-D and Pendimethaline herbicides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6495.429	2	3247.714	259.817	.000
Within Groups	50.000	4	12.500		
Total	6545.429	6			

H_0: There is no statistical difference in number of colonies within herbicidal treatment group in day 135.

H_1: There is statistical difference in number of colonies within herbicidal treatment group in day 135.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Herbicidal treatment affects the number of colonies of *B. subtilis* on soil in day 135.

ANOVA

Bacterial colonies (45 DAI) of Mancozeb and Carbendazim fungicides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7861.333	2	3930.667	287.610	.000
Within Groups	54.667	4	13.667		
Total	7916.000	6			

H_0: There is no statistical difference in number of colonies within fungicidal treatment group in day 45.

H_1: There is statistical difference in number of colonies within fungicidal treatment group in day 45.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Fungicidal treatment affects the number of colonies of *B. subtilis* on soil in day 45.

ANOVA

Bacterial colonies (90 DAI) of Mancozeb and Carbendazim fungicides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2088.095	2	1044.048	55.436	.001
Within Groups	75.333	4	18.833		
Total	2163.429	6			

H_0: There is no statistical difference in number of colonies within herbicidal treatment group in day 90.

H_1: There is statistical difference in number of colonies within herbicidal treatment group in day 90.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Herbicidal treatment affects the number of colonies of *B. subtilis* on soil in day 90.

ANOVA

Bacterial colonies (135 DAI) of Mancozeb and Carbendazim fungicides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3719.048	2	1859.524	111.571	.000
Within Groups	66.667	4	16.667		
Total	3785.714	6			

H_0: There is no statistical difference in number of colonies within fungicidal treatment group in day 135.

H_1: There is statistical difference in number of colonies within fungicidal treatment group in day 135.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Fungicidal treatment affects the number of colonies of *B. subtilis* on soil in day 135.

ANOVA

Bacterial colonies (45 DAI) of Imidachlorpid and Emamactin benzoate insecticides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4388.762	2	2194.381	75.236	.001
Within Groups	116.667	4	29.167		
Total	4505.429	6			

H_0: There is no statistical difference in number of colonies within insecticidal treatment group in day 45.

H_1: There is statistical difference in number of colonies within insecticidal treatment group in day 45.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Insecticidal treatment affects the number of colonies of *B. subtilis* on soil in day 45.

ANOVA

Bacterial colonies (90 DAI) of Imidachlorpid and Emamactin benzoate insecticides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2774.667	2	1387.333	42.254	.002
Within Groups	131.333	4	32.833		
Total	2906.000	6			

H_0: There is no statistical difference in number of colonies within insecticidal treatment group in day 90.

H_1: There is statistical difference in number of colonies within insecticidal treatment group in day 90.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Insecticidal treatment affects the number of colonies of *B. subtilis* on soil in day 90.

ANOVA

Bacterial colonies (135 DAI) of Imidachlorpid and Emamactin benzoate insecticides

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2893.524	2	1446.762	108.507	.000
Within Groups	53.333	4	13.333		
Total	2946.857	6			

H_0: There is no statistical difference in number of colonies within insecticidal treatment group in day 135.

H_1: There is statistical difference in number of colonies within insecticidal treatment group in day 135.

Result: $p<0.05$, so the result is statistically significant

Conclusion: Insecticidal treatment affects the number of colonies of *B. subtilis* on soil in day 135.